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Genomics of Volvocine Algae

James G. Umen and

Donald Danforth Plant Science Center, 975 North Warson Rd., St. Louis, MO 63132 USA

Bradley J.S.C. Olson

Molecular Cellular and Developmental Biology, Ecological Genomics Institute, Division of Biology, Kansas State University, Manhattan, KS 66506 USA

Abstract

Volvocine algae are a group of chlorophytes that together comprise a unique model for evolutionary and developmental biology. The species *Chlamydomonas reinhardtii* and *Volvox carteri* represent extremes in morphological diversity within the Volvocine clade. *Chlamydomonas* is unicellular and reflects the ancestral state of the group, while *Volvox* is multicellular and has evolved numerous innovations including germ-soma differentiation, sexual dimorphism, and complex morphogenetic patterning. The *Chlamydomonas* genome sequence has shed light on several areas of eukaryotic cell biology, metabolism and evolution, while the *Volvox* genome sequence has enabled a comparison with *Chlamydomonas* that reveals some of the underlying changes that enabled its transition to multicellularity, but also underscores the subtlety of this transition. Many of the tools and resources are in place to further develop Volvocine algae as a model for evolutionary genomics.

1. Introduction

Ever since van Leeuwenhoek first described *Volvox* he had collected from a roadside ditch (van Leeuwenhoek, 1700), this elegant organism and its cousins, the Volvocine algae, have fascinated biologists. Just over 300 years after their first published description Volvocine algae entered the genomic era with genome sequencing of the unicellular species *Chlamydomonas reinhardtii* (Merchant et al., 2007) followed by that of *Volvox carteri* (Prochnik et al., 2010). These two species are at the forefront of research in diverse areas including evolution, motility, photosynthesis, and development before their genomes were completed. It has now become possible to add an arsenal of -omics tools to speed progress and answer new questions. Genomics research with *Chlamydomonas* has shed light on several areas of biology by helping define conserved genes related to both plant and animal biology. The addition of the *Volvox* genome makes possible comparisons within Volvocine algae aimed at deciphering the genetic changes that underlie a major transition in biological complexity represented by differences between unicellular *Chlamydomonas* and multicellular *Volvox*. These comparisons have revealed how remarkably similar these two species are at the genome level, and have prompted a reconsideration of how much change is required to drive a major evolutionary transition. In this chapter we introduce the Volvocine algae and highlight key areas of genomics research to which they have contributed.

2. The Biology of the Volvocales

The Volvocales (or Volvocine algae) are a sub-group of chlorophytes (green algae) comprising dozens of species in seven major genera (Fig. 1). The two best-known species are unicellular *Chlamydomonas reinhardtii,* and multicellular *Volvox carteri* (Fig. 2). These two species represent two extremes in a range of developmental complexity observed within the Volvocales (Fig. 2). Each of the major genera is defined by its level of organization such as colony size and morphology, cell number, cell differentiation, and the degree of germ-soma specialization (Fig. 2). A brief introduction to the biology, taxonomy, ecology, and culture resources for Volvocine algae follows.

2a. *Chlamydomonas* is a prototypical Volvocine alga

Chlamydomonas reinhardtii has been used as a model for many decades to investigate diverse areas of biology (Harris, 2001; 2001; Grossman et al., 2003; 2003; Harris, 2004; Peers and Niyogi, 2008; Stern et al., 2009; 2009). Molecular genetic tools and resources have been developed for *Chlamydomonas* including nuclear and organellar transformation, insertional mutagenesis, RNAi-mediated silencing, microarrays, EST libraries, BAC libraries, cDNA libraries, and numerous strains and mutants (see Tables 1 and 2). The following section provides a brief overview of *Chlamydomonas.*

2a.i *Chlamydomonas* taxonomy—*Chlamydomonas reinhardtii* belongs to the chlorophycean family *Chlamydomonaceae* (Fig. 1). The genus *Chlamydomonas* encompasses over 600 species, but is now recognized as being polyphyletic and the genus will need substantial revision (Lemieux et al., 1985; Buchheim et al., 1997; Morita et al., 1999; Pröschold et al., 2001; Hoham et al., 2002; Stern et al., 2009). Molecular phylogenies place *Chlamydomonas reinhardtii* and a few other *Chlamydomonas* species as the closest unicellular relatives of colonial Volvocine algae that are together part of a coherent, monophyletic grouping (Fig. 2) (Nozaki and Itoh, 1994; Nozaki et al., 1995; Coleman, 1999; Nozaki et al., 2000; 2002; Nakada et al., 2010) with an estimated divergence time of ~200 MY (Herron et al., 2009). We refer here to Volvocine algae as the group containing colonial Volvocales plus their closest unicellular relatives including *Chlamydomonas reinhardtii* (Fig. 2).

2a.ii *Chlamydomonas* cell structure—Each *Chlamydomonas* cell is 5–10 μM in diameter and is shaped like a pear or ovoid (Fig. 3). Surrounding the plasma membrane is a cell wall composed of distinct layers of crosslinked hydroxyproline-rich glycoproteins (Woessner and Goodenough, 1994; Stern et al., 2009). A pair of basal bodies at the anterior end of the cell nucleates two flagella that provide motility and chemosensory functions. The basal bodies also nucleate and organize the microtubule cytoskeleton within the cell body (Dutcher, 2003; Pearson and Winey, 2009) (Fig. 3). The flagella and basal bodies are structurally and functionally homologous to cilia and basal bodies of animal cells and *Chlamydomonas* serves as a model for investigating these organelles (Dutcher, 2003; Pedersen and Rosenbaum, 2008). The nucleus is positioned below the basal bodies, while the posterior of each cell is occupied by a single large cup-shaped chloroplast that encompasses over half of the total cell volume (Fig. 3). *Chlamydomonas* is of particular

interest for evolutionary genomics because it has attributes of land plants (chloroplast, photosynthetic metabolism) and of animals, lower plants and protists (flagella and basal bodies) (Harris, 2001; Grossman et al., 2003; Merchant et al., 2007). Indeed, as described below, the unique biology of this duality is reflected in its protein coding gene repertoire and has been exploited to identify new candidate genes that are involved in plant- and animal-specific processes.

2a.iii *Chlamydomonas* vegetative and sexual cycles—*Chlamydomonas* cells are haploid and have two mating types, *plus* and *minus*, that are specified by a mating locus (*MT*) (Ferris et al., 2002; Goodenough et al., 2007; Ferris et al., 2010). Under nutrient replete conditions *Chlamydomonas* cells proliferate mitotically using a modified cell cycle termed multiple fission (also called palintomy) that is shared by most other Volvocine algae and many other chlorophytes (Kirk, 1995; 2005; Graham et al., 2009). This mode of reproduction involves a long growth period followed by a rapid series of S phases and mitoses to produce 2^n daughters (2, 4, 8, 16 …) where *n* is typically 1, 2, 3 or 4 in *Chlamydomonas* (Fig. 4). In a diurnal environment the cell cycle becomes synchronized with growth occurring during the day and division occurring at night (Harris, 2001). In *Chlamydomonas* the division number (*n*) is variable but in colonial Volvocine algae it is more stereotyped with variability of usually just one cycle within a species.

When starved of nitrogen, vegetative *Chlamydomonas* cells differentiate into mating-competent gametes. While gametes are morphologically similar to vegetative cells, they express a genetic program that allows them to recognize cells of the opposite mating type and to fuse into a zygote that differentiates to become a dormant diploid zygospore. Upon return to light and nutrients the spores undergo meiosis and germinate to yield four haploid progeny that reenter the vegetative reproductive cycle (Goodenough et al., 1995; 2007; Stern et al., 2009) (Fig. 4).

2a.iv *Chlamydomonas* Metabolism—*Chlamydomonas* cells can grow phototrophically on simple mineral nutrient media, but can also grow heterotrophically using external acetate as a carbon source for respiration. Its ability to grow heterotrophically has made *Chlamydomonas* an important model for photosynthesis research since photosynthetically inactive mutants are viable when supplied with acetate (Harris, 2001; Grossman et al., 2003; Dent et al., 2005). Moreover, *Chlamydomonas* also has evolved anaerobic metabolic pathways that are of significant interest for understanding biological hydrogen (H_2) production (Rupprecht, 2009; Kruse and Hankamer, 2010; Costa and de Morais, 2011). Pathways for key nutrients and metabolites such as nitrogen, sulfur, phosphorous, iron, copper, starch lipids and others have been investigated extensively (Harris, 2001; Grossman et al., 2003; Stern et al., 2009).

2b. Colonial Volvocine algae show convergent evolution of form and function

2b.i Overview of colonial Volvocine forms—The colonial Volvocine algae come in a diverse forms that can be organized based on colony size, cell type specialization and other morphological attributes. These attributes were traditionally used to group the Volvocine algae into genera and are a useful means of describing different types; but as described

below and in Fig. 2, the morphological classification scheme is misleading with respect to phylogenetic relationships within the Volvocales where multiple instances of convergent evolution have occurred.

The simplest colonial genera is *Gonium*. Morphologically, *Gonium* looks like a group of 4, 8 or 16 *Chlamydomonas* cells that are stuck together. Indeed, each *Gonium* colony arises from a single cell that undergoes multiple fission and whose daughters remain attached after division (Hoops et al., 2006) (Fig. 4). *Gonium* colonies also have polarity with cells arranged in a slightly curved sheet with flagella on the apical surface that corresponds to the convex side of the colony Fig. 2. This configuration is achieved through a post-mitotic contortion termed inversion that bends the cell sheet outward so that the flagella (that originally faced inward after division) end up on the outside face of the colony. Polarity is also evident in the orientation of flagella in 16-cell *Gonium* colonies where the inner four cells have their flagella arranged as those in *Chlamydomonas* (with opposed beat directions), while the outer twelve cells have rotated basal bodies that allow the two flagella to beat with a more parallel stroke that is characteristic of all the larger colonial genera (Fig. 6). Partial inversion in *Gonium* is functionally similar to the process of full inversion in the remaining spheroidal genera that involves a complete turning inside-out of the post-mitotic colony (Kirk, 2005).

The remaining genera include *Pandorina* that contain 8, 16 or 32 similar-sized cells in a compact ball with flagella pointing outward (Fig. 2). *Eudorina* colonies have 16, 32 or 64 cells but are much larger than *Pandorina* due to the secretion of an extensive glycoprotein-rich extracellular matrix in which cells are embedded. *Pleodorina* colonies are larger still (32, 64 or 128 cells) and show partial cell type specialization with some small somatic cells on the anterior of the spheroid that never grow or divide, but only provide motility. The remaining cells are flagellated and motile, but continue to grow. Eventually these cells withdraw their flagella and divide into new colonies.

Volvox is the most morphologically complex Volvocine in terms of size and cell-type specialization. Each spherical colony is up to 0.5 mm in diameter and contains 2000–4000 small sterile somatic cells around the outside embedded in an extensive network of ECM (Fig. 6). The somatic cells provide motility for the colony and secrete ECM, but do not grow or reproduce. Within each colony are large reproductive cells termed gonidia that grow and undergo a morphogenesis program that is described in more detail below for the model species *Volvox carteri* (see section 6a, below).

Colonial Volvocine algae have sexual cycles similar to that of *Chlamydomonas* with the smaller colonial forms producing gametes of equal size like *Chlamydomonas*. Larger colonial genera such as *Eudorina*, *Pleodorina* and *Volvox* are anisogamous or oogamous with males producing packets of small sperm and females producing large eggs (Fig. 5) (Nozaki et al., 2000).

2b.ii Phylogenetic relationships among Volvocine algae—Morphological criteria that led to grouping the various Volvocine species into the genera described above also suggested a simple, step-wise progression in developmental complexity termed the

Volvocine lineage hypothesis (Larson et al., 1992; Kirk, 1998). In its simplest form this hypothesis predicts a nested phylogenetic tree with each new genera arising once from a less complex ancestral form. Molecular systematics reveals a more complex history in this group with multiple independent transitions between levels of organization. For example, colonials with morphology similar to *Eudorina* (Fig. 2) have arisen independently at least three times to create the genera now termed *Eudorina, Volvulina* and *Yamagishiella* (Nozaki et al., 2000; Herron et al., 2009). Likewise, *Volvox*-like colonial forms have also evolved at least three times independently with distinct developmental patterning mechanisms that give rise to germ-soma differentiation (Desnitski, 1995). Even more problematic for the Volvocine lineage hypothesis are trait losses such as the absence of expanded ECM in some subgroups of *Eudorina* that are morphologically closer to *Pandorina* than to other members of their clade (Fig. 2). While complicating the task of classification, convergent evolution within the Volvocine algae makes them appealing as a model system because it allows comparative studies of how similar developmental innovations arose independently within a well-defined clade.

2c. Ecology of the Volvocine algae

Volvocine algae are freshwater species that are distributed world-wide. Long distance dispersion is possible due to the production of dormant sexual zygospores that can survive for years in desiccated environments and are highly resistant to stress. Wind, attachment to animals or ingestion by animals provides a means for the spores to travel and allows for maintenance of genetic continuity between geographically distant populations. *Chlamydomonas* is often isolated from soil samples though it can also be found in ponds and temporary bodies of water. The methods for isolating *Chlamydomonas* and other algae from soils favor germinating zygospores, so it is not clear whether significant populations of vegetative *Chlamydomonas* are present in soils where they are collected. In contrast, colonial Volvocine algae are typically isolated as vegetative forms from transient puddles, rice paddies and warm ponds. The presence of vegetative colonial Volvocine algae in temporary bodies of water implies the presence of zygospores in the soil prior to inundation as vegetative forms do not survive desiccation. Collection method biases notwithstanding, there are several potential advantages to a colonial lifestyle in an open water environment. The first is increased size. Colonies can be resistant to grazing predators that eat unicells but which have an upper limit to the size of prey they can ingest (Bell, 1985; Kirk, 1998; Bonner, 2000). The second potential advantage of being colonial is increased efficiency in resource utilization. In general such efficiency is thought to be of greatest benefit for multicellular species in environments that fluctuate from highly nutrient-rich to nutrient poor. Under these conditions a multicellular organism can collect and store nutrients allowing it to outcompete unicellular species whose growth is more tightly coupled to immediately available nutrients (Kirk, 1998). A third potential benefit of multicellularity is increased mobility. For example, the combined effort of 4000 somatic flagella beating on the surface of a *Volvox* spheroid supports motility at rates of ~500–900 μm s^{-1} (Solari et al., 2006; 2008; Ueki et al., 2010) while the two flagella on a *Chlamydomonas* cell allow movement at a rate of ~100–200 μm s^{-1} (Ojakian and Katz, 1973). The faster speed of a *Volvox* spheroid would allow it to travel much greater distances through a stratified water column in search of mineral nutrients. Moreover, the second advantage (efficiency of

resource utilization) may be enhanced by the third advantage (motility) in the action of flagellar beating that disrupts the diffusion boundary layer around each spheroid and thereby increases the efficiency of nutrient uptake (Koufopanou, 1994; Short et al., 2006).

2d. Culture resources for Volvocine algae

Chlamydomonas and other Volvocine algae are maintained and distributed from any of several culture collections. Some of the major sources of Volvocine culture stocks are summarized in Table 1. The *Chlamydomonas* Stock Center has wild-type strains, including inbred isogenic reference strains, many published and unpublished mutants, and several independent wild isolates. In addition to cultures, the *Chlamydomonas* Stock Center also distributes cDNA libraries and plasmids. The UTEX algal collection at the University of Texas Austin has a good representation of Volvocine algal strains. UTEX also maintains a comprehensive database of freshwater algal media recipes and protocols. Additional collections of Volvocine algae are SAG at the University of Gottingen in Germany, CCAP in UK, and NIES in Japan. Currently, two web-based resources provide such information for Volvocine algae and other groups. Protist Images (http://protist.i.hosei.ac.jp/Protist_menuE.html) has a large collection of photos and taxonomy of protists, including Volvcine algae. Algaebase (http://www.algaebase.org/) includes a comprehensive taxonomy for many Volvocine species.

3. *Chlamydomonas* and *Volvox* Genome structure and content

The genome sequence of *Chlamydomonas reinhardtii* was published in 2007 (Merchant et al., 2007), and that of *Volvox carteri* in 2010 (Prochnik et al., 2010). These two sequences have provided new insights into the biology of Volvocine algae and have enabled a host of additional “omics” and systems-level approaches (Gonzalez-Ballester, 2005; Eberhard et al., 2006; Jamers et al., 2009; Rolland et al., 2009; Schmidt et al., 2010; Castruita et al., 2011). The majority of genomics research on Volvocine algae has been done with *Chlamydomonas*; however, the genome of *Volvox* has already provided new perspectives on the evolution of multicellularity and of Volvocine sexual cycles (Ferris et al., 2010; Prochnik et al., 2010), and we can expect the area of comparative Volvocine algal genomics to be one of increasing activity and interest in the near future. In this review we focus first on *Chlamydomonas* genomics and the insights it has provided on the evolution of a remarkably complex and sophisticated unicellular eukaryote. We then focus on the emerging area of comparative genomics of *Chlamydomonas*, *Volvox* and soon-to-be sequenced species of Volvocine algae that will be of increasing future interest.

3a. *Chlamydomonas* Nuclear Genome Structure

The *Chlamydomonas reinhardtii* nuclear genome sequence is ~117 MB distributed on 17 chromosomes. The nucleotide composition is GC biased (64% GC) as is 3rd position codon usage (Merchant et al., 2007). Protein coding gene density is relatively high with 16.7% exonic sequence and 12.5% total repeats. *Chlamydomonas* chromosomes have genetically mappable centromeres that are not well-characterized, but likely contain repeats (Preuss and Mets, 2002; Merchant et al., 2007). Telomeric repeats are around 300–350 bp and are composed of the sequence $(TTTTAGGG)_n$ (Petracek et al., 1990).

3a.i. The *Chlamydomonas* Mating-type Locus—*Chlamydomonas* has two mating types, *plus* and *minus*, that are governed by a mating locus with haplotypes designated *MT+* and *MT−*. Although it segregates as a single Mendelian trait, *MT* is a large multigenic region of around 200–300 kb (Fig. 6). Sequence rearrangements (inversions and transpositions) between *MT+* and *MT−* suppress recombination in this region and keep the genes within and around *MT* in linkage disequilibrium. This rearranged configuration keeps sex-related genes together, but also keeps over a dozen housekeeping genes trapped within a non-recombining region (Umen, 2011). Thus, the *MT* locus has acquired some properties of a haploid sex chromosome and its expansion in *Volvox* is one of the most notable differences between the two algal genomes (Ferris et al., 2010). The comparative genomics of the *Chlamydomonas* and *Volvox* mating loci are discussed in more detail below.

3a.ii Protein coding genes—Various strategies have been employed for protein coding gene predictions that are homology based or use empirical data. One of the most successful gene prediction programs for *Chlamydomonas* is AUGUSTUS that combines evidence-based and *de novo* gene model predictions (Stanke and Morgenstern, 2005; Stanke et al., 2006; 2008). The *de novo* gene prediction program GreenGenie2 has been trained on a large set of known *Chlamydomonas* cDNA sequences from which it has generated high-quality gene models (Kwan et al., 2009) that are available pre-formatted for processing with Cufflinks (Trapnell et al., 2010), a popular tool for transcript assembly and differential gene expression analysis.

3a.iii Genes for RNAs—*Chlamydomonas* encodes 259 tRNAs, over half of which are present in clusters that arose through serial gene duplication (Merchant et al., 2007). Compared to other eukaryotes *Chlamydomonas* tRNAs are relatively intron rich, with 60% intron-containing tRNA genes, and contain an unusually large number that encode a 3′ terminal CCA sequence that is usually added post-transcriptionally in eukaryotes (Merchant et al., 2007).

Cytosolic ribosomal RNA (rRNA) encoding gene clusters (18S, 5.8S, 28S) are found at two locations: one end of Chromosome 14, and at one end of Chromosome 8. A single cytosolic 5S rRNA cluster is located on chromosome 8 about 200 kb from 18S/5.8S/28S cluster with several dozen predicted protein coding genes in between the two loci (J. Umen, unpublished observation). The highly repetitive nature of these clusters makes their exact size difficult to determine.

The nuclear genome encodes 322 snoRNAs that are involved in rRNA processing and base modifications. *Chlamydomonas* snoRNAs are notable for being clustered and for the high proportion that are encoded within spliceosomal introns of protein coding genes (Merchant et al., 2007; Chen et al., 2008).

Chlamydomonas contains a complex repertoire of small 22–24 nt RNAs (sRNAs), some of which appear to be microRNAs (miRNAs) (Molnár et al., 2007; Zhao et al., 2007). The *Chlamydomonas* genome also encodes key proteins involved in sRNA biogenesis and function including Argonaute proteins and Dicer nuclease, though it does not appear to encode any RNA-dependent RNA polymerases that are potentially involved in generation or

amplification of double stranded RNAs that act as sRNA precursors (Schroda, 2006; Casas-Mollano et al., 2008; Cerutti et al., 2011). As discussed below, RNAi-based gene silencing has proven to be a powerful reverse genetics tool in Volvocine algae.

3b. *Chlamydomonas* Organellar Genomes

The *Chlamydomonas* chloroplast genome is typical for those in the green algal/land plant lineage. It is a 200 kb circle with two large inverted repeats that contain rRNA genes and the *psbA* gene. In total the chloroplast encodes around 99 genes including protein coding genes, tRNAs and rRNAs (Maul et al., 2002; Grossman et al., 2003). The chloroplast DNA (cpDNA) is present in around 80 copies per cell and is packaged into nucleoids containing around 10 copies each. Chloroplast DNA replication occurs continuously through the cell cycle and is not synchronized with the nuclear division cycle, while chloroplast division is tightly coordinated with cytokinesis (Chiang and Sueoka, 1967; Johnson and Porter, 1968; Turmel et al., 1980; Adams et al., 2008). Though typical in size and protein coding capacity for a chloroplast genome, the *Chlamydomonas* cpDNA is relatively rich in non-conserved short intergenic repeats that may contribute to its high rate of rearrangement (Maul et al., 2002; Odom et al., 2008).

The *Chlamydomonas* mitochondrial (mt) genome is a 15.8 kb linear molecule with inverted repeats at each end (Vahrenholz et al., 1993). The mt genome encodes 8 proteins, mitochondrial rRNAs, and three tRNAs (Grant and Chiang, 1980; Gray and Boer, 1988; Michaelis et al., 1990). The *Chlamydomonas* mt genome is notable in being linear in structure and significantly smaller than the circular mt genomes found in other algae and most plants (Grant and Chiang, 1980; Boer et al., 1985; Stern et al., 2009). Other notable features of the mt genome are fragmentation of its rRNA genes that are separately transcribed as pieces that assemble non-covalently into intact mt ribosomes (Boer and Gray, 1988; Denovan-Wright et al., 1996) and are rapidly evolving with respect to other mt genes cytosolic rRNAs (Popescu and Lee, 2007).

The *Chlamydomonas* organellar genomes are inherited uniparentally through postzygotic mechanisms that are still not well understood. In meiotic progeny cpDNA is inherited from the *MT*+ parent while mitohondrial DNA is inherited from the *MT*− parent (Boynton et al., 1988). cpDNA inheritance involves targeted destruction of *MT*− derived cpDNA in early zygotes (Nishimura et al., 1998; Kuroiwa, 2010), and may also involve differential replication in germinating zygotes mediated by cpDNA methylation (Umen, 2001). Loss of female mtDNA appears to occur during meiosis as zygotes germinate (Aoyama et al., 2006).

3c. The *Volvox* Nuclear Genome

Previous genetic mapping defined 19 linkage groups for *Volvox* that probably correspond to chromosomes (Kirk, 1998), though exact chromosome number remains to be established. The ~131 Mbp *Volvox* nuclear genome sequence assembly (version 2) contains 434 scaffolds, 100 of which are >50 kbp and contain over 98% of the sequence. Updated assemblies and annotations are available on the Phytozome web site (Table 2) (Goodstein et al., 2011). The extra ~14 Mbp of sequence in *Volvox* compared with *Chlamydomonas* is composed of repeats that are interspersed within and between genes (Prochnik et al., 2010).

A second difference between the two algae is that the *Volvox* genome is ~56% GC while *Chlamydomonas* is ~64% GC. However, with respect to coding capacity *Volvox* is highly similar to *Chlamydomonas* with ~15,000 predicted protein coding genes (Prochnik et al., 2010). A more detailed comparison of gene content between the two species is in sections 5 and 6 below.

3d. The *Volvox* Organellar Genomes

Compared with *Chlamydomonas* and with most other green algae, the *Volvox carteri* organellar genomes are atypically large and filled with non-coding sequences, most of which are palindromic repeats (Smith and Lee, 2009). The cp genome is 420 kb and the mt genome is 30 kb. The cp genome is the largest and most repeat-rich within the green lineage (Chlorophytes and Streptophytes), while the *Volvox* mt genome is also repeat rich with few genes. Other than their expanded repeat content, the organellar protein coding genes of *Volvox* are very similar to those of *Chlamydomonas* (Smith and Lee, 2009). The increased non-coding content of all three *Volvox* genomes compared with *Chlamydomonas* (nuclear, cp and mt) is consistent with less efficient natural selection on a smaller, and possibly fragmented population of *Volvox carteri* (Smith and Lee, 2010).

3d.i Organelle DNA inheritance in *Volvox*—Like *Chlamydomonas*, the *Volvox* organellar genomes are inherited uniparentally, but unlike *Chlamydomonas*, both derive from the maternal parent (Adams et al., 1990). The presence of the putative mating type specification gene *MID* in males of colonial Volvocine algae makes them formally equivalent to the *MT*− mating type of *Chlamydomonas* that also has a *MID* gene (Ferris and Goodenough, 1997; Nozaki et al., 2006; Hamaji et al., 2008; Ferris et al., 2010). Therefore, with respect to mating type there has been at least one switch in mtDNA inheritance patterns in the lineage. In *Gonium pectorale* organellar DNA inheritance was found to follow a similar pattern to that of *Chlamydomonas* with cpDNA inherited from the *MT*+ parent and mtDNA from the *MT*− parent (Hamaji et al., 2008; Setohigashi et al., 2011). These findings are consistent with the *Chlamydomonas/Gonium* organellar inheritance patterns being ancestral, but more data from other Volvocine species will be required to confirm this idea.

Moreover, even though cpDNA is inherited maternally in all three Volvocine algal species described above, *Volvox* female *MT* is missing *EZY1*, *EZY2* and other *MT*+ specific genes that are thought to be associated with uniparental cpDNA inheritance in *Chlamydomonas* (Fig. 6) (Armbrust et al., 1995; Ferris et al., 2002; Goodenough et al., 2007). The absence of these genes in *Volvox* suggests that the underlying mechanism that specifies uniparental cpDNA inheritance in the two species may differ as well. A default hypothesis for uniparental organelle DNA inheritance in oogamous species such as *Volvox* is by a passive mechanism based on differential contributions of organellar DNA from eggs versus sperm (as opposed to active elimination that occurs in *Chlamydomonas*). Nonetheless, additional active mechanisms to ensure uniparental organelle DNA inheritance may also operate in *Volvox* as they do in *Chlamydomonas* and other eukaryotic taxa (Shitara et al., 2000; Zouros, 2000; Rawi et al., 2011; Sato and Sato, 2011; DeLuca and O'Farrell, 2012).

3d.ii The *Volvox* Mating Locus—*Volvox* and other large colonials (*Pleodorina*, *Eudorina*) transitioned from an isogamous ancestral mating system to an anisogamous or oogamous one with eggs and sperm. *Volvox* sexual development occurs via a modified embryogenesis program that differs between males and females and is controlled by a mating locus with two haplotypes, *MTF* (female) and *MTM* (male).

There are striking similarities and differences between the mating loci of *Chlamydomonas* and *Volvox*. They both are relatively large regions with rearrangements that suppress recombination (Fig. 6). Somewhat surprisingly, the location of *MT* in both species is in the same region of a syntenic chromosome (chromosome 6 in *Chlamydomonas reinhardtii*, scaffold 1 in *Volvox carteri*) indicating that *MT* has not moved during the ~200 Mya since the *Chlamydomonas* and *Volvox* lineages last shared a common ancestor (Fig. 7A) (Ferris et al., 2010). Unlike *Chlamydomonas MT*, *Volvox MT* is repeat-rich with low gene density compared with the rest of the genome, making it similar in most respects to classical sex chromosomes (Ferris et al., 2010). However, unlike diploid heteromorphic sex chromosomes, haploid sex chromosomes are not sheltered from gene loss and are less likely to lose essential genes (Bull, 1978). At >1Mbp the *Volvox MT* region is about five times larger than *Chlamydomonas MT* and unlike the case for *MT+/MT−*, *MTF* and *MTM* contain almost no residual synteny (identical gene order) between the 70+ shared genes that they have in common (Fig. 6C).

3e. Additional Volvocine algal genomes

Chlamydomonas and *Volvox* represent two extremes of organismal complexity in the Volvocales. Many questions about evolution in the Volvocine algae can only be answered by sequencing additional genomes of intermediate species. Projects to do so are underway and include genomes of *Gonium pectorale* and *Pleodorina starrii* (B. Olson, H. Nozaki, P. Durrand, R. Michod, personal communication).

4. Molecular genetics and genomics tools for Volvocine algae

Genomics resources for *Chlamydomonas* and *Volvox* are steadily growing and include informatics sites that provide access to browsers and computational tools as well as repositories for strains and cDNA or genomic libraries. These resources are described in Tables 1 and 2.

4a. The *Chlamydomonas* molecular genetics toolkit

4a.i Transformation—All three genomes (nuclear, chloroplast, mitochondrial) of *Chlamydomonas* are transformable (Kindle et al., 1989; Kindle, 1990). Transformed nuclear genes insert randomly, a property that can be exploited for making tagged mutants (Dent et al., 2005; Galván et al., 2007). Transformation of the nuclear genome by electroporation with either a circular or linearized plasmid is a routine procedure typically resulting in hundreds to thousands of independent transformants (Keller, 1995; Shimogawara et al., 1998). *Chlamydomonas* can also be transformed by vortexing gametolysin-treated cells with glass beads or silicon carbide whiskers (Kindle, 1990; Dunahay et al., 1997). Chloroplast and mitochondrial transformation occurs by homologous recombination. It is now possible

to transform not just one or a few genes into the *Chlamydomonas* chloroplast, but to re-engineer the entire chloroplast genome ex-vivo and then reintroduce it to obtain gene replacements and modifications at multiple loci (O'Neill et al., 2012).

4a.ii. Promoters for nuclear transgene expression—Unlike the case in higher plants, heterologous promoters have so far not been successful in *Chlamydomonas*. A handful of endogenous promoters have been successfully used to drive transgene expression (Neupert et al., 2012). These include an engineered hybrid promoter from the *HSP70A* and *RBCS2* genes that also contains the first intron of RBSC2 (Schroda et al., 2000; 2002; Lodha et al., 2008), The *HSP70a* and *HSP70b* promoters alone (Schroda et al., 2000), and the *PSAD* promoter that is derived from an intronless gene and well-suited to drive expression of cDNAs (Fischer and Rochaix, 2001).

4a.iii. Regulated Promoters—Several regulated promoters have been described that utilize environmentally responsive transcriptional responses to drive transgene expression. These include a copper-responsive promoter from the cytochrome b_6 gene *CYC6* that can be induced using Ni^{2+} (Quinn et al., 2003; Ferrante et al., 2008), the nitrate inducible/ammonium repressible promoter from the nitrate reductase gene *NIT1*, and the low-CO_2-inducible promoter from the carbonic anhydrase gene *CA1* (Ohresser et al., 1997; Villand et al., 1997). The activities of these promoters are coupled to environmental conditions, a property that might limit their utility. Synthetic or heterologous regulated promoters would be useful for expanding the molecular genetic and genomic toolkits of both *Chlamydomonas* and *Volvox*.

4a.iv. Selectable markers in *Chlamydomonas*—Two classes of selectable markers have been utilized for nuclear transformation in *Chlamydomonas*; endogenous genes that complement auxotrophies, and dominant antibiotic resistance makers (Neupert et al., 2012). Complementable mutations include *nit1* (nitrate requiring) (Kindle et al., 1989), *arg7* (arginine requiring) (Debuchy et al., 1989), *nic7* (nicotinamide requiring) (Ferris, 1995), and *thi10* (thiamine requiring) (Ferris, 1995). Dominant antibiotic resistance markers that have been engineered for *Chlamydomonas* include *ble* (zeocin resistance) (Stevens et al., 1996), *aphVIII* (paromomycin resistance) (Sizova et al., 2001), *aph7″* (hygromycin resistance) (Berthold et al., 2002), *aadA* (spectinomycin resistance) (Cerutti et al., 1997), *cry1-1* (emetine resistance) (Nelson et al., 1994), and *ppx1* (protoporphyrinogen oxidase mutant conferring resistance to the herbicide S-23142) (Randolph-Anderson et al., 1993).

Chloroplast transformants are typically selected by either complementation of an *atpB* mutation to restore phototrophic growth (Boynton et al., 1988) or with a dominant spectinomycin/kanamycin resistance gene (*aadA*) (Purton, 2007; Ramesh et al., 2011). Mitochondrial transformants are selected by complementation of the respiration deficient mutant *dum1* (Boynton and Gillham, 1996; Yamasaki et al., 2005; Hu et al., 2011).

4a.v. Reporter genes—Green fluorescent protein (GFP) has been codon optimized for use in *Chlamydomonas* (Fuhrmann et al., 1999) and has been expressed transgenically from the nucleus, chloroplast and mitochondrial genomes (Fuhrmann et al., 1999; Purton, 2007; Hu et al., 2011; Ramesh et al., 2011). Live cell imaging with GFP in algae is a challenge due

to high background fluorescence from chlorophyll and other auto-fluorescent compounds, but can be effective in cases where the GFP is well expressed or concentrated in a specific subcellular location (Fuhrmann et al., 1999; Yoshihara et al., 2008; Diener, 2009; Hayashi and Shinozaki, 2011; Neupert et al., 2012). Luciferase reporters have also been developed for *Chlamydomonas* (Fuhrmann et al., 2004; Mayfield and Schultz, 2004; Shao and Bock, 2008), and adapted for various purposes including extracellular secretion (Eichler-Stahlberg et al., 2009) and for monitoring circadian gene expression (Minko et al., 1999; Matsuo et al., 2008) (Mayfield and Schultz, 2004).

4a.vi. RNAi and antisense technology in Chlamydomonas—RNA interference (RNAi) is an effective tool for gene silencing in *Chlamydomonas* that has all the essential components of the RNAi machinery (see section 4a). Two strategies that have proven successful for generating targeted gene knockdowns are double strand hairpin-forming constructs (Rohr et al., 2004) and artificial microRNAs (Molnar et al., 2009; Zhao et al., 2009) that can also be coupled to regulated promoters (Schmollinger et al., 2010).

4b. Forward genetics and functional genomics in *Chlamydomonas*

Positional cloning of *Chlamydomonas* mutants can be accomplished using PCR mapping makers (Kathir et al., 2003; Rymarquis et al., 2005), that are available from the *Chlamydomonas* Resource Center (http://www.chlamycollection.org). Next-generation re-sequencing-based strategies for mutant gene identification are also now feasible and should allow more rapid identification of point mutants (Dutcher et al., 2012).

Insertional mutagenesis is a powerful tool for isolating tagged mutants in *Chlamydomonas* (Galván et al., 2007), but its potential is even greater when combined with high-throughput methods for identifying and cataloging insertion sites. This potential is gradually being realized. One approach for doing so involves pooling sets of mutant DNA and screening for insertions in genes of interest using PCR with a gene-specific and insert-specific pair of primers. When optimized this method has a very high success rate (Pootakham et al., 2010; González-Ballester et al., 2011). A variant approach involves screening insertion mutants that have been enriched for specific phenotypes such as photoprotection or nitrogen utilization, and then identifying mutants en masse by sequencing the insertion border in all of the identified strains (Dent et al., 2005; Gonzalez-Ballester, 2005). A next-generation approach to insertional mutant screening using bar-coded sequencing of selected pools may be on the horizon. A drawback of using insertional lines in a haploid strain is that essential genes will be highly underrepresented. A diploid insertion library is a feasible way around this problem, but has yet to be generated as a reverse genetics resource.

4c. The *Volvox* toolkit

4c.i *Volvox* transformation—The *Volvox* nuclear genome can be transformed by particle bombardment using the *nitA* gene (encoding nitrate reductase) to complement a *nitA*$^{-}$ strain (Schiedlmeier et al., 1994; Gruber et al., 1996). Dominant antibiotic resistance markers including *aphVIII* (conferring paromomycin resistance) and *ble* (conferring zeocin resistance) have also been adapted for use in *Volvox* (Hallmann and Rappel, 1999; Jakobiak et al., 2004). An sex pheromone inducible promoter system has also been developed for

regulated gene expression (Hallmann and Sumper, 1994). Some *Volvox* transformation vectors have also been used successfully to transform another Volvocine alga, *Gonium pectorale* (Lerche and Hallmann, 2009). GFP fusion proteins expressed in *Volvox* have been used to localize proteins within the ECM (Ender et al., 2002; Ishida, 2007) and in nuclei (Pappas and Miller, 2009). Gene expression can be knocked down in *Volvox* using antisense constructs as demonstrated for *GlsA* (Cheng et al., 2003) and a photoreceptor, Volvox rhodopsin (Ebnet, 1999).

4c.ii Forward genetics by transposon tagging—it is challenging to create and maintain thousands of insertion tagged mutants in *Volvox* due to its relatively low transformation efficiency and the difficulty of maintaining so many transformed lines (Kirk, 2000). An alternative strategy for forward genetics is transposon tagging mutagenesis using the cold-inducible transposons *Idaten* (Ueki and Nishii, 2008) or *Jordan* (Miller et al., 1993). This method relies on endogenous transposons that can occasionally excise to yield revertants. While successful, the method is relatively time consuming and inefficient. The development of artificial transposons would be a useful advance.

Classical genetics has been used successfully in *Volvox* to identify developmental mutants, but was done in a pre-genome era with few molecular markers (Huskey et al., 1979). The decreasing costs of resequencing may reopen this route for identifying important developmental regulators in *Volvox*.

5. The *Chlamydomonas* genome as a window into plant and animal evolution

In this section we highlight areas where genomic information from *Chlamydomonas* has shed light on eukaryotic cell biology and the evolution of the green plant lineage. While every species is in some ways specialized, *Chlamydomonas* is particularly interesting in that it does not have a reduced genome size as do marine picoalgae such as *Ostreococcus* and *Micromonas* (Derelle et al., 2006; Misumi et al., 2007; Palenik et al., 2007; Worden et al., 2009), and it has retained features that were likely present in the last common eukaryotic ancestor such as flagella and basal bodies. In addition, *Chlamydomonas* is part of the Archeaplastidia or green lineage that includes a diverse group of green algae and land plants (Fig 1A), all of which are descended from a unicellular eukaryote that underwent a primary endosymbiotic event of engulfing a cyanobacterium that subsequently evolved into the chloroplast. The tractability of *Chlamydomonas* as an experimental model makes it highly attractive for genomic studies that can then lead to experimentally testable hypotheses about gene function. Some examples of genomics-based discoveries in *Chlamydomonas* are described below.

5a. Cell motility and the flagella

Long before its genome was sequenced, it was recognized that the flagella and basal bodies of *Chlamydomonas* are structurally and functionally homologous to those in other eukaryotes including animals (Preble et al., 1999; Silflow and Lefebvre, 2001). Indeed, a key process for flagellar biogenesis that was discovered in *Chlamydomonas*, intraflagellar

transport (IFT) (Kozminski et al., 1993), is now widely recognized for its role in human ciliary signaling and a variety of genetic diseases (Sharma et al., 2008; Hildebrandt et al., 2011). *Chlamydomonas* is also a valuable resource for identifying new basal body and flagellar genes. Part of its utility for this approach stems from it having one of the best annotated sets of flagellar and basal body protein coding genes, many of which were validated by empirical proteomics (Table 2) (Keller et al., 2005; Pazour et al., 2005; Merchant et al., 2007).

Comparative approaches have been successful at identifying gene sets enriched for those encoding flagella and basal body proteins. This approach is based on the fact that higher plants, fungi and slime molds have lost flagella and basal bodies whereas most animals and unicellular eukaryotes have retained these organelles. By focusing on protein families where ciliated species have at least one member, but where non-ciliated species do not, new cilia or basal body proteins were identified (Avidor-Reiss et al., 2004; Li et al., 2004). These studies identified known proteins, but also new ones such as BBS5 whose human homolog is encoded by a gene associated with the genetic disease Bardet-Biedl syndrome (Li et al., 2004). A *Chlamydomonas*-centric comparative genomics approach was also used to subclassify flagellar genes by identifying those that are common to organisms with different cilia subtypes. Using a similar comparative approach as above, a more comprehensive analysis of flagella genes was undertaken. Starting with a set of proteins that are present in flagellated/ciliated organisms but not in those without flagella, a more refined subset of classifications were made. For example, the nematode *C. elegans* has non-motile sensory cilia, and proteins found in *Chlamydomonas* and other species with motile cilia but not in *C. elegans* were provisionally designated as MOT genes. Similar functional comparisons were made with the moss *Physcomitrella patens* and diatom *Thalassiosira pseudonana*, each of which have structurally modified motile cilia that are missing or thought to be missing structural components found in the *Chlamydomonas* flagella that has a standard 9+2 microtubule doublet structure with inner and outer dynein arms and radial spokes (Merchant et al., 2007).

5b. Green Genes

Genomic analyses in *Chlamydomonas* have aided progress in understanding photosynthetic metabolism and cell biology in the green lineage. A green lineage specific tool based on the predicted *Chlamydomonas* proteome was used to identify gene families that are specific to photosynthetic species. Indeed, 83% of the Green Cut proteins from *Chlamydomonas* whose functions were already known were chloroplast localized (Merchant et al., 2007). However, the Green Cut set also includes proteins that evolved specifically in the green lineage but may not be directly related to photosynthesis. These include proteins with predicted functions in signaling and nuclear transcription factors that may be specialized within the green lineage. Since they were first identified, a number of Green Cut protein family members were characterized and functionally verified (Grossman et al., 2003; Karpowicz et al., 2011) and this process will likely accelerate in the next few years as functional genomic resources such as insertional library screening are applied to *Chlamydomonas* and other green organisms.

Large-scale approaches such as Green Cut have inherent limitations that are complemented by more directed studies. For example some chloroplast import machinery proteins were missed by Green Cut, probably due to sequence divergence or incomplete gene models, but could be identified in directed searches based on screening for *Arabidopsis* Tic (translocon in the inner chloroplast envelope) and Toc (translocon in the outer chloroplast envelope) homologs (Kalanon and McFadden, 2008). Homologs of all but two *Arabidopsis* Tic and Toc proteins were identified in *Chlamydomonas*, and the two missing proteins (Tic62 and Toc64) are also missing from one or more other algal species. Comparison of the chloroplast transit peptide receptor complex proteins CrToc34 and CrToc159 indicated differences in amino acid composition that may reflect differences in composition of transit peptides between *Chlamydomonas* and higher plants (Franzén et al., 1990; Patron and Waller, 2007; Kalanon and McFadden, 2008).

5c. Selenoproteins

One interesting surprise in the *Chlamydomonas* genome was the presence of selenocysteine-containing proteins that are absent from land plants and fungi, but present in animals (Novoselov et al., 2002). Selenosysteine (Sec) is inserted into translating polypeptides by a modified tRNA-Sec that recognizes the codon UGA (normally a translation terminator) whose insertion is specified by a stem-loop forming sequence element (termed SECIS) in the 3′ UTR of the message containing the tRNA-Sec codon (Novoselov et al., 2002). *Chlamydomonas* was found to encode a single tRNA-Sec gene (RAO et al., 2003) as well as the enzymes and factors needed to produce and insert tRNA-Sec into elongating polypeptide chains (Grossman et al., 2007). Twelve putative selenoproteins have been identified in *Chlamydomonas*, five of which are involved in redox biochemistry (Grossman et al., 2007). Five other selenoproteins have no known function, but do have orthologs in mammals. This finding makes *Chlamydomonas* a potential new model for investigating selenoprotein-related biology.

5d. Carbon concentrating mechanism

Inorganic carbon (Ci) is often a limiting nutrient for aquatic photosynthetic microbes such as *Chlamydomonas.* Unlike terrestrial environments with relatively stable atmospheric CO_2 concentrations, aquatic Ci exists in gaseous (CO_2) and ionic (HCO_3, CO_3^{2-}) forms that are not always in equilibrium with atmospheric CO_2 and whose ratios fluctuate depending on pH. Aquatic algae have evolved carbon concentrating mechanisms (CCMs) to help overcome this limitation CCM proteins are part of an energy mediated transport system to move Ci from outside the cell and concentrate it in the chloroplast where it serves as the substrate for RuBisCo in photosynthetic dark reactions. CCM proteins include carbonic anhydrases (CAs) that interconvert CO_2 and HCO_3^{2-}, and transporters that move HCO_3 or CO_2 across membranes. While forward genetic screens have identified many CCM proteins (Spalding, 2008; Wang et al., 2011), genome-wide homology searches have revealed additional candidate genes that may play a role in the CCM or related processes. These include a large repertoire of twelve CAs that localize to different subcellular compartments or the periplasmic space. They also include additional paralogs of LCI proteins (LCIA, LCIB, LCIC, LCID, LCIE), that are thought to be involved in CO_2 transport or uptake (Wang and Spalding, 2006; Grossman et al., 2007) and which have homologs in other algae

such as *Ostreococcus*. A second observation from the *Chlamydomonas* genome sequence is that several CCM genes cluster in a single 75 kb region of chromosome 4 (Merchant et al., 2007). These include CCP1, CCP2, LCID, LCIE and two carbonic anhydrase paralogs, CAH1 and CAH2. Whether such clustering is connected to gene regulation remains to be determined.

EST and genome sequencing identified a medically relevant protein family, Rh, as another possible connection to CO_2 physiology. Rh is a red blood cell antigen and integral plasma membrane protein that is part of an ammonium transporter superfamily (PFAM 00909). The discovery of Rh proteins in non-metazoans such as *Chlamydomonas* was unexpected (Huang and Liu, 2001), as is their overall distribution in a few select non-metzoan taxa that include green algae (*Chlamydomonas*, *Coccymyxa*, *Chlorella*), oomycetes (*Phytophthora*), slime molds (*Dictyostelium*, *Polysphondylium*), Choanoflagellates (*Monosiga*, *Salpingoeca*), Incertae sedis (*Capsaspora*) and Heteroloboseans (*Naeglaria*). Whether Rh transports NH_4/NH_3^+ or CO_2/HCO_3 is not fully resolved, however the *Chlamydomonas* Rh paralogs are linked to high CO_2 acclimation possibly by acting as a bidirectional CO_2 gas exchange channel (Soupene et al., 2004; 2004).

5e. Vitamin biosynthesis

Many algae require one or more of vitamins B1 (thiamine), B7 (biotin) and B12 (cobalamin) for growth (Croft et al., 2006). Vitamin auxotrophies are distributed across algal taxa in a manner that suggests multiple independent losses of genes or enzymes requiring the co-factors. *Chlamydomonas* does not require any vitamin supplements. It has biosynthetic pathways for thiamine and biotin, and its single cobalamin-dependent enzyme, the methionine synthase METH, is redundant with a cobalamin-independent enzyme, METE (Croft et al., 2006). Interestingly, *Volvox carteri* requires B12 supplementation for growth suggesting that it has lost its cobalamin-independent methionine synthase gene, METE (or that it has acquired another essential enzyme that is cobalamin dependent). Indeed, the sequence of *Volvox* METE has multiple frame-shift mutations and a premature stop indicating that it has become a pseudogene, thus explaining the dependence of *Volvox* on B12 (Helliwell et al., 2011). Moreover, METE loss has occurred at least one other time in the Volvocine lineage. The 16-cell colonial species *Gonium pectorale* METE was also found to be a pseudogene whose loss appears to have occurred independently from that in *V. carteri* (Helliwell et al., 2011), though some strains of *Gonium pectorale* are B12 independent suggesting a very recent loss of METE in some lines (Stein, 1966). The loss of METE implies that *Volvox carteri* and some *Gonium pectorale* strains acquire B12 environmentally and raise the question of whether they do so in specific association with other microbes.

Volvox carteri has orthologs of the thiamine and biotin biosynthetic enzymes identified in *Chlamydomonas* (Croft et al., 2006) (J. Umen, unpublished observation), but their expression and function have not been carefully examined. The question of whether *Volvox carteri* truly requires biotin and thiamine supplementation merits reexamination in light of this observation.

5f. Cell cycle

The non-canonical multiple fission cell cycle of *Chlamydomonas* might be expected to require innovation in cell cycle control machinery compared to organisms that divide by binary fission. In addition, multiple-fission is likely to have been an important factor in facilitating the evolution of colonialism in Volvocine algae (see section 6e). A genomic survey of cell cycle regulatory proteins in *Chlamydomonas* revealed a typical repertoire of eukaryotic cell division control proteins that are similar to those in land plants, though with far less gene duplication than is seen in plants (Bisova et al., 2005). A similar situation was observed for another alga, the Prasinophyte *Ostreococcus taurii* (Robbens et al., 2005). A few notable observations arose from these studies. First, both algae encode homologs of the plant-lineage-specific cyclin dependent kinase (CDK) CDKB that is expressed during cell division (Bisova et al., 2005; Corellou, 2005). This finding broadens the possible roles for CDKB and suggests an ancestral function that encompasses both algae and land plants. A notable feature of *Chlamydomonas* cell cycle genes is the presence of novel cyclin dependent kinases, CDKG1 and CDKG2, that are not found in species outside of Volvocine algae and may be important for the multiple fission cycle found in the Volvocales. Genomic level analysis also confirmed the presence of key components of the retinoblastoma (RB) tumor suppressor pathway that are conserved in the green lineage and in animals, but have been lost from fungi (Xie et al., 1996; Umen and Goodenough, 2001; Bisova et al., 2005; Robbens et al., 2005; Grafi et al., n.d.). Interestingly, the only cell cycle gene family in *Chlamydomonas* that underwent expansion is the D-type cyclins which are presumed to activate CDKs that regulate RB-related proteins through phosphorylation. *Chlamydomonas* has four D-type cyclins that are diverged from each other enough to suggest an ancient duplication event. This idea is supported by the finding that *Volvox* has orthologs for each of the four D-type cyclin subfamilies that are found in *Chlamydomonas* plus additional paralogous duplications whose potential significance is discussed in section 6e (Prochnik et al., 2010). This finding indicates that a D-cyclin family expansion occurred early in the Volvocine lineage and has been maintained in two independent branches suggesting an important role for each of the D-type cyclin sub-types in Volvocine algal cell cycle control.

5g. Scavenger receptors

Scavenger receptor and cysteine rich domain (SRCR) proteins are found in metazoans where they have diverse roles in the immune system (e.g. pathogen recognition by macrophages) and elsewhere such the sea urchin sperm flagellar receptor for the egg peptide speract (Cardullo et al., 1994; Resnick et al., 1994). SRCR proteins contain a ~100 amino acid repeat module with conserved cysteine pairs that form disulfide bridges (Hohenester et al., 1999). SRCR proteins are abundant in metazoans, but are absent from higher plants, and most algae. Remarkably, *Chlamydomonas* encodes 35 SRCR domain containing proteins, many of which were described previously (Wheeler et al., 2008), and few of which have appeared in a more updated genome assembly from Phytozome (Goodstein et al., 2011). *Volvox* has at least 21 SRCR domain-containing proteins, though its genome annotation is not as comprehensive as that of *Chlamydomonas*, so this family awaits a true interspecies comparison in Volvocine algae. SRCR domains are often repeated multiple times in a protein with a range of 1–11 copies in *Chlamydomonas* SRCR proteins. A notable feature of

several of the *Chlamydomonas* SRCR proteins is their enormous size, with the largest containing 8671 amino acids and encoded by a gene that spans almost 60 kb. Several of the *Chlamydomonas* SRCR proteins also contain lectin domains that bind to carbohydrates and are widespread in eukaryotes (Wheeler et al., 2008). The presence of these large metazoan-like adhesion molecules or receptors in *Chlamydomonas* and *Volvox* raises many interesting questions about their function(s) and provides a new avenue for investigating their evolutionary origins.

6. The Volvox genome as a window into multicellular evolution

6a. Volvox development

Volvox carteri has a complex developmental program that involves conserved processes that are shared with *Chlamydomonas* and other features with no obvious analogs in *Chlamydomonas* (Fig. 4 and 5). Insights into the evolution of *Volvox* have come from genomics and from isolation of developmental mutants (Kirk, 1998; Nishii and Miller, 2010; Prochnik et al., 2010). Below we outline key stages of *Volvox* development and discuss what genetic and genomic approaches have revealed about the origins of multicellularity in Volvocine algae. More comprehensive reviews of *Volvox carteri* development are also available (Kirk, 1998; 2005).

Each *Volvox carteri* vegetative spheroid is composed of ~2000 sterile somatic cells and sixteen large reproductive cells called gonidia, all of which are embedded within a complex compartmentalized ECM that occupies 99% of the spheroid volume. Somatic cells do not normally grow or reproduce but provide motility for the spheroid and secrete extracellular matrix. They may also help concentrate nutrients in the interior to support growth of reproductive cells (Koufopanou, 1994). The vegetative gonidial cells undergo embryogenesis involving successive cleavage divisions that follows a modified program of multiple fission in which incomplete cytokinesis leaves post-mitotic cells attached through a network of cytoplasmic bridges. At cycle 6 (32-->64 cell stage transition) 16 cells on the anterior of the embryo divide asymmetrically to produce a large and a small cell while the remaining cells continue to divide a total of twelve times. The 16 large cells from the asymmetric division divide one or two more times asymmetrically, and then withdraw from cleavage. By the end of embryonic division these 16 large cells will end up differentiating into the next generation of germ cells, while the small cells become somatic. Interestingly, germ-soma differentiation is controlled by cell size rather than by partitioning of specific cell fate determinants into either of the two cell types (Kirk et al., 1993). At the end of cleavage the embryo is inside out with respect to its final configuration with gonidial cells on the outside of the spheroid and flagella of somatic cells pointing inwards. Through the remarkable process of inversion the embryo turns itself right-side out into its adult configuration with gonidial precursor cells on the interior of the spheroid and the flagella of the somatic cells facing the exterior (Viamontes and Kirk, 1977; Viamontes et al., 1979; Kirk and Nishii, 2001; Hallmann, 2006a). Juvenile spheroids remain inside their mother where they continue to grow for another day or two before hatching out to repeat the vegetative growth cycle.

In addition to its vegetative asexual reproductive cycle *Volvox carteri* has a sexual development cycle (Fig. 5) that is under control of its mating locus (Fig. 6). While vegetative males and females of *Volvox carteri* are indistinguishable, the male and female sexual forms are different from each other and from vegetative *Volvox*. Sexual differentiation in *Volvox* is triggered by a glycoprotein sex-inducer produced by males that causes modified embryogenesis programs in males and females (Fig. 5) (Kochert, 1968; Tschochner et al., 1987; Mages et al., 1988). Females treated with sex inducer produce sexual offspring with 32–48 egg cells instead of vegetative gonidia, while males treated with sex inducer produce sexual offspring containing a 1:1 ratio of 128 somatic cells and 128 sperm packets, each containing 64–128 sperm. Sperm packets travel as a unit and upon encountering a sexual female, break apart and tunnel inside the ECM where they find and fertilize eggs to produce a diploid zygote spore. Upon germination the spore undergoes meiosis and produces 3 polar bodies plus one new viable haploid progeny spheroid. While not described in Kirk's twelve steps (see below), innovations in the *Volvox* sexual cycle have been recently summarized (Ferris et al., 2010; Umen, 2011).

6b. Kirk's twelve steps of multicellular evolution in the Volvocales

David Kirk proposed a twelve-step model of multicellular evolution in the Volvocales (Kirk, 1998; 1999; 1999; 2005) that posited the minimum changes or innovations required for a unicellular *Chlamydomonas*-like ancestor to evolve into a multicellular *Volvox carteri*-like spheroid (Fig. 2A). When broken into discrete steps it becomes clear that half of the innovations occur in the first transition to form the simplest colonial genus exemplified by species such as *Gonium* (Kirk, 1998; 1999; 1999; 2005; Herron and Michod, 2008; Herron et al., 2009; Nishii and Miller, 2010). This finding implies that the most difficult transition is evolving the minimal functional colonial form, a finding that is partly supported by the monophyly of colonial Volvocines as a whole (i.e., only one original instance of colonialism) compared to polyphyly of several genera within the Volvocine algae (i.e. more than one instance of a transition to a more complex form) (Fig. 1).

Evolving *Gonium*-like colonies from *Chlamydomonas*-like unicells requires a minimum of six changes: 1. incomplete cytokinesis to maintain linkage between daughter cells (i.e. cytoplasmic bridges), 2. partial inversion to reorient post-mitotic daughters within the new colony. 3. basal body rotation by ninety degrees in peripheral cells to align flagella, (Kirk, 1998; 2005). 4. establishment of organismal polarity as evidenced by the difference in basal body and flagellar orientations of central versus peripheral cells in the colony, 5. modification of the cell wall to keep daughters attached 6. genetic control of cell number. After the evolution of a colonial morphology typified by *Gonium*, the Volvocales show a significant increase is colony size/cell number. The spherical genera starting with *Pandorina* undergo full inversion corresponding to Kirk's step 7. Eudorina is representative of step 8--expansion of the extracellular matrix (ECM). *Pleodorina* represents Kirk's step 9--partial division of labor between germ and somatic cells. Finally, *Volvox carteri* represents Kirk's steps 11 and 12–asymmetric cell divisions and a bifurcated cell division program. Notably, these last two steps are found in only one subgroup of *Volvox* species from the section Merrillosphaera. Other, species of *Volvox* develop in a different manner through progressive

binary divisions that represent an alternative evolutionary solution to colony growth (Desnitski, 1995; Kirk, 1998; Coleman, 2012).

6c. Comparing the content of the *Chlamydomonas* and *Volvox* genomes

It has long been assumed that an extensive new genetic toolkit would be required for multicellular evolution (King, 2004; Ruiz-Trillo et al., 2007; Rokas, 2008a; 2008b). Previous genomic comparisons reinforced this idea (Putnam et al., 2007; Srivastava et al., 2010), though in no case had two closely related species with such divergent morphology, such as *Chlamydomonas* and *Volvox*, been compared (Prochnik et al., 2010). On a global level the genomes of these two algae are remarkably similar, including extensive regions of synteny (Ferris et al., 2010; Prochnik et al., 2010). The total protein coding gene count for *Chlamydomonas* is ~14,516 (Merchant et al., 2007) while that for the first version of the *Volvox* genome was ~14,520 genes (Prochnik et al., 2010). Protein domain content is also similar between the two species: *Chlamydomonas* has 2,354 PFAM domains while *Volvox* has 2,431 PFAM domains that are largely overlapping (Prochnik et al., 2010). Of the ~15,000 genes found in *Chlamydomonas* and *Volvox*, ~64% can be assigned into protein families that are shared with other eukaryotes (Prochnik et al., 2010). Of these, 80% show a 1:1 orthology between the two algae. In summary, with a few exceptions described below, large-scale comparisons of *Chlamydomonas* and *Volvox* reveal very few differences that can be tied to the substantial differences in biological organization that distinguish them.

The overall similarity between the two genomes is mirrored by results of forward genetics that identified key developmental regulators in *Volvox*. Mutants that affect somatic cell specification (*regA*) (Kirk et al., 1999), asymmetric cell division (*glsA*) (Miller and Kirk, 1999), and inversion (*invA*, *invB*, invC) (Nishii et al., 2003; Ueki and Nishii, 2008; 2009) have all been identified and cloned. Remarkably, four of the genes underlying these processes that are unique to *Volvox* have orthologs in *Chlamydomonas* (*glsA*, *invA*, *invB*, *invC*), and the fifth, *regA*, is part of a complex gene family that has members in both species. Moreover, two of the *Volvox* mutants, *invA* and *glsA*, can be complemented by their *Chlamydomonas* orthologs (Cheng et al., 2003; Nishii et al., 2003; Duncan et al., 2007; Ueki and Nishii, 2008; 2009). Thus, it appears that many ancestral functions carried out by proteins in *Chlamydomonas* could be modified or coopted to perform developmental functions in *Volvox*, suggesting that the genetic toolkit required for multicellularity may have been largely present in ancestral unicells (King, 2004; Prochnik et al., 2010).

26% (1,835) of the 15,000 genes in *Chlamydomonas* and *Volvox* are only found in these algae, and are thus Volvocine specific. This latter group of Volvocine algal protein families is of special interest since they show asymmetric expansion/contraction patterns between *Chlamydomonas* and *Volvox*. These Volvocine algal protein families tend to be larger in *Volvox* than in *Chlamydomonas* (Prochnik et al., 2010). Moreover, this asymmetric distribution of expansions/contractions does not extend to protein families that are shared with species outside Volvocine algae. This latter group of more ancient proteins shows similar degrees of loss/gain between the *Volvox* and *Chlamydomonas* lineages (Prochnik et al., 2010).

What might be responsible for the preferential expansion/retention of Volvocine algal specific protein families in *Volvox*? While answers to this question are speculative, it is striking that among these protein families are two whose members are components of the *Volvox* ECM (see below), which is itself a massive elaboration of the ancestral cell wall of its *Chlamydomonas*-like ancestor. Extrapolating further, one can speculate that the entire group of Volvocine-algal-specific proteins might be highly evolvable. By definition they have been around long enough to remain established in both algal lineages, but are still young enough to be amenable to evolutionary experimentation and elaboration. Unfortunately, the functions of most Volvocine-algal-specific proteins are unknown since they are restricted to this lineage, but they may be a rich source of evolutionary plasticity and novelty that merits further examination. Supporting this idea are recent findings indicating that the origins of many metazoan proteins associated with multicellular functions (e.g. cell adhesion) extend back to their last unicellular ancestor that resembled a choanoflagellate, and are also clade-specific proteins that are not found outside of holozoa (e.g. integrins, cadherins, tyrosine kinases) (King et al., 2008; Abedin and King, 2010; 2010).

6c.i Gene family expansions in *Chlamydomonas*—Notable gene family expansions in *Volvox* relative to *Chlamydomonas* are discussed below, but there are a few instances where *Chlamydomonas* has more gene family members than *Volvox* (Prochnik et al., 2010). *Chlamydomonas* has ~35 histone gene clusters while *Volvox* has less than half this number (Prochnik et al., 2010). The *Chlamydomonas* histone content is also exceptionally high compared with plants and other algae. Increased gene copy number is a possible means of increasing the rate of gene product synthesis which might be important for multiple fission where rapid successive divisions occur, but it is not clear why *Chlamydomonas* would need more histone clusters than *Volvox* since their cell division rates during mitosis are similar (Harper and John, 1986; Kirk, 1998). Proteins with ankyrin repeats were reported to be highly enriched in *Chlamydomonas* versus *Volvox* (~40 versus 12) but a reevaluation of protein domain content using more sensitive criteria in the new version of the *Volvox* genome (Goodstein et al., 2011) shows that *Chlamydomonas* has 146 ankyrin repeat proteins versus 80 in *Volvox* (J. Umen, unpublished observation). This is still a biased ratio, but no longer appears exceptional and may be a result of genetic drift.

6c.i. Cell cycle modifications - key steps in Kirk's twelve steps—Four of Kirk's twelve steps of multicellular evolution in the Volvocales are closely or directly related to cell cycle modifications: incomplete cytokinesis (1), genetic modulation of cell number (6), Asymmetric cell division (11), Bifurcated cell division program (12) (Kirk, 2005). In addition, sexual development in *Volvox* entails further cycle modifications that include altered timing of embryonic asymmetric cell division in males and females, and a set of post-embryonic germ cell divisions to produce sperm packets in males (Ferris et al., 2010; Umen, 2011). As described above *Volvox* utilizes a *Chlamydomonas*-like multiple fission cell cycle for embryonic and post-embryonic divisions. Unlike the case in *Chlamydomonas* where division number is flexible, the number of divisions in *Volvox* and other colonial Volvocine algae is more stereotyped so that cell number per colony stays relatively constant and is always a power of 2 (e.g. 4, 8, 16, 32 …). The triggering of asymmetric cell division

at a specific embryonic cell cycle number and the subsequent bifurcated division program for germ cell and somatic cell precursors in *Volvox* is not understood, but may be coupled in some manner to cell size, as is the final process of germ-soma differentiation (Kirk et al., 1993; Kirk, 2005).

Forward genetics in *Chlamydomonas* identified the retinoblastoma (RB) tumor suppressor pathway as a central regulator of size control during multiple fission (Umen and Goodenough, 2001; Fang et al., 2006). It might be expected that this pathway is modified or elaborated in *Volvox* and perhaps other Volvocine species to accommodate alterations in colony size and/or developmental timing. While the cell division cycle protein coding genes in *Volvox* are overall very similar to those in *Chlamydomonas*, there are two intriguing differences uncovered by genome sequencing that suggest possible modifications in *Volvox*. First, an expansion of the D1 cyclin subfamily was found in *Volvox* that has single orthologs of the other *Chlamydomonas* cyclins (D2, D3, D4) but four cyclin D1 paralogs (Prochnik et al., 2010). D type cyclins are the activator subunits of cyclin dependent kinases that have retinoblastoma tumor suppressor related proteins (RBRs) as their substrates and key targets for controlling cell cycle progression. The elaboration of D1 cyclins in *Volvox* may enable more fine-tuned developmental control over multiple fission than in *Chlamydomonas*, an idea that merits further investigation.

A second change in the Volvox RB pathway compared with *Chlamydomonas* is the incorporation of the RBR homolog *MAT3* into the *Volvox* mating locus and subsequent divergence of the male and female alleles (*vcMAT3f* and *vcMAT3m*) (Fig. 6) (Ferris et al., 2010). The large divergence between *MAT3f* and *MAT3m* as well as their sex-regulated alternative splicing patterns suggests that this protein has acquired a role in controlling developmental patterning differences that distinguish male and female sexual forms of *Volvox*.

6c.ii. Asymmetric cell division - Kirk's 11th step—*Chlamydomonas* does not divide asymmetrically, so it might be expected that *Volvox* would have evolved one or more proteins that facilitate this function, perhaps through modifications to its cytoskeletal protein repertoire. On the contrary, no major differences in cytoskeletal proteins were detected between the two species (Prochnik et al., 2010). Moreover, the single identified mutant that affects asymmetric division in *Volvox*, *glsA*, has a *Chlamydomonas* ortholog that complements its phenotype (Miller and Kirk, 1999; Cheng et al., 2003). *glsA* encodes a DnaJ-domain protein that interacts with heat shock protein HSP70 to promote asymmetric cell division in an unknown manner (Cheng et al., 2005; 2006). The investigation of GAR1, the *Chlamydomonas* glsA ortholog, may shed light on the ancestral role of this protein in *Chlamydomonas* cell division.

6c.iii. Inversion - Kirk's 7th step—Like the case for asymmetric cell division, there is no obvious correlate of inversion in *Chlamydomonas* and no candidate cytoskeletal genes that emerged from genomic comparisons as candidate mediators of this process. Forward genetic screens have identified three genes required for *Volvox* inversion– *invA*, *invB* and *invC*--that encode a kinesin, a sugar transporter, and a glycosyl transferase respectively (Nishii et al., 2003; Ueki and Nishii, 2008; 2009). InvA is thought to interact with the

microtubule cytoskeleton at cytoplasmic bridge attachment sites to effect cell-shape changes required for inversion (Nishii et al., 2003). InvB and InvC are required for expansion of the vesicle that surrounds the inverting embryo and which physically restricts inversion in the *invB* and *invC* mutants. Each of the above proteins has a highly similar ortholog in *Chlamydomonas* indicating that the pathways they control have cognate processes in *Chlamydomonas* from which they were coopted. The invB and invC functions might have ancestral roles in cell wall expansion that must occur in *Chlamydomonas* to accommodate cell growth. The potential role of the *Chlamydomonas* invA ortholog, IAR1, is harder to imagine but suggests the existence of cytoskeletal processes that have not yet been characterized.

6c.iv. Evolution of an elaborated ECM—Cell-cell adhesion is a critical aspect of multicellularity (Bonner, 2000; Abedin and King, 2010). In the Volvocales adhesion is accomplished by ECM proteins that are related to cell wall proteins from *Chlamydomonas* (Kirk et al., 1986; Adair et al., 1987; Hallmann, 2006b). Indeed, the importance of cell-cell adhesion and ECM modifications in colonial Volvocine algae is reflected in two of Kirk's twelve steps of multicellular evolution that are changes to the ECM (Kirk, 2005). Step 5 is modification of the cell wall to maintain adhesion between post-mitotic daughters, while step 8 is expansion of the ECM that allows for spheroid growth in the absence of cell growth and division.

The *Chlamydomonas* cell wall is composed of three major layers of hydroxyproline rich glycoproteins that can be divided into seven sub-layers (Goodenough and Heuser, 1985). The outermost of these layers can be used to nucleate the assembly of *Volvox* cell walls demonstrating the evolutionary conservation of Volvocine cell wall structural components (Adair et al., 1987; Adair, 1988). The expanded ECM in *Volvox* is organized and compartmentalized as revealed by various structural and localization studies (Fig. 7) (Kirk et al., 1986; Ertl et al., 1989; Godl et al., 1995; Hallmann and Kirk, 2000; Hallmann, 2006b).

Paralleling the expansion of its ECM, the Volvox genome codes a much larger number of ECM-related proteins than does *Chlamydomonas*. Two families of ECM proteins whose members are known constituents of *Volvox* ECM underwent notable expansion in the *Volvox* lineage. These are a family of hydroxyproline-rich glycoproteins called pherophorins and a family of metalloproteases called VMPs (Sumper and Hallmann, 1997; Hallmann, 2002; 2007; Prochnik et al., 2010). *Chlamydomonas* has 27 predicted pheophorins versus 45 in *Volvox*, and 8 VMPs versus 42 in *Volvox*.

ECM proteins in *Volvox* also play a role in the sexual cycle. The *Volvox* sex inducer is a pherophorin-related protein (Mages et al., 1988), and expression of several pherophorin genes is up-regulated during sexual differentiation (Hallmann, 2006b; Prochnik et al., 2010). Likewise, three related *Volvox* ECM metalloproteinases are also sexually regulated (Hallmann, 2006b; Prochnik et al., 2010). The remarkable organizational properties of the *Volvox* ECM and the potential roles of ECM proteins in signaling and nutrient storage/ transport are all exciting topics for future investigation.

6c.v. Terminal differentiation of somatic cells and the origins of VARL Genes—Somatic cell fate in *Volvox* is controlled by r*egA*, whose protein product is a nuclear localized putative transcriptional repressor (Tam et al., 1991; Kirk et al., 1999; Meissner et al., 1999; Duncan et al., 2006; 2007). RegA belongs to a family of proteins in Volvocine algae called VARL (Volvocine Algae RegA Like) that all share a putative DNA binding domain related to the SAND domain found in some transcriptional repressors (Duncan et al., 2006; 2007). Unlike the previously described *glsA*, *invA*, *invB* and *invC* genes, *Volvox regA* has no clear ortholog in *Chlamydomonas*. Instead, both algae have multiple VARL paralogs that arose from a complicated pattern of duplications and/or losses in each of the two species (Duncan et al., 2006; 2007). *regA* is part of a four gene paralogous duplication that is unique to *Volvox*. Interestingly, it is the only one of the four paralogs that has been identified as a developmental mutant (Kirk et al., 1999). In *Chlamydomonas* the closest VARL protein to *Volvox* regA is called RLS1 whose expression has been linked to stress and environmental responses (Nedelcu and Michod, 2006; Nedelcu, 2009), but whose function is not known. These data again point to the unicellular origins of proteins associated with multicellular development in the Volvocine algae.

6c.vi *Chlamydomonas* and *Volvox* microRNAs are not homologous—Both *Chlamydomonas* and *Volvox* have RNAi machinery (Cerutti et al., 2011) and it might be expected that they contain homologous microRNA genes as do many higher plants and animals (Berezikov, 2011; Cuperus et al., 2011). On the contrary, when *Chlamydomonas* microRNAs were compared to those predicted from *Volvox*, there was very little overlap (Zhao et al., 2007), though there are caveats to this conclusion. The comparison was based on empirical data for small RNAs and predicted microRNAs in *Chlamydomonas* from studies that captured only a fraction of possible stages in the life cycle. An empirical evaluation of *Volvox* miRNAs and small RNA targets will be required to know if any underlying homology exists in the small RNA networks of the two algae. Equally important will be an improved understanding of how miRNAs and other small regulatory RNAs contribute to the developmental programs of Volvocine algae.

6c.vi. The Volvox mating locus and evolution of sexual dimorphism—As discussed above, the sexual cycles of *Chlamydomonas* and *Volvox* are under the control of a mating locus (*MT*) whose location is similar in the two species, but whose size, content, and evolutionary dynamics are very different (Ferris et al., 2010; Umen, 2011). The structure of the *MT* region from *Volvox* and *Chlamydomonas* is described in Section 3. Besides its relative expansion and loss of residual synteny between shared genes (those with an allele in both mating types), the divergence rates of *Volvox* mating locus genes are up to two orders of magnitude higher than those in *Chlamydomonas* (Ferris et al., 2010). Whereas shared genes in *Chlamydomonas MT* retain high nucleotide identity (typically 99% in coding regions, and >90% in introns), shared genes are in *Volvox* have coding region identities as low as 40–50% and typically have no similarity in their intron sequences. This large divergence is the result of completely blocked recombination that can be traced back through speciation events in sister species of *Volvox* (Ferris et al., 2010). Paradoxically, these differences with *Chlamydomonas MT* makes *Volvox MT* look older even though it is part of a presumably younger and more recently-derived lineage (Charlesworth and

Charlesworth, 2010; Umen, 2011). One hypothesis that needs testing is whether cryptic recombination might occur in *Chlamydomonas MT* that acts to maintain homogeneity between shared genes in the two *MT* haplotypes (Umen, 2011). The effect of long-term isolation of female and male alleles of *Volvox* mating locus genes is divergence in both primary sequence and expression patterns, with a remarkable number of genes having acquired sex-regulated expression including female or male biases, and up- or down-regulation during sexual differentiation (Ferris et al., 2010). Thus, even shared genes in the *Volvox* mating locus have diverged enough to acquire male or female specific functions.

Another remarkable feature of *Volvox MT* is the presence of numerous male-limited and female-limited genes that do not have an allelic counterpart in the opposite mating type (Fig. 6). Of fifteen such genes (five female-specific, ten male-specific) only two, *VcMID* and *VcMTD*, have any similarity to *Chlamydomonas* genes, and only one, encoding an HMG-box protein, shows similarity to genes in any other species (Ferris et al., 2010). The number of "new" genes in the *Volvox* mating locus stands in contrast to the paucity of *Volvox*-specific autosomal genes (Prochnik et al., 2010) (and see below). This finding implies that the *Volvox* mating locus has evolved into a hotbed of evolutionary experimentation (Ferris et al., 2010), an idea that might be confirmed by obtaining sequences from mating loci of related Volvocine species and additional wild isolates of *Volvox carteri*..

VcMID and *VcMTD* are in male *MT* and have homologs *CrMId* and *CrMTD* in the *Chlamydomonas MT*− locus. *MId* and *MTD* homologs have been found in *MT*− strains of *Gonium pectorale* (Hamaji et al., 2008; 2008), and *MId* has also been found in males of *Pleodorina starrii* (Nozaki et al., 2006). Mid proteins are related to RWP-RK family putative transcription factors, while MTD proteins are Volvocine-algal-specific. In *Chlamydomonas MID* is a key sex determining gene whose presence or absence dictates differentiation as *minus* or *plus* gametes (Ferris and Goodenough, 1997). *MTD* appears to play an auxiliary role in augmenting the expression of *MID* (Lin and Goodenough, 2007). Functional studies of *MID* have not been reported outside of *Chlamydomonas*, but two observations suggest that sexual differentiation in *Volvox* will involve additional factors besides *VcMId* and *VcMTD*. First, *VcMId* mRNA is expressed constitutively in both vegetative and sexually induced males, and is not controlled by sex inducer or by nitrogen starvation as is the case for Mid orthologs in *Chlamydomonas*, Gonium and *Pleodorina* (Ferris and Goodenough, 1997; Nozaki et al., 2006; Lin and Goodenough, 2007; Hamaji et al., 2008). Second, *VcMTD* appears to be a pseudogene in *Volvox* with no start codon anywhere near the 5′ end of its mRNA (Ferris et al., 2010). These observations suggest that other genes in *MT* or elsewhere contribute to sexual differentiation in *Volvox*.

7. Future Perspectives

Genomics research with Volvocine algae is still far from reaching its potential. While the unexpectedly similar genome sequences of *Chlamydomonas* and *Volvox* have not led to a simple list of genes that underlie each major trait difference that distinguishes them, they have provided a framework for more in-depth studies. Genomics has also refocused investigations into the origin of complexity towards the unicellular species *Chlamydomonas* that is remarkably sophisticated and appears to possess most of the genetic toolkit that was

required for Volvocine algae to become multicellular. Critical for understanding how Volvocine algae and their genomes evolve will be sequencing additional species. Such sequencing will allow important genomic attributes such as gene gains and losses to be polarized in order to determine the direction of change within the group. Given the complex evolutionary history within Volvocine algae (Fig. 2) additional genomes will provide insights into how convergent traits evolved in different subgroups. As genomes from closely related Volvocine algal subgroups are compared the key genetic changes that distinguish them may come into focus. The decreasing cost of sequencing will soon make it possible to do population genomics on Volvocine algae that will begin to shed light on how population structure and natural variation relate to long-term evolutionary change and speciation. Low cost genome sequencing is an enabling technology for large-scale forward genetics and functional genomics that are of increasing importance for *Chlamydomonas* and will soon be so for *Volvox* and other species. Sequencing is also revolutionizing the ability to rapidly create new model systems from previously unstudied species. Efforts to develop additional Volvocine algal models such as *Gonium pectorale* (whose genome is currently being sequenced) will greatly expand the utility of Volvocine algae as a premier model for evolution and development.

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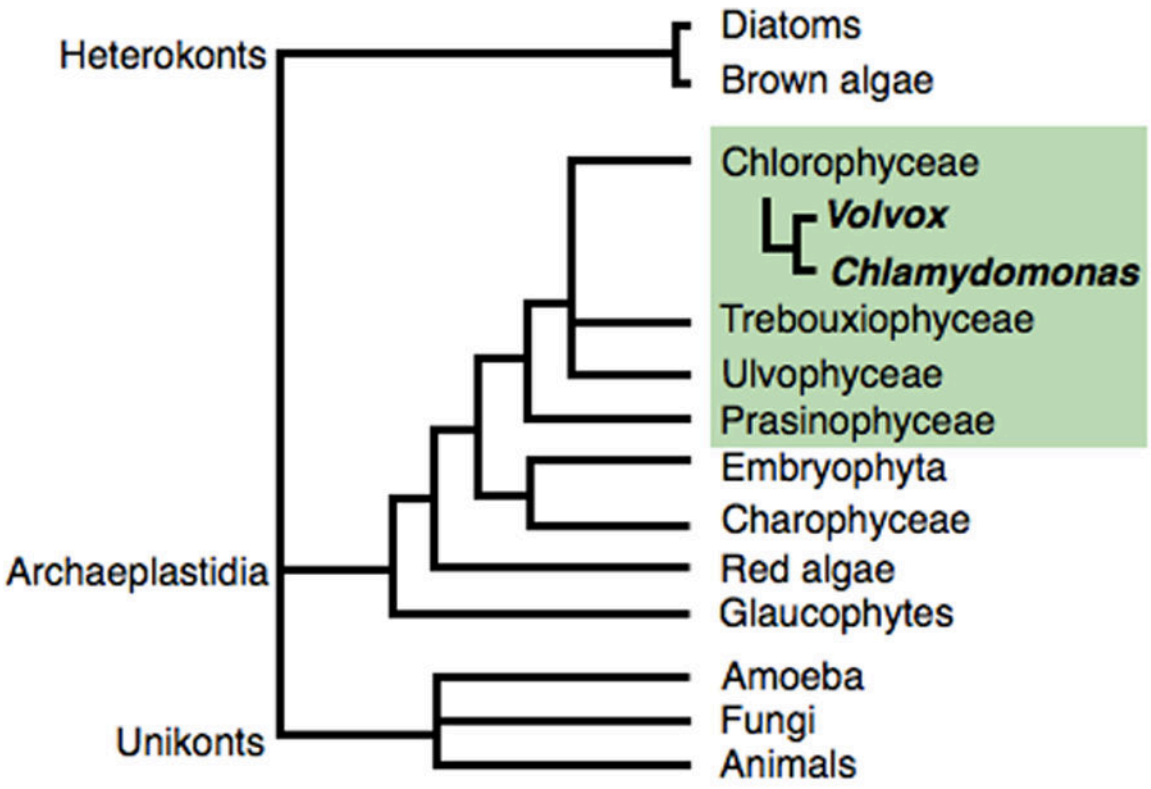

Figure 1. The phylogenetic relationship of the Volvocales to other algae, plants and eukaryotes

The Chlorophyceae, with C*hlamydomonas* and *Volvox* indicated, are members Chlorophyta, or green algae, one of the five major groups of Archaeoplastidia. The unrooted cladogram is adapted from (Baldauf, 2003).

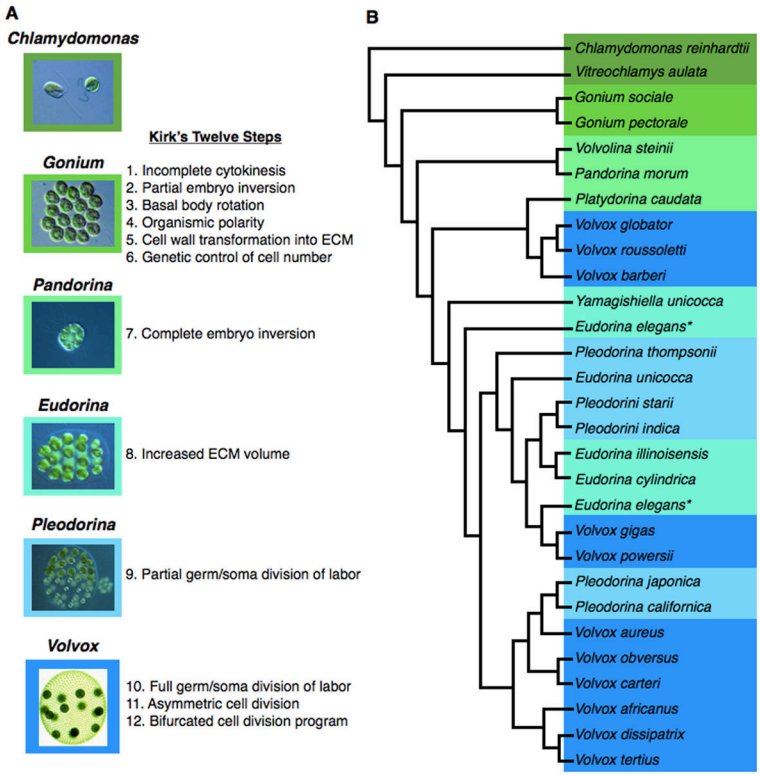

Figure 2. The morphology and phylogeny of the Volvocales

(A) Micrographs of Volvocine species and their relationship to each of David Kirk's twelve steps of multicellular evolution (Kirk, 2005). **(B)** Phylogeny of selected Volvocales adapted from (Nozaki, 2003; Kirk, 2005). Species are color-coded based on morphology as indicated in panel **(A)**. Asterisks indicate two isolates of *Eudorina elegans* that subsequently were reclassified as distinct species (Nozaki, 2003; Yamada et al., 2008).

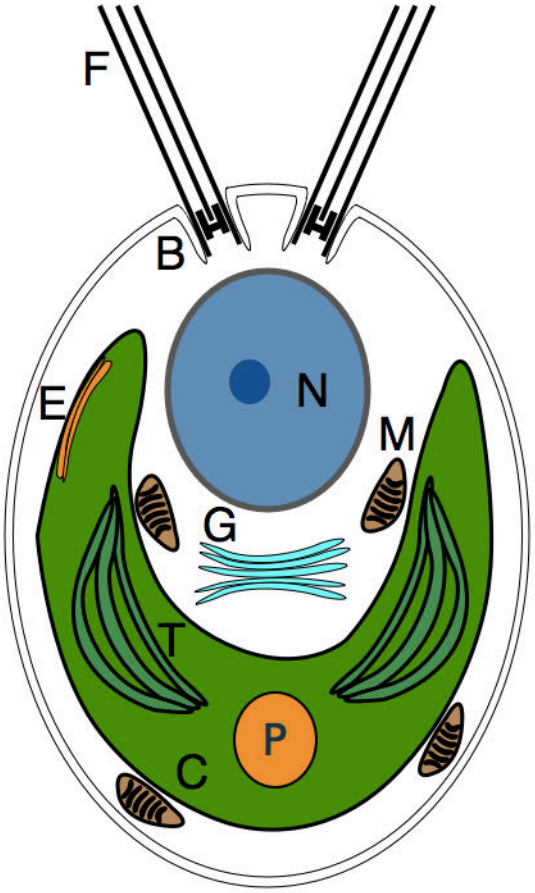

Figure 3. Cell structure of *Chlamydomonas reinhardtii*

Schematic representation of the subcellular components of a typical *Chlamydomonas* cell; flagella (F), basal body (B), nucleus (N), eye spot (E), golgi apparatus (G), mitochondria (M), chloroplast (C), thylakoids (T), and the pyrenoid (P).

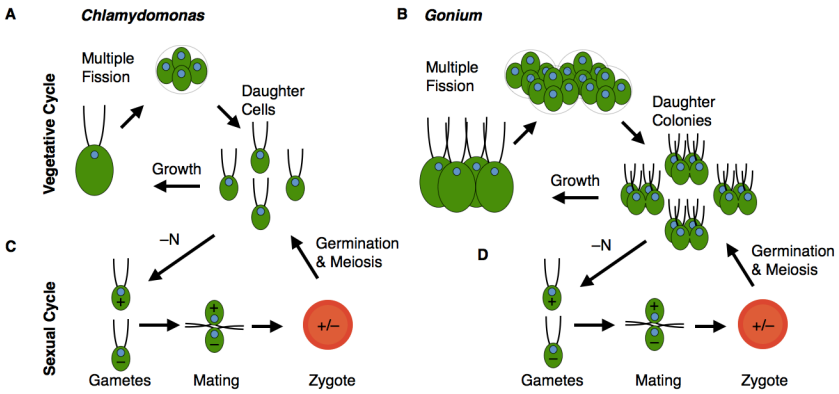

Figure 4. The multiple fission cell cycle and sexual cycles of *Chlamydomonas* and *Gonium*

During vegetative growth **(A)**, *Chlamydomonas* cells may grow many fold size with the extent of growth somewhat indeterminate. Cells then divide multiple times to produce uniform-sized daughters. Two rounds of cell division are shown in this panel, with division numbers ranging from one to four in a typical culture. *Gonium* colonies **(B)** follow a similar growth and division pattern as *Chlamydomonas*, but the daughter cells remain attached to each other through cytoplasmic bridges and ECM (see Fig. 6). The *Chlamydomonas* sexual cycle **(C)** is induced by lack of nitrogen (-N) that causes cells to differentiate into gametes. Gametes from each mating type (*plus* and *minus*) are similar in size. Flagellar adhesion between gametes of opposite mating type precedes cell fusion to form a diploid zygote. Upon germination four meiotic progeny are produced that can reenter the vegetative cycle. In *Gonium* –N also triggers gametogenesis that involves colony dissolution into unicellular gametes. Post-meiotic *Gonium* progeny are single cells that produce a new vegetative colony after their first passage through the cell cycle.

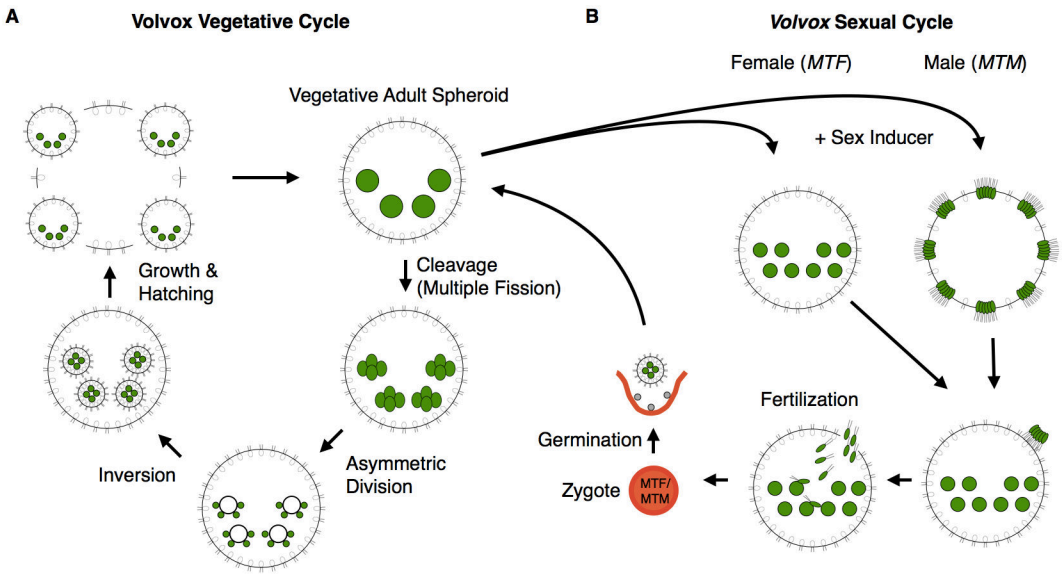

Fig. 5. Vegetative and sexual developmental in *Volvox*

(A) The *Volvox* vegetative life cycle for males and females is identical and begins with a mature adult (upper right) whose gonidia (large green cells) undergo cleavage to begin embryogenesis. During the sixth cleavage cycle asymmetric division occurs and leads to production of 16 large anterior cells that are destined to form the germ cells in the next generation. After a total of 12 cleavage cycles the ~2000-celled embryo undergoes inversion to place the gonidial precursors inside the spheroid and the flagella of the somatic cells pointing outside in their final adult configuration. After a period of growth the daughter spheroids hatch and continue to grow and mature into adults that can restart the vegetative cycle. **(B)** Sexual development begins when immature gonidia are exposed to sex-inducer protein. Their subsequent embryogenesis is then altered to produce egg-bearing females or sperm-packet-bearing males (Hallmann et al., 1998; Ferris et al., 2010). Females contain 32–48 eggs and ~2000 somatic cells, while males contain 128 somatic cells and 128 packets of 64–128 sperm. Sperm packets are released whereupon they swim as a single unit until they encounter a sexual female. Upon interacting with a sexual female the sperm packet dissolves into individual cells that enter the female ECM through a fertilization pore and swim until an individual sperm encounters and fuses with an egg to form a diploid zygote. When a *Volvox* zygospore germinates, only one of the four meiotic products survives and differentiates into a new vegetative spheroid.

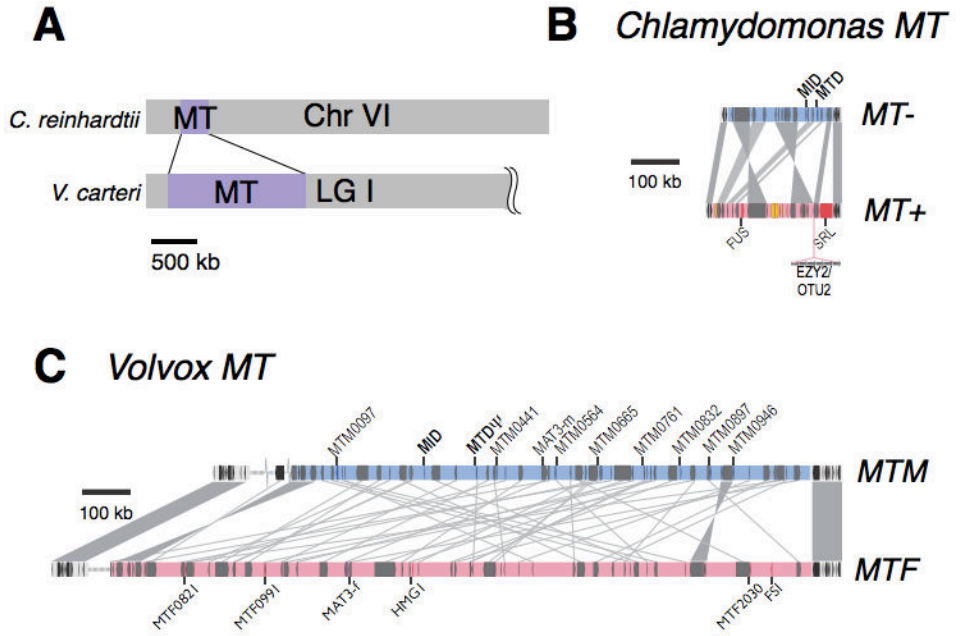

Figure 6. The mating-type loci of *Chlamydomonas* and *Volvox*

(A) The mating-type locus for both species is near the telomere of a syntenic chromosome (chromosome 6 in *Chlamydomonas*, linkage group I of *Volvox*). **(B)** *MT+* and *MT−* mating haplotypes of *Chlamydomonas*. Rearrangements between the two haplotypes are indicated by gray shading. Locations of the sex determining genes *MID* and *MTD1* and the sex limited gene gamete fusion gene *FUS1* are shown. Also indicated are the *EZY2/OTU2* regions that may be important for uniparental chloroplast inheritance (Ferris et al., 2002; Goodenough et al., 2007). **(C)** The *Volvox* male and female mating-type loci are about six times larger than *Chlamydomonas MT* and contain very little syntenic gene order between haplotypes. Several novel genes are shown as well as genes encoding homologs *MID*, *MTD*, and the retinoblastoma tumor suppressor homolog, *MAT3*. Figure adapted from (Ferris et al., 2010; Umen, 2011).

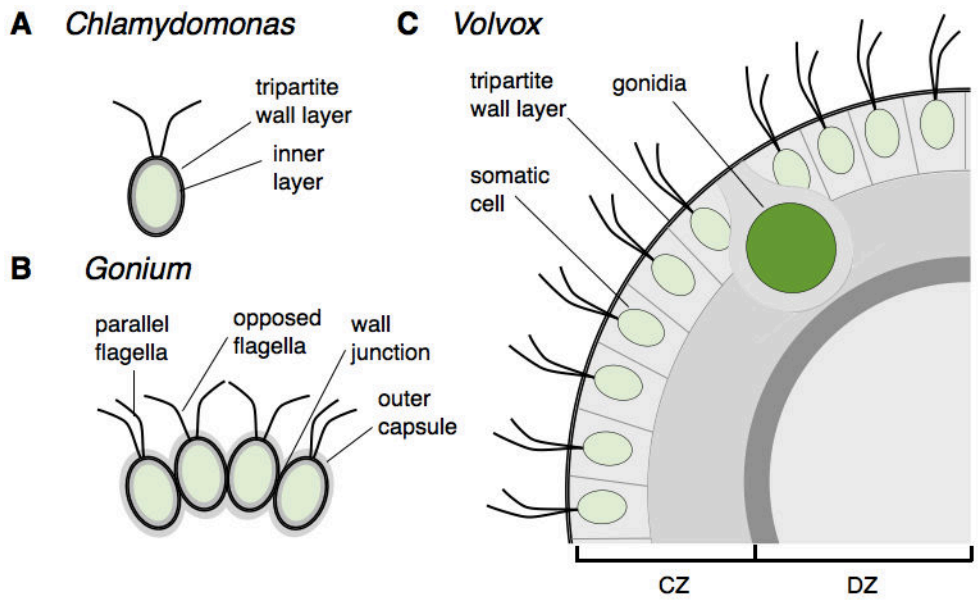

Figure 7. Comparison of the ECM structure of *Chlamydomonas*, *Gonium* and *Volvox*

The *Chlamydomonas* cell wall **(A)** is composed of an inner layer and an outer tripartite layer that is conserved with other Volvocales (indicated as a black layer around the cell). Individual *Gonium* cells **(B)** have an identical tripartite layer and the entire colony is surrounded by an additional outer capsule layer of ECM. Cells are held together by specialized attachments at their wall junctions. **(C)** *Volvox* cells are completely embedded in an expanded and compartmentalized ECM with a conserved tripartite boundary layer surrounding the entire colony (instead of individual cells). Inside the tripartite layer of the somatic cells are the cellular zones of ECM and the deep zone of the interior.

Table 1

Volvocales Culture Collections

Culture Collection	Species available	Media	Other	Accepts New Cultures?	Web Site
Chlamydomonas collection	*Chlamydomonas reinhardtii*, some other chlamydomonadaceae	Recipes and for sale	Plasmids cDNA libraries, Genomic libraries	Yes, all chlamydomonadaceae	http://chlamycollection.org/
UTEX	Some *Chlamydomonas*, many Volvocales, other Chlorophytes and protists	Recipes	Genomic DNA for PCR analysis	Yes, with prior approval	http://web.biosci.utexas.edu/utex/
SAG	Many Volvocales, many other *Chlorophytes* and Protists	References		Yes, with prior approval	http://epsag.uni-goettingen.de/cgi-bin/epsag/website/cgi/show_page.cgi?kuerzel=start
CCAP	Many Volvocales, many other *Chlorophytes* and Protists	Recipes and for sale		Yes, also accepts private collections	http://www.ccap.ac.uk/index.htm
NIES	A wide array of algal strains, extensive collection of the Volvocales	Recipes		Yes, with prior approval	http://mcc.nies.go.jp/

Table 2

Genomic Resources for *Chlamydomonas* and *Volvox*

Resource	Description	Reference(s)	URL
General Resources			
Chlamy.org	General landing page for all information about *Chlamydomonas*		http://www.chlamy.org
Chlamy Collection	Strain and plasmid stock center and general information about *Chlamydomonas*		http://www.chlamycollection.org
Sequences			
Phytozome	Latest assembly and annotations of the *Chlamydomonas* and *Volvox* genomes and comparison tools.		http://www.phytozome.net
JGI Genome Browser for *Chlamydomonas*	Version 4 of the *Chlamydomonas* genome including use annotations. All future assemblies will be deposited on Phytozome		http://genome.jgi-psf.org/Chlre4/Chlre4.home.html
Chlamydomonas reinhardtii minus mating type locus	GenBank Accession GU814015	Ferris, Olson et al. 2010	http://www.ncbi.nlm.nih.gov/nuccore/GU814015
Chlamydomonas reinhardtii plus mating type locus	GenBank Accession GU814014	Ferris, Olson et al. 2010	http://www.ncbi.nlm.nih.gov/nuccore/GU814014
Chlamydomonas reinhardtii chloroplast genome	GenBank Accession GU814014	Maul et al. 2002	http://www.chlamy.org/chloro/default.html
Chlamydomonas reinhardtii mitochondrial genome	GenBank Accession FJ423446	Ma et al. 1992	http://www.ncbi.nlm.nih.gov/nuccore/NC_001638
Chlamydomonas incerta EST database	GenBank Accessions GI106792973-GI106798104	Popescu et al. 1996	http://megasun.bch.umontreal.ca/pepdb/pep.html
JGI Genome Browser for *Volvox*	Version 1 of the Volvox carteri genome including user annotations. All future versions of the genome will be deposited on Phytozome		http://genome.jgi.doe.gov/Volca1/Volca1.info.html
Volvox carteri Female mating type locus	GenBank Accession GU784915	Ferris, Olson et al. 2010	http://www.ncbi.nlm.nih.gov/nuccore/GU784915
Volvox carteri Male mating type locus	GenBank Accession GU784916	Ferris, Olson et al. 2010	http://www.ncbi.nlm.nih.gov/nuccore/GU784916
Volvox carteri chloroplast genome	GenBank Accession GU084820.1	Smith and Lee 2009	http://www.ncbi.nlm.nih.gov/nuccore/GU084820.1
Volvox carteri mitochondrial genome	GenBank Accession EU760701	Smith and Lee 2009	http://www.ncbi.nlm.nih.gov/nuccore/EU760701
Libraries			
Chlamydomonas reinhardtii genomic BAC library			http://www.genome.clemson.edu
Volvox carteri Female genomic BAC libraries			http://www.genome.arizona.edu/
Volvox cateri Male genomic BAC libraries		Ferris, Olson et al. 2010	http://www.genome.clemson.edu/
Chlamydomonas EST database		Asamizu et al. 2004	http://est.kazusa.or.jp/en/plant/chlamy/EST/

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Resource	Description	Reference(s)	URL
Chlamydomonas 454 read database	2 Gb of 454 sequencing of Chlamydomonas cDNAs from various growth conditions		http://genomes.mcdb.ucla.edu/Cre454/
Functional Annotation and Expression Tools			
Algal Functional Annotation Tool	Comprehensive algal genomics, annotation and expression tool	Lopez et al. 2011	http://pathways.mcdb.ucla.edu/algal/index.html
GreenGenie2	Gene predictions for version 4 of the *Chlamydomonas* genome	Kwan et al 2009	http://stormo.wustl.edu/GreenGenie2/
ChlamyCyc	*Chlamydomonas* centric, integrated annotation tool that integrates with other related plant databases		http://chlamyto.mpimp-golm.mpg.de/chlamycyc/index.jsp
Plant Genome Database	Plant genome annotation comparison tool that includes *Chlamydomonas reinhardtii*		http://www.plantgdb.org/search/misc/
PLAZA	Tools for comparative plant genomics	Van Bel et al. 2012	http://bioinformatics.psb.ugent.be/plaza/
Proteomic Databases			
Chlamydomonas flagellar proteome			http://labs.umassmed.edu/chlamyfp/protector_login.php
Plant Transcription Factor Databases			
Plant Transcription Factor Database	Includes both *Chlamydomonas reinhardtii* and *Volvox carteri.* Version 2.0	Zhang et al. 2010	http://planttfdb.cbi.edu.cn/
Plant Transcription Factor Database	Includes *Chlamydomonas reinhardtii*. Version 3.0	Pérez-Rodríguez et al. 200	http://plntfdb.bio.uni-potsdam.de/v3.0/
Microarrays			
Chlamydomonas reinhardtii microarray	Agilent *Chlamydomonas reinhardtii* microarray, number 024664	Toepel et al. 2011	https://earray.chem.agilent.com/earray/
Chlamydomonas reinhardtii UNIGENE cDNA array	cDNA array based on the first *Chlamydomonas* UNIGENE set superseded by the Agilent arrays	Jain et al. 2007	
Chlamydomonas reinhardtii oligo array	Oligonucleotide array based on the first *Chlamydomonas* EST set superseded by the Agilent arrays	Eberhard et al. 2006	
Chlamydomonas sRNA Database			
SiLoDb sRNA Locus Database	Database of sRNA locations in the *Chlamydomonas* genome		http://silodb.cmp.uea.ac.uk/