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Evolution of an Expanded Sex-Determining Locus in *Volvox*

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Although dimorphic sexes have evolved repeatedly in multicellular eukaryotes, their origins are unknown. The mating locus (*MT*) of the sexually dimorphic multicellular green alga *Volvox carteri* specifies the production of eggs and sperm and has undergone a remarkable expansion and divergence relative to *MT* from *Chlamydomonas reinhardtii*, which is a closely related unicellular species that has equal-sized gametes. Transcriptome analysis revealed a rewired gametic expression program for *Volvox MT* genes relative to *Chlamydomonas* and identified multiple gender-specific and sex-regulated transcripts. The retinoblastoma tumor suppressor homolog *MAT3* is a *Volvox MT* gene that displays sexually regulated alternative splicing and evidence of gender-specific selection, both of which are indicative of cooption into the sexual cycle. Thus, sex-determining loci affect the evolution of both sex-related and non–sex-related genes.

Sexually dimorphic gametes have evolved in every major group of eukaryotes, and are thought to be selected when parents can differentially allocate resources to progeny (*1*). However, the origins of oogamy (large eggs and small sperm) and the contribution of sex-determining loci to such evolution are largely unknown (*2*, *3*) [see the glossary of terms (*4*) for further explanation of terminology].

The Volvocine algae are a group of chlorophytes comprising unicellular species, such as *Chlamydomonas reinhardtii* (hereafter *Chlamydomonas*), and a range of multicellular species of varying complexity, such as *Volvox carteri* (hereafter *Volvox*). *Volvox* has a vegetative reproductive form containing 16 large germ cells (gonidia) and ~2000 terminally differentiated somatic cells (fig. S1) (*4*, *5*).

Chlamydomonas and other Volvocine algae also undergo a sexual cycle in which a large, haploid mating locus (*MT*) controls sexual differentiation, mating compatibility, and zygote development (*6*). *MT* in *Chlamydomonas* is a 200- to 300-kb multigenic chromosomal region (Fig. 1A) within which gene order is rearranged between the two sexes (*MT*+ and *MT*–) and meiotic recombination is suppressed, thus leading to its inheritance as a single Mendelian trait. Within each *MT* allele are gender-limited genes (allele present in only one of the two sexes), which are required for the sexual cycle, as well as shared genes (alleles present in both sexes), most of which have no known function in sex or mating (*7*). The rearrangements that suppress recombination serve to maintain linkage of gender-limited genes, but they also reduce genetic exchange between shared genes, leading to their meiotic isolation. Thus, *Chlamydomonas MT* bears similarity to sex chromosomes and to expanded mating-type regions of some fungi and bryophytes (*8*–*10*).

Although *Chlamydomonas* is isogamous (producing equal-sized gametes), *Volvox* and several other Volvocine genera have evolved oogamy that is under the control of female and male *MT* loci (fig. S1) (*11*). Moreover, the *Volvox* sexual cycle is characterized by a suite of other traits not found in *Chlamydomonas*, such as a diffusible sex-inducer protein rather than nitrogen deprivation (–N) as a trigger for gametogenesis (table S1). A detailed characterization of *MT* in *Volvox* would be expected to shed light on the transition from isogamy to oogamy and on other properties of the sexual cycle that evolved in this multicellular species (table S1).

The *MT*+ allele of *Chlamydomonas* was previously sequenced and resides on chromosome 6 (Fig. 1A and fig. S2) (*12*). To enable a comparison of mating loci evolution between two related species with markedly different sexual cycles, we sequenced *Chlamydomonas MT*– and both alleles of *Volvox MT* (Fig. 1 and table S2) (*4*). *Volvox MT* was previously assigned to linkage group I (LG I) (*5*), but the locus had not been further characterized. We mapped *Volvox MT* to the genome sequence and assembled most of LG I (table S3) (*4*). Extensive synteny with *Chlamydomonas* chromosome 6 indicates that *MT* has remained on the same chromosome in both lineages for ~200 million years since their estimated divergence, despite numerous intrachromosomal rearrangements between the two (fig. S2) (*13*).

Although the haploid *Volvox* genome is ~17% larger than that of *Chlamydomonas* (138 Mb versus 118 Mb) and the two have very similar predicted proteomes (*12*, *14*), *Volvox MT* is ~500% larger than *Chlamydomonas MT* and contains over 70 protein-coding genes in each allele (Fig. 1B and tables S4 and S5). Compared with autosomes, *Volvox MT* is unusually repeat-rich (greater than three times the genomic average), has lower gene density, and has genes with more intronic sequence (table S6), all of which are properties that suggest an unusual evolutionary history and distinguish it from *Chlamydomonas MT*.

Only two gender-limited genes from *Chlamydomonas MT*–, *MID* and *MTD1*, have recognizable homologs in *Volvox* that are both in male *MT*

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(Fig. 1 and figs. S3 and S4). *MID* is a conserved RWP-RK family transcription factor whose expression in other Volvocine algae is induced by –N (*15*–*17*), as is also the case for *MTD1* (*18*, *19*). Both *MTD1* and *MID* are expressed constitutively in *Volvox* (fig. S5), indicating that their transcription is uncoupled from sexual differentiation. This result suggests that additional *MT* genes might play a role in gametogenesis.

We used differential deep transcriptome sequencing (tables S7 and S8) (*4*) to identify *MT* genes in *Volvox*, a method that helped to mitigate problems associated with automated gene prediction in atypical genomic regions such as *MT*. We identified transcripts for five new female-limited and eight new male-limited genes that do not have detectable homologs in *Chlamydomonas* and found that most of these gender-limited genes are sex-regulated (expression was induced or repressed during sexual differentiation) (Fig. 1C and table S9) (*4*). *HMG1* encodes a female-limited HMG domain protein (figs. S6 and S7 and table S10) that belongs to a family of DNA-binding proteins whose members regulate mammalian and fungal sex determination (*20*, *21*). However, HMG proteins had not been previously implicated in the sexual cycles of green algae or plants. A second previously unknown female-limited gene, *FSI1*, is strongly induced during gametogenesis and encodes a small predicted transmembrane protein with no identifiable homologs (Fig. 1C and fig. S7).

Besides identification of new gender-limited genes, our transcriptome data provided empirical support for 51 of 52 single-copy shared genes in *Volvox MT* that previously had limited expressed sequence tag (EST) support for the female allele (33 of 52) and no EST support for the male allele. Moreover, some of these shared genes showed patterns of expression that suggest co-option into the *Volvox* sexual cycle. These patterns include gender-biased expression (male:female expression ratio ≠ 1) and sex-regulated expression (Fig. 1C and fig. S7) (*4*). This set of genes encodes putative signaling, extracellular matrix, and chromatin-associated proteins with known or potential roles in gametogenesis and fertilization and are candidates for further investigation (fig. S7).

In diploid species, heterogametic sex chromosomes evolve rapidly (*22*) and lose genes that are not related to sex (*23*). Because of suppressed recombination, genes within large haploid mating loci are predicted to accumulate mutations more rapidly than would genes in autosomal regions, but they are continuously exposed to selection (*24*). Suppressed recombination also appears to have played a role in diversification of mating locus–linked genes in haploid fungi and bryophytes (*8*–*10*). Our data allowed us to compare the evolutionary history of *Volvox MT* genes from this oogamous species to each other and to genes from *MT* of its isogamous relative *Chlamydomonas*.

Divergence was measured from synonymous (d_S) and nonsynonymous (d_N) substitutions (Fig.

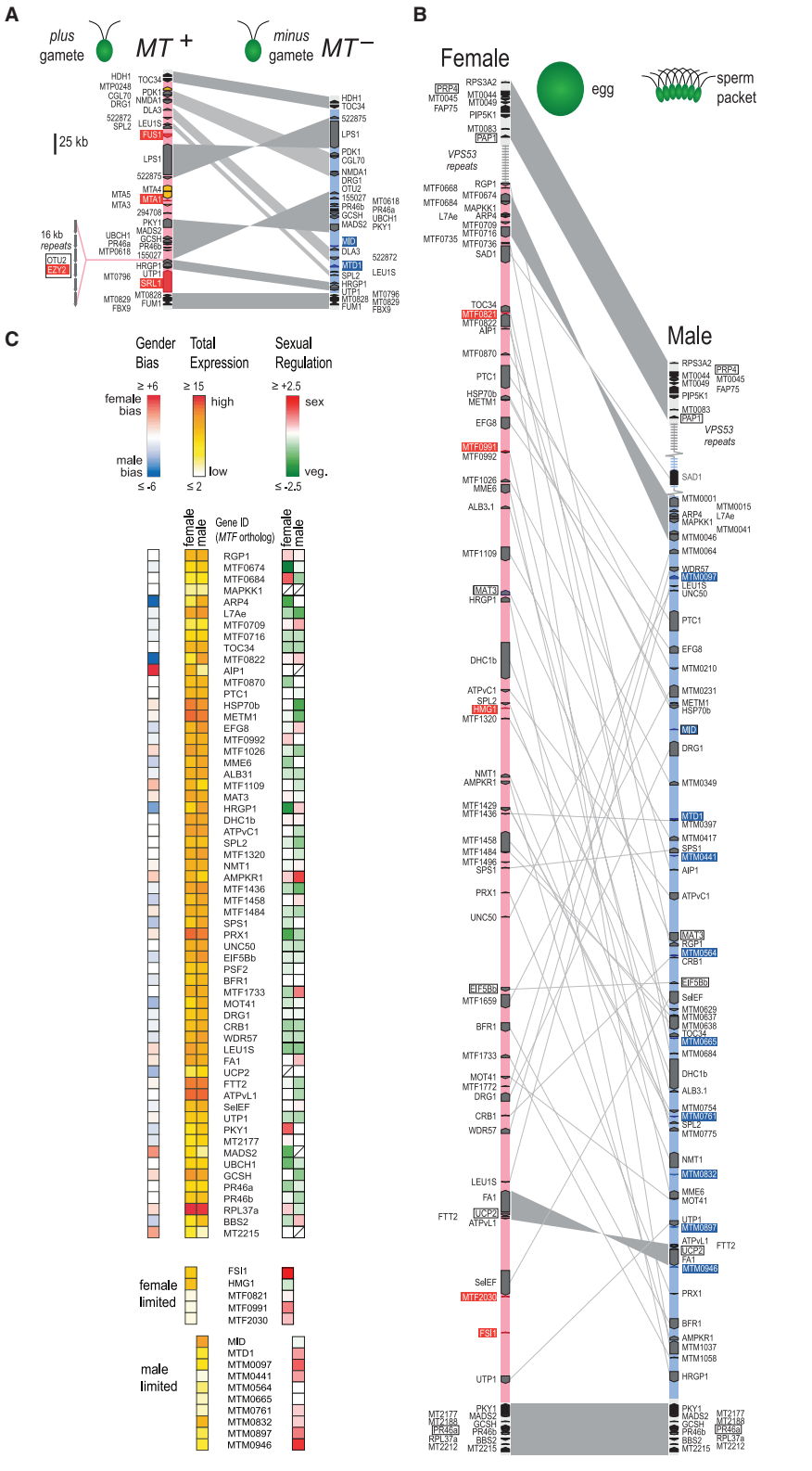

Fig. 1. Expansion of *Volvox MT* and sex-regulated gene expression. (**A**) Schematic of *Chlamydomonas* mating locus with rearranged domains in light blue or pink. *MT*+-limited genes are shaded red if unique or orange if they have an autosomal copy. *MT*−-limited genes are shaded blue. Flanking and shared genes are shaded black and gray, respectively. Synteny is indicated by gray shading. (**B**) Schematic of *Volvox MT* scaled as in (A). Boxed genes were used for mapping. The broken segment represents a transposon repeat region containing copies of *VPS53*. (**C**) Expression heat maps of *Volvox MT* genes. (Left) Female/male expression ratio. (Middle) Total expression. (Right) Sexual induction (Sex) or repression (Veg). Diagonal hatch indicates insufficient data.

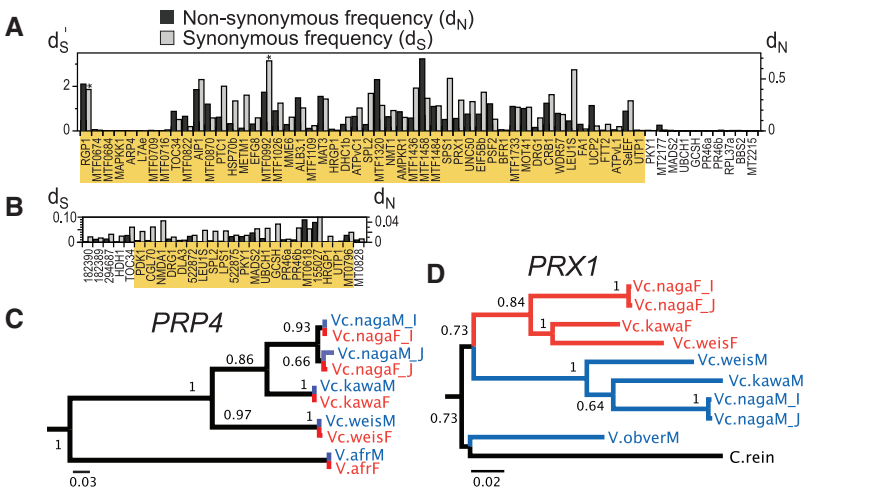

Fig. 2. Divergence of *MT* genes. (**A** and **B**) d_N and d_S for shared *Volvox* (A) or *Chlamydomonas* (B) genes within *MT* (orange shading) or flanking *MT*. Asterisks indicate saturated d_S values. (**C** and **D**) Maximum likelihood phylogenies for *PRP4* (C) and *MT* gene *PRX1* (D). Red and blue respectively indicate female and male strains and clades.

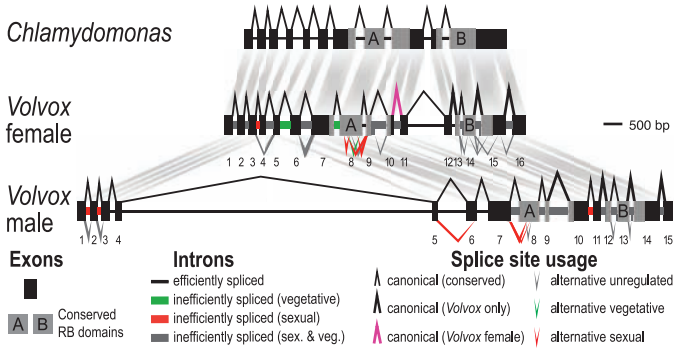

Fig. 3. Gender-specific divergence and splicing of *MAT3*. Shown is a schematic of *MAT3* from *Chlamydomonas* (top), *Volvox* female (middle), and *Volvox* male (bottom). *Volvox* exons are numbered.

2, A and B) and from total nucleotide distances for shared genes (tables S11 and S12) (*4*). Unexpectedly, divergence for *Volvox MT* allelic pairs is up to two orders of magnitude larger than for allelic pairs in *Chlamydomonas MT*, suggesting that *Volvox MT* alleles may have been subject to more intense and/or more prolonged recombinational suppression than *Chlamydomonas MT* alleles have been. In contrast, two internal syntenic blocks within *Volvox MT* are relatively similar (Figs. 1B and 2A), suggesting that they were acquired more recently in an ongoing stratification process as first described for the human X chromosome (*25*). *Volvox MT* genes also showed reduced codon usage bias relative to autosomal genes (fig. S8), which is most likely due to suppressed recombination (*26*).

We sequenced three *MT* genes and a flanking gene, *PRP4*, from a set of related *Volvox* species in order to determine the extent of *MT* gene isolation (*4*). Phylogenies revealed the expected pattern for *PRP4*, which grouped by species and geographical location (Fig. 2C). In contrast, the *MT* genes grouped by gender (Fig. 2D and fig. S9). These data demonstrate that the shared genes in *Volvox MT* have essentially become gender-specific and have remained genetically isolated during speciation. Thus, the *MT* locus in *Volvox* has become a repository of genetic diversity that is linked to the sexual cycle.

In *Chlamydomonas*, the retinoblastoma (RB) tumor suppressor pathway controls cell division in response to cell size (*27*), and the RB homolog encoded by *MAT3* is adjacent to *MT* (*28*). *Volvox MAT3*, on the other hand, is within *MT* (Fig. 1B), and we investigated its evolution and expression as a candidate regulator of sexually dimorphic cell divisions (fig. S1). The *Volvox* male and female MAT3 proteins are exceptionally diverged from each other (figs. S9 to S11). Moreover, male and female *Volvox MAT3* have different structures: The female allele contains an intron that is absent from males, whereas the male allele contains an unusually large fourth intron as compared with that of females (Fig. 3). Although *MAT3* shows signs of having undergone purifying selection (d_N/d_S = 0.23) (table S11), several short sequences in the male and female proteins are asymmetric in their conservation pattern, suggesting that the two alleles are under different selective constraints (figs. S10 and S11). We also found dozens of alternatively processed *MAT3* mRNAs from both *Volvox* sexes, representing most types of alternative splicing (Fig. 3 and fig. S12) (*29*). In addition, sex-regulated pre-mRNA splicing of *MAT3* was found for both genders and might be controlled by the *MT*-encoded splicing factor *SPL2*, whose expression level is sex-regulated in males (Fig. 1C and fig. S13). The predominant *MAT3* isoform in sexual males retains the first two introns, leading to inclusion of an early termination codon (Fig. 3 and fig. S12). *mat3* mutants in *Chlamydomonas* produce tiny gametes (*28*), and down-regulation of *MAT3* in *Volvox* males through alternative splicing may be linked to the production of small-celled sperm.

The accelerated divergence of sex chromosomes is usually associated with gene loss and degeneration (*23*), although adaptive evolution of sex chromosomes is an emerging theme (*30*). Our data suggest that expansion, loss of recombination, and rapid divergence can be mutually reinforcing properties of sex-determining regions that facilitate cooption into the sexual cycle and provide previously undiscovered sources of developmental innovation (fig. S14).

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Supporting Online Material

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Resolving Mechanisms of Competitive Fertilization Success in *Drosophila melanogaster*

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Our understanding of postcopulatory sexual selection has been constrained by an inability to discriminate competing sperm of different males, coupled with challenges of directly observing live sperm inside the female reproductive tract. Real-time and spatiotemporal analyses of sperm movement, storage, and use within female *Drosophila melanogaster* inseminated by two transgenic males with, respectively, green and red sperm heads allowed us to unambiguously discriminate among hypothesized mechanisms underlying sperm precedence, including physical displacement and incapacitation of "resident" sperm by second males, female ejection of sperm, and biased use of competing sperm for fertilization. We find that competitive male fertilization success derives from a multivariate process involving ejaculate-female and ejaculate-ejaculate interactions, as well as complex sperm behavior in vivo.

Remating with different males by females generates sexual conflict over paternity (*1*) and sets the stage for postcopulatory sexual selection (*2*–*4*), which can drive diversification of both male and female biochemistry, physiology, morphology, and behavior (*4*, *5*). Most investigations of postcopulatory sexual selection have focused on the pattern of sperm precedence, such as the proportion of progeny sired by the second of two males subsequent to female remating (P_2). However, without knowledge of underlying mechanisms, these patterns reveal little about the intensity of selection or sex-specific adaptation (*4*–*6*). Consequently, even with *Drosophila melanogaster*, there is contention over the mechanisms giving rise to the roughly 80% last-male sperm precedence observed (*7*–*12*). Our understanding of these and other phenomena has been constrained by the technical challenge of directly observing sperm dynamics within the female reproductive tract and our limited ability to discriminate between sperm of different males (*7*, *13*, *14*).

We have overcome these challenges by transforming *D. melanogaster* to express a protamine labeled with green fluorescent protein (GFP) or red fluorescent protein (RFP) in sperm heads, which can be easily observed and unambiguously differentiated within the female reproductive tract (figs. S1 and S2 and movies S1 to S3). These lines enable direct visualization of sperm competition in vivo, in real time, and over extensive periods of time, allowing us to discriminate among alternative hypothesized mechanisms of sperm precedence. Multiple indices of male fitness relevant to sperm and/or ejaculate function were assayed, with transgenic males compared with each other and with a wild-type LH_m strain (into which the GFP and RFP constructs were backcrossed for six generations). Although some significant differences were found, with transformed lines performing less well, equivalent to, or better than the wild type in different fitness assays, all three strains fell within the typical range of values reported in the literature for all assays (figs. S3 to S7), which suggested there was no dysfunction of transformant sperm. Moreover, results of the sperm precedence experiments reported below are unbiased, because GFP and RFP males (i) were competed using a reciprocal mating order design and (ii) did not differ in female remating interval, P_1, or P_2 in those experiments (*15*).

We quantified (i) spatiotemporal patterns of sperm storage and use by the female after remating, (ii) the extent and timing of sperm ejection by females, and (iii) the influence of remating on resident sperm motility. Unless otherwise specified, in all experiments, 3-day-old, virgin LH_m females were randomly assigned to all treatment groups, initially mated to a GFP or an RFP male, and, beginning 3 days later (the typical remating latency for *D. melanogaster*), provided a daily, 6-hour opportunity to remate to a male of the alternative genotype (reciprocal male mating order balanced). All copulations were observed, and copulation durations recorded (*15*).

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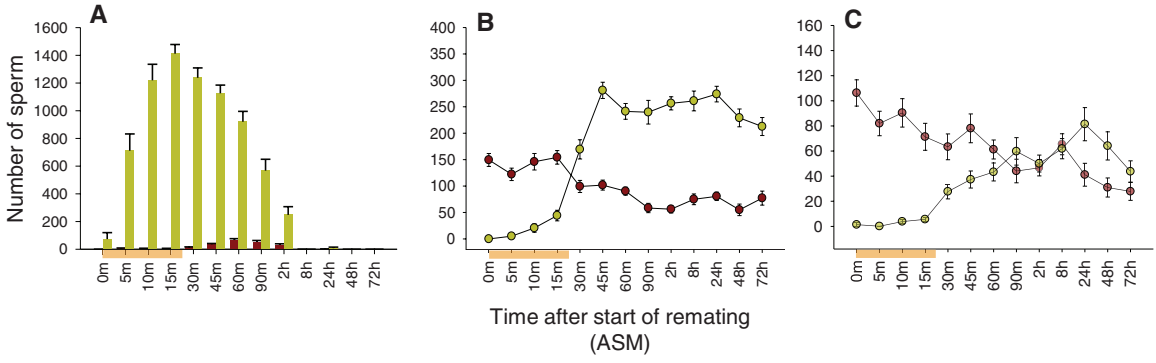

Fig. 1. Numbers of first-male (red) and second-male (yellow) sperm in the (**A**) bursa, (**B**) seminal receptacle, and (**C**) spermathecae for 13 time points ASM, averaged over two experimental replicates and both reciprocal mating orders. Error bars represent SEM.

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