

# Chapter 19

## Origin of Female/Male Gender as Deduced by the Mating-Type Loci of the Colonial Volvocalean Greens

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**Abstract** Colonial Volvocales (green algae) are a model lineage for the study of the evolution of sexual reproduction because isogamy, anisogamy, and oogamy are recognized within the closely related group, and several mating type (sex)-specific genes were identified in the closely related unicellular *Chlamydomonas reinhardtii* during the past century. In 2006, we first identified a sex-specific gene within the colonial Volvocales using the anisogamous colonial volvocalean alga *Pleodorina starrii*, namely, a male-specific gene called “*OTOKOGI*,” which is a homologue of the *minus* mating type-determining gene *MID* of the isogamous *C. reinhardtii*. Thus, it was speculated that the derived or *minus* mating type of *C. reinhardtii* is homologous to the male in the anisogamous/oogamous members of the colonial Volvocales. The discovery of the male-specific gene facilitated comparative studies of the mating-type locus (*MT*) (primitive sex chromosomal region) because it must be localized in *MT*. Recently, our international research group determined the genome sequence of *MT* in the oogamous *Volvox carteri*. *V. carteri* *MT* shows remarkable expansion and divergence relative to that from *C. reinhardtii*. Five new female-limited “*HIBOTAN*” genes and ten male-limited genes (including “*OTOKOGI*”) were identified in *V. carteri* *MT*. These observations suggest that the origins of femaleness and maleness are principally affected by the evolution of *MT*, which has undergone a remarkable expansion and gain of new male- and female-limited genes. Our recent results regarding the evolution of the volvocalean *MT* gene *MAT3/RB* are also discussed in relationship to the evolution of male–female sexual dimorphism.

**Keywords** Gametes • Male–female sexual dimorphism • Mating-type locus • Sex-specific gene • Volvocales

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## 19.1 Introduction

There is a great deal of interest in sexual reproduction because we are the progeny of our parents who produced sperm and immotile eggs (oogamy), which differ markedly in size and motility. In contrast, primitive organisms sometimes exhibit isogamy (equally sized gametes); in this case, we cannot distinguish between males and females. Therefore, evolutionary transition from isogamy to anisogamy (small and large motile gametes) to oogamy (Bold and Wynne 1985), or the origin of male and female sexes, is one of the most interesting issues in biological research. Although such male–female sexual dimorphism has arisen repeatedly in the evolution of eukaryotes (Kirk 2006), no molecular genetic evidence had been reported for the evolutionary link between male and female and mating types of isogamy until our recent study (Nozaki et al. 2006a), possibly because animals and land plants have no extant isogamous relatives (Rokas et al. 2005; Laurin-Lemay et al. 2012).

Colonial volvocalean algae represent a useful model lineage for the study of such an evolutionary link because isogamy, anisogamy, and oogamy can be recognized within the closely related organisms (Nozaki et al. 2000; Herron et al. 2009), and several mating type-specific genes had been identified in the related unicellular model species, *Chlamydomonas reinhardtii* (Ferris and Goodenough 1994, 1997). This review focuses on studies of sexual reproduction of the colonial Volvocales as well as the related *C. reinhardtii*.

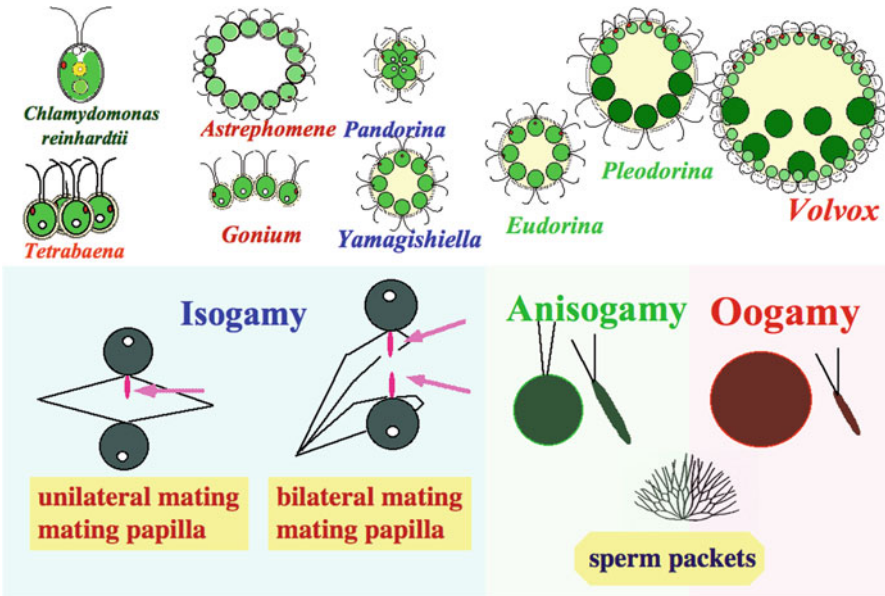
## 19.2 Sexual Reproduction in the Colonial Volvocales

Figure 19.1 presents a summary of the vegetative organization and modes of sexual reproduction in volvocine algae. It is interesting that, in this lineage, the primitive colonial organization also has a primitive type of sexual reproduction (Nozaki and Ito 1994).

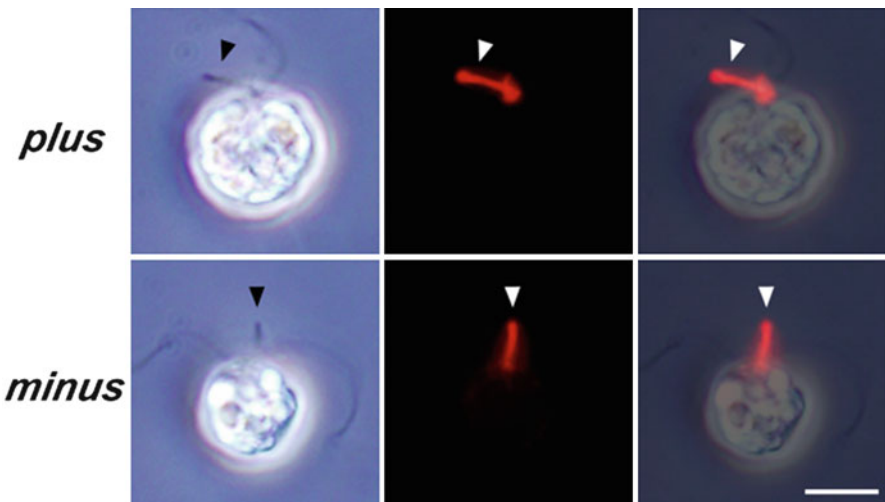
In the colonial volvocalean *Gonium pectorale*, vegetative spheroids are flattened with 8 or 16 cells, and sexual reproduction is isogamous with no differentiation between female and male gametes. Both the two conjugating isogametes bear a mating papilla, which is called the bilateral mating papilla (Nozaki 1984, 1996; Mogi et al. 2012) (Figs. 19.1, 19.2). Plasmogamy is initiated by the union of the tips of the mating papillae from mating-type *plus* and *minus* gametes.

In *Eudorina elegans*, vegetative spheroids are spheroidal with 16 or 32 cells, and sexual reproduction is anisogamous with flagellate female and male gametes. In sexual reproduction, bundles of male gametes called “sperm packets” are formed. Sperm packets swim to a female spheroid and dissociate into individual male gametes that penetrate the female for gametic union (Goldstein 1964; Nozaki 1983, 1996) (Fig. 19.3).

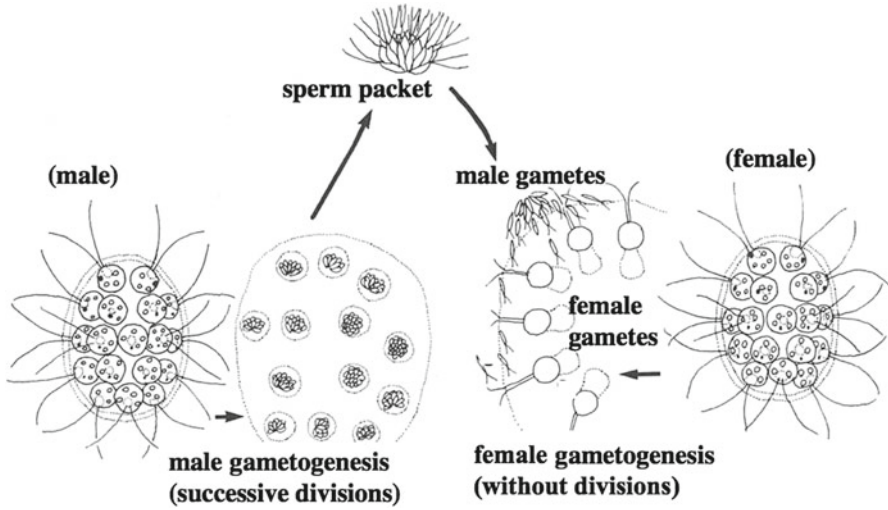
*Volvox carteri* is oogamous and has more than 500 cells in a spheroid. When inducing sexual reproduction, dwarf male and special female sexual spheroids are formed. The male spheroid produces sperm packets that swim to the female spheroid, which contains a number of eggs (Nozaki 1988) (Fig. 19.4). *V. carteri* shows prominent male–female differentiation (Kirk 1998; Nozaki 1996).



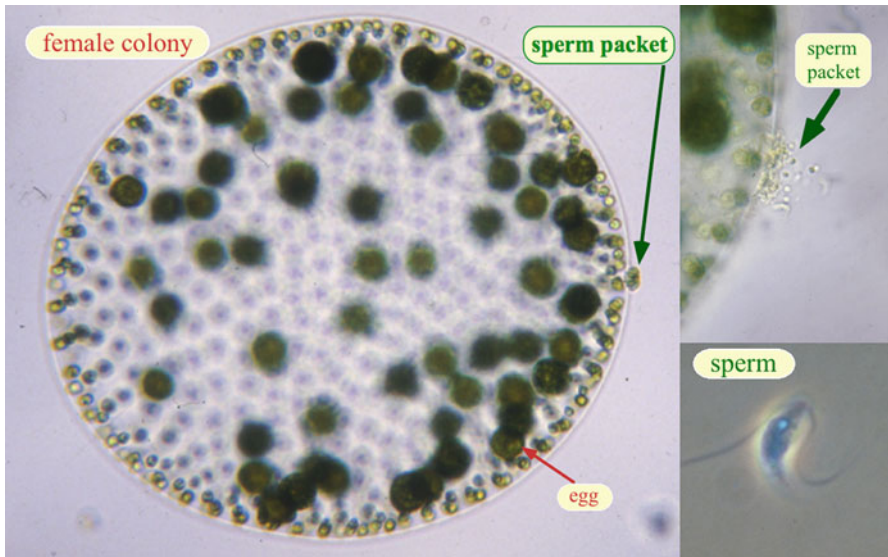
**Fig. 19.1** Diagrams of vegetative organization and sexual reproduction in various members of the colonial Volvocales and *Chlamydomonas reinhardtii*. (Based on Nozaki and Ito 1994)



**Fig. 19.2** Light microscopy of *Gonium pectorale* isogametes induced in separate mating type plus (*plus*) and mating type minus (*minus*) cultures. All adjacent panels show the same cell. Bar 10  $\mu$ m. Phase-contrast (*left*) and immunofluorescence (actin) (*middle*) images are merged in the *right* panels (merge). Note naked gametes of both mating types bearing a tubular mating structure (*arrowheads*). Note the accumulation of actin (*red*) in the mating structure. (From Mogi et al. 2012; reproduced by permission of John Wiley and Sons, License No. 3079280348215)



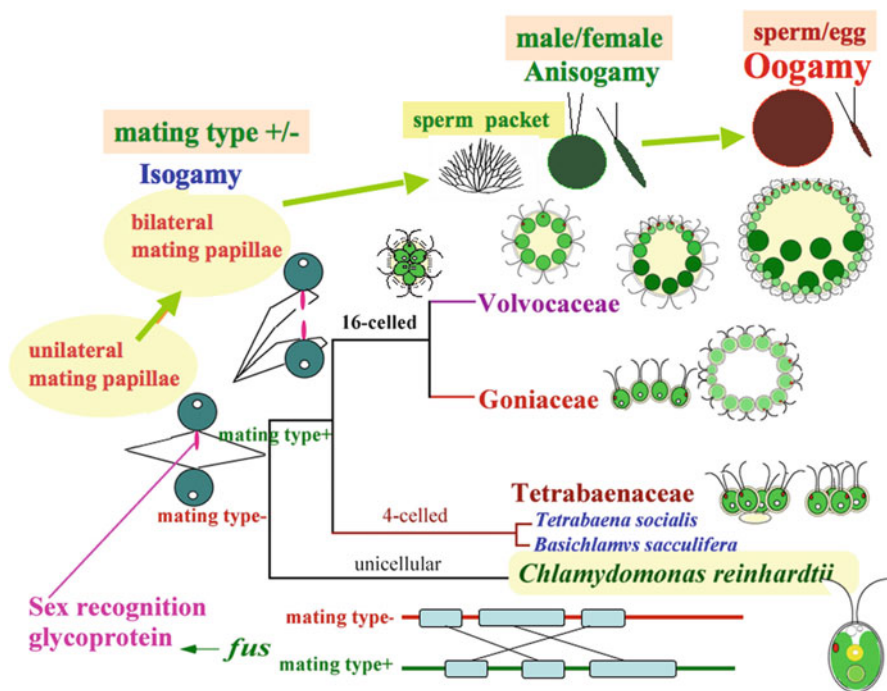
**Fig. 19.3** Diagrams of anisogamous sexual reproduction in *Eudorina elegans*. (Based on Nozaki 1983)



**Fig. 19.4** Oogamous sexual reproduction of the multicellular green alga *Volvox carteri f. kawasakiensis*. Female spheroids and male spheroids with sperm packets develop in female and male strains, respectively, when sexually induced. The sperm packet attaching to the female spheroid containing many eggs (*left panel*). Sperm packet (*arrow*) dissociates into individual sperm that penetrate the female spheroid (*right upper panel*). Sperm (*right lower panel*). (Original photographs of the strains used by Nozaki 1988)

### 19.3 Determining the Evolutionary Process of Sexual Reproduction and Tracing Gender-Specific Genes of the Colonial Volvocales

To determine the evolutionary process of the sexual reproduction characteristics in colonial Volvocales, detailed and robust phylogenetic relationships of the members are needed. Therefore, we carried out cladistic analysis of morphological characteristics of vegetative and reproductive phases (Nozaki and Ito 1994; Nozaki et al. 1996) and multigene phylogeny of plastid protein-coding genes (Nozaki et al. 2000; Nozaki 2003). Based on our phylogenetic analyses of morphological and multigene sequence data, a possible evolutionary scenario of sexual reproduction could be deduced. In relationship to the increase in spheroid cell number, female and male genders evolved with the formation of sperm packets from isogamy with bilateral mating papillae (Fig. 19.5). Therefore, it was of interest to determine the genetic basis underlying this evolution of sex. However, mating type-specific genes had not



**Fig. 19.5** Schematic representation of phylogenetic relationships within the colonial Volvocales as inferred from morphological and multigene sequence data (Nozaki and Ito 1994; Nozaki et al. 2000; Nozaki 2003). A possible evolutionary scenario of sexual reproduction is also shown

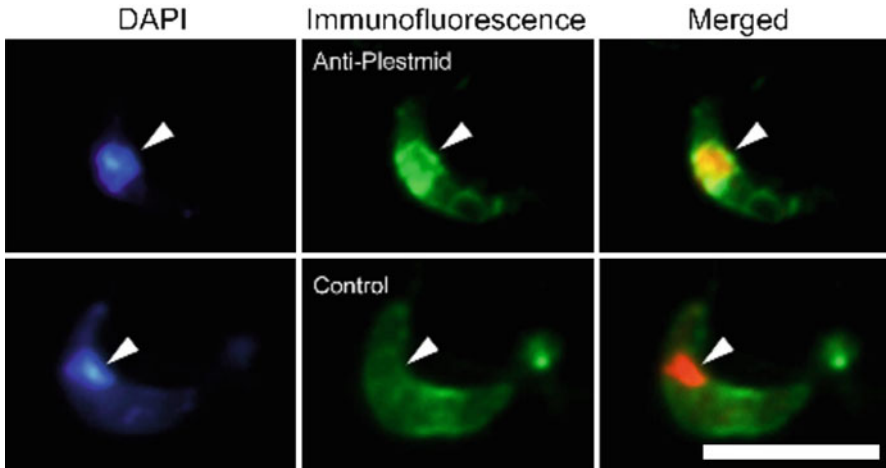
been identified within colonial Volvocales, despite studies of the mating type-specific genes, including the sex-determining minus dominance (*MID*) gene in the close relative *C. reinhardtii* (Ferris and Goodenough 1994, 1997).

There are two possible explanations for difficulties in identifying mating type-specific genes from colonial Volvocales. The first is the rapid evolution of the sex-related genes. Ferris et al. (1997) could not isolate *MID* homologues from *G. pectorale* and *V. carteri* based on Southern blotting, and they discussed the rapid evolution of sex-related genes. The second is loss of fertility or sexual activity during long-term maintenance of culture strains (Coleman 1975). Strains obtained from the culture collections that have been maintained in living culture for more than 10 years cannot generally be induced to reproduce sexually to study sexual reproduction, especially in anisogamous and oogamous species. Therefore, we used newly designed degenerate polymerase chain reaction (PCR) primers and new strains of the anisogamous volvoclean *Pleodorina starrii* to identify gender-specific genes. *P. starrii* was described by Nozaki et al. (2006a) based on samples collected in Japanese lakes in 2000–2001, and it is heterothallic with sperm packet formation occurring even within the male strain (Nozaki et al. 2006a, b).

## 19.4 Problems Regarding the Evolutionary Link Between Isogametic Mating Types and Male–Female Differentiation

In volvocine algae, sex is determined by a single mating-type locus (*MT*) with two haplotypes that specify sexual differentiation. Although *MT* segregates as a single Mendelian trait, it is a complex genomic region containing both shared and sex-limited genes that are rearranged with respect to each other and do not undergo meiotic recombination (Umen 2011). Sexual reproduction of the unicellular volvoclean *C. reinhardtii* is isogametic, with *plus* and *minus* mating types. The *minus* mating type of this species represents a “dominant sex” because occasionally produced diploid cells exhibit the *minus* mating phenotype (Ebersold 1967); when the *minus* mating type-determining gene *MID* is lacking, the phenotype of the sex changes from *minus* to *plus*, forming a fertilization tubule (Goodenough et al. 1982). Therefore, mating type *minus* in *C. reinhardtii* can be considered a “derived type of sex.” However, the evolutionary link between isogamous mating type *plus/minus* and female–male differentiation cannot be deduced, even based on comparison of modes of uniparental inheritance of the organelle genomes. In *C. reinhardtii*, the plastid genome is inherited from the *plus* mating type parent, whereas the mitochondrial genome is inherited from the *minus* parent (Boynton et al. 1987). In contrast, both the plastid and mitochondrial genomes are inherited from the female parent in the oogamous alga *V. carteri* (Adams et al. 1990). Thus, homologues of some *C. reinhardtii* mating type-specific genes were needed to be identified in the anisogamous or oogamous members of colonial Volvocales.





**Fig. 19.7** Visualization of male-specific “OTOKOGI” protein in male gamete after release from sperm packets. “OTOKOGI” expression is obvious in nucleus (arrowheads in DAPI images). Specimens were double stained with 4',6-diamidino-2-phenylindole (DAPI) and anti-“OTOKOGI” antibodies. All panels show identical cells. DAPI (pseudo-colored) and immunofluorescence images are merged (right panels). Bar 5  $\mu\text{m}$ . (From Nozaki et al. 2006a,b; reproduced by permission of Elsevier Ltd., License No. 3079280808611)

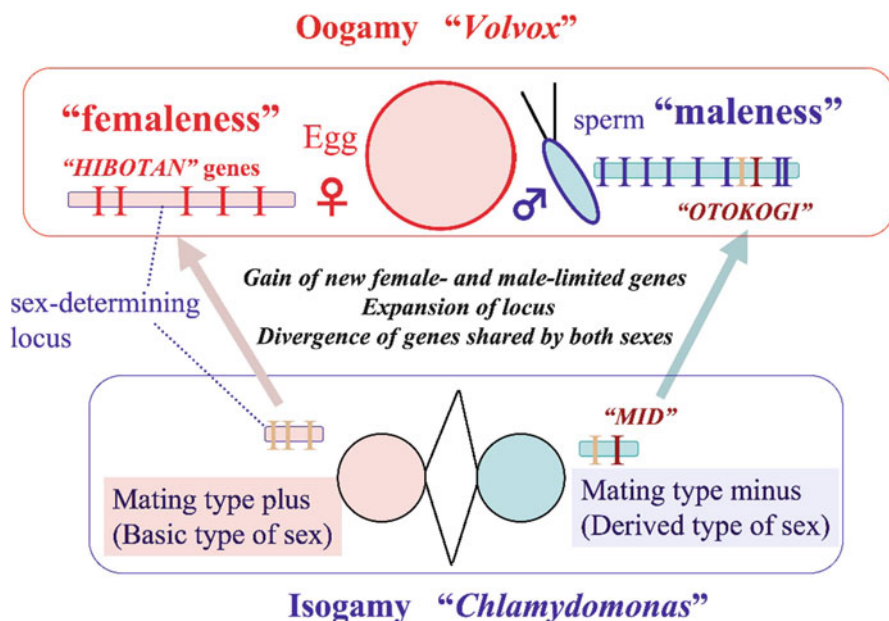
## 19.6 Female-Limited “HIBOTAN” Genes Resolved in the *Volvox carteri* Genome

Even though the evolutionary link between male–female differentiation and isogamic mating types was determined (Nozaki et al. 2006a), genes contributing to the evolution of femaleness and maleness from isogamic mating types remained unclear. In colonial Volvocales, the female sex evolved from the original type of sex (*plus* mating type) in isogamy (Nozaki et al. 2006a), and female-specific genes are lacking or rare in higher animals and land plants. Thus, the female-specific gene “HIBOTAN” may be lacking in the evolution of femaleness of colonial Volvocales. Genomic data regarding *MT* or the sex chromosomal region of the anisogamous or oogamous members were needed.

In 2010, we published a paper on the genome analyses of the *MT* of *V. carteri* that exhibits the production of eggs and sperm (Ferris et al. 2010). This publication was based on co-research between the United States (USA) and Japan, which originally started in 2005 when Dr. Patrick Ferris came to Japan and became a guest researcher at the Graduate School of Science, University of Tokyo. The Japanese team contributed to cloning of the *V. carteri* “OTOKOGI” gene and construction of a male *V. carteri* BAC library.

The *V. carteri* *MT* shows remarkable expansion and divergence relative to that from *C. reinhardtii*, which has equal-sized gametes, and is about five times larger and contains more genes than the *C. reinhardtii* *MT*. Our transcriptome analysis



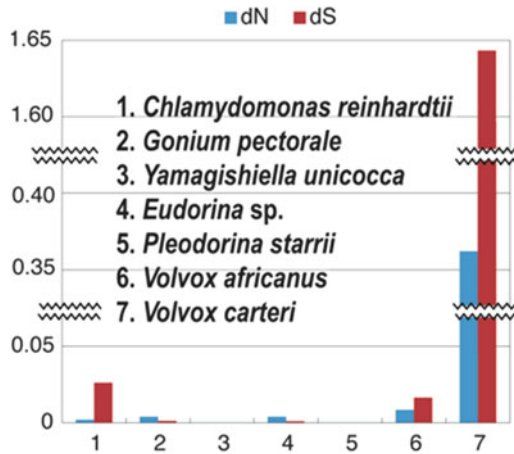


**Fig. 19.8** Diagram of evolution of oogamy from isogamy and mating type (sex-determining) loci in the volvocalean algae. (Based on Nozaki et al. 2006a and Ferris et al. 2010)

using next-generation sequencing identified five new female-limited "HIBOTAN" genes and ten male-limited genes, including "OTOKOGI," in the *MT* locus of *V. carteri* (Fig. 19.8). None of the five "HIBOTAN" genes was identified in the *C. reinhardtii* *MT* (Ferris et al. 2010). Thus, the origins of femaleness and maleness are principally affected by the evolution of the *MT* or sex chromosome that has undergone remarkable expansion and gain of new male- and female-limited genes. However, our study (Ferris et al. 2010) was based on the comparison of only two evolutionary extremes, the isogamous unicellular *Chlamydomonas* and the oogamous multicellular *Volvox* (Fig. 19.5). Thus, genome information on the *MT* from intermediate colonial Volvocales is needed to determine the evolutionary significance of expansion of the *MT* as well as the male- and female-limited genes newly identified in *V. carteri* (Charlesworth and Charlesworth 2010).

## 19.7 Origin of the Gender-Based Divergence of the Mating Locus Gene *MAT3* from Oogamous *Volvox carteri*

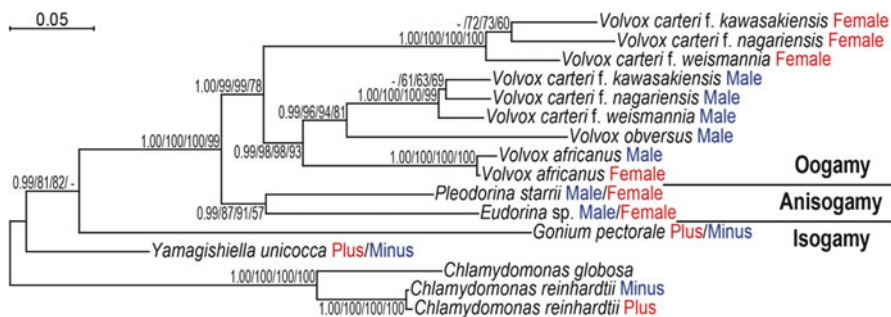
Our recent comparative study of *MT* from oogamous *V. carteri*, with *MT* from an isogamous species, *C. reinhardtii*, revealed major differences in the size and sex-based differentiation of *MT* genes (Ferris et al. 2010). *V. carteri* *MT* was found to show a much higher degree of sex-based differentiation in its shared genes (those



**Fig. 19.9** Gender-based divergence of mating-type locus *MAT3* genes from the colonial Volvocales and *Chlamydomonas reinhardtii*. Bar graph depicts dN (number of substitutions per nonsynonymous site) and dS (number of substitutions per synonymous site) between *MAT3* alleles from each of the two mating types or sexes. (From Hiraide et al. 2013; reproduced by permission of Oxford University Press, License Number 3079271441882)

with an allele in both mating haplotypes or sexes). Shared genes that have become masculinized and feminized in sequence or expression, as occurred in *V. carteri*, are candidates for contributing to male–female sexual dimorphism. However, there had been no investigations of *MT* genes in the context of the isogamy to anisogamy/oogamy transition in volvocine algae other than the previously described comparison of *C. reinhardtii* and *V. carteri* (Ferris et al. 2010; Charlesworth and Charlesworth 2010).

One candidate regulator of gamete size is the mating locus gene *MAT3*, which encodes a homologue of the retinoblastoma (RB) tumor suppressor protein. In *C. reinhardtii*, *MAT3* is closely linked to *MT* and regulates cell size and cell-cycle progression (Umen and Goodenough 2001; Fang et al. 2006). In contrast to *C. reinhardtii*, in which the *minus* and *plus* *MAT3* alleles are nearly identical and function interchangeably (Umen and Goodenough 2001; Merchant et al. 2007), high degrees of male–female sequence differentiation and sex-regulated alternative splicing were observed for *V. carteri* *MAT3* (Ferris et al. 2010). These observations led Ferris et al. (2010) to suggest that *MAT3* homologues may be related to control of gamete size in colonial volvocine algae, as predicted earlier by the gamete size regulator recruitment model for the evolution of anisogamy/oogamy from isogamous mating types (Charlesworth 1978). Thus, Hiraide et al. (2013) sequenced the full-length coding regions of *MAT3* from *plus* and *minus* mating types of isogamous *Gonium pectorale* and *Yamagishiella unicocca*, from males and females of anisogamous *Eudorina* sp. and *Pleodorina starrii*, and from males and females of oogamous *Volvox africanus*. In contrast to *V. carteri*, *MAT3* homologues from the five colonial species examined had almost identical nucleotide sequences between the two sexes (Fig. 19.9). Our phylogenetic analysis of *MAT3* sequences suggested that the extensive *MAT3*



**Fig. 19.10** Phylogeny of deduced amino acids of mating-type locus *MAT3* genes from colonial Volvocales and *Chlamydomonas*. Branch labels indicate, from left to right: posterior probabilities ( $\geq 0.90$ ) from Bayesian inference/bootstrap values ( $\geq 50\%$ ) obtained using 1,000 replicates with RAxML/Bootstrap values ( $\geq 50\%$ ) using 1,000 replicates with maximum parsimony. (From Hiraide et al. 2013; reproduced by permission of Oxford University Press, License Number 3079271441882)

divergence in the *V. carteri* lineage may have occurred recently in the ancestor of the three *V. carteri* forms after their divergence from the anisogamous lineage containing *P. starrii* and *Eudorina* sp. (Fig. 19.10). These observations suggest the roles of genetic determinants other than or in addition to *MAT3* in the evolution of anisogamy in colonial volvocalean algae.

## 19.8 Conclusions

The considerable expansion of oogamous *V. carteri* *MT* is based mostly on the increase in the noncoding DNA region, but *V. carteri* *MT* contains more coding regions or genes than *C. reinhardtii* *MT* (Ferris et al. 2010). Similar expansion of noncoding DNA regions was recently recognized in the mitochondrial and plastid genomes from colonial Volvocales (Smith and Lee 2009, 2010; Hamaji et al. 2013; Smith et al. 2013). Smith et al. (2013) demonstrated that ratios of noncoding DNA regions in the mitochondrial and plastid genomes increase in relationship to the increase in spheroid cell number (from unicellular *Chlamydomonas* to *Gonium* to *Pleodorina* to *Volvox*) (see Fig. 19.1). This increase may be explained by the mutational hazard hypothesis (Lynch 2007), which argues that genome expansion is a product of a low effective population size ( $N_e$ ), which results in increased random genetic drift, or a low mutation rate ( $\mu$ ), which reduces the burden of harboring excess DNA. Therefore, a gradual increase in *MT* size as a function of the increase in spheroid cell number, which may result in a decrease in  $N_e$ , may also be considered within colonial Volvocales. However, the origins and evolution of the many gender-specific genes found in *V. carteri* *MT* remain unexplained on the basis of the present limited data from colonial volvocalean *MT*.

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