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EVOLUTION OF COMPLEXITY IN THE VOLVOCINE ALGAE: TRANSITIONS IN INDIVIDUALITY THROUGH DARWIN'S EYE

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The transition from unicellular to differentiated multicellular organisms constitutes an increase in the level complexity, because previously existing individuals are combined to form a new, higher-level individual. The volvocine algae represent a unique opportunity to study this transition because they diverged relatively recently from unicellular relatives and because extant species display a range of intermediate grades between unicellular and multicellular, with functional specialization of cells. Following the approach Darwin used to understand "organs of extreme perfection" such as the vertebrate eye, this jump in complexity can be reduced to a series of small steps that cumulatively describe a gradual transition between the two levels. We use phylogenetic reconstructions of ancestral character states to trace the evolution of steps involved in this transition in volvocine algae. The history of these characters includes several well-supported instances of multiple origins and reversals. The inferred changes can be understood as components of cooperation–conflict–conflict mediation cycles as predicted by multilevel selection theory. One such cycle may have taken place early in volvocine evolution, leading to the highly integrated colonies seen in extant volvocine algae. A second cycle, in which the defection of somatic cells must be prevented, may still be in progress.

KEY WORDS: ***Chlamydomonas*, *Gonium*, multicellularity, multilevel selection, *Pleodorina*, Volvocaceae, *Volvox***

The four billion or so years during which life has existed on Earth have seen some millions of speciation events and innumerable instances of evolutionary change within populations. In contrast, only a few dozen examples are known in which such changes have led to the reorganization of the very units of adaptation and evolution. These reorganizations, in which groups of previously existing evolutionary individuals form a new kind of individual, include the transitions from groups of replicating molecules to prokaryotic cells, from associations of prokaryotic cells to eukaryotic cells, from groups of cells to multicellular organisms, and from individual organisms to societies. Collectively, these transitions have been called changes in the level of complexity (Maynard Smith 1988), transitions in the units of fitness (Michod and Roze 1999), and evolutionary transitions in individuality (ETIs; Buss 1987; Maynard Smith and Szathmáry 1995; Michod 1996, 1997; Michod and Roze 1997).

One of the best-studied ETIs is that from unicellular organisms to differentiated multicellular organisms in the volvocine green algae (Chlorophyta) (Kirk 1998, 2005; Solari 2005; Nedelcu and Michod 2006; Solari et al. 2006a). The volvocine algae are biflagellated, photosynthetic, facultatively sexual, haploid eukaryotes, comprising both unicellular species (*Chlamydomonas* and *Vitreochlamys*), and colonial forms with widely varying colony sizes, colony structures, and degrees of cellular specialization (see Fig. 4). Except in discussions of individuality, we will refer to any species with more than one cell as "colonial" (see Kirk 1998 pp. 115–116 for a discussion of terminology). Among the colonial forms, organization can be as simple as clumps of four *Chlamydomonas*-like cells that fail to separate after cytokinesis (*Basichlamys*, *Tetrabaena*). Colonial forms comprising 8–32 undifferentiated cells can be organized as flat or slightly curved sheets in a single layer (*Gonium*), as spheroidal colonies

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(*Pandorina, Volvulina, Eudorina* and *Yamagishiella*), or as a flat double layer of undifferentiated cells (i.e., a flattened sphere; *Platydorina)*. Larger colonies, consisting of 32–128 cells, are all spheroidal and exhibit both undifferentiated (*Chlamydomonas*-like) and terminally differentiated (somatic) cells (*Pleodorina* and *Astrephomene*). The largest forms—up to up to 50,000 cells (various *Volvox* species)—are spherical and consist mostly of somatic cells and a much smaller number of large germ cells.

One member of this lineage, *Volvox carteri* forma *nagariensis*, has been developed as a model system for the evolution of multicellularity and cellular differentiation (Kirk 1998). Like other major groups of multicellular eukaryotes, *V. carteri* begins development as a single cell and exhibits determinate development and functional specialization of cell types. In addition, *V. carteri*'s close relatives, described above, represent several intermediate grades between unicellular and truly multicellular, with functional specialization of cells.

The unicellular to multicellular transition, like the other transitions described above, constitutes an increase in the level of complexity (Maynard Smith 1988). In each case, previously existing individuals are combined to form a new, more inclusive, higher-level individual. The new individual must contain all of the complexity of the previously existing individuals of which it is composed as well as many new properties (such as the spatial relationship of the components and the structure that holds them together). How can such evolutionary jumps in the levels of complexity and individuality come about?

When confronted with a similar challenge, that of understanding "organs of extreme perfection" such as the human eye, Darwin (1872) reasoned that in the evolution of a complex trait there must be a series of small steps, each advantageous in itself, leading to the more complex state. This approach of deconstructing the complex into the simple is now commonplace in evolutionary biology, but it has never been done in sufficient detail for any of the specific cases of the ETIs mentioned above. In "A twelve-step program for evolving multicellularity and a division of labor," Kirk (2005) identified a number of morphological and developmental changes that must have occurred between the most recent unicellular ancestor of the colonial volvocines and *V. carteri*. By identifying a series of plausibly small steps that cumulatively span the transition between a unicellular ancestor and the differentiated, multicellular *Volvox*, Kirk has set up a gradualist framework in which an ETI can occur. As in Darwin's example of the eye, many of the stages in the transition are reflected in the traits of extant species, demonstrating their plausibility.

Retaining Kirk's original numbering, the proposed steps are (Table 1): (1) incomplete cytokinesis, which creates cytoplasmic bridges among cells and is presumably involved in holding the cells of colonial forms together; (2) incomplete inversion of the embryo, by which *Gonium* colonies change shape so that the flagella are on the convex side; (3) rotation of the basal bodies, which causes the flagella of a given cell to beat in the same direction; (4) establishment of organismal polarity, center-edge in the case of *Gonium* and anterior–posterior in spheroidal colonies; (5) transformation of cell walls into an extracellular matrix (ECM), a presumed homology between the outer and inner cell walls of *Chlamydomonas* and volvocalean colonial boundaries and ECM, respectively; (6) genetic modulation of cell number, which determines the maximum number of cells in a colony; (7) complete inversion of the embryo, a gastrulation-like process by which spheroidal colonies invert, moving their flagella from inside to outside; (8) increased volume of ECM, which makes up most of the volume of large volvocine colonies; (9) partial germ-soma division of labor, defined by the appearance of sterile somatic cells; (10) complete germ-soma division of labor, defined by soma and specialized, nonflagellated germ cells; (11) asymmetric cell division, which occurs during the development of *V. carteri* and *V. obversus*; and (12) bifurcation of the cell division program, which determines the fate (somatic or reproductive) of cells in these two species based on asymmetric division.

Table 1. Derived character states and the taxa in which they are present. Numbers are from Kirk (2005).

	Character	Taxa
1	Incomplete cytokinesis	Volvocaceae+Goniaceae
2	Partial inversion of the embryo	*Gonium*
3	Rotation of the basal bodies	Volvocaceae+Goniaceae
4	Establishment of organismal polarity	Volvocaceae+Goniaceae
5	Transformation of cell walls in to ECM	Volvocaceae+Goniaceae+ Tetrabaenaceae
6	Genetic modulation of cell number	Volvocaceae+Goniaceae+ Tetrabaenaceae
7	Complete inversion of the embryo	Volvocaceae
8	Increased volume of ECM	Goniaceae+Volvocaceae (except *Pandorina*, *Eudorina cylindrica*)
9	Partial germ/soma division of labor	*Astrephomene*, *Pleodorina*, *Volvox* (except *V. carteri*, *V. obversus*)
10	Complete germ/soma division of labor	*Volvox carteri*, *V. obversus*
11	Asymmetric division	*Volvox carteri*, *V. obversus*
12	Bifurcated cell division program	*Volvox carteri*, *V. obversus*

In reconstructing the history of these 12 steps, Kirk (2005) used the "volvocine lineage hypothesis" as a first-order

Table 2. Models selected for data partitions. GTR=general time reversible (Rodriguez et al. 1990); SYM (Zharkikh 1994) is a restriction of the GTR model with equal base frequencies. I=proportion of invariant sites; G=gamma distribution of among-site rate variation (Yang 1993).

Partition	Number of taxa	Number of characters	AIC Model
all	124	6021	GTR+I+G
all position 1	124	2007	GTR+I+G
all position 2	124	2007	GTR+I+G
all position 3	124	2007	GTR+I+G
atpB	91	1128	GTR+I+G
atpB position 1	91	376	GTR+I+G
atpB position 2	91	376	GTR+I+G
atpB position 3	91	376	GTR+I+G
psaA	63	1491	GTR+I+G
psaA position 1	63	497	GTR+I+G
psaA position 2	63	497	GTR+I+G
psaA position 3	63	497	GTR+I+G
psaB	86	1494	GTR+I+G
psaB position 1	86	498	GTR+I+G
psaB position 2	86	498	GTR+I+G
psaB position 3	86	498	GTR+I+G
psbC	63	780	GTR+I+G
psbC position 1	63	260	GTR+I+G
psbC position 2	63	260	SYM+I+G
psbC position 3	63	260	GTR+I+G
rbcL	121	1128	GTR+I+G
rbcL position 1	121	376	GTR+I+G
rbcL position 2	121	376	SYM+I+G
rbcL position 3	121	376	GTR+I+G

approximation of volvocine phylogeny (see, for example, his figure 6). The evolution of volvocine algae has often been viewed in the framework of this hypothesis, which holds that the members of this group represent a progressive increase in size and complexity from unicellular *Chlamydomonas* to *Volvox* and that the phylogeny of the group reflects this progression (Lang 1963; Van de Berg and Starr 1971; Pickett-Heaps 1975). Although it was not a part of the original formulation, this hypothesis is often interpreted as implying that ancestral species in the lineage leading to *V. carteri* were morphologically and developmentally similar to extant volvocines of intermediate size and complexity (e.g., Nozaki and Itoh 1994, Nozaki et al. 1999, 2000; Kirk 2005). The volvocine lineage hypothesis is known to be a simplified picture of volvocine phylogeny (Larson et al. 1992; Kirk 1998; Nozaki et al. 2000; Nozaki 2003), and it is therefore likely that the history of morphological and developmental changes for the entire lineage is more complicated than those in the single lineage leading to *V. carteri* would indicate.

The steps Kirk has outlined constitute a detailed account of the cellular, and in some cases molecular, changes by which some volvocine algae underwent an ETI from unicellular to differentiated multicellular forms. In so doing he has effectively shown that the seemingly difficult and complicated transition from one level of complexity to another can be reduced to a modest number of changes, each conceivably adaptive by itself. The order in which these changes occurred, though, can only be inferred in an explicit phylogenetic context. The main goal of our article is to understand the history of these changes and how they relate to changes in the level of complexity in the volvocine green algae.

Here we reconstruct the evolutionary history of the characters identified in Kirk's 12 steps (2005) using phylogenetic inference of ancestral character states. In so doing, we hope to address several questions related to the evolution of multicellularity: (1) How many times has each of the identified changes in character state occurred? (2) Is there any evidence of reversals, that is, that derived character states have reverted to ancestral ones? (3) Are there identifiable characters (preadaptations) in the ancestors of colonial volvocines that may have made this lineage, among hundreds of related lineages, likely to evolve the kind of differentiated multicellularity exhibited by *V. carteri*? (4) Is there any evidence of constraints, that is, that some changes necessarily precede others? (5) Which of these characters and changes can be understood in terms of existing theory regarding ETIs?

To address these questions, we examined phylogenetic relationships within the volvocine algae using previously published nucleotide sequences from five chloroplast genes. We used the resulting phylogenetic hypotheses to infer ancestral character states for nodes of interest and to trace the evolution of characters related to the evolution of multicellularity. By accounting for uncertainty in the phylogenetic reconstruction and in models of character state change, we provide, for the first time in this group, estimates of confidence levels for inferred ancestral character states.

Numerous studies of volvocine phylogeny, based on both morphological and molecular characters, have been published (e.g., Larson et al. 1992; Buchheim et al. 1994; Nozaki et al. 1995, 1999, 2000, 2002; Coleman 1999; Nozaki 2003), and the present study will add little new information on the evolutionary relationships among taxa. However, we chose to infer a phylogeny ourselves, rather than base our analyses on a previously published phylogeny, to include as many relevant taxa as possible in a single tree. This goal might also have been achieved by using supertree methods to combine published trees, but generating our own trees using Bayesian methods had the additional benefit of generating a large sample of trees that could be used to explicitly account for phylogenetic uncertainty in ancestral character state reconstructions.

Materials and Methods

PHYLOGENETIC ANALYSES

We based our phylogenetic analyses on previously published nucleotide sequences of five chloroplast genes (See online Supplementary Appendix S1): beta subunit of ATP synthase (atpB), P700 chlorophyll a-apoprotein A1 (psaA), P700 chlorophyll a-apoprotein A2 (psaB), photosystem II CP43 apoprotein (psbC), and large subunit of Rubisco (rbcL). Because they are propagated clonally, the members of a given strain were treated as the same genetic individual, allowing us to combine sequence data from different genes even when the nominal species included multiple lineages. Because the sister taxon to the colonial volvocines is not known with certainty, we included a large number of related unicellular taxa, with *Chlorella vulgaris* as the ultimate outgroup (See online Supplementary Appendix S2).

We estimated phylogenetic relationships using Bayesian Markov chain Monte Carlo analysis (MCMC) in MrBayes version 3.0b4 (Ronquist and Huelsenbeck 2003) with vague priors and model parameters estimated as part of the analyses as per the program's defaults. We used three heated chains and a single cold chain in all MCMC analyses, and initiated runs with random trees. The combined results of four independent Bayesian analyses of 2×10^7 generations each were used to generate consensus trees and estimate posterior probabilities (PP). We sampled trees every 100 generations and assembled majority rule consensus cladograms and calculated posterior probabilities for nodes from all sampled post burn-in trees. DNA sequences from the chloroplast genome of *C. vulgaris* (GenBank accession number NC001865) were used for outgroups in all analyses. Convergence of posterior probability estimates among runs was tested by comparing posterior probability estimates from subsets of the sampled trees using the "comparetrees" function in MrBayes. Runs were considered to have adequately sampled solution space if posterior probability estimates were 95% correlated between each pair of runs (Smith and Farrell 2005).

We chose a partitioning strategy based on the approach outlined in Brandley et al. (2005). Partitions were based on gene identity and codon position. In all, 34 partitioning strategies were tested: all data combined, partitioned by codon position, partitioned by gene, and all 31 combinations partitioned by gene with one or more genes partitioned by codon. The strategies combining partitioning by gene and by codon position were denoted by five digit binary codes in which each digit represents a gene (in order, atpB, psaA, psaB, psbC, and rbcL), with a "1" indicating that the gene in question was partitioned by codon position. For example, the partitioning strategy represented by "01000" includes the following partitions: atpB, psaA position 1, psaA position 2, psaA position 3, psaB, psbC, and rbcL.

For each of the 34 partitioning strategies tested, we conducted four independent Bayesian analyses of 2×10^7 generations each (5×10^6 generations burn-in). We chose this run length because shorter runs (with correspondingly shorter burn-in periods) failed to reach stationarity by the end of the burn-in period. We chose the partitioning strategy for use in further analyses by estimating Bayes factors (BF; Jeffreys 1961) as twice the difference in the logarithm of harmonic mean likelihood. A Bayes factor threshold of 10 performs well in model selection for Bayesian phylogenetic analysis (Brown and Lemmon 2007).

The model used for each partition was chosen using Akaike information criterion (AIC; Akaike 1974) implemented in MrModelTest (Nylander 2002). A substitution model was inferred for each of the 24 partitions: all data combined, three codon positions, five genes, and three codon positions for each of five genes (Table 2). Likelihood scores for all partitions were estimated using a single initial tree inferred for the entire dataset using neighbor joining with the JC model (Jukes and Cantor 1969).

Because sequence data for all five genes were not available for all taxa, we independently analyzed a dataset consisting only of those taxa for which all five gene sequences were available (See online Supplementary Appendix S1). The results of this analysis were compared to those from the full dataset to evaluate the robustness of our analyses to missing data.

ANCESTRAL CHARACTER STATE RECONSTRUCTION

Character state data were compiled from published reports (See online Supplementary Appendix S3). We inferred ancestral character states using sum-of-squared-changes parsimony in Mesquite (Maddison and Maddison 2004) and using MCMC in BayesTraits (Pagel et al. 2004; program available at www.evolution.rdg.ac.uk). Support for character state reconstructions was evaluated, considering uncertainties in both the phylogeny and the process of character change, using a Markov chain model (Ronquist 2004). For characters that are only feasible for colonial taxa (inversion, differentiation, and expanded ECM), the presumed unicellular most recent common ancestor (MRCA) of *C. reinhardtii* and *V. carteri* f. *nagariensis* was constrained to the absence of the trait. Hypothesized character states at internal nodes were tested by estimating BF comparing MCMC runs in which the node in question was constrained to one state versus the other. Each Bayes factor was based on the difference between the highest harmonic mean log likelihood from three independent MCMC runs for each state.

To explicitly account for phylogenetic uncertainty, we based the comparative analyses on a subsample of the trees sampled during the MCMC runs. The subsample included every 1000th post-burn-in tree from each of the four codon-partitioned MrBayes runs, for a total of 600 trees (out of a total sample of 6×10^5 trees). The adequacy of the subsample as a representation of the overall sample was evaluated by comparing the 95% majority rule consensus tree of the subsample to that of the overall sample. Outgroup taxa were pruned from these trees for character state reconstructions.

We used uniform priors, ML priors, and gamma-distributed hyperpriors seeded from a uniform distribution for Bayesian ancestral character state reconstructions. Uniform priors and gamma-distributed hyperpriors were initially based on a range of 0 to 100, and in cases in which transition probability distributions were truncated, the upper end of this range was doubled successively until transition probability distributions were not truncated. Run lengths and burn-in periods were started at 5×10^6 and 10^4 generations, respectively, and both were doubled until the effective sample size for all parameters (estimated in Tracer; Rambaut and Drummond 2003) exceeded 100. 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

PHYLOGENETIC ANALYSES

Comparison of BF (Fig. 1) led us to employ the partitioning strategy identified as "codon" – one partition for each codon position across the entire alignment (three total partitions), with a separate GTR+I+Γ model for each partition: a general time reversible model (Tavaré 1986) with a proportion of sites invariant and a gamma distribution of among-site rate variation (Yang 1993). All four independent MCMC runs for the full dataset converged with regard to chain likelihood scores and topology (50% majority rule consensus identical among runs). Posterior probability estimates for clade support were at least 98% correlated in each of the six pairwise comparisons between runs by the end of the burn-in period. Estimates of substitution model parameters for all four independent runs also converged at stationarity. Based on this evidence for convergence among runs, we pooled all post burn-in trees (representing a total of 6×10^7 post burn-in generations) to estimate the posterior probabilities for clades. The 95% consensus tree from the reduced dataset (including only taxa for which all five gene sequences were available) did not contradict, in any case, the 95% consensus tree from the full dataset, although some nodes resolved in one analysis were not resolved in the other.

The consensus cladogram of 6×10^5 post burn-in trees (pooled from four MrBayes runs) is shown in Figure 2. Several unicellular species in the genera *Chlamydomonas* and *Vitreochlamys* are more closely related to the colonial forms, with high support (PP 1.0), than is *C. reinhardtii*. The colonial forms are monophyletic, as is each of the three colonial families (Tetrabaenaceae, Goniaceae, and Volvocaceae). Goniaceae is well supported as the sister clade to Volvocaceae. However, every nominal genus that is not monotypic (*Eudorina*, *Pandorina*, *Pleodorina*, *Volvox*, and *Volvulina*) is para- or polyphyletic. In addition, several nominal species, notably *P. morum* and *E. elegans*, appear to represent convergent morphologies rather than monophyletic relationships.

ANCESTRAL CHARACTER STATES

Inferred character state histories are shown in Figure 3, and changes in character states are summarized in Figure 4. Several of the characters in question are found exclusively and universally in well-supported monophyletic groups, and it is trivial to conclude that the most likely history for these characters involves a single origin with no reversals. Characters in this

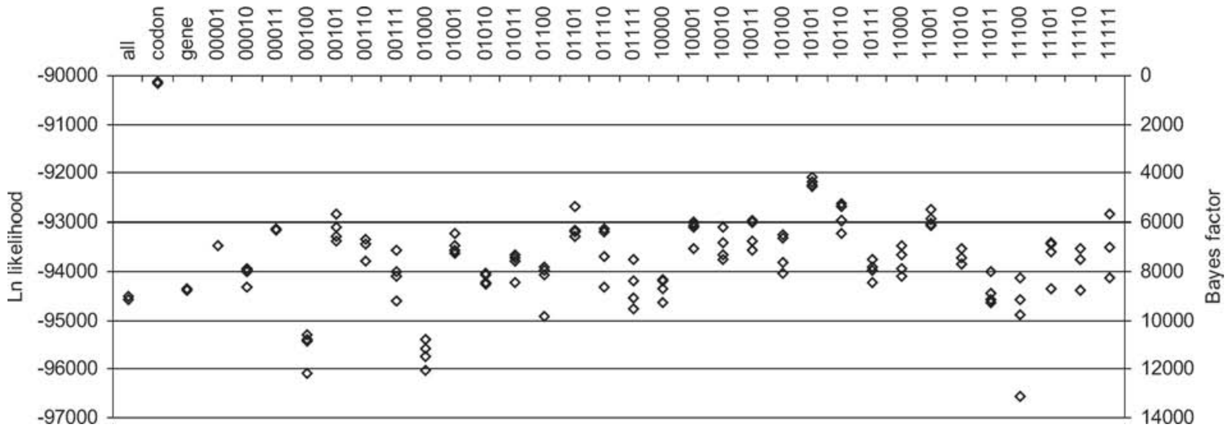


Figure 1. Comparison of partitioning strategies for Bayesian phylogenetic inference. "All," all data combined; "codon," partitioned by codon position across all five genes; "gene," one partition per gene. Models represented by binary codes combine partitioning by gene and by codon; each number represents a gene (in order, atpB, psaA, psaB, psbC, and rbcL), with a "1" indicating that the gene in question was partitioned by codon. For example, the model designated 00010 includes the following partitions: atpB, psaA, psaB, psbC position 1, psbC position 2, psbC position 3, rbcL. Bayes factors are relative to the "codon" model; a Bayes factor >10 indicates robust support for rejecting the model tested in favor of the preferred model (Brown and Lemmon 2007).

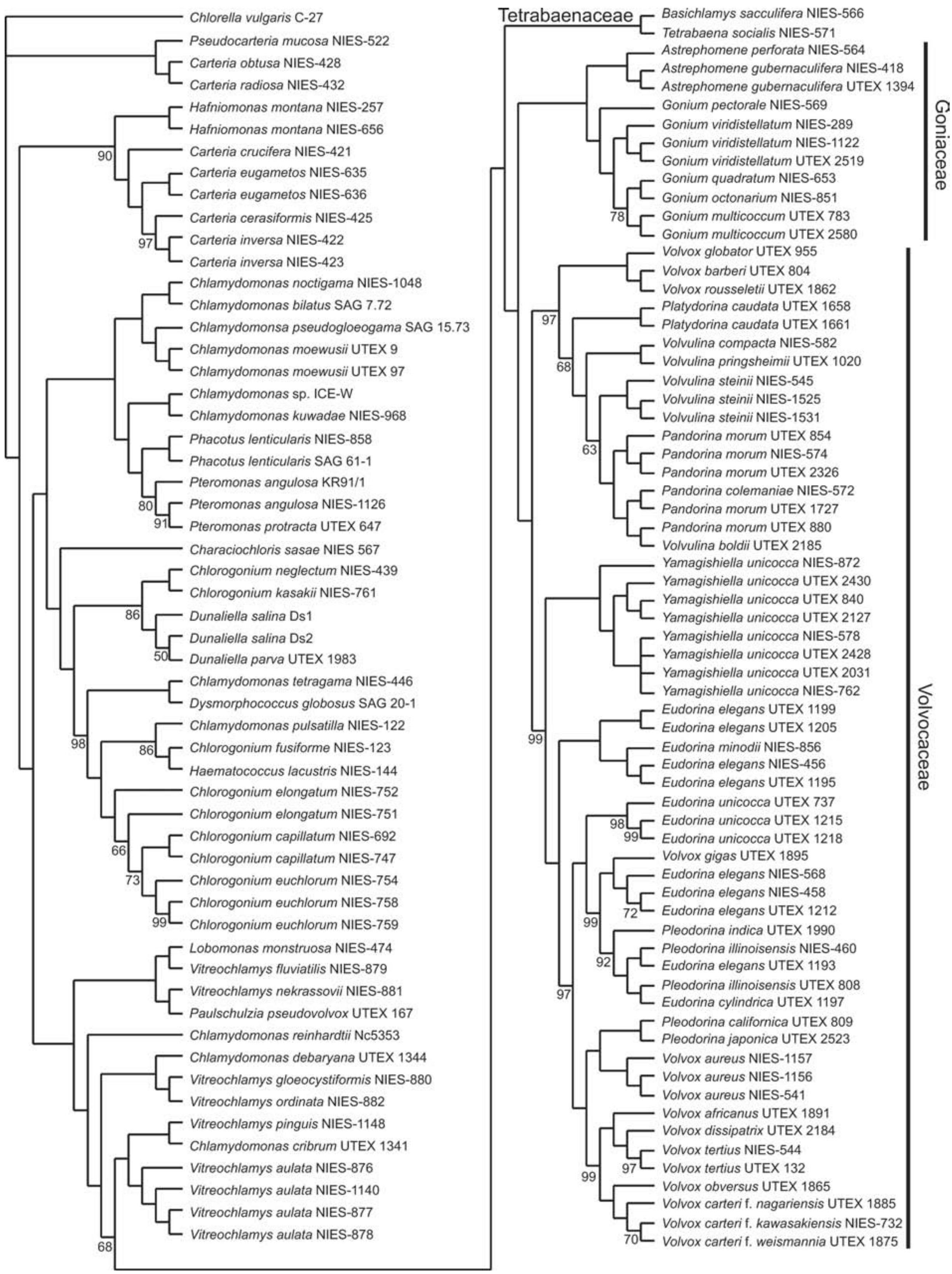


Figure 2. Consensus cladogram from four independent MCMC runs. Numbers are posterior probabilities; unlabeled nodes are supported with posterior probability of 1.0.

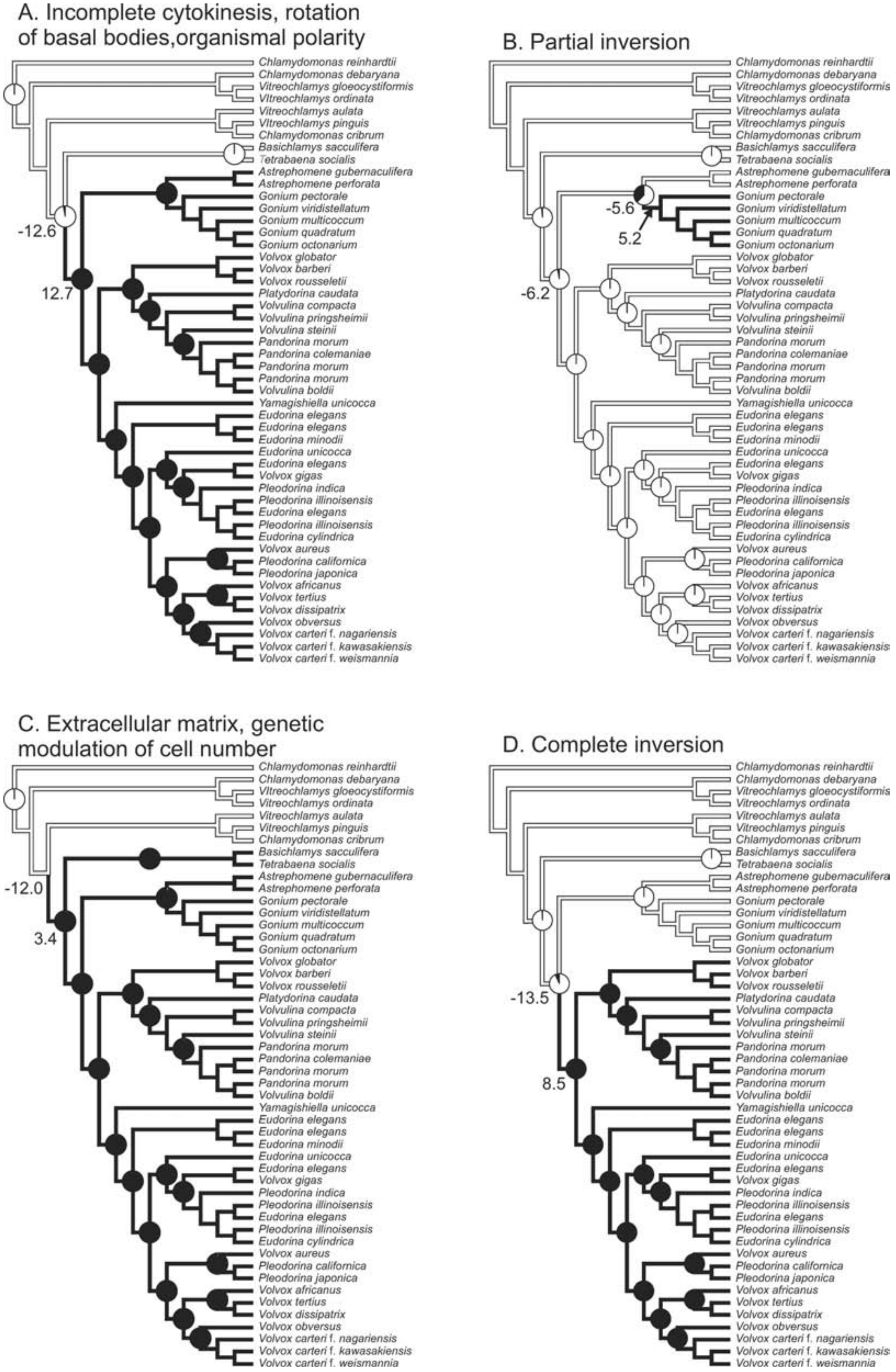
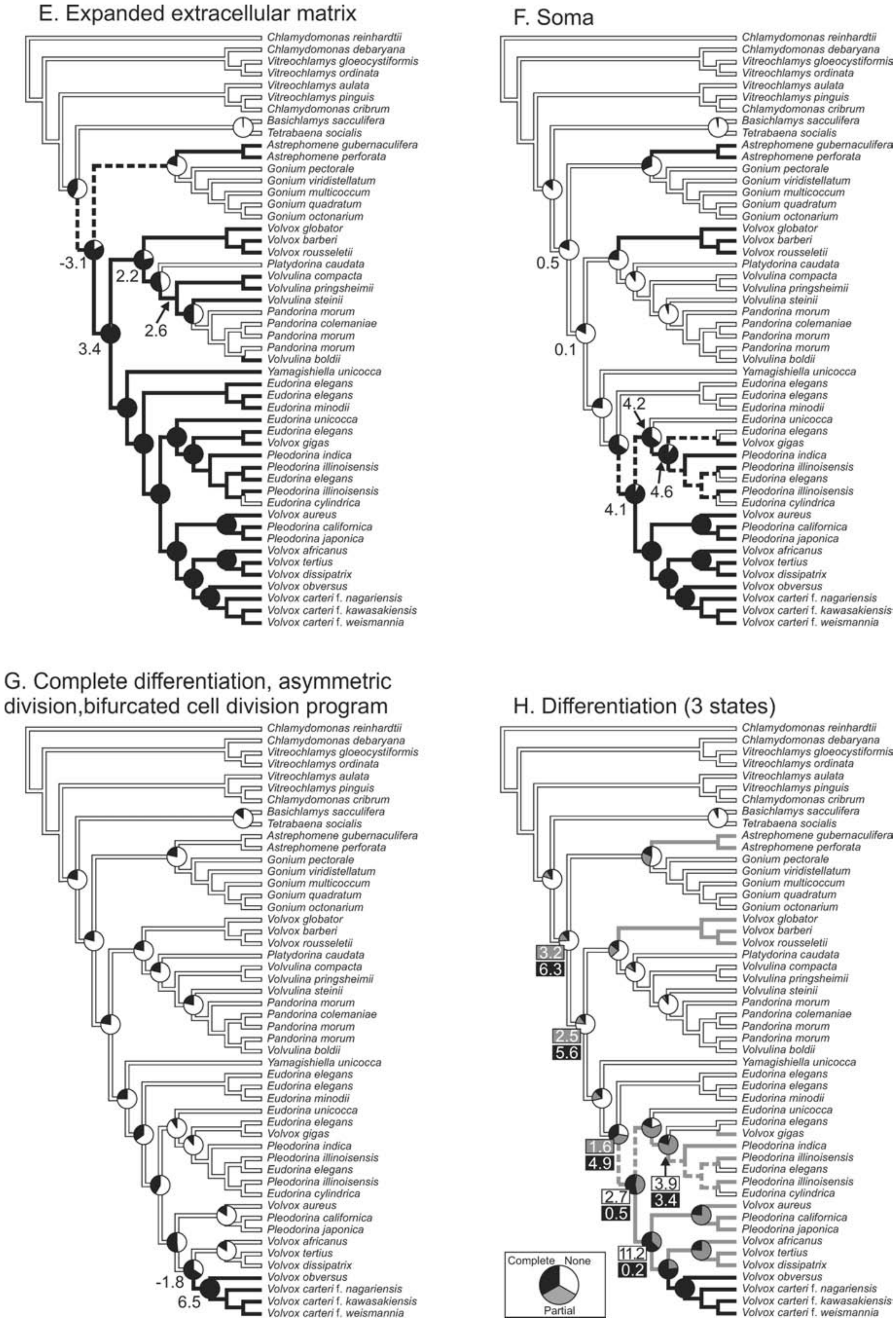


Figure 3. Reconstructed ancestral character states. Branch shading indicates maximum parsimony reconstruction (white, absent; black, present; dashed, ambiguous in A–G, I). Pie charts indicate Bayesian posterior probabilities at selected nodes. Numbers indicate Bayes factors at selected nodes: positive, support for trait presence; negative, support for trait absence. In H and J, the background shade behind the numbers indicates the character state against which the most likely state is supported. Interpretation of 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.



E. Expanded extracellular matrix

F. Soma

G. Complete differentiation, asymmetric division,bifurcated cell division program

H. Differentiation (3 states)

Figure 3. Continued.

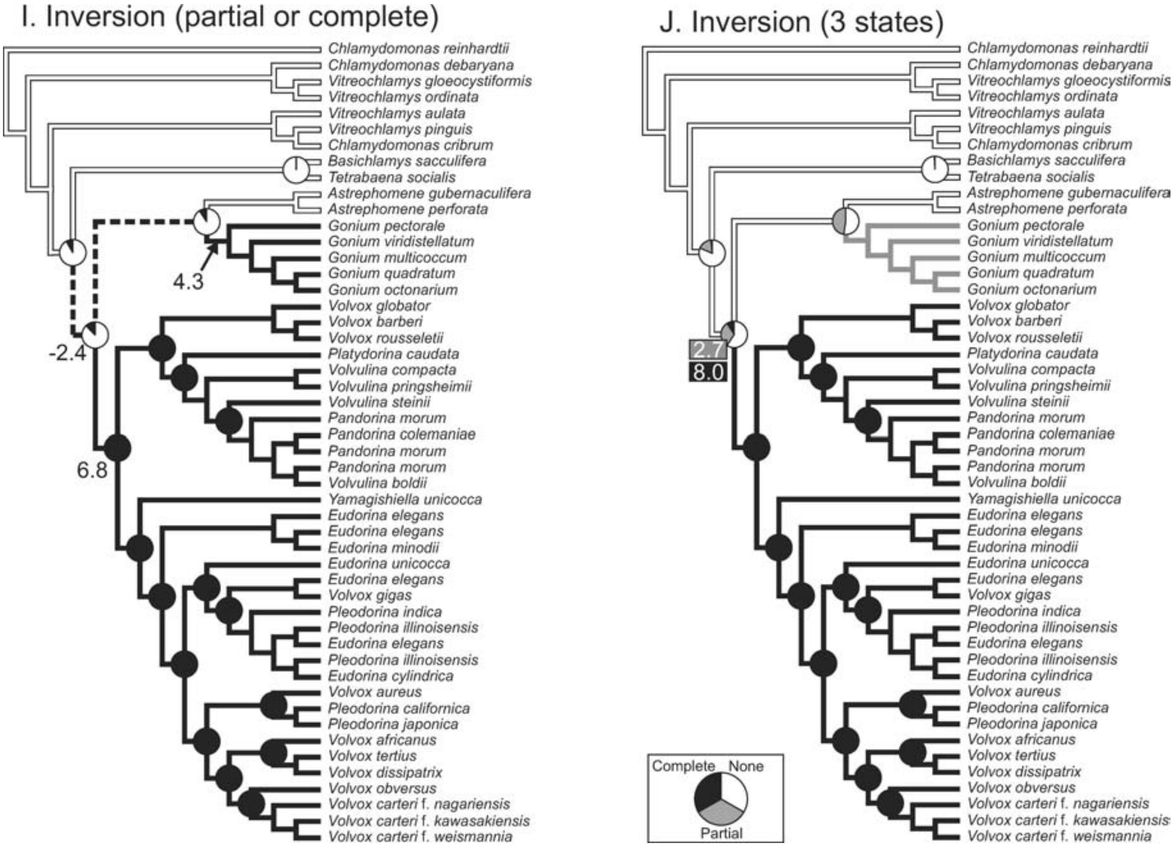


Figure 3. Continued.

category include incomplete cytokinesis, rotation of the basal bodies, and the establishment of organismal polarity (present in all Goniaceae + Volvocaceae; Fig. 3A); partial inversion of the embryo (present in all *Gonium*; Fig. 3B); transformation of the cell wall into ECM and genetic modulation of cell number (present in all colonial forms; Fig. 3C); complete inversion of the embryo (present in all Volvocaceae; Fig. 3D); and complete differentiation, asymmetric division, and a bifurcated cell division program (present in *V. carteri* and its sister species, *V. obversus*; Fig. 3G). With the exceptions of complete differentiation, asymmetric division, and bifurcated cell division program, these inferences of single origins are supported by hypothesis tests, which show each of these characters ancestral to its respective clade and absent from the MRCA with its sister clade (Figs. 3A–D). In the case of complete differentiation, asymmetric division, and bifurcated cell division program the absence of these characters in the MRCA of *V. carteri* + *V. obversus* and *V. africanus* + *V. tertius* + *V. dissipatrix* is only weakly supported (BF 1.8; Fig. 3J).

Distributions of the remaining characters are more complicated. At least two expansions of the ECM are supported (Fig. 3E): one in the lineage leading to *Astrephomene* and one in the lineage leading to the Volvocaceae. *Platydorina*, *Pandorina*, and *Eudorina cylindrica* represent losses of this character, or reductions in ECM volume. This scenario, supported by hypothesis tests, differs somewhat from that depicted by the MCMC reconstruction, in which the MRCA of Goniaceae + Volvocaceae more likely had expanded ECM (0.82 PP), in which case *Gonium* would represent another reduction in ECM volume.

For partial and complete inversion of the embryo, different treatments of these characters lead to different inferred histories. Treating each as a binary character leads to the trivial conclusion that partial inversion originated once in the MRCA of all *Gonium* species (Fig. 3B) and that complete inversion originated once in the MRCA of Volvocaceae (Fig. 3D). The former conclusion is supported by hypothesis tests that show partial inversion present in the MRCA of *Gonium*, absent in the MRCA of Goniaceae, and absent in the MRCA of Goniaceae + Volvocaceae. A single origin of complete inversion is strongly supported, with hypothesis tests showing this trait present in the MRCA of Volvocaceae and absent in the MRCA of Volvocaceae + Goniaceae. Inversion can also be treated as a single character with three states (absent, partial, complete), either ordered (transitions directly from absent to complete forbidden) or unordered (all transitions allowed). Treating inversion as an ordered character effectively assumes that some ancestor of the Volvocaceae (all of which have complete inversion) had partial inversion. If, however, inversion is treated as an unordered character, the most likely reconstruction shows no ancestor of the Volvocaceae with partial inversion (Fig. 3J). This

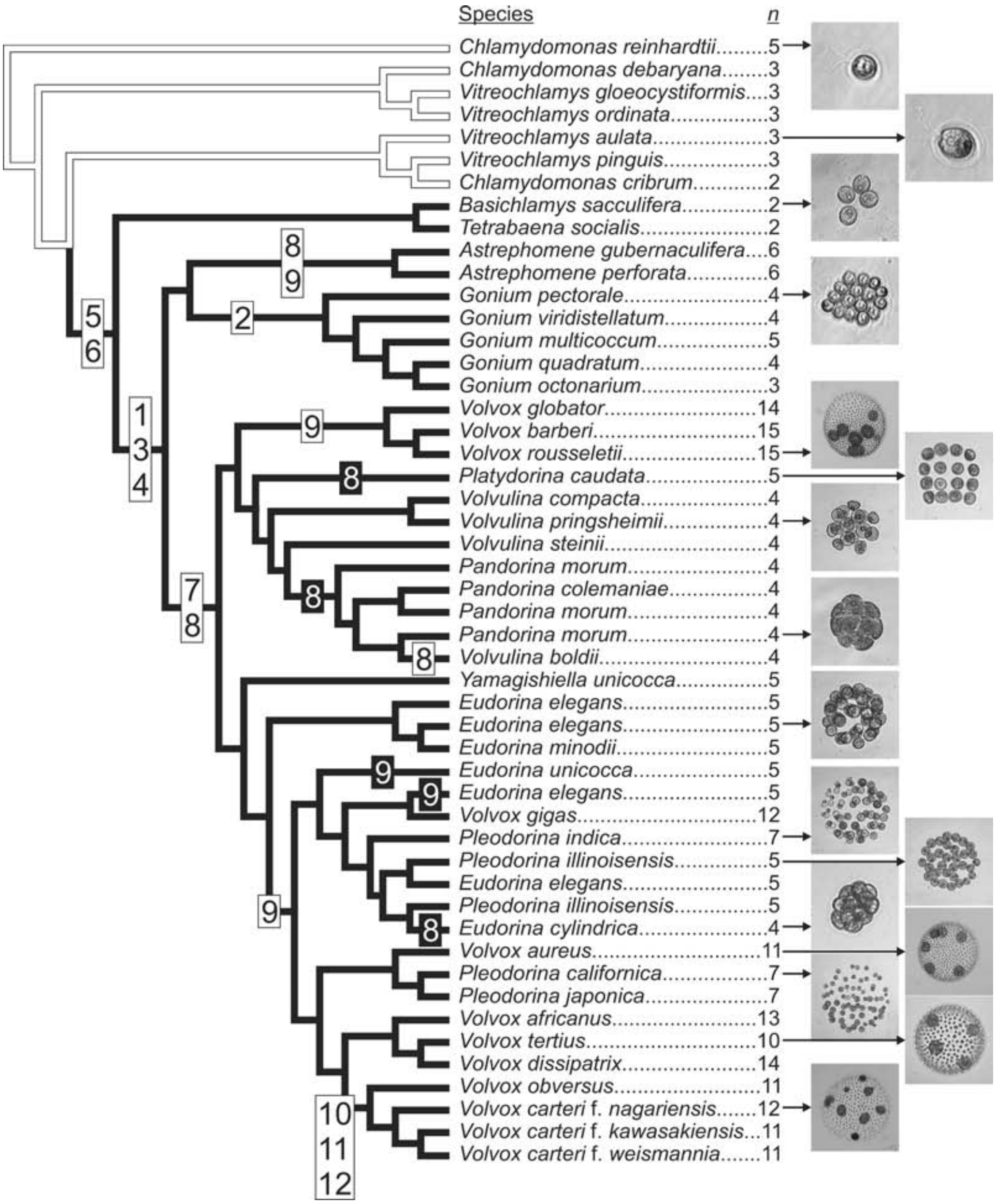


Figure 4. Character state changes supported by hypothesis tests. Gains of derived character states are indicated by black numbers on white background, losses by white numbers on black background. Numbering follows Kirk (2005): (1) incomplete cytokinesis, (2) partial inversion, (3) rotation of basal bodies, (4) establishment of organismal polarity, (5) transformation of cell wall into ECM, (6) genetic control of cell number, (7) complete inversion, (8) increased volume of ECM, (9) partial differentiation (soma), (10) complete differentiation (specialized germ), (11) asymmetric division, (12) bifurcated cell division program. Representative maximum number of cell divisions (n) is shown at right; unicellular species (white branches) produce up to 2^n offspring, and colonial species (black branches) contain up to 2^n cells per colony. These numbers often vary among reports by different authors, especially for larger species, and are given here to show the overall pattern rather than to provide precise values. Photographs of representative species are not to scale.

conclusion is supported by hypothesis tests showing the MRCA of Volvocaceae + Goniaceae significantly more likely to have had no inversion than partial inversion. This would mean that partial and complete inversion likely arose independently of one another.

As with inversion, partial and complete differentiation can be treated as separate characters or as a single character with three states. The characters that define the levels of differentiation are the presence or absence of sterile somatic cells and the presence or absence of specialized, unflagellated germ cells. Species with both are considered completely differentiated; those with soma but no specialized germ are partially differentiated, and those lacking both characters are defined as undifferentiated. Species with specialized germ but lacking soma are conceivable, but no extant species has this combination of characters, and there are biological reasons to suspect that it would be selected against (Michod et al. 2006), so we will not consider it further. Because partial differentiation is not a single character but the presence of one (soma) and the absence of another (specialized germ), we instead reconstructed these two characters separately. The character designated as complete differentiation is defined as the presence of specialized (nonflagellated) germ cells.

The maximum parsimony reconstruction shows at least three separate origins of sterile somatic cells (in the lineages leading to *Astrephomene*, to *Volvox globator* + *V. barberi* + *V. rousseletii*, and to the clade including all other *Volvox* and all *Pleodorina*; Fig. 3F). Losses of this character may have occurred in the ancestors of *Eudorina unicocca*, some lineages of *E. elegans*, and *E. cylindrica*, but these are all ambiguous in the MP reconstruction. Hypothesis tests do not resolve the number of origins of soma but do support a minimum of three losses, one in *E. unicocca* and at least two in different lineages of *E. elegans*.

When differentiation is treated as an unordered character with three states, the MP reconstruction shows at least three separate origins of partial differentiation from undifferentiated ancestors and one transition from partially to completely differentiated (Fig. 3H). Hypothesis tests support a minimum of three origins of partial differentiation from undifferentiated ancestors.

Discussion

ANCESTRAL CHARACTER STATES

As shown in Figure 4, the history of characters related to the evolution of multicellularity in the volvocine algae is considerably more complicated than the traditional view of a linear increase in complexity as embodied in the volvocine lineage hypothesis used by Kirk (2005). Numerous previous studies have rejected the phylogenetic relationships implied by the volvocine lineage hypothesis (e.g., Larson et al. 1992; Buchheim et al. 1994; Coleman 1999; Nozaki 1996, 2003; Nozaki et al. 1997, 2000, 2003, 2006; Nozaki and Krienitz 2001). The volvocine lineage hypothesis can be considered a reasonable approximation of evolutionary relationships only if the majority of volvocine diversity is ignored.

Because taxonomic assignments within this clade are typically based on morphological, developmental, and ultrastructural characters, it is not surprising that the taxonomy does not accurately reflect phylogenetic relationships. Similar conclusions have been reached in numerous previous studies (e.g., Larson et al. 1992; Nozaki 1996, 2003; Nozaki et al. 1997, 2000, 2003, 2006; Coleman 1999; Nozaki and Krienitz 2001).

The only traits that are common to, and probably ancestral to, all extant colonial species are transformation of the cell wall into ECM and genetic modulation of cell number. These traits thus preceded incomplete cytokinesis, rotation of the basal bodies, and establishment of organismal polarity, which are present in all extant Goniaceae + Volvocaceae. There is no evidence that any ancestor of the Volvocaceae had a *Gonium*-like morphology and development with partial inversion. The compact morphology characteristic of *Pandorina* species and *E. cylindrica* appears to be derived from ancestors with expanded ECM, as in the extant *E. elegans*, rather than the other way around. Finally, the undifferentiated *E. unicocca, E. cylindrica*, and some lineages of *E. elegans* appear to have descended from partially differentiated ancestors.

The criteria used to assign some of the characters analyzed in this study require further explanation. We defined expanded ECM as present in those species in which the cells are arranged at the periphery of the colony, with ECM filling the space between cells, as opposed to a *Pandorina*-like morphology in which the cells fill nearly the entire volume of the colony. Genetic, rather than environmental, control of cell number is based on the inference of “an additional layer of genetic regulation” (Kirk 2005, p. 307) that determines the maximum number of division cycles in the development of a given species. Smaller numbers of division cycles are possible and are often seen under suboptimal growth conditions. It could be argued that the unicellular relatives of the colonial volvocine algae also have genetic control of maximum cell (offspring) number, in which case this character would likely have been present in the MRCA of the taxa included in this study. Different species of *Chlamydomonas* and *Vitreochlamys* have different characteristic maximum numbers of offspring (see for example Iyengar and Desikachary 1981; Ettl 1983), and it is possible that the same mechanism that controls maximum offspring number in the unicellular species controls cell number in the colonial species (as suggested by Koufopanou 1994). On the other hand, the maximum number of rounds of cell division is lower in some colonial species (e.g., two in the Tetrabaenaceae, three in *Gonium octonarium*) than in some unicells (e.g., five in *C. reinhardtii*; Lien and Knutsen 1979). The establishment of an additional layer of genetic regulation early in volvocine evolution is one possible explanation of this observation. Alternatively, it could be a lineage-specific step or an artifact of the much lower effort that has been devoted to studying small colonial species compared to *C. reinhardtii*. In our analyses, we have followed Kirk (2005) in considering genetic control of cell number as restricted to the colonial volvocines.

PREADAPTATIONS

Inferences that particular characters may have served as preadaptations to the evolution of differentiated multicellularity are necessarily speculative. The palintomic, or multiple fission, mode of cell division found in most colonial volvocines and all close unicellular relatives has often been considered as a possible preadaptation to multicellularity (e.g., Kirk 1998, 2005). This trait provides for the simultaneous production of a large number of genetically related cells at once (relative to binary fission); all that is needed for an initial step toward multicellularity is a mechanism to keep the cells together. The resulting high kinship likely facilitated the evolution of cooperation among cells in a volvocine group.

Incomplete cytokinesis provides one such mechanism to keep cells together and results in the creation of cytoplasmic bridges, which are crucial to the process of inversion. Inversion may in turn be needed for the development of large, spherical colonies (although the example of *Astrephomene*, which lacks inversion, argues against this as an absolute requirement).

Another possible preadaptation is the tripartite cell wall found in extant *Chlamydomonas*. The conversion of the cell wall into ECM is another mechanism by which the products of multiple fission are bound together and likely preceded incomplete cytokinesis in volvocine evolution. The close relationship of some species of *Vitreochlamys* to the colonial volvocines suggests an interesting possibility. In *Vitreochlamys*, the gelatinous middle layer of the cell wall is expanded relative to that in *Chlamydomonas* (Nakazawa et al. 2001). This may have been an early step in the conversion of the cell wall into ECM.

CONSTRAINTS

Reconstructions of ancestral character states allow us, in some cases, to conclude that one character state change preceded another, but this is not sufficient to conclude that a constraint exists, that is, that the second change cannot occur without the first. Arguments for constraints, such as that characters 1–5 " . . . probably evolved almost simultaneously, because abrogation of any one of these five traits in a modern volvocine alga results in a failure to produce a colony of defined shape that is capable of swimming directionally and maintaining its place in the sun," (Kirk 2005, p. 306), can, however, be falsified. If some of the traits in question can be shown to have occurred before others, or if some of the traits but not others are present in extant taxa, the argument that the traits cannot exist independently fails. From the reconstructions of ancestral character states given here, as well as from the distribution of traits in extant taxa, we can conclude that characters 1–5 are not all required to arise simultaneously. Transformation of the cell wall into an ECM likely preceded the remaining four characters, and is present without the others in the extant Tetrabaenaceae. Partial inversion is unlikely to have existed in any ancestor of the Volvocaceae, and so the MRCA of Goniaceae + Volvocaceae likely had the other four traits without any form of inversion, as does the modern *Astrephomene*.

Generally speaking, arguments of this form—that a set of traits must have evolved simultaneously because modern taxa do not function well if one is disrupted—should always be viewed with caution. Phylogenies that are missing taxa, either because of extinction or incomplete sampling, will often appear to show that multiple traits arose simultaneously. This effect is demonstrated by the example of the Tetrabaenaceae: any phylogenetic reconstruction missing these two species will appear to show that ECM and genetic modulation of cell number arose at the same time as incomplete cytokinesis, basal body rotation, and establishment of organismal polarity. Another way of showing this is to remove taxa from a known phylogeny. For example, if *Gonium* is omitted, expanded volume of ECM will appear to have arisen simultaneously with the establishment of organismal polarity in the MRCA of Goniaceae + Volvocaceae (Figs. 3A, E). We might then wrongly conclude that a spherical colony with cells arranged on the periphery is required for the establishment of organismal polarity. If enough taxa are removed, any number of traits will appear to have arisen simultaneously. Character state changes do not occur in isolation, and numerous unmeasured characters likely change to accommodate each change in a measured character. We should not, therefore, be surprised when disruption of a trait that arose some millions of years ago disrupts the functioning of the organism, nor should we conclude from this that the trait arose simultaneously with other changes that have occurred in the same lineage.

THE TRANSITION TO A NEW LEVEL OF COMPLEXITY

In his example of the vertebrate eye, Darwin (1872 pp. 143–146; extended in Dawkins 1996) demonstrated that the evolution of a complex, integrated trait can be reduced to a series of plausibly small steps, each conceivably adaptive in itself. By citing examples of extant species with eyes similar to each of the proposed steps, he showed convincingly that each stage could result in a functional eye. In outlining the steps by which a unicellular alga evolved into a differentiated, multicellular species, Kirk (2005) has begun the extension of Darwin's approach to the eye to an evolutionary transition in individuality. By extending this approach to the diversity of volvocine species, we can infer how and to what degree this transition has occurred in multiple lineages.

It is clear that *V. carteri* and *V. obversus*, as multicellular organisms with deterministic development and a complete germ-soma division of labor, have undergone a transition in individuality from unicellular ancestors. Even with an understanding of the steps involved, though, it is difficult to pinpoint when (and in which taxa) this transition should be considered complete. The answer to this question will ultimately depend on the criteria by which individuality is defined. Other than the Tetrabaenaceae, all colonial species have at least some cellular differentiation (reviewed in Kirk 2005). These differences establish an anterior–posterior polarity (center-peripheral in *Gonium*) and include orientation of the flagella, size of the eyespots, and size of cells (in species lacking germ-soma differentiation). *Gonium*, traditionally considered to be among the simplest of the colonial forms, is nonetheless a tightly integrated unit capable of coordinating processes such as phototaxis and inversion. By the criterion of indivisibility and as a unit of selection, a case can be made that *Gonium* is an individual. If, however, germ-soma differentiation is taken to be a prerequisite for an ETI (Griesemer 2000; Maynard Smith and Szathmáry 1995, p. 227; Michod 2005, 2006), the various *Astrephomene*, *Pleodorina*, and *Volvox* species meet this criterion to different degrees. As with many other phenomena, biological reality indicates a continuum even when the vocabulary used to describe it indicates a dichotomy.

Most ETIs of which we are aware occurred so far in the past that evidence bearing on how they happened is hard to come by, and the nature of intermediate forms can only be imagined. The differences between levels of complexity, for example between multicellular taxa and their unicellular relatives, are so profound in most cases that it is difficult to imagine how such transitions might have occurred. In contrast, the colonial volvocine algae diverged from unicellular relatives only 30–70 MYA (Rausch et al. 1989), and extant species represent a diverse array of cell numbers and levels of differentiation.

The diversity of volvocine forms provides a sample of plausible intermediates between unicellular and differentiated multicellular levels of complexity. We cannot assume that extinct ancestors were similar to extant relatives (assuming, for example, that the MRCA of the Volvocaceae was *Gonium*-like is the logical equivalent of assuming that the MRCA of placental mammals was kangaroo-like). By analyzing the distribution of traits in a phylogenetic context, though, we can infer what combination of traits was likely present in a given ancestor. Furthermore, when a combination inferred for an ancestor is also found in an extant species, we know that such a combination is viable.

As an ETI, the unicellular to multicellular transition requires a reorganization of fitness: as cells relinquish their autonomy in favor of the group, traits such as fitness heritability, indivisibility, and evolvability shift their frame of reference from the level of the cell to that of the group. These shifts are best understood in the framework of multilevel selection theory, which predicts a series of cooperation, conflict, and conflict mediation cycles that lead to greater integration and greater individuality of the group (Michod 1999; Michod and Roze 2001; Michod and Nedelcu 2003). In particular, under certain conditions groups may form and costly forms of cooperation, altruism, may increase. This leads to the opportunity for cheating and conflict that must be mediated if the group is to evolve into a new higher-level individual. In this view, ETIs involve the evolution of traits that reduce within-group selection or variation and enhance between-group selection, increasing the individuality of the group. Using this approach an in principle set of stages can be hypothesized to explain, for example, the transition from unicellular life to multicellular life (Michod 2007) or the origin of the eukaryotic cell (Michod and Nedelcu 2003).

Some features of the volvocine life cycle, particularly the relatively short cell lineages and high relatedness among cells, may limit the opportunities for within-colony conflict and, consequently, the need for mechanisms of conflict mediation. Nevertheless, selfish mutants are known to recur spontaneously (Nedelcu and Michod 2006). The assumptions and parameter ranges under which conflict mediation mechanisms can evolve have been modeled (Michod 1996, 1997, 2003; Michod and Roze 1999; Roze and Michod 2001; Michod et al. 2003). These models predict that conflict mediation can evolve in groups derived from single-celled propagules and short cell lineages (such as the volvocine algae) given sufficiently high rates of selfish mutation, benefits of defection at the cell level, and costs of defection at the colony level. As the values of these parameters are unknown in the volvocine algae, we acknowledge that the following scenarios are speculative and may not be feasible given some combinations of parameters.

The steps identified by Kirk (2005) can be interpreted as components of the cooperation–conflict–conflict mediation cycles predicted by the theory of multilevel selection. For example, one of the first of these steps to occur was the transformation of the cell wall into ECM. Volvocine ECM is made up primarily of glycoproteins and presumably has a metabolic cost, so its production by cells in a colony is potentially a form of altruism. In addition, the ECM may be kind of "commons" (Hardin 1968) in which nutrients are stored (Bell 1985). As with all shared resources, there is the potential for conflict and cheating: cells that commit fewer resources to building ECM, or disproportionately take resources from the ECM, could potentially have more resources, energy, and time left for reproduction.

All extant volvocines with ECM also have genetic control of cell number. The order in which these two traits evolved, or if they evolved simultaneously, is not resolved by our analyses. If ECM evolved first, genetic control of cell number may have been a mechanism of conflict mediation: defecting cells would have less to gain if the number of daughter cells (i.e., size of the daughter colony) were fixed or limited (Michod 2003).

The next few steps inferred relate to the increasing integration of cooperative interactions within the colony. Incomplete cytokinesis created cytoplasmic bridges connecting the cells. Rotation of the basal bodies presumably played a role in allowing colonies of more than a few cells to swim directionally, thus changing the framework of organismal polarity from the level of the cell to that of the colony. The integration provided by the cytoplasmic bridges furnished a mechanism by which cells could coordinate the processes of partial inversion (in the ancestors of *Gonium*) and complete inversion (in the ancestors of the Volvocaceae).

These developments resulted in tightly integrated but undifferentiated colonies, as in the modern genera *Eudorina*, *Gonium*, *Pandorina*, *Volvulina*, and *Yamagishiella*. Except for inversion, the characters involved appear to have been present in the MRCA of Goniaceae + Volvocaceae, suggesting that a considerable degree of integration preceded the subsequent diversification in these families. Thus it is possible that one cycle of cooperation, conflict, conflict mediation, and subsequent integration was completed early in volvocine evolution.

The last four of Kirk's (2005) steps are related to germ-soma differentiation. Michod argued that a necessary component of an ETI is specialization of group members in reproduction and viability (Michod 2005, 2006). When cells completely specialize at the two basic fitness components, reproduction and viability, the cells lose fitness and the capacity to function as evolutionary individuals. As a consequence, the fitness of the group is no longer related to the average of fitness of the individual cells. By virtue of their specialization in only one of the two necessary components of fitness, cells will have low fitness, whereas the fitness of the group may be quite high. Maynard Smith and Szathmáry put the matter this way (Maynard Smith and Szathmáry 1995, p. 227), ". . . entities that were capable of independent replication before the transition can replicate only as part of a larger whole after it . . ." As discussed by Griesemer (2000), this criterion for an ETI implies division of labor and specialization of the lower-level entities at reproduction.

Hypotheses for the origin of cell specialization at reproduction and viability (not necessarily mutually exclusive) include division of labor (Bell 1985; Koufopanou and Bell 1993; Queller 2000; Schlichting 2003; Wahl 2004; Michod et al. 2006) and mitigation of trade-offs between motility and fecundity (Koufopanou 1994; Michod 2006; Solari et al. 2006b). The results of our study do not resolve which of these explanations, or which combination of explanations, may have driven this development. Body size and cell number could be relevant to either of the above hypotheses. Division of labor is most beneficial and least costly when there are a large number of components (cells in our case), and the trade-off between motility and fecundity becomes more exaggerated (i.e., motility becomes more costly) as cell number increases (Koufopanou 1994; Solari et al. 2003, 2006a).

Regardless of the ultimate causes of its origin, the development of terminally differentiated somatic cells is an example of an altruistic trait. Somatic cells, by definition, give up their reproduction and thus their direct fitness. The benefit to the colony is likely in terms of enhanced and permanent motility (Koufopanou 1994; Solari et al. 2006b). Somatic cells, due to their smaller size, contribute less negative buoyancy and can be packed more closely on the colonial surface than the larger reproductive cells, and unlike reproductive cells, they remain flagellated throughout development and reproduction. Germ-soma differentiation only appears in spheroidal colonies with 32 or more cells and expanded ECM. As mentioned above, production of ECM can be seen as a form of altruism, and prodigious amounts are required for colonies such as *Pleodorina* and *Volvox*. The potential for conflict among cells in these large colonies is great: the costs to individual cells of sacrificing reproduction and of ECM production create an opportunity for cheating. Furthermore, the large number of cells means both that the opportunity for selfish mutations is high and that the defection of a single cell is unlikely to have a large impact on colony fitness. Early germ-line segregation has been proposed as a mechanism for reducing the likelihood of selfish mutations (Michod 1996; Michod et al. 2003). The processes of asymmetric division and the bifurcated cell division program, which together cause germ cells in *V. carteri* and *V. obversus* to withdraw from the cell division program early, may therefore serve as mechanisms of conflict mediation.

To summarize, the changes that are inferred to have occurred in the evolution of multicellularity in the volvocine algae can be understood within the framework of multilevel selection theory and the cooperation–conflict–conflict mediation cycles it predicts. The formation of ECM, which occurred early in volvocine evolution, is altruistic from the point of view of the individual cells, and, as with any form of altruism, the potential for cheating exists. Genetic control of cell number may have served as a mechanism for mediating this conflict, paving the way for further integration, such as the rotation of basal bodies, the resulting organismal polarity, and inversion. Terminal differentiation of somatic cells, which appeared only after this first cycle of cooperation, conflict, and conflict mediation was completed, is an extreme form of altruism on the part of individual cells. The potential for cheating in differentiated species is therefore great, especially in large colonies, in which failure of one cell to cooperate would have little impact on the colony. Early segregation of the germ line may serve to mediate the resulting conflict, but this is a lineage-specific trait only seen in two extant sister species (which are not among the largest *Volvox* species). Thus it is possible that a second cycle, in which the defection of somatic cells must be prevented before further integration can be achieved, is ongoing in extant volvocine species.

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Supplementary Material

The following supplementary material is available for this article:

Appendix S1. Accession numbers of ingroup taxa.
Appendix S2. Accession numbers of outgroup taxa.
Appendix S3. Character states for ingroup taxa and source references.

This material is available as part of the online article from:
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