

EVOLUTION OF DEVELOPMENTAL PROGRAMS IN *VOLVOX* (CHLOROPHYTA)¹

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The volvocine green algal genus *Volvox* includes ~20 species with diverse sizes (in terms of both diameter and cell number), morphologies, and developmental programs. Two suites of characters are shared among distantly related lineages within *Volvox*. The traits characteristic of all species of *Volvox*—large (>500) numbers of small somatic cells, much smaller numbers of reproductive cells, and oogamy in sexual reproduction—have three or possibly four separate origins. In addition, some species have evolved a suite of developmental characters that differs from the ancestral developmental program. Most multicellular volvocine algae, including some species of *Volvox*, share an unusual pattern of cell division known as palintomy or multiple fission. Asexual reproductive cells (gonidia) grow up to many times their initial size and then divide several times in rapid succession, with little or no growth between divisions. Three separate *Volvox* lineages have evolved a reduced form of palintomy in which reproductive cells are small and grow between cell divisions. In each case, these changes are accompanied by a reduction in the rate of cell division and by a requirement of light for cell division to occur. Thus, two suites of characters—those characteristic of all *Volvox* species and those related to reduced palintomy—have each evolved convergently or in parallel in lineages that diverged at least 175 million years ago (mya).

Key index words: cellular differentiation; development; evolution; palintomy; *Volvox*

Abbreviations: BF, Bayes factor; MCMC, Markov chain Monte Carlo; MRCA, most recent common ancestor; mya, million years ago; UTEX, University of Texas Culture Collection

The colonial volvocine algae include three families of haploid, facultatively sexual eukaryotes—Tetrabaenaceae, Goniaceae, and Volvocaceae—in the chlorophycean order Volvocales (Nozaki et al. 2000, Nozaki 2003a). Together with their close unicellular relatives in the genera *Chlamydomonas* and *Vitreochlamys*, members of this group span a great diversity of morphological forms, developmental programs, body sizes, and levels of complexity (Iyengar and Desikachary 1981, Ettl 1983, Nozaki 2003b).

Unicellular forms include *C. reinhardtii*, an important model organism for genetics, chloroplast biogenesis, and cell-cycle regulation. The smallest colonial forms are in the family Tetrabaenaceae (*Basichlamys* and *Tetrabaena*) and are made up of four *Chlamydomonas*-like cells held together in a common extracellular matrix. Members of the Goniaceae (*Gonium* and *Astrephomene*) range from 8 to 64 cells arranged in a flat or slightly curved plate (*Gonium*) or on the perimeter of a spheroid (*Astrephomene*). The diverse Volvocaceae (sensu Nozaki and Kuroiwa 1992) include seven genera (*Eudorina*, *Pandorina*, *Platydorina*, *Pleodorina*, *Volvox*, *Volvulina*, and *Yamagishiella*) ranging from 8 to 16 cells up to 50,000 cells. Members of this family are mostly spheroidal (with the exception of *Platydorina*) and may have complete, partial, or no division of labor between reproductive and somatic cells.

The largest members of the Volvocaceae, in the genus *Volvox*, have a large number (~500 to 50,000) of small somatic cells, which provide the flagellar action for the colony's motility, and a few much larger reproductive cells, or gonidia. In some cases, the division of labor between somatic and reproductive cells is complete, and reproductive cells do not contribute to motility. *Volvox* includes at least 18 recognized species, which display a diversity of sizes, patterns of development, and details of colonial organization. The great morphological and ontogenetic diversity within this small group (Starr 1970a) led to their classification in as many as six genera in the early 20th century (Shaw 1922). These genera were reduced by Printz (1927) to the rank of the taxonomic sections of a single genus, *Volvox*.

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Later, Smith (1944) reduced the number of sections from six to four (see Table 1). *Volvox*, like several other nominal genera of volvocine algae, is polyphyletic (Larson et al. 1992, Coleman 1999, Nozaki et al. 2000, Nozaki 2003a), but the correspondence between the sections and evolutionary lineages is imperfect. Although a number of *Volvox* ontogenetic traits have been used as taxonomic criteria (Smith 1944, Starr 1970b, Desnitskiy 1997), comparison with molecular data shows that some such characters have evolved convergently and are not reliable indicators of phylogeny.

Volvox, particularly *V. carteri*, has been developed as a model for the developmental genetics of multicellularity and cellular differentiation (Kirk 1998, 2001). Very few recent publications have dealt with the development of other representatives of this genus. Kirk (2005) defined a sequence of developmental changes, most stages of which are represented in extant volvocine species, that would have been required for a unicellular ancestor, presumably similar to *C. reinhardtii*, to evolve into a large, completely differentiated colony such as *V. carteri*. Herron and Michod (2008) traced the evolution of these characters through the volvocine phylogeny and concluded that some had complex histories with multiple origins and reversals. Here we apply a similar approach to the evolution of developmental characters found only in the genus *Volvox*.

Taking into account the size of mature gonidia, the rate of their division, and specific features of the formation of cell lineages, four programs (types) of asexual development of *Volvox* can be distinguished (Desnitski 1995). The first developmental program is characteristic of *V. powers-*

ii, *V. spermatozphaera*, *V. gigas*, and *V. pocockiae*. These species have large gonidia, a high rate of cell division, and no determination of cell fate by means of unequal (asymmetric) division. Their development is characterized by an unusual pattern of cell division known as palintomy, or rapid multiple fission. This term is sometimes used in the protistological literature (Sleigh 1989, Desnitski 1992, 1995) to designate the process during which a large parental cell undergoes a rapid sequence of repeated divisions without intervening growth. Other terms have been used to describe cell division in the volvocine algae, such as “schizogonous division” (Ettl 1981, 1988) and “sporulation” (Sluiman et al. 1989), but none of these terms is as pertinent to our work as “palintomy,” because, as we discuss below, not all species of *Volvox* are palintomic.

Outside of the genus *Volvox*, all colonial volvocine genera (e.g., *Gonium*, *Pandorina*, *Eudorina*, *Pleodorina*) are palintomic; they have large asexual reproductive cells, which divide rapidly and without asymmetric division (Coleman 1979). Therefore, all of these other colonial algae are characterized by the first developmental program. *Chlamydomonas* and other closely related unicellular volvocine algae are also characterized by rapid light-independent multiple fission (Spudich and Sager 1980). Such a palintomic asexual cycle closely resembles the first developmental program of *Volvox* (except, of course, for the absence of differentiation into two cell types).

The second program, characteristic of *V. carteri* and *V. obversus*, differs from the first program in having an asymmetric division, which forms presumptive reproductive and somatic cells of the next generation. The genetics of asymmetric division and

TABLE 1. *Volvox* species included in this study.

Species	Section	Developmental program	Asymmetric division	Slow division, light-dependent division	Small gonidia, growth between divisions	Maximum reported cell number
<i>V. africanus</i> G. S. West	<i>Merillosphaera</i>	2	Yes	No	No	6,000 ^d
<i>V. aureus</i> Ehrenb.	<i>Janetosphaera</i>	4	No	Yes	Yes	3,200 ^d
<i>V. barberi</i> W. Shaw	<i>Volvox</i>	4	No	Yes	Yes	47,800 ^d
<i>V. carteri</i> f. <i>kawasakensis</i> Nozaki	<i>Merillosphaera</i>	2	Yes	No	No	3,000 ^c
<i>V. carteri</i> f. <i>nagariensis</i> M. O. P. Iyengar	<i>Merillosphaera</i>	2	Yes	No	No	8,000 ^a
<i>V. carteri</i> f. <i>weismannia</i> (Powers) M. O. P. Iyengar	<i>Merillosphaera</i>	2	Yes	No	No	7,500 ^c
<i>V. dissipatrix</i> (W. Shaw) Printz	<i>Copelandosphaera</i>	4	No	Yes	Yes	31,800 ^d
<i>V. gigas</i> Pocock	<i>Merillosphaera</i>	1	No	No	No	3,000 ^c
<i>V. globator</i> L.	<i>Volvox</i>	4	No	Yes	Yes	22,000 ^a
<i>V. obversus</i> (W. Shaw) Printz	<i>Merillosphaera</i>	2	Yes	No	No	4,000 ^b
<i>V. rousseletii</i> G. S. West	<i>Volvox</i>	4	No	Yes	Yes	42,000 ^d
<i>V. tertius</i> Meyer	<i>Merillosphaera</i>	3	No	Yes	No	2,000 ^d

Sections are from Smith (1944) as modified by Kirk (1998); developmental programs are defined in Desnitski (1995). “Small gonidia” refers to the size of mature asexual reproductive cells (just before the onset of cleavage). Reported cell numbers vary among authors and are intended to illustrate the overall pattern rather than to provide precise values for natural and laboratory populations.

^aIyengar and Desikachary (1981).

^bKarn et al. (1974).

^cNozaki (1988).

^dSmith (1944).

^eVande Berg and Starr (1971).

that of the resulting determination of cell fate have been worked out in great detail for *V. carteri* forma *nagariensis* (Kirk 1998, 2001, Cheng et al. 2003, 2005, Kirk and Kirk 2004). Very little is known about the genetics of cell-fate determination in any other species of *Volvox*.

While the first and second programs are palintomic (without and with asymmetric division respectively), the third and fourth programs represent losses or reductions of characters associated with palintomy (Table 1). The third program is characteristic of *V. tertius*, in which gonidia are large (as in palintomic species), but the rate of cell division is low and division takes place only in the light. No asymmetric division takes place.

The fourth developmental program is characteristic of *V. aureus* and *V. globator* but is also probable for *V. dissipatrix* and the majority of other species. These species are characterized by small gonidia that divide slowly and without asymmetric division. In the cycle of asexual development of *V. carteri* and several other palintomic *Volvox* species, the interval between two consecutive gonidial divisions at 20°C–30°C is 1 h or less. However, in species with the fourth developmental program, the interval between two divisions occupies ~3–4 h, and cells grow measurably between divisions. This growth does not fully compensate a halving of cell volume during each division (i.e., cells still decrease in volume over subsequent divisions, but by <2-fold), so the origin of growth between divisions represents a reduction, rather than a loss, of palintomy (Desnitski 1992).

The first program, with large gonidia, fast division, and differentiation into two cell types without unequal division, is presumed to be ancestral. The second, third, and fourth programs are each distinguished by a unique combination of derived features: asymmetric division; slow, light-dependent divisions; and small gonidia that grow between divisions, respectively. In the case of *V. tertius*, only some palintomic traits are reduced; some features of its asexual development are similar to those of *V. aureus*, but the others are more similar to *V. carteri* (Desnitski 1995). It has been suggested (Kirk 1998) that from the viewpoint of contemporary cladistics, the first developmental program is symplesiomorphic (ancestral), the second synapomorphic (derived, shared by descent), the third autapomorphic (derived, unique), and the fourth homoplastic (derived, shared by convergence).

It has long been appreciated that the developmental programs resulting from reduction of palintomy (programs 3 and 4) are not found exclusively within a monophyletic group. However, the history of these characters has not previously been explicitly reconstructed in a phylogenetic context. Here, we investigate these characters in three poorly studied species and reconstruct their evolutionary history, as well as that of specialized reproductive cells and asymmetric division. In each case, we have

tested hypotheses about the number of times the change may have occurred and, accounting for phylogenetic uncertainty, assessed confidence levels of alternate states in particular ancestors.

MATERIALS AND METHODS

The same set of 50 species of colonial and unicellular volvocine algae analyzed in the previous work of Herron and Michod (2008) (including 10 representatives of the genus *Volvox*—see Table 1 including taxonomic authors) have been included in this research. We used previously published data on asexual reproduction of various species of *Volvox* (Smith 1944, McCracken and Starr 1970, Starr 1970a, Vande Berg and Starr 1971, Karn et al. 1974, Desnitskiy 1984, 1997, 2008, Desnitski 1992, 1995, Ransick 1993, Kirk 1998). We have obtained additional evidence on details of asexual reproduction in the cultures of *V. africanus* (UTEX 2907), *V. barberi* (Carolina Biological), and *V. rousseletii* (UTEX 1861). The algae have been routinely maintained at 20°C and a light:dark (L:D) regime of 16:8 (~35 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). To observe details of the segregation of cell lines during gonidial cleavage process, we made microphotographs of developing colonies of *V. africanus* using a digital camera (Moticam 2300, Motic Industries, China). The goal of experiments with *V. barberi* and *V. rousseletii* was to specify the rate of gonidial cleavage and the role of light for this process. In each species, 16–20 colonies with embryos at two- to eight-celled stages of cleavage have been taken from asynchronously growing cultures by means of a glass micropipette under the microscope. One half of the material was placed in the dark, and the other in the light. Eighteen to 20 h later, the colonies were checked under the microscope again.

To confirm species identification of *V. barberi*, we amplified and sequenced the chloroplast gene for the LSU of RUBISCO (*rbcl*) and region 2 of the nuclear ribosomal internal transcribed spacer (ITS2). DNA was phenol extracted as described in Nedelcu (2006). For amplification by PCR and sequencing of *rbcl*, we used primers *rbcl* 1-20F, *rbcl* 320-341F, *rbcl* 803-782R, *rbcl* 1181-1160R, and *rbcl* 1421-1402R, all from Nozaki et al. (1995), and *rbcl* upstream from Karol et al. (2001). For ITS2, we used G-FOR (Coleman and Vacquier 2002) and G-REV (Mai and Coleman 1997). The following conditions were used for the PCR reactions: 4 μL total genomic DNA, 2.5 units Eppendorf MasterTaq (Westbury, NY, USA) with 5x enhancer and 10x buffer, 0.2 mM dNTP mix, 0.1 mM each primer (50 μL reaction volume); initial denaturation at 94°C for 1 min; 35 cycles of denaturation at 94°C for 45 s, primer annealing at 43°C for 1 min, and extension at 72°C for 3 min; and a final extension at 72°C for 10 min.

We reconstructed the evolutionary histories of several traits. When two or more traits were present in the same set of extant taxa, we inferred their history as a single trait. We inferred ancestral character states using sum-of-squared-changes parsimony in Mesquite (Maddison and Maddison 2004) and using reversible-jump Markov chain Monte Carlo (MCMC) in BayesTraits (Pagel et al. 2004; program available at <http://www.evolution.rdg.ac.uk>). To explicitly account for phylogenetic uncertainty, we based the MCMC analyses on a sample of trees based on five chloroplast genes generated in a previous study (Herron and Michod 2008). The sample included every 1,000th postburn-in tree from each of four codon-partitioned MrBayes (Ronquist and Huelsenbeck 2003) runs, for a total of 600 trees (out of a total sample of 6×10^5 trees). Outgroup taxa were pruned from these trees for character state reconstructions.

Support for character state reconstructions was evaluated, considering uncertainties in both the phylogeny and the

process of character change, using a Markov chain model (Metropolis et al. 1953, Hastings 1970) in the Multistate package of BayesTraits (Pagel et al. 2004). The sample of trees described above was used for these reconstructions. Hypothesized character states at internal nodes were tested by estimating Bayes factors (BF) comparing MCMC runs in which the node in question was constrained to one state versus the other. Each BF was based on the difference between the highest harmonic mean log likelihood from three independent MCMC runs for each state.

We began Bayesian ancestral character state reconstructions with uniform priors on transition rates with a range of 0 to 100. We chose uniform, or uninformative, priors because they require the fewest assumptions, basing conclusions on the data rather than incorporating prior beliefs. Run lengths and burn-in periods were 10^7 and 10^6 generations, respectively, and transition probability distributions and effective sample size for all parameters were estimated in Tracer (Rambaut and Drummond 2003). In the cases of asymmetric division and specialized reproductive cells, the marginal posterior distributions appeared to be truncated, so the upper end of the prior range was reset to 200 in each of these cases, at which point no further truncation was observed. The effective sample sizes of all parameters exceeded 100 for all BayesTraits analyses. For complete differentiation, the presumed unicellular most recent common ancestor (MRCA) of *C. reinhardtii* and *V. carteri* f. *nagariensis* was constrained to the absence of the trait. Deviation of the normal distribution from which proposed parameter changes are drawn (RateDev command in BayesTraits) was adjusted by trial-and-error to achieve a mean acceptance rate between 0.28 and 0.32. For hypothesis tests, we used the same set of conditions (prior distribution, run length, burn-in) used in the ancestral character state reconstructions for the character being tested.

RESULTS

Our observations on *V. africanus* confirmed those of Smith (1944) that in this species (unlike many others) the gonidia do not begin dividing simultaneously (Fig. 1, A and B). Moreover, we corroborated Ransick's (1993) preliminary data that *V. africanus* is characterized by segregation of two cell lines due to an asymmetric division during the series of consecutive divisions (Fig. 1, C–E). Ransick's data are in a certain sense contradictory, though. In the text (p. 56), he indicated the similarity of early asymmetric division in *V. africanus*, *V. carteri*, and *V. obversus*. However, in his table 1 (p. 57), Ransick indicated the occurrence of asymmetric division in *V. africanus* during late cleavage only (i.e., considerably later than in the two other species). Our data suggest an explanation: the stage of cleavage during which asymmetric division occurs is inconsistent in *V. africanus*. In some embryos, gonidial initials are already formed at the 16-celled stage (Fig. 1, C and D), whereas many other embryos reach 64- and 128-celled stages (Fig. 1, A and B) without asymmetric division, which occurs at a later stage (as in Fig. 1E). In spite of these differences, it is now certain that *V. africanus*, like *V. carteri* and *V. obversus*, has the second program of asexual development, as defined by Desnitski (1995).

In *V. barberi* and *V. rousseletii*, on the other hand, gonidia divide slowly: an interval between two

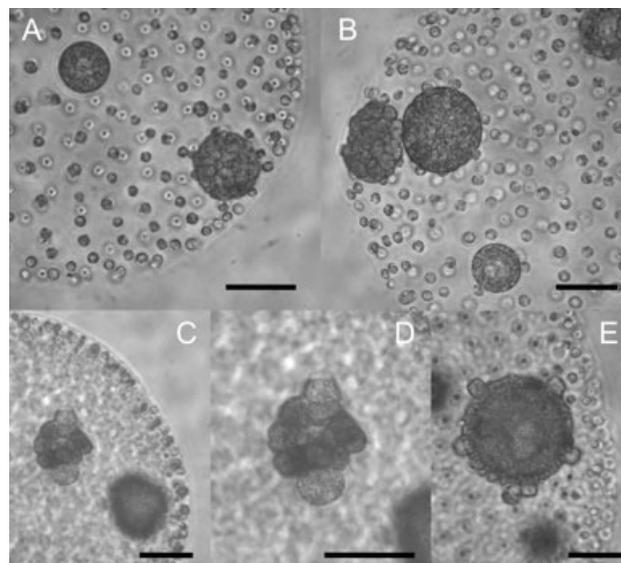
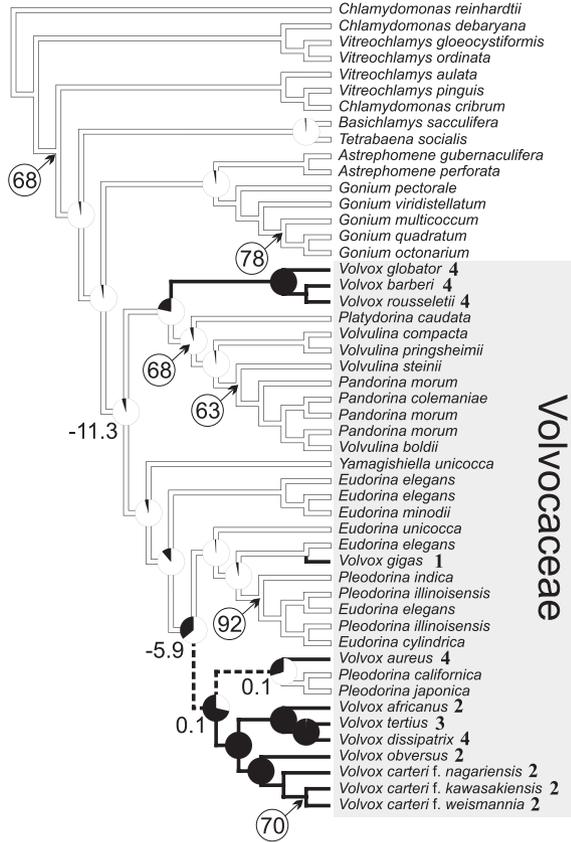


FIG. 1. *Volvox africanus* development (scale bars, 50 μ m). (A) Part of a *V. africanus* colony with a unicellular gonidium and an embryo at the 64-celled stage. No morphological differentiation into two cell types is visible in the embryo. (B) Part of a *V. africanus* colony with two unicellular gonidia at various stages of growth and a pair of cleaving embryos (left: the 64-celled stage; right: >100 cells). No morphological differentiation into two cell types is visible in these embryos. (C) Part of a *V. africanus* colony with an embryo at the 16-celled stage. Two large gonidial initial cells are visible, the result of an asymmetric division. Contrast enhanced. (D) Enlarged view of the embryo in (C). Contrast enhanced. (E) Part of a *V. africanus* colony with an embryo in the period of late cleavage. Large gonidial initial cells have formed by this stage as a result of recent asymmetric division.

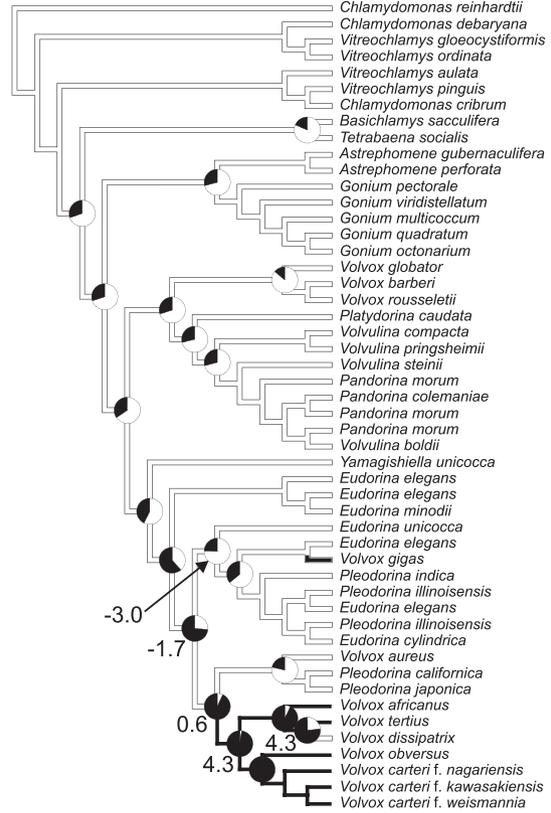
consecutive divisions is ~ 3 –4 h (on average, approximately five or six divisions pass during 18–20 h in the light). The process of cleavage rapidly stops in the darkness (usually no more than one division occurs). Therefore, these two species are characterized by the same asexual development traits as their close relative, *V. globator* (Desnitski 1995), and they also have the fourth program of development. The newly generated *V. barberi* ITS2 and *rbdL* sequences were identical to those of the UTEX 804 strain sequenced by Mai and Coleman (1997) and Nozaki et al. (1997), respectively, confirming that the Carolina Biological strain of *V. barberi* is correctly identified. The newly generated sequences were deposited in GenBank with accession numbers GQ253917 (ITS2) and GQ253918 (*rbdL*).

Inferred character state histories are shown in Figure 2. The traits characteristic of the genus *Volvox*—asexual forms with >500 cells, only a few of which are reproductive, and oogamy in sexual reproduction—have arisen at least three times independently: once in the section *Volvox* (represented by *V. globator*, *V. barberi*, and *V. rousseletii*), once in *V. gigas*, and once or possibly twice in the remaining *Volvox* species (Fig. 2A). Because the states of these characters in the common ancestor of *V. aureus* and

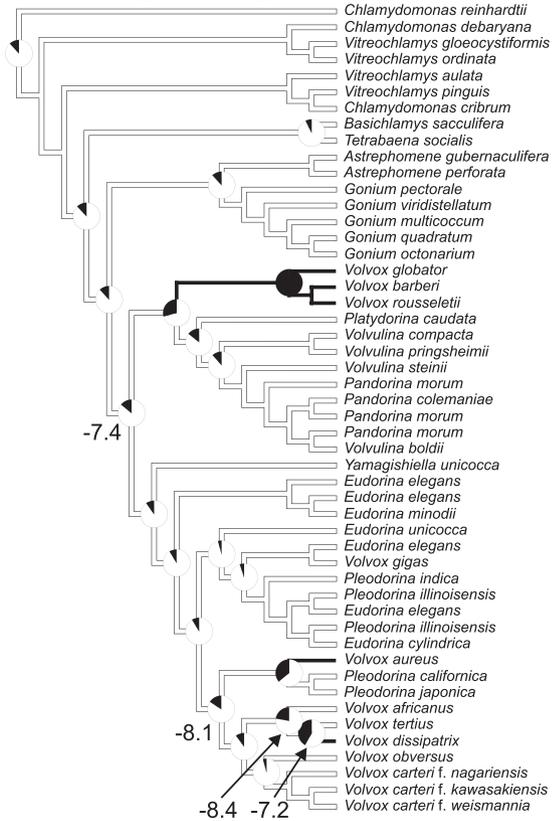
A. *Volvox*



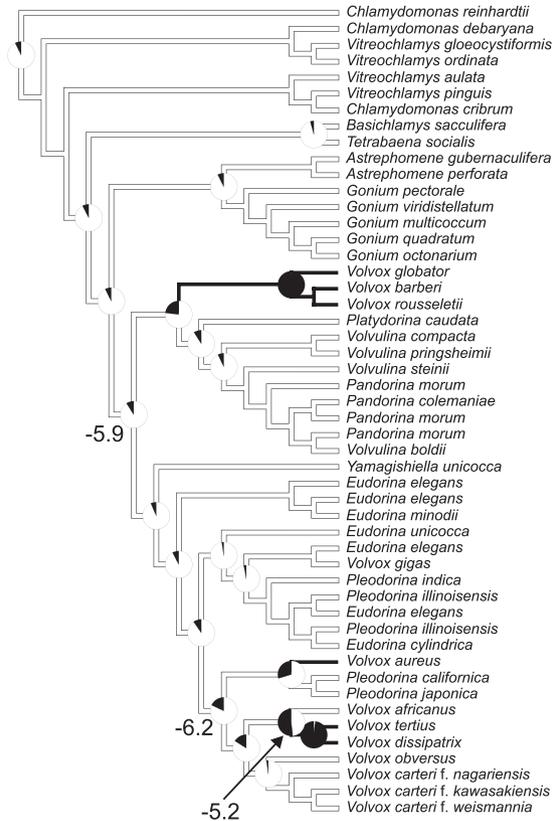
B. Specialized reproductive cells



C. Small gonidia, growth between divisions



D. Slow division, light-dependent division



E. Asymmetric division

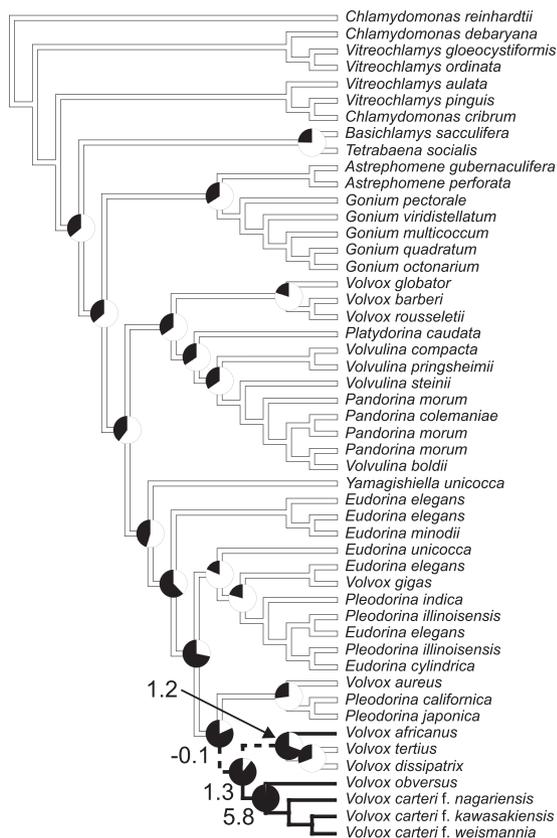


FIG. 2. Ancestral character states reconstructed on the 50% consensus cladogram from four independent Markov chain Monte Carlo (MCMC) runs (Herron and Michod 2008) in MrBayes (Ronquist and Huelsenbeck 2003). Branch shading indicates maximum-parsimony reconstruction (white = absent, black = present, dashed = ambiguous). Pie charts indicate Bayesian posterior probabilities at selected nodes. Numbers to the left of cladograms indicate log-Bayes factors at selected nodes: positive = support for trait presence, negative = support for trait absence. Interpretation of log-Bayes factors is based on Kass and Raftery's (1995) modification of Jeffreys (1961): 0 to 2, barely worth mentioning; 2 to 6, positive; 6 to 10, strong; >10, very strong. Boldface numbers following species names in (A) indicate *Volvox* developmental programs following Desnitski (1995). Circled numbers in (A) are Bayesian posterior probabilities (expressed as percentages) <95%.

(*V. africanus* + *V. tertius* + *V. dissipatrix* + *V. obversus* + *V. carteri*) are ambiguous, our analyses do not resolve whether *V. aureus* represents an independent origin of *Volvox*.

Specialized reproductive cells, which do not contribute to motility, are restricted to the members of section *Merillosphaera* (Table 1). This form of specialization arose separately in *V. gigas* and in the MRCA of the remaining members of the *Volvox* section *Merillosphaera* and was apparently lost in *V. dissipatrix* (Fig. 2B). Small gonidia and growth between divisions originated three times independently, in the lineages leading to section *Volvox*, to *V. aureus*, and to (*V. tertius* + *V. dissipatrix*) (Fig. 2C). The ancestral state (large gonidia without growth between divi-

sions) is strongly supported for the MRCA of *V. tertius* and *V. dissipatrix*. Slow division and light-dependent division also originated three times independently, in the lineages leading to section *Volvox*, to *V. aureus*, and to *V. dissipatrix* (Fig. 2D).

The reconstruction of asymmetric division is ambiguous for the MRCA of the three species with this trait (Fig. 2E). The maximum-parsimony analysis shows the state at this node as ambiguous: a single origin (with a loss in *V. tertius* + *V. dissipatrix*) and two origins (*V. africanus*, *V. obversus* + *V. carteri*) are equally parsimonious. The Bayesian MCMC analysis assigns a 90% probability of a single origin, but hypothesis testing provides only weak support (BF 1.3) for this scenario.

DISCUSSION

A shared name conceals a surprising diversity within the nominal genus *Volvox*: a range of >10-fold in diameter and nearly 100-fold in cell number, with a variety of morphological, ultrastructural, and developmental differences among species. In addition, divergences among *Volvox* species are the deepest even within a group badly in need of taxonomic revision, in which para- and polyphyletic genera are the norm. The most distantly related species of *Volvox* share no common ancestor more recent than that of the family Volvocaceae.

Two suites of traits are shared among distantly related lineages within the genus *Volvox*. All *Volvox* species have in common a large (>500) number of small, terminally differentiated somatic cells, a much smaller number of much larger reproductive cells, and oogamy in sexual reproduction (Nozaki 1996). These traits have evolved convergently (though not necessarily simultaneously) in at least three, and possibly four, lineages. If there were three origins of these traits (i.e., if the MRCA of *V. aureus* and *V. carteri* was *Volvox*-like), then these traits must have been lost in the lineage leading to *P. californica* and *P. japonica*, as suggested by Larson et al. (1992) and Nozaki et al. (2000) but later dismissed by Nozaki (2003a). Otherwise, *V. aureus* represents a fourth origin of the traits characteristic of *Volvox*. We were not able to conclusively support either of these scenarios, so the question of whether the ancestors of *P. californica* and *P. japonica* were *Volvox*-like remains unresolved.

Herron and Michod (2008) found that two characters, sterile somatic cells and large volume of extracellular matrix, had undergone reversals from derived to ancestral states in the volvocine algae. The strongly supported loss of specialized germ cells in *V. dissipatrix* means that this character can be added to this list of reversals, further undermining the view of volvocine evolution as a progressive increase in complexity. Herron and Michod (2008) took asymmetric division and the resulting early segregation of reproductive and somatic cells as the

criteria for complete differentiation, but from the point of view of division of labor, the most important criterion is whether reproductive cells contribute to motility. In all members of the section *Merillosphaera*, reproductive cells are specialized in this sense, in contrast to the partially differentiated members of the genera *Pleodorina*, *Astrephomene*, and the other *Volvox* sections (*Copelandosphaera*, *Janetosphaera*, and *Volvox*), in which the reproductive cells contribute to motility for part of the life cycle. In this study, we have therefore treated specialized reproductive cells, those that do not contribute to motility, separately from asymmetric division.

In addition to the traits characteristic of the genus *Volvox*, several other derived traits are shared among species that span the deepest division within the genus. Small gonidia that grow between divisions, cytoplasmic bridges, and slow, light-dependent cell divisions are found in members of the section *Volvox*, in *V. aureus*, and in *V. dissipatrix*, each of which clearly represents a separate origin of these traits. A single species prevents a perfect correspondence among these traits: *V. tertius* has slow, light-dependent division but lacks small gonidia, growth between divisions, and cytoplasmic bridges.

Thus, two suites of traits each have a perfect or near-perfect correspondence across at least three separate *Volvox* lineages: large numbers (>500) of cells always occur in species with high proportions of somatic cells and oogamous sexual reproduction, while small gonidia are always accompanied by growth between divisions and by slow, light-dependent division. The convergence of these traits is surprising in light of the distant relationships among the lineages involved: the extant species of *Volvox* last shared a common ancestor at least 175 mya (Herron et al. 2009).

Several previous authors have addressed the possible adaptive significance of the suite of traits that define the genus *Volvox*. The proportion of cells differentiated as soma increases as total cell number increases across species, and this is generally interpreted as a requirement for maintaining motility (Koufopanou 1994, Solari et al. 2006b). Gravity imposes an increasing cost to motility as colony size increases, requiring an increased investment into small, flagellated somatic cells. Another factor may be the need to keep the layer of water surrounding a colony stirred in order to maximize exchange of dissolved nutrients and gases across the colonial boundary (Solari et al. 2006a). Like motility, maintaining sufficient exchange becomes more of a problem as colony sizes increase and surface area: volume ratios decrease.

The ancestral, palintomic program of development (program 1) imposes an unavoidable trade-off. As there is little or no growth between successive rounds of cell division, essentially all of the initial cytoplasmic volume of a daughter colony must be contained in the reproductive cell from which it develops. Producing large offspring therefore

requires large reproductive cells, which require time and resources to grow to their final size. Reproductive cells start at the same size as somatic cells, so producing (for example) a 1,000-celled colony requires 1,000-fold growth. In addition, the very large reproductive cells required to produce colonies with thousands of cells are likely to encounter difficulties because of their low ratio of surface area to volume.

The second developmental program, characteristic of *Volvox carteri*, *V. obversus*, and *V. africanus*, avoids much of the need for growth through the mechanism of asymmetric division. Reproductive cells start much larger than somatic cells and therefore need to grow much less to reach their final size. In *V. carteri* f. *nagariensis*, for example, gonidia grow ~140-fold before they begin dividing, much less than the 2,000-fold growth that would be required if they started at the same size as somatic cells (Kirk 1998). This program has also been interpreted as an adaptation for mediating conflict among cells; by segregating the germ line earlier in development, species with program 2 reduce the opportunities for selfish mutations that break down the germ-soma division of labor (Michod 1996, Michod et al. 2003).

The presence of asymmetric division in *V. africanus* requires that this trait either evolved in parallel with the *V. carteri* + *V. obversus* lineage or was lost in the lineage leading to *V. tertius* and *V. dissipatrix*. It is unlikely that phylogenetic analysis alone will conclusively distinguish between these two possibilities. The molecular-genetic basis of this trait has been well characterized in *Volvox carteri* forma *nagariensis* (Kirk 1998, 2001, Cheng et al. 2003, 2005, Kirk and Kirk 2004). Similar analyses of *V. africanus*, *V. obversus*, and the other two forms of *V. carteri* might reveal whether the same genes are involved, possibly helping to resolve the number of times this trait has originated.

The fourth developmental program, characteristic of *V. aureus*, *V. dissipatrix*, and the members of section *Volvox*, avoids the surface area:volume scaling problems that affect very large cells by growing between cell divisions. This allows members of these species to produce offspring with large numbers of cells (and large total volumes of cytoplasm) from small gonidia.

Volvox tertius, the sole species with the third developmental program, differs from palintomic species in having slow, light-dependent cell division, but it has retained the ancestral traits of large gonidia that do not grow between divisions. The strongly supported absence of small gonidia (and, therefore, the absence of growth between divisions) in the MRCA of *V. tertius* and *V. dissipatrix* suggests that, in this lineage at least, the fourth developmental program was derived from the third (i.e., that the evolutionary origin of slow, light-dependent cell division preceded the reduction of gonidial size and the insertion of growth between consecutive divisions). The possibility of extinct species with the

third program in other lineages makes it impossible to say whether the other instances of the fourth developmental program went through a similar intermediate step or if they were derived directly from the first (palintomic) program.

Unlike asymmetric division, very little is known about the molecular-genetic basis of the characters associated with a reduction of palintomy. For example, we do not know whether the genes causing reduction in the size of gonidia, growth between divisions, reduced rate of cell division, and light-dependence of cell division are the same in the three lineages in which these changes have occurred. Application of the techniques worked out by the labs of D. Kirk and R. Schmitt over the last four decades for *V. carteri* f. *nagariensis*, as well as molecular techniques that have become available more recently, might yield surprising insights on the causes, mechanisms, and history of ontogenetic change if applied to representatives of these related lineages.

In conclusion, two suites of characters have evolved convergently or in parallel in at least three lineages of *Volvox*. The members of section *Volvox*, *V. gigas*, and the remaining *Volvox* species represent three separate origins of the traits that define the genus (>500 cells, high proportion of soma, and oogamy), and *V. aureus* may or may not represent a fourth origin. Four characters associated with a partial loss of the ancestral, palintomic program of cell division (small gonidia, growth between divisions, reduced rate of cell division, and light-dependent cell division) have evolved convergently or in parallel at least three times (in the lineages leading to section *Volvox*, to *V. aureus*, and to *V. dissipatrix*). In both cases, the lineages in which these traits are shared last shared a common ancestor at least 175 mya.

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