Phylotranscriptomics points to multiple independent origins of multicellularity and cellular differentiation in the volvocine algae

Charles Ross Lindsey¹, Frank Rosenzweig¹,² and Matthew D. Herron¹*

Abstract

Background: The volvocine algae, which include the single-celled species *Chlamydomonas reinhardtii* and the colonial species *Volvox carteri*, serve as a model in which to study the evolution of multicellularity and cellular differentiation. Studies reconstructing the history of this group have by and large relied on datasets of one to a few genes for phylogenetic inference and ancestral character state reconstruction. As a result, volvocine phylogenies lack concordance depending on the number and/or type of genes (i.e., chloroplast vs nuclear) chosen for phylogenetic inference. While multiple studies suggest that multicellularity evolved only once in the volvocine algae, that each of its three colonial families is monophyletic, and that there have been at least three independent origins of cellular differentiation in the group, other studies call into question one or more of these conclusions. An accurate assessment of the evolutionary history of the volvocine algae requires inference of a more robust phylogeny.

Results: We performed RNA sequencing (RNA-seq) on 55 strains representing 47 volvocine algal species and obtained similar data from curated databases on 13 additional strains. We then compiled a dataset consisting of transcripts for 40 single-copy, protein-coding, nuclear genes and subjected the predicted amino acid sequences of these genes to maximum likelihood, Bayesian inference, and coalescent-based analyses. These analyses show that multicellularity independently evolved at least twice in the volvocine algae and that the colonial family Goniaceae is not monophyletic. Our data further indicate that cellular differentiation arose independently at least four, and possibly as many as six times, within the volvocine algae.

Conclusions: Altogether, our results demonstrate that multicellularity and cellular differentiation are evolutionarily labile in the volvocine algae, affirming the importance of this group as a model system for the study of major transitions in the history of life.

Keywords: Phylotranscriptomics, Volvocine algae, Green algae, Phylogeny, Multicellularity

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Background
The evolution of multicellularity is widely considered a major transition in the history of life [1–4]. Multicellularity not only gave rise to most of the visible life forms on the planet, but also opened the door to cellular differentiation, including that between somatic and reproductive cells, a hallmark feature of sexual reproduction in eukaryotes that exhibit morphological complexity [3, 5, 6]. Questions regarding the evolution of multicellularity and cellular differentiation have been approached using the fossil record [7–9], laboratory evolution [10–13], and comparative approaches that include superimposing cell biology upon molecular phylogeny [14–16]. The last of these approaches is predicated on the assumption that the cell biology and molecular phylogeny are mutually informative, an assumption that requires the phylogeny itself to be accurate.

The volvocine green algae have proved especially useful for investigating the major transition leading to multicellularity. The group consists of ~ 50 extant species, which exhibit a range of body plans, cell numbers, sizes, and forms of sexual reproduction. The smallest of these are single-celled (e.g., Chlamydomonas reinhardtii); the largest, at up to 3 mm in diameter and up to 50,000 cells, are spheroidal, swimming colonies in the genus Volvox. Since the initial “very pleasant sight” of swimming Volvox colonies described by Van Leeuwenhoek more than 300 years ago [17], the volvocine algae have come to be accepted as a useful model system in which to address questions related to the origins of multicellularity and cellular differentiation [18, 19]. Multiple species have now had their genomes sequenced [20–24], and those of unicellular C. reinhardtii and multicellular V. carteri forma nagariensis are well-annotated [23, 24]. However, the volvocine algae encompass more than two organisms representing alternative forms of life in terms of size and development. Vegetative forms range in characteristic cell number from 1 to ~ 50,000 and exhibit intermediate degrees of complexity likely similar to extinct ancestors. Further, multicellularity and cellular differentiation arose within the volvocine algae much more recently than those traits arose in animals: ~ 220 million years ago [25] versus ~ 600 million years ago [26], respectively.

Evolution of the volvocine algae has sometimes been viewed as a linear progression in size and complexity [27, 28]. Unicellular taxa such as Chlamydomonas occupy one end of this continuum, while fully differentiated, multicellular taxa such as Volvox occupy the other. This concept, the “volvocine lineage hypothesis”, used a streamlined phylogeny of the volvocine algae to help explain how a multicellular species with complete germ-soma differentiation such as Volvox might evolve from a unicellular, Chlamydomonas-like ancestor. However, morphological and molecular phylogenetic studies suggest that the history of the volvocine algae may be more complicated, as cellular differentiation, different modes of sexual reproduction, and varying body plans appear to have evolved multiple times within the group [29, 30].

Current understanding of the major evolutionary relationships within this group has often been based on the analysis of five chloroplast gene sequences [14, 25, 31–35]. Chloroplast gene-based phylogenies have also been used to carry out ancestral-state reconstructions [14, 29, 30, 36], opening a window on how multicellularity and cellular differentiation evolved within the volvocine algae. Overall, the branching order of most chloroplast gene-based phylogenies is defined by two related groups: (i) a set of unicellular species (e.g., Chlamydomonas reinhardtii) that are paraphyletic with respect to (ii) a clade that encompasses the three major families of colonial volvocine algae: Tetrabaenaceae (Tetraebaena and Basichlamys), Goniacese (Gonium and Astrephomene), and Volvocaceae (Colemanosphera, Eudorina, Pandorina, Platydorina, Pleodorina, Volvox, Volvulina, and Yama- gishiiela) (Fig. 1a, d). In this scheme, the Tetrabaenaceae is a sister group to the clade formed by the Goniacese and Volvocaceae. Although this framework only takes into account family-level relationships, several conclusions can be drawn. First, the colonial species form a clade. Second, each of the three families is monophyletic. Third, monophyly among the colonial species implies that multicellularity evolved only once within that group with no reversion to unicellularity.

Two recent studies have called into question the monophyly of the colonial volvocine algae (Fig. 1c). Pröschold et al. [37] based their inferences on two datasets: one consisting of SSU rDNA sequences plus internal transcribed spacer (ITS) sequences 1 and 2, the other consisting of ITS sequences alone. Nakada et al. [38] used a single-gene 18S rRNA dataset. Both studies inferred that the colonial species are paraphyletic with respect to certain unicells in the genera Chlamydomonas and Vitreochlamys.

The taxonomic status of the Goniacese has also been called into question by studies (Fig. 1b) that indicate the group is either not monophyletic [39] or that there is low support for a sister relationship between Astrephomene and Gonium [33, 37, 38]. Moreover, a number of recent volvocine algal phylogenies leave uncertainty as to how many times cellular differentiation evolved within the group. Chloroplast sequence data suggest at least 3 independent origins of cellular differentiation: in Astrephomene, in Volvox section Volvox (sometimes referred to as Euvolvox), and in the Eudorina, Volvox, Pleodorina (EVP) clade (Fig. 1b–d). Within the EVP clade it is unclear whether cellular differentiation in
Pleodorina thompsonii, Volvox gigas and V. powersii, and Pleodorina starrii and P. indica arose independently from that in V. carteri (Fig. 1b–d).

The foregoing uncertainties highlight the need for a new and more robust molecular phylogeny of the volvocine algae. These uncertainties may arise from incomplete taxonomic sampling, limited genetic sampling, or both. While five volvocine algal species have had their genomes sequenced, most taxonomically comprehensive phylogenetic inferences about this evolutionarily important group have been constructed using relatively small datasets. Most consist of the sequence of five chloroplast genes [14, 31, 34, 40] representing an aggregate of ~6000 nucleotide positions. Others consist of small (≤6) multi-gene datasets consisting of chloroplast gene(s), ribosomal molecular markers, or both [37, 38]. Moreover, the use of chloroplast genes in phylogenetic reconstruction can be problematic because they are effectively a single linkage group, they vary little among recently diverged species [41], and they are at increased risk of incomplete lineage sorting due to the retention of ancestral polymorphisms [42, 43].

Of special concern is the observation that volvocine phylogenies inferred using chloroplast genes (Fig. 1d) conflict with those constructed using nuclear genes (Fig. 1b, c) [37–39]. While conflicts between chloroplast and nuclear phylogenies are not unusual [44–46], they do foster ambiguity.

Here, we seek to resolve volvocine relationships using taxonomically dense sampling of multiple, unlinked loci. We have adopted a phylotranscriptomic approach that uses a concatenated amino acid alignment of 40 nuclear protein-coding, single-copy genes. We sequenced whole transcriptomes of 55 strains encompassing 47 nominal species and used previously published RNA-Seq data for 9 strains and amino acid alignments for 4 strains that were shared with our group by the De Clerck laboratory. Our goal was to derive a robust phylogeny of the volvocine algae that would enable inferences about the evolution of multicellularity, cellular differentiation, sexual dimorphism, and other traits in this group. Our results represent the most taxonomically comprehensive phylogeny yet produced of the volvocine algae using a nuclear dataset, including all described genera and multiple representatives of all genera that are not monotypic. Our results show that the colonial species do not form a clade, that the Goniaceae are not monophyletic, and that multicellularity has independently evolved at least twice and cellular differentiation at least four times within the volvocine algae.

Results and discussion
De novo transcriptome data makes possible 40 single-gene alignments
We sampled 68 taxa representing all presumed major lineages of the colonial volvocine algae and 9 of their nearest unicellular relatives. Because the phylogenetic position of Chlamydomonas reinhardtii has recently been called into question [37, 38], we used a member of the Trebouxiophyceae, Chlorella variabilis, as an out-group (Table 1). All described volvocine genera were included, with multiple species represented for every genus that is not monotypic. Truly comprehensive taxon sampling was not possible, since several described species, especially in the genus Volvox, are no longer available in culture collections. While our main focus was to resolve relationships within the colonial volvocine algae, our study included several closely related unicellular taxa from the genera Chlamydomonas and Vitreochlamys in
Table 1 List of taxa used in this study and summary of sequencing and assembly. Under Strain or Pubmed ID, “CC” refers to Chlamydomonas Culture Collection at the University of Minnesota (CC, USA), “F” refers to Culture Collection of Freshwater Algae at the Institute of Hydrobiology, Chinese Academy of Sciences (FACHB, China), “N” refers to National Institute for Environmental Studies (NIES, Japan), “S” refers to Culture Collection of Algae at the University of Göttingen (SAG, Germany), and “U” refers to The Culture Collection of Algae at the University of Texas at Austin (UTEX, USA). “QRPMK” and “TR” under RNA Extraction Method refer to QIAGEN RNeasy Plant Mini Kit and TRizol RNeasy, respectively. Strains assigned an asterisk represent data from previously published studies, with accession numbers shown in Supplementary Materials: Table S1 [21, 47].

| Taxa                          | Strain or Pubmed ID | RNA extraction method | Total # of raw reads | Total # of clean PE reads | Total # of Contigs | N50 G/C % |
|-------------------------------|---------------------|-----------------------|----------------------|--------------------------|-------------------|-----------|
| Astrephomene gubernaculifera  | N-418               | QRPMK                 | 61460551             | 44350080                 | 78105             | 3894      | 0.61     |
| A. perforata                  | N-564               | QRPMK                 | 63350620             | 46811342                 | 72913             | 3613      | 0.61     |
| Basichlamys sacculifera       | N-566               | QRPMK                 | 66205749             | 48327288                 | 134880            | 1945      | 0.69     |
| Colemanosphaera angeleri      | F 2363*             |                       |                      |                          |                   |           |          |
| C. charkowiensis              | F 2326*             |                       |                      |                          |                   |           |          |
| C. debaryana                  | S 11-55a            | QRPMK                 | 87455695             | 64552440                 | 105096            | 2776      | 0.67     |
| C. charkowiensis              | F 2326*             |                       |                      |                          |                   |           |          |
| C. charkowiensis              | N-3383              | TR                    | 58777161             | 40550696                 | 119848            | 2523      | 0.62     |
| Colemanosphaera angeleri      | N-3383              | TR                    | 58777161             | 40550696                 | 119848            | 2523      | 0.62     |
| Chlamydomonas debaryana       | S 70.81             | QRPMK                 | 50406749             | 42095108                 | 103962            | 2858      | 0.64     |
| C. globosa                    | S 81.72             | TR                    | 73820020             | 49419928                 | 120062            | 2079      | 0.65     |
| C. moewusii                   | S 11-16f            | QRPMK                 | 54728573             | 40436359                 | 118833            | 2498      | 0.6     |
| C. reinhardtii                | CC-503*             |                       |                      |                          |                   |           |          |
| C. schloesseri                | S 2486              | QRPMK                 | 70163393             | 49255021                 | 64233             | 3094      | 0.66     |
| Chlorella variabilis NC64a    | 20852019*           |                       |                      |                          |                   |           |          |
| Eudorina cylindrica           | F 2322*             |                       |                      |                          |                   |           |          |
| E. elegans                    | N-722               | QRPMK                 | 44479173             | 36297801                 | 105029            | 2927      | 0.64     |
| E. illinoisensis              | N-456               | TR                    | 84549961             | 56756776                 | 122975            | 2108      | 0.63     |
| E. illinoisensis              | N-458               | QRPMK                 | 67451343             | 46725012                 | 131335            | 2944      | 0.65     |
| E. illinoisensis              | N-568               | QRPMK                 | 83433797             | 69539684                 | 120487            | 3614      | 0.64     |
| E. illinoisensis              | N-717               | QRPMK                 | 47625429             | 37616922                 | 89118             | 2094      | 0.64     |
| E. illinoisensis              | N-719               | QRPMK                 | 57157324             | 43565312                 | 98180             | 2976      | 0.63     |
| E. schloesseri                | N-460               | QRPMK                 | 62485922             | 49396583                 | 97014             | 2615      | 0.63     |
| E. minodii                    | N-856               | QRPMK                 | 83574258             | 66413244                 | 128802            | 4127      | 0.61     |
| E. peripheralis               | N-725               | QRPMK                 | 46607143             | 36528930                 | 100934            | 2322      | 0.62     |
| E. unicocca                   | S 24-1c             | TR                    | 54482446             | 37679863                 | 110391            | 2267      | 0.62     |
| Gonium multicoccum            | N-737               | QRPMK                 | 65984190             | 44301578                 | 122279            | 3275      | 0.64     |
| G. pectorale                  | N-2863*             |                       |                      |                          |                   |           |          |
| G. octonarium                 | N-851               | TR                    | 60029472             | 39826211                 | 104857            | 2302      | 0.65     |
| G. quadratum                  | N-653               | QRPMK                 | 62431743             | 48230702                 | 100468            | 3789      | 0.64     |
| G. viridistellatum            | N-654               | QRPMK                 | 43286980             | 31852410                 | 97991             | 2837      | 0.65     |
| Pandorina colemaniae          | F 2361*             |                       |                      |                          |                   |           |          |
| P. morum                      | F 2362*             |                       |                      |                          |                   |           |          |
| P. morum                      | N-890               | QRPMK                 | 39333752             | 24490235                 | 133742            | 3123      | 0.61     |
| Platydorina caudata           | N-728               | QRPMK                 | 46702213             | 36896556                 | 125206            | 3503      | 0.61     |
| Pleodorina indica             | N-736               | QRPMK                 | 57564627             | 44082516                 | 192570            | 2006      | 0.63     |
order to provide better phylogenetic resolution of the volvocine algae as a whole.

The total number of raw reads generated from RNA sequencing for each species ranged from 25,665,262 to 87,455,695 reads with an average of 60,194,849 reads per species. After quality trimming of the raw reads (see “Methods”), the total number of clean paired-end reads ranged from 20,161,297 to 69,539,684 with an average of 44,416,935 reads per species (Table 1). From the RNA-seq data, we assembled a total of 40 single-gene alignments that were later concatenated to a single alignment representing an aggregate of 12,650 amino acids, equivalent to 37,950 nucleotide positions, with a total of 5972 parsimony-informative sites. Numbers of informative positions in the single-gene alignments ranged from 40 to 446. Trees inferred using maximum likelihood (ML),

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| Taxa                      | Strain or Pubmed ID | RNA extraction method | Total # of raw reads | Total # of clean PE reads | Total # of Contigs | N50 G/C % |
|---------------------------|---------------------|-----------------------|----------------------|--------------------------|--------------------|------------|
| *P. japonica*             | U 2523              | QRPMK                 | 64897803             | 49495315                 | 83662              | 3499 0.61  |
| *P. starrii*              | N-1362              | TR                    | 68790016             | 45935282                 | 87641              | 2438 0.64  |
| *P. thompsonii*           | N-4126              | QRPMK                 | 81077847             | 61986735                 | 118940             | 4005 0.62  |
| Tetrabaena socialis       | N-571*              |                       | 42302450             | 41232209                 | 62014              | 1012 0.65  |
| Vitreochlamys aulata      | N-878               | QRPMK                 | 80710519             | 54651118                 | 132115             | 2673 0.65  |
|                          | S 80.81             | QRPMK                 | 41008694             | 32585611                 | 84834              | 2100 0.65  |
| V. nekassovii             | S 11-10             | QRPMK                 | 65568295             | 48341108                 | 109360             | 3304 0.62  |
| V. ordinata               | N-882               | TR                    | 60631467             | 42981449                 | 75434              | 2778 0.69  |
| Volvox africanus          | N-863               | QRPMK                 | 70739371             | 52378499                 | 110066             | 4586 0.55  |
| V. aureus                 | N-541               | QRPMK                 | 80821130             | 64315625                 | 106245             | 4065 0.55  |
| V. barbei                 | N-730               | QRPMK                 | 49805303             | 37197105                 | 152910             | 2217 0.58  |
| *V. carteri* f. kawasakiensis* | N-732              | TR                    | 53994112             | 34239609                 | 86004              | 1982 0.58  |
| *V. carteri* f. nagariensis* | 206612680*         |                       | 65624222             | 50641434                 | 117314             | 3947 0.57  |
| *V. carteri* f. weismannia* | N-866               | QRPMK                 | 25913610             | 20987137                 | 78230              | 2636 0.57  |
| V. dissipatrix             | N-4128              | QRPMK                 | 77137081             | 51045569                 | 112190             | 5146 0.55  |
| V. ferrissii               | N-3986              | QRPMK                 | 49810357             | 37522413                 | 91045              | 3281 0.57  |
| V. gigas                  | N-867               | QRPMK                 | 50554912             | 37592146                 | 151302             | 2499 0.63  |
| V. globator               | S 19980             | QRPMK                 | 67413919             | 52861449                 | 100861             | 4005 0.56  |
| V. kirkiorum              | N-543               | QRPMK                 | 44941633             | 34650729                 | 89533              | 3081 0.56  |
| V. obversus               | N-868               | QRPMK                 | 67394604             | 50823546                 | 99171              | 3881 0.58  |
| V. ovalis                 | N-2569              | QRPMK                 | 52242003             | 35319700                 | 107999             | 3643 0.53  |
| V. powersii               | N-4127              | QRPMK                 | 55232784             | 42410688                 | 140237             | 2908 0.62  |
| V. tertius                | N-544               | QRPMK                 | 86222840             | 62563240                 | 108551             | 5454 0.54  |
| Volvulina boldii          | N-893               | QRPMK                 | 25665262             | 20161297                 | 158265             | 1593 0.62  |
| V. compacta               | F 2337*             |                       | 27543035             | 26374210                 | 90261              | 3550 0.61  |
| V. pringsheimii           | N-582               | QRPMK                 | 53045384             | 38991781                 | 112583             | 3380 0.63  |
| V. steini                 | S 90-1              | QRPMK                 | 46133638             | 37696635                 | 89515              | 3635 0.62  |
| Yamagishihia unicocca     | F 2364*             |                       | 30922957             | 29574420                 | 64415              | 3609 0.63  |
Bayesian inference (BI), and coalescence-based (CB) analyses were generally well-supported with some topological differences between the ML and BI analyses relative to the CB analysis, as described below.

Our results conflict with prior volvocine algal phylogenies in four respects

First, we find that the colonial volvocine algae are paraphyletic with respect to some unicellular species. Second, monophyly of the family Goniaceae is not supported. Third, section Volvox is inferred to be sister to the remaining Volvocaceae. Fourth, cellular differentiation independently arose at least four and perhaps as many as six times within the volvocine algae.

Colonial volvocine algae are not monophyletic

All three of our phylogenetic analyses indicate that the colonial volvocines are not monophyletic (Figs. 2 and 3); further, an approximately unbiased (AU) test strongly rejected monophyly for this group (p = 2.82e−38) (Additional file 1: Fig. S1a). These findings represent a major departure from earlier chloroplast gene-based volvocine phylogenies [14, 25, 31–34, 40, 48], phylogenies based on morphological...
characters [49, 50], phylogenies inferred using ITS 1 and 2 sequences [39], as well as less taxonomically comprehensive phylogenies inferred using nuclear data [51], all of which suggest that the colonial volvocine algae are monophyletic.

Consistent with Pröschold et al. [37], our results support the view that multicellularity evolved independently in the Tetrabaenaceae and in the Goniaceae + Volvocaceae. In each analytical framework, the Tetrabaenaceae was found to be sister to *Vitreochlamys ordinata* rather than to the Goniaceae + Volvocaceae (Maximum Likelihood Bootstrap [MLBS] = 100, Bayesian Posterior Probabilities [BPP] = 1.0, Coalescent Posterior Probabilities [CPP] = 1.0). A sister relationship between the Tetrabaenaceae and *V. ordinata* was inferred in 17/39 of our single-gene phylogenies and in 27/39 of our 4-taxon, unrooted, single-gene phylogenies (Fig. 4). These results imply one independent origin of multicellularity in the Tetrabaenaceae and another origin in the Goniaceae + Volvocaceae.

Our results differ in key respects from a recent volvocine algal phylogeny inferred by Zhang et al. [51], which like ours is based on single-copy nuclear genes. Zhang et al. [51] sought to understand the evolutionary relationships between two psychrophilic algae: *Chlamydomonas* sp. ICE-L and *Tetraebaena socialis* N-691. To do so, they constructed a phylogeny consisting of ICE-L, N-691, three colonial Volvox strains, and eight unicellular species, including *C. reinhardtii*. Among their conclusions was that *T. socialis* N-691 is sister to the Volvocaceae, which is at odds with results shown in Figs. 2 and 3. These results indicate that the Tetrabaenaceae is sister to *V. ordinata*, and together they are sister to *C. reinhardtii* + Goniaceae + Volvocaceae.

![Fig. 3 Phylogeny of the volvocine algae inferred using a coalescent-based analysis of 40 single-gene phylogenies. Numbers on branches represent posterior probabilities (support values not shown are CPP = 1.0). Members of the Tetrabaenaceae, Goniaceae, and Volvocaceae are denoted in orange, purple, and green, respectively; unicellular species are denoted in black](image-url)
outgroup species, we sampled *Chlorella variabilis*. Multiple studies have shown that the accuracy of phylogenetic reconstruction can be improved by increasing the number of taxa sampled [54–56]. When we added more taxa and performed ML analysis on the new dataset, the three colonial volvocine families were no longer monophyletic. The Tetrabaenaceae were sister to *Vitreochlamys ordinata*, and this clade appeared sister to *C. reinhardtii* + Goniacae + Volvocaceae (Additional file 1: Fig. S2b) [51]. These analyses confirm that the placement of *T. socialis* N-691 as sister to the Volvocaceae is an artifact of limited taxon sampling. From this, we draw three conclusions: First, the colonial volvocine algae are not monophyletic; second, at least two independent origins of multicellularity occurred within the volvocine algae; third, once multicellularity evolved no extant lineage reverted to the ancestral unicellular state (see Figs. 2 and 3).

The family Goniacae is not monophyletic

Multiple volvocine phylogenies have concluded that the Goniacae is monophyletic [14, 25, 29, 31, 33, 37, 38, 49, 50, 57, 58]. Our analyses suggest otherwise (Figs. 2 and 3): we find that *Astrephomene* is sister to the Volvocoaceae (MLBS = 98, BPP = 1.0, CPP = 0.81), with *Gonium* sister to *Astrephomene* + Volvocaceae (MLBS = 100, BPP = 1.0, CPP = 0.86). Furthermore, we performed an AU test where the monophyly of the Goniacae was tested against our finding of paraphyly for the Goniacae. The null hypothesis, monophyly of the Goniacae, was rejected (p = 0.0446) (Additional file 1: Fig. S1b). The inferred sister relationship between *Astrephomene* and the Volvocaceae is also consistent with the apparent synapomorphy of zygote germination producing a single gone cell, which is unique to these two taxa [50]

Prior studies have produced mixed results regarding monophyly of the Goniacae, sometimes with low support values for the relevant relationships. Nozaki and colleagues [59] published four phylogenies inferred using a single chloroplast gene and different inference methods; all four trees either showed low support for monophyly of the Goniacae or suggested a topology where *Astrephomene* is sister to *Gonium* + Volvocaceae. Coleman [39] inferred a volvocine phylogeny based on ITS-1 and ITS-2 sequences that showed *Astrephomene* sister to Tetrabaenaceae + *Gonium* + Volvocaceae; however, the bootstrap support for this suggested relationship was between 50 and 75%, indicating weak support for the branching order. Other phylogenies suggesting monophyly in the Goniacae do so with weak or contradictory support [33, 37, 38].

Our inference that the Goniacae are not monophyletic is consistent with some — but not all — of the analyses recently reported by Pröschold et al. [37] and Nakada et al. [38]. However, we should not disregard past
morphological and ultrastructural studies suggesting a close relationship between Astrephomene and Gonium [50, 60, 61]. These taxa differ from the Volvocaceae in that each cell, rather than the entire colony, is surrounded by a tripartite boundary [62]. This feature distinguishes their mode of colony formation from all other colonial algae within the Volvocaceae; our results suggest that it is ancestral to the Goniaceae + Volvocaceae and lost in the Volvocaceae.

Volvox section Volvox is sister to the remaining Volvocaceae

Our data indicate that Volvox section Volvox is not a subclade within either the Pandorina + Volvulina + Colemanosphaera (PVC) or Eudorina + Volvox + Pleodorina (EVP) subclades. Older studies based on the rbcL chloroplast gene [49], ITS-1 and ITS-2 sequences [39], and morphology [50] suggest that section Volvox belongs to a clade that encompasses Eudorina, Pleodorina, and other Volvox species. More recent studies of the volvocine algae based on 5 chloroplast genes, or based on multiple datasets that include 1 chloroplast gene [37], suggest that section Volvox belongs to a clade that includes Pandorina, Volvulina, and Platydorina [14, 31], and (in the studies where it was included) Colemanosphaera [34, 40]. By contrast, all of our analyses indicate that section Volvox is monophyletic and sister to the remaining Volvocaceae (MLBS = 83, BPP = 1.0, CPP = 0.73). AU tests rejected the monophyly of section Volvox + Colemanosphaera + Platydorina (p-AU = 4.64e−88) and the monophyly of section Volvox + the

Fig. 5 A Phylogeny of the volvocine algae highlighting the lineages in which soma differentiation has evolved (peach). This tree indicates a minimum of four and maximum of six independent origins of cellular differentiation. B Phylogeny of the volvocine algae highlighting the lineages that are isogamous (black), anisogamous (blue), and oogamous (names in pink only). Both phylogenies were inferred using maximum likelihood
These results bolster our finding that section Volvox is sister to the remaining Volvocaceae (Figs. 2 and 3).

**Cellular differentiation independently arose at least four times in the volvocine algae**

The last major difference between our results and earlier phylogenies concerns the number of independent origins of cellular differentiation. Prior literature suggests that cellular differentiation independently evolved at least three times: once in *Astrephomene*, once in section *Volvox*, and at least once in the EVP clade [14, 36]. By contrast, our results show a *minimum* of four independent origins of cellular differentiation: one in *Astrephomene*, one in section *Volvox*, and at least two in the EVP clade (Fig. 5a). We cannot exclude the possibility of two additional independent origins in the branches leading to *Pleodorina starrii* and *Volvox gigas* (Fig. 5a). In *Astrephomene*, section *Volvox*, *Pleodorina*, and *Volvox dissipatix*, differentiated cells carry out the function of motility, whereas undifferentiated cells participate in both motility and reproduction [15]. The remaining *Volvox* species within the EVP clade have all evolved specialized germ cells for reproduction and somatic cells for motility [25, 30].

**Isogamy is the ancestral mode of sexual reproduction**

Consistent with past studies, our results suggest that isogamy, the production of similar sized, motile gametes, is the ancestral mode of sexual reproduction among the volvocine algae (Fig. 5b and Additional file 1: Table S2). Isogamy is present in the unicellular genera *Chlamydomonas* and *Vitreochlamys* and is retained within the multicellular genera *Astrephomene*, *Basichlamys*, *Gonium*, *Pandorina*, *Platydorina*, *Volvulina*, and *Yamagishiella*. *Colemanosphaera*, *Eudorina*, *Pleodorina*, and *Volvox* have all evolved either isogamy or oogamy [34, 63–65]. Anisogamy appears to have independently evolved at least three times from an isogamous ancestor: in section *Volvox* and in both *Colemanosphaera* and EVP. Conventional anisogamy, which consists of two motile gamete types of unequal size, appears in *Colemanosphaera*, *Eudorina*, and *Pleodorina*. This finding differs from those of Hanschen et al. [29], who reported that anisogamy independently evolved twice among the volvocine algae from isogamous ancestors. Oogamy, a specialized form of anisogamy where the female gamete is immotile and significantly larger than the motile, male gamete, is inferred to have independently evolved at least three times in lineages leading to section *Volvox*, *V. gigas* + *V. powersii*, and in the clade containing *V. africanus*, *V. aureus*, *V. carteri*, *V. dissipatix*, *V. obversus*, *V. ovalis*, and *V. tertius* [34, 63, 64]. This last finding confirms results from Hanschen et al. [29], who also reported at least three independent origins of oogamy among the volvocine algae.

*Platydorina caudata* is sister to *Colemanosphaera*, and *Pandorina* is paraphyletic with respect to *Volvulina*

Within the PVC clade, our results add further support to the view that *Pandorina* is paraphyletic with respect to *Volvulina* (Figs. 2 and 3) [14, 25, 29, 33, 34, 39, 66]. Also, consistent with other multi-gene analyses *Colemanosphaera* appears to be monophyletic with high support (MLBS = 100, BPP = 1.0, CPP = 1.0) and sister to *Platydorina* (MLBS = 100, BPP = 1.0, CPP = 1.0) (Figs. 2 and 3) [31, 34, 35].

**The genera *Eudorina*, *Volvox*, and *Pleodorina* are polyphyletic**

*Yamagishiella unicocca* is sister to the *Eudorina* + *Volvox* + *Pleodorina* (EVP) clade, which encompasses two large subclades (MLBS=99, BPP=1.0, CPP=0.88) (Figs. 2 and 3). Our results support prior work suggesting that the genera *Volvox*, *Eudorina* and *Pleodorina* are not monophyletic [14, 25, 29–31, 33–36, 39, 67, 68]. The genus *Volvox* appears to be polyphyletic, with members represented across the two EVP subclades and the section *Volvox* clade. Members of both the *Pleodorina* and *Eudorina* genera are inferred to be polyphyletic across the two EVP subclades.

Historically, the genus *Volvox* has been divided into 4 sections – *Copelandosphaera*, *Janetosphaera*, *Merrillosphaera*, and *Volvox* – based on morphological [69] and molecular data [67]. A recent section-level revision of the genus *Volvox* [35] resulted in the creation and deletion of sections *Besseysphaera* and *Copelandosphaera*, respectively. Hereafter, we will only refer to the revised taxonomic sections proposed by Nozaki et al. [35], with which our maximum likelihood, Bayesian inference, and coalescent-based results are in agreement (Additional file 1: Fig. S3) [35]. Our coalescent-based analysis suggests that each of the four sections is monophyletic, and that none encompass novel taxa not listed by Nozaki et al. [35] (Fig. 3). The branching order of our ML and BI analyses, however, suggests that section *Merrillosphaera* is not monophyletic (Additional file 1: Fig. S3) [35]. Our ML and BI analyses indicate that *V. africanus*, *V. dissipatix*, *V. ovalis*, and *V. tertius* form a clade with *V. aureus* and *P. japonica* that is separate from the other *Merrillosphaera* taxa (MLBS=65, BPP=0.99) (Additional file 1: Fig. S3) [35]. In contrast, our CB analysis provides strong support (CPP=0.99) for the inference that the *Merrillosphaera* species are monophyletic (Fig. 3). Heeding our support values rather than only the branching order, we propose that the taxonomic system of the genus *Volvox* as outlined by Nozaki and colleagues [35] be retained.
Unicellular taxa are nested within the clade containing the colonial volvocine algae

Of the unicellular taxa, Chlamydomonas debaryana, C. globosa, C. reinhardtii, C. schloesseri, and Vitreochlamys ordinata are nested within the clade containing the colonial volvocine algae. Our results confirm prior studies showing the genus Vitreochlamys to be polyphyletic [38, 48]. The closest unicellular relative to the clade that contains the colonial algae + C. reinhardtii is suggested to be V. auvula (Figs. 2 and 3). This suggests that at least some members of Vitreochlamys are very closely related to the colonial volvocine algae. This relationship had been previously suggested by other studies [38, 70] including Nakazawa et al. [48], whose ultrastructural studies uncovered striking similarities in how these taxa formed pyrenoids and eyespot apparatus (stigma), and established their tripartite cell walls.

Chlamydomonas is a polyphyletic genus [20, 38, 71, 72] composed of at least 500 species [72]. Although we sampled only a handful of Chlamydomonas species, our data support this view and broadly agree with the Chlamydomonas relationships inferred by Pröschold et al. [37], who used a combination of molecular phylogenetic analyses, sporangium wall lysis tests, and ultrastructural analyses. Our data strongly support C. schloesseri being sister to C. reinhardtii + C. globosa (MLBS = 100, BPP = 1.0, CPP = 1.0) and designating C. schloesseri as a “true” Chlamydomonas species, as suggested by Pröschold et al. [37]. Our study is also in agreement with a recent study by Craig et al. [20] that shows C. schloesseri being sister to C. reinhardtii + C. globosa. Also, like Pröschold et al. [37], our analyses indicate that C. debaryana SAG 70.81 is sister to Chlamydomonas schloesseri and its relatives (MLBS = 100, BPP = 1.0, CPP = 1.0). However, unlike the Pröschold et al. [37] study, which proposed that strain C. debaryana/Edaphochlamys debaryana (SAG 11-55a) is sister to the Tetrabaenaceae, our analyses support the view that C. debaryana/Edaphochlamys debaryana is more closely related to C. reinhardtii (MLBS = 91, BPP = 1.0, CPP = 0.81) than to the colonial algae. Our finding is further supported by Craig et al. [20] who inferred that C. debaryana/Edaphochlamys debaryana + Chlamydomonas sphaeroides is sister to the clade containing C. schloesseri + C. reinhardtii + C. globosa. Our placement of C. debaryana (SAG 11-55a) could be a result of limited (N = 6) sampling within the Chlamydomonas genus, which was more extensively sampled by Pröschold et al. [37] (N > 30). Consistent with a prior study, C. moewusii appears to be more distantly related to the colonial volvocines than is Vitreochlamys nekrassovii [14].

Conclusions

Using a 40-protein dataset, we have shown that the Tetrabaenaceae and the Goniaceae + Volvocaceae likely represent two independent origins of multicellularity and that cellular differentiation has independently evolved at least four, and possibly six times within the volvocine algae. The separate origin of multicellularity within the Tetrabaenaceae highlights the need for certain volvocine genomes, such as Vitreochlamys ordinata, to be sequenced, assembled and annotated. Because Vitreochlamys ordinata is the unicellular sister taxon to the multicellular Tetrabaenaceae, detailed analysis of its genome could give future researchers insight into how the simple form of multicellularity observed among the Tetrabaenaceae might have evolved.

Our results suggest that both multicellularity and cellular differentiation are evolutionarily labile traits within the volvocine algae. We have established a robust phylogeny of this group, which we hope will assist future efforts aimed at re-evaluating ancestral character states and understanding the origins of multicellularity and cellular differentiation in the volvocine green algae. The fruit of such efforts could then be used to carry out ancestral-state reconstruction of traits related to cellularity, differentiation, and gamete size as well as to discern the evolutionary history of gene families across the volvocine algae as a whole and within its major clades.

Methods

Strains and culture conditions

Algal strains used in this study were obtained from the National Institute for Environmental Studies (NIES, Japan), the Culture Collection of Algae at the University of Göttingen (SAG, Germany), and the Culture Collection of Algae at the University of Texas at Austin (UTEX, USA). Strain provenance and culture collection ID numbers are shown in Table 1, with previously published data designated with an asterisk. All cultures were grown at 20–26 °C under cool-white LED lamps (4300K) with an intensity of 2500–2700 lux under a 14-h light/10-h dark cycle. A detailed description of each strain’s morphology, degree of cellular differentiation, and gamete size, as well as the medium used to culture each strain is provided in Additional file 1: Tables S2 and S3 [73–77], respectively.

RNA extraction procedures

Two protocols were used to isolate total RNA: a modified version of the TRizol RNeasy method described by Matt and Umen [78] and a slightly modified QIAGEN RNeasy Plant Mini Kit protocol. For a detailed description of each, please see Additional file 2: RNA extraction
procedures. Information on the protocol used for each strain is provided in Table 1.

Library preparation and sequencing
Before generating a sequencing library, RNA quality and quantity were assessed by Nanodrop and Qubit (Thermo Fisher Scientific, Waltham, MA 02451 USA). RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA 95051, USA). mRNA was isolated using poly T beads, whereafter Illumina libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit. Library concentrations were determined fluorometrically; sequencing was carried out on the Illumina NovaSeq 6000 platform (Illumina, Inc., San Diego, CA 92122 USA) to generate 151 bp paired-end reads.

Quality control of reads
Raw read quality was assessed through FastQC v.0.11.8 with an additional FastQC assessment post-trimming. Quality control of the raw reads was completed with Trimmomatic v.0.39 [79] where the bases at the 5′ and 3′ end of each read are trimmed if found to be below a quality score of 3. A 4-base sliding window approach was used to trim the rest of the read once average quality fell below a score of 15; reads that were below a minimum length of 36 bases were discarded (LEADING: 3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36). If adapter content was detected by FastQC the additional ILLUMINA CLIP step was used with the “TruSeq3-PE-2.fa” file provided by the Trimmomatic developers. If performed, the following ILLUMINA CLIP parameters were used: 2:30:10 at the beginning of each command line. This allows for 2 “seed” mismatches where the seed is a short segment of the adapter that is being aligned in every section of the read. If more than 2 mismatches occurred, no trimming of the read occurred. Additionally, there had to be at least 30 matched bases in the paired-end palindrome read alignment and at least 10 matched bases between an adapter sequence and read.

De novo assembly
SOAPdenovo-Trans v1.0.4 [80] was used to assemble de novo transcriptomes from the quality filtered, paired-end reads using a k-mer size of 25 (SOAPdenovo-Trans 31mer all -s <config input file> -o <outfile> -K 25). GapCloser from the SOAPdenovo package was utilized to close gaps in each transcriptome using the same configuration file, which contains read-specific information and file paths, from the previous step (-b <config file> -a <scafSeq file output by SOAPdenovo-Trans> -o <outfile> -l <max read length, int value> -t <thread number>). Default parameters were used for CD-HIT v4.8.1 [81] to reduce redundant transcripts from our de novo transcriptomes.

Orthologous gene identification for phylotranscriptomic analysis
The evolutionary history of the volvocine algae dates back at least 200 million years [25]. Over this timescale nucleotide sequences become saturated with substitutions, diminishing their phylogenetic utility [82]. Amino acid sequences were therefore chosen for our alignments, as they are known to be more reliable for ascertaining distant evolutionary relationships [83]. De Clerck and colleagues identified 58 nuclear protein-coding, single-copy genes that were members of highly conserved gene families across the green algae (Chlorophyceae, Prasinophyta, and Trebouxio-phyceae) and land plants (Streptophyta) [84]. Their amino acid alignment of the 58 nuclear protein-coding genes that includes Chlamydomonas reinhardtii CC-503, Chlorella variabilis NC64A, Gonium pectorale NIES-2863, and Volvox carteri HK10 was kindly shared with our research team. Out of the 58 genes shared, we used 40 for our gene alignments. In order to identify those specific genes in the de novo transcriptomes of our taxa, a Basic Local Alignment Search Tool (BLAST) server was established in our lab, and a unique BLAST database for each taxon was created following the instructions in the BLAST manual. A BLASTP search using the C. reinhardtii CC-503, G. pectorale NIES-2863, and V. carteri HK10 genes from De Clerck et al. [84] as our query sequences enabled us to identify the orthologous genes for each of our taxa.

Gene sequence alignments and phylotranscriptomic analysis
The BLASTP results were used to identify the scaffold and open read frame where each gene was located in a strain’s transcriptome. Using a custom Python script (Additional file 3), each scaffold was extracted from its transcriptome and translated in the appropriate reading frame; then, the translated scaffold was added to an alignment file. For consistency, we generated de novo transcriptomes since we lacked a reference genome for most of our sequenced strains. At times, a gene was found to be incomplete for a given taxon due to assembler or sequencing error after manual examination. When this was determined to be the case, the gene was manually stitched together. This was done in a highly conservative manner: if we could not ascertain whether or not a gene was incomplete due to assembler or sequencing error, then it was excluded from the alignment for the given species. We treated the data from previously published studies in the same fashion as data generated in our lab by filtering the raw reads through quality trimming, then assembling de novo transcriptomes using the same programs and parameters (Table 1).
Amino acid sequences were aligned using MUSCLE v3.8.31 [85]. Alignments were also subjected to manual alignment in Aliview v1.26 [86]; extraneous data were trimmed, leaving only the aligned genes. Ambiguously aligned regions were eliminated from each alignment leaving only conserved and reliably aligned regions for phylogenetic analysis using the following parameters in Gblocks v0.91b [87]: -t+p -b3=8 -b4=2 -b5=h -b6=y. Phylutility v2.7.1 [88] was used to concatenate all gene alignment files.

Single-gene alignments were subjected to ML and BI analyses in order to infer single-gene phylogenies. Single-gene phylogenies were then further analyzed using a coalescent-based approach. The concatenated multi-gene alignment was partitioned so that the appropriate model of protein substitution was applied to each gene for the supermatrix phylogenetic approach under ML and BI.

The ML and BI analyses of the concatenated dataset used a partitioning strategy where the best evolutionary model for each gene was predicted by ProtTest v3.4.2 under the Akaike Information Criterion (AIC). For information regarding each predicted evolutionary model, please refer to Additional file 1: Table S4 [84, 89–91]. The ML analysis was conducted using IQTree v1.6.12 [92] under partition models [93]. Support values reported for the IQtree ML analysis were estimated through the bootstrap technique where 1000 ultrafast bootstrap replicates were generated [94]. The BI analysis was performed with MrBayes 3.2.7a [95] with 3 heated and 1 cold Markov chains, where trees were sampled every 1000 generations for a total of 1, 000,000 generations with 1000 trees discarded at the beginning of each chain (ngen = 100000000, sample-freq = 1000, burnin = 1000, rnruns = 4, nchains = 4, starttree = random).

ASTRAL [96] was used to perform the coalescent-based analysis where all 40 single-gene phylogenies produced by IQtree were used as the input after collapsing branches with low bootstrap support (< 10) using Newick Utilities v1.6 [97]. Posterior probabilities were assessed for the Bayesian and coalescent-based analyses in MrBayes and ASTRAL, respectively. Lastly, approximately unbiased (AU) tests with 100,000 RELL resamplings were conducted to test certain key topologies and hypotheses using IQtree (-zw 100000 -au) (Additional file 1: Fig. S1).

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Authors’ contributions

Performed the experiments (CRL), carried out the analyses (CRL, MDH), and wrote the manuscript (CRL, MDH, RFR). The authors have read and approved the final manuscript.

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Availability of data and materials

All raw data and materials used for this study have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under BioProject PRJNA701495 [98]. Accession numbers for our raw RNA-Seq reads range from SRR13719233 to SRR13719287, and accession numbers for our assembled contigs have been provided in Additional file 1: Table S4. For detailed information regarding accession number assignment to a specific taxon, please refer to Additional file 1: Table S5. Files containing our single-gene phylogenies and amino acid alignments, our IQtree partition file, and MrBayes configuration file have been uploaded to Dryad [99]. Previously published data used in this study from Hu et al. [47] and Featherston et al. [21] can be found under BioProject number PRJNA532307 [100] and PRJNA393411 [101], respectively.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12915-021-01087-0.

Additional file 1 Table S1. Accession numbers of previously published RNA-seq data. Table S2. Information on sampled genera regarding cellularity, typical cell number, differentiation, and gamete size. Table S3.
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