

On the rediscovery of *Volvox perglobator* (Volvocales, Chlorophyceae) and the evolution of outcrossing from self-fertilization

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ABSTRACT

Hypothesis: Genetic recombination underlies the frequent origins of self-fertilization and rare reversions to outcrossing.

Organisms: *Volvox perglobator*, *Gonium pectorale*, volvocine green algae.

Methods: We report the rediscovery of a volvocine alga, obligate outcrossing *V. perglobator*, including morphological characteristics and molecular phylogeny. We use ancestral-state reconstruction and phylogenetic simulation to demonstrate multiple transitions between self-fertilization and outcrossing. We sequence sex-determining genes in multiple volvocine algae.

Results: We find significant support for numerous origins of self-fertilization and multiple reversions to outcrossing. *Volvox perglobator* is one of these reversions to outcrossing. Combination of ancestrally sex-restricted and sex-determining genes into the same genotype correlates with the evolution of self-fertilization. Re-segregation of these same sex-determining loci into separate sexes correlates with the evolution of outcrossing. These results suggest that recombination underlies the evolutionary transitions between self-fertilization and outcrossing in the volvocine algae.

Keywords: homothallism, phylogenetics, reversion, self-fertilization, sexual reproduction, volvocine green algae.

INTRODUCTION

The evolutionary transition from outcrossing to self-fertilization is common throughout the Tree of Life (Whitehouse, 1949; Yun *et al.*, 1999; Jarne and Auld, 2006; McDaniel *et al.*, 2013; Wright *et al.*, 2013; Luthringer *et al.*, 2014). However, the reverse transition, the evolution of outcrossing from self-fertilizing ancestors, is hypothesized to be evolutionarily difficult and thus rare (Takebayashi and Morrell, 2001; Igc and Busch, 2013). The evolution of outcrossing from self-fertilization has occurred infrequently in fungi (Lee *et al.*, 2010; Billiard *et al.*, 2011), plants (Bawa, 1980; Ross, 1980; Shore and Barrett, 1985;

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Barrett and Shore, 1987; Barrett, 2003; Igic *et al.*, 2004), and the volvocine green algae (Hanschen *et al.*, 2018b). Why might outcrossing evolve from self-fertilization? What is the genetic basis for transitions between outcrossing and self-fertilization?

The evolution of obligate outcrossing from predominant self-fertilization is thought to be unlikely for four reasons. First, selfing produces homozygous offspring and, in homozygous offspring, selection readily purges deleterious alleles (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987; Crnokrak and Barrett, 2002). Purging greatly reduces inbreeding depression (Lande and Schemske, 1985), thus negating a major advantage of outcrossing, the avoidance of inbreeding depression. Purging prevents a selfing population from accumulating the deleterious mutations that select for outcrossing (Lande and Schemske, 1985). Thus, the transition to outcrossing is putatively rare because selfing can remove a major advantage of outcrossing. Second, self-fertilizing individuals show increased genetic transmission to their offspring (Fisher, 1941; Nagylaki, 1976). The offspring of an outcrossing individual contain 50–100% of alleles identical to either parent [depending on inbreeding frequency (Lehtonen and Kokko, 2012)]. In contrast, self-fertilizing individuals contribute all of the genetic material in their offspring. Therefore, the transmission advantage of self-fertilization relative to outcrossing can be as large as 50%. Third, outcrossing individuals lose the reproductive assurance of self-fertilization. If a species is locally rare, lives in an inherently patchy habitat, or is colonizing a new habitat, selfing individuals are more likely to reproduce than strictly outcrossing individuals (Darwin, 1877; Baker, 1955; Schoen *et al.*, 1996; Barrett, 2010). Finally, the complex genetic mechanisms underlying outcrossing may be lost through mutation and are not easily re-gained.

While these factors can make it difficult for outcrossing individuals to invade a selfing population, self-fertilization is not without its costs. Purging is a considerable fitness cost to individuals whose offspring are purged. Even in populations purged of recessive mutations, new deleterious mutations may accumulate in selfing populations if mutation effect sizes are small (Heller and Maynard Smith, 1979; Kondrashov, 1984; Schultz and Lynch, 1997) or if the mutation rate is elevated (Morran *et al.*, 2009). Moreover, new adaptive mutations cannot spread quickly through a selfing population, reducing the adaptive potential of selfing lineages (Stebbins, 1957; Felsenstein, 1974; Barton and Otto, 2005) and increasing the extinction risk of selfing lineages (Stebbins, 1957; Goldberg *et al.*, 2010).

Here, we investigate the evolution of obligate outcrossing from self-fertilizing ancestors in the Chlorophycean volvocine green algae. These algae are a tractable model system for studying the evolution of mating systems (Ferris and Goodenough, 1997; Nozaki *et al.*, 2006; Ferris *et al.*, 2010; Geng *et al.*, 2014; Hanschen *et al.*, 2018b). Volvocine algae are facultatively sexual with numerous rounds of asexual reproduction typically occurring between occasional rounds of sexual reproduction (Coleman, 1979; Kirk, 1998). Sexual reproduction generates a diploid zygote, which undergoes meiosis during germination (Fig. S1, see evolutionary-ecology.com/data/3149Appendix.pdf). Volvocine algae exhibit multiple mating systems (Fig. S1), including obligate outcrossing (heterothallism) and two forms of self-fertilization [monoecious and dioecious homothallism (Smith, 1944)]. Outcrossing and self-fertilization are defined at the genotype level; heterothallic species cannot self because mating types are different genotypes, while homothallic species can self-fertilize because a single genotype can produce both mating types. In obligate outcrossing species, distinct genotypes sexually differentiate into separate mating types or sexes (Fig. S1).

In homothallic monoecious species, a single individual is capable of sexually differentiating into both mating types or sexes and producing both gamete types in a single individual (Fig. S1). Monoecious colonies contain both sperm and eggs (in the case of anisogamy)

and are self-fertile (Overton, 1889; Ferris and Goodenough, 1997; Isaka *et al.*, 2012; Geng *et al.*, 2014; Nozaki *et al.*, 2015). Both intra-colony and inter-colony (within a single genetic strain) self-fertilization have been observed in homothallic monoecious species (Starr *et al.*, 1980; Nozaki *et al.*, 2015). In homothallic dioecious species (Fig. S1), a single genotype is capable of producing both types of gametes, but not in the same colony. In facultatively sexual volvocine algae, dioecy is not necessarily outcrossing. A selfing dioecious genotype can undergo asexual reproduction, and genetically identical daughter colonies may sexually differentiate as opposite sexes or mating types and mate, as in *V. aureus* (Darden, 1966).

Very little is known about self-fertilization rates in natural volvocine populations. Unfortunately, because the diploid phase of the life cycle is a single-celled, dormant zygospore, measuring rates of self-fertilization or homozygosity is challenging. Some selfing *Volvox* species are protandrous [sperm develop and liberate before egg development (Smith, 1944)], which may indicate a mixed-mating strategy [both self-fertilization and outcrossing (Goodwillie *et al.*, 2005)]. In *Volvox* section *Volvox* (studied here), almost all zygotes of homothallic monoecious *V. ferrisii*, *V. kirkiorum*, *V. barberi*, *V. capensis*, and *V. globator* are the result of intra-colony self-fertilization [in laboratory conditions (Dr. Hisayoshi Nozaki, personal communication; Starr *et al.*, 1980; Nozaki *et al.*, 2015)], suggesting these algae may be predominantly selfing. Given the potential for both self-fertilization and outcrossing in homothallic species, these species may represent a mixed-mating system (Goodwillie *et al.*, 2005); however, given the lack of selfing rate estimates, we distinguish homothallic species as predominantly self-fertilizing. Self-incompatibility has never been described in homothallic volvocine algae.

The genes underlying sexual differentiation have been investigated in six obligately outcrossing (heterothallic) volvocine species: *Chlamydomonas reinhardtii* (Ferris and Goodenough, 1997; Ferris *et al.*, 2002, 2010; Merchant *et al.*, 2007), *Gonium pectorale* (Hamaji *et al.*, 2016), *Yamagishiella unicocca* (Hamaji *et al.*, 2018), *Eudorina* sp. (Hamaji *et al.*, 2018), *Pleodorina starrii* (Nozaki *et al.*, 2006), and *Volvox carteri* (Ferris *et al.*, 2010). In these species, genes *FUS1* and *MID* play critical roles in sexual differentiation. *FUS1* is a (+) (in the absence of male and female sexes, mating types are denoted (+) and (–)) or female-restricted gene required for gamete fusion (Ferris *et al.*, 2002; Hamaji *et al.*, 2018), while *MID* is a (–) or male-restricted gene necessary for gametic differentiation (Ferris and Goodenough, 1997; Nozaki *et al.*, 2006; Ferris *et al.*, 2010; Hamaji *et al.*, 2013; Geng *et al.*, 2014). These loci are contained in non-recombining, sex-determining loci (potentially incipient sex chromosomes) in outcrossing species (Ferris *et al.*, 2002, 2010; Geng *et al.*, 2014; Hamaji *et al.*, 2016). Furthermore, low-level expression of *MID* in a (+) or female colony results in self-fertile hermaphroditic colonies (Ferris and Goodenough, 1997; Geng *et al.*, 2014). Therefore, we hypothesize that recombination of *FUS1* and *MID* into the same genotype occurs with the evolution of self-fertilization, and re-segregation of these alleles into separate sexes occurs during the transition from self-fertilization to outcrossing.

The evolutionary history of self-fertilization and outcrossing in the volvocine green algae includes eleven inferred independent origins of self-fertilization (including an independent origin in *Gonium pectorale* Russia) and two inferred reversions (*V. rousseletii* and *V. perglobator*) from self-fertilization to outcrossing (Hanschen *et al.*, 2018b). Both of these reversions occurred in a group of mostly selfing *Volvox* referred to as *Volvox* section *Volvox* (Fig. S2) (Hanschen *et al.*, 2018b). However, *Volvox* section *Volvox* also includes four described species that were lost from all known culture collections before genetic sequencing. Two of these species, *V. perglobator* and *V. prolificus*, are heterothallic outcrossing. The other two species, *V. amboensis* and *V. merrillii*, are homothallic selfing. Two synapomorphies (presence of thick cytoplasmic bridges in the adult colony and spines on zygote spores)

place lost *Volvox* species within *Volvox* section *Volvox* (Smith, 1944; Isaka *et al.*, 2012) and morphological and geographic differences support treating these described algae as separate species from those with sequence data (Smith, 1944; Isaka *et al.*, 2012). As both outcrossing and selfing are represented in lost species, their phylogenetic placement substantially affects ancestral-state reconstructions (Fig. S3). Therefore, further analysis, taking lost species of *Volvox* section *Volvox* into account, is required.

In this study, we report the rediscovery of ‘lost’ *V. perglobator* (Powers, 1908), which was lost from all known culture collections sometime after 1944 (Smith, 1944). We first detail the morphology and phylogenetic placement of *V. perglobator*. We next use ancestral-state reconstruction to infer the evolutionary history of self-fertilization in the volvocine algae. However, this reconstruction does not account for the effect of other described but lost species of *Volvox* on ancestral-state reconstruction of self-fertilization. Therefore, we use phylogenetic simulations to account for other lost species of *Volvox*, including both outcrossing and selfing species. Lastly, we investigate the genetic changes underlying evolutionary transitions between outcrossing and selfing. We show that recombination resulting in the combination and re-segregation of sex-determining loci is the genetic mechanism underlying the frequent transitions from outcrossing to self-fertilization and the rare transitions from self-fertilization to outcrossing in the volvocine green algae.

MATERIALS AND METHODS

Species characterization

Cultures of *Volvox* were isolated from two connected duck ponds in Reid Park, Tucson, Arizona (GPS coordinates: 32°12'35.6"N, 110°55'21.2"W) on 27 October 2012 (males), in September 2014 (females), and on 19 October 2017 (males and females). On 27 October 2012, the water temperature was 21°C and the pH was approximately 7.5; on 19 October 2017, the water temperature was 25°C and the pH was approximately 8.0. Single colonies were isolated and grown in standard *Volvox* medium (SVM) at 25°C on a 16/8 hour light/dark cycle at approximately 35 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Attempts to generate axenic strains through washes and antibiotic media resulted in algal death. Sexual differentiation into male and female colonies occurred spontaneously, usually 7 days after inoculation into 2 mL SVM (well plates). Every isolate produced either sperm or eggs, never both [heterothallic and dioecious (Hanschen *et al.*, 2018a, 2018b)]. Asexual, male, and female colonies were observed using a Nikon SMZ800 stereomicroscope and a Nikon Eclipse Ti-E inverted microscope (Nikon, Tokyo, Japan) throughout their life cycle. Zygotes, somatic cells, cytoplasmic bridges, and parthenospores were observed with a Leica DMI6000 inverted microscope (Leica, Wetzlar, Germany). Cell sheaths were observed using 10 μL of 1% (weight/volume in distilled water) methylene blue (Sigma-Aldrich, St. Louis, MO, USA), added to 380 μL of exponentially growing *V. perglobator* and mixed for 45 seconds prior to imaging on a Nikon Eclipse Ti-E inverted microscope (Nikon, Tokyo, Japan).

The number of somatic cells present in asexual colonies, male colonies, and female colonies was evaluated by counting the number of cells on the circumference (n) and inferring the total number of somatic cells (N) by assuming cells are hexagonally arranged (Fig. 1F) (Smith, 1944). We followed Janet (1912), setting the surface area of a sphere equal to the surface area of N hexagonal cells and using the equivalent formula for circumference to solve for N :

$$N = \frac{2}{\pi\sqrt{3}} n^2 = 0.367n^2.$$

We performed an experiment to investigate the occurrence, prevalence, and cause of embryos that cease to develop (Smith, 1944). A 100 μL aliquot of colonies, previously transferred to fresh SVM (10 mL tubes) every 7 days, was inoculated into 1.5 mL of SVM. To ensure sufficient sample size, four pseudo-replicates for each of two treatments were inoculated. After 4 days (approximately two generations), the number of daughter colonies and embryos that ceased to develop was counted for 100 haphazardly selected mature adults (treatment ‘Fresh’ in Fig. 1O; appearance similar to Fig. 1D). After a further 6 days (10 days in total since inoculation), the numbers of daughter colonies and embryos that ceased to develop were counted for 100 haphazardly selected mature adults (treatment ‘Older’ in Fig. 1O; appearance similar to Fig. 1P). A second experiment was conducted to ensure embryos that cease to develop do not reproduce. Twelve haphazardly selected colonies from the second treatment above were placed in 1.5 mL of fresh SVM and observed for several days. As predicted, embryos identified as having ceased to develop did not reproduce (appearance similar to Fig. 1Q).

To measure the distribution of colony dimensions and daughter colony dimensions at hatching/liberation for asexual colonies, a small aliquot of colonies, previously transferred to fresh SVM every 7 days (10 mL tubes), was transferred into 100 mL SVM and grown for 6 days before imaging ($n = 85$) using a Benchtop FlowCam model VS-IV (Fluid Imaging Technologies, Scarborough, ME) with 4 \times objective on Trigger mode.

All data and measurements collected from *V. perglobator* are in Dataset 1, available in the [Appendix](#).

Phylogenetic simulations

We used simulation to phylogenetically account for lost *Volvox* section *Volvox* species (*V. merrillii*, homothallic selfing; *V. amboensis*, homothallic selfing; *V. prolificus*, heterothallic outcrossing) in ancestral-state reconstructions of outcrossing and selfing. The steps of these simulations are summarized in Fig. S5 in the [Appendix](#).

First, a lost species was added to the *Volvox* section *Volvox*, diverging along a random edge. This was repeated for the second and third species (Fig. S5). Three methods were used to choose the edge a species was added to: (1) the probability of choosing any edge is equal; (2) the probability of choosing any edge is proportional to edge length; and (3) the probability of choosing any edge is inversely proportional to edge length. Given the effect of species relationships on ancestral-state reconstruction (Fig. S3), this approach provides a comprehensive sampling of possible *Volvox* section *Volvox* phylogenetic relationships. Once an edge was chosen, where along that edge the species was added was randomly calculated using a uniform distribution, which follows a Yule process [macroevolutionary process with a constant speciation rate, λ , for all species and no extinction (Yule, 1925)]. It was possible to choose the edge ancestral to all *Volvox* section *Volvox* species. When this occurred, the divergence of that lost species to the remaining *Volvox* section *Volvox* species was calculated from an exponential distribution with $\lambda = 1/\hat{a}$, where \hat{a} is the mean of internal edge lengths (Yule, 1925). Because the probability of adding the second species to any given edge is different from the probability of adding the first species to the same edge, the order of species added was randomized for each iteration.

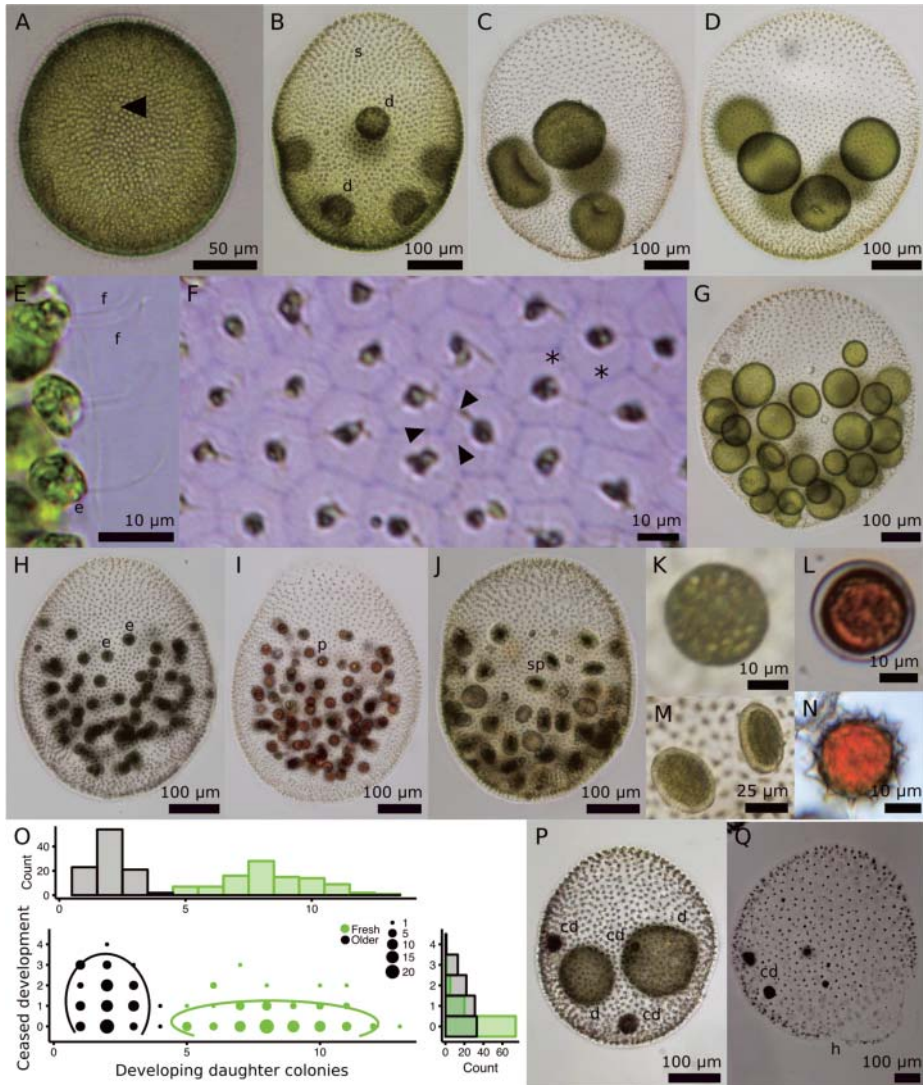


Fig. 1. Micrographs of *Volvox perglobator*. (A) Recently hatched asexual adult with reproductive gonidia visible (arrow). (B) Asexual adult with numerous somatic cells (s); daughter colonies (d) are visible. (C) Asexual adult with embryos near completion of inversion. (D) Asexual adult with daughter colonies near hatching. (E) Biflagellate (f) somatic cells with an eyespot (e). (F) Cytoplasmic bridges (arrows) in an asexual adult; hexagonal pattern of somatic cells (asterisks) visible after staining with methylene blue. (G) Adult asexual colony grown in SVM supplemented with sodium acetate. (H) Female colony with eggs (e). (I) Female colony containing parthenospores (p). (J) Male colony with sperm packets (sp). (K) Egg. (L) Parthenospore. (M) Sperm packets. (N) Diploid zygotic spore with straight, rounded-tip spines. (O) Bivariate distributions of daughter colonies and embryos ceasing development in fresh cultures (green) and one week older cultures (black). Univariate distributions are shown in top and right histograms. Ellipses represent 95% confidence regions. (P) Colony from a one-week-old culture with three embryos that ceased development (cd) and two daughter colonies (d). (Q) Post-hatching colony with four embryos that ceased development (cd), and the hole (h) where daughter colonies left the colony.

Second, an ancestral-state reconstruction was performed on all 69 volvocine species [with genetic data (Hanschen *et al.*, 2018b)] and three lost volvocine species (Fig. S5). The ancestral-state reconstruction was performed in two ways: (1) equal rates for transitions between states (ER) and (2) all rates different for transitions between states (ARD). Model fit was compared using the Akaike Information Criterion [AIC (Akaike, 1974)], corrected for small sample size (Burnham and Anderson, 2002), which should reveal the best-fitting model without including unnecessary parameters. The root prior was set to the outcrossing (heterothallic) state, which is consistent with previous results (Hanschen *et al.*, 2018b) and allows a more conservative test of reversals to outcrossing in *Volvox* section *Volvox*. Alternative root priors are statistically indistinguishable and do not affect ancestral-state reconstructions (Hanschen *et al.*, 2018b).

Third, four parameters were calculated from this ancestral-state reconstruction (Fig. S5): (1) the ancestral state for *Volvox* section *Volvox*; (2) the distribution of statistical confidence for all nodes in the *Volvox* section *Volvox* tree; (3) the number of *Volvox* section *Volvox* transitions from outcrossing to selfing; and (4) the number of *Volvox* section *Volvox* transitions from selfing to outcrossing. A total of 2500 iterations were visually inspected to ensure accuracy of these inferences.

Lastly, this simulation was repeated for 331,500 iterations for each method of choosing which edge to add a species to (Fig. S5). This number of iterations was derived by the number of possible edges (13 for the first species, 15 for the second species, 17 for the third species: $13 \times 15 \times 17 = 3315$), multiplied by 100 to ensure comprehensive sampling and to account for variation in where along each branch a species is added. In total, 994,500 simulations were performed.

A state-dependent speciation and extinction (SSE) approach, which estimates state-dependent speciation and extinction rates [Binary State Speciation and Extinction (Maddison *et al.*, 2007)], was not implemented. Previous BiSSE reconstructions of self-fertilization in the volvocine algae have resulted in unusual ancestral-state reconstructions, with numerous large clades of mostly outcrossing species having a strongly supported selfing ancestor (Hanschen *et al.*, 2018b). In these previous BiSSE analyses, two reversions from selfing to outcrossing are still observed in *Volvox* section *Volvox* (Figure S2 in Hanschen *et al.*, 2018b).

RESULTS

Description of *Volvox perglobator*

Isolates of *Volvox* were identified as the lost *V. perglobator* (Powers, 1908) based on dioecious, heterothallic sexual reproduction (Fig. 1H–M), males with a relatively large number of sperm packets (bundles of 64 sperm, Fig. 1J), and zygotes that form straight, rounded-tip spines (Fig. 1N) (Isaka *et al.*, 2012). The observation of embryos that cease to develop (Fig. 1O–Q) is also consistent with identification as *V. perglobator* (Smith, 1944).

Our isolates of *V. perglobator* exhibited a similar morphology to other *Volvox* section *Volvox* species (Fig. S2). Colonies were ovoid with 2500–7500 small, non-reproductive somatic cells and 4–13 larger, reproductive gonidia in the posterior of the colony (Fig. 1A–D, Table 1). Reproductive gonidia underwent numerous rounds of division to produce daughter colonies (Fig. 1B). Mature adult colonies (Fig. 1D) exhibited a size range of 466–687 μm (length) \times 449–667 μm (width) (Table 1). Daughter colonies grew (Fig. 1B) and underwent inversion (Fig. 1C) before hatching from the mother colony. Somatic cells had

Table 1. Morphological comparison of outcrossing (heterothallic) species of *Volvox* section *Volvox*

	<i>V. perglobator</i> Tucson	<i>V. perglobator</i>	<i>V. prolificus</i>	<i>V. rousseletii</i>
Size of asexual spheroid	466–687 × 449–667 μm, average 599 × 566 μm	550–980 × 610–1100 μm	975–1275 μm	690–2180 μm
Diameter of asexual spheroid at liberation	63–201 μm, average 153 μm	250–275 μm	345–450 μm	300–370 μm
Number of cells in asexual spheroid	2500–7500, average 3800	6500–12,000	9000–16,000	14,000–50,000
Number of gonidia in asexual spheroid	4–13, average 8.3	3–9	4–20, usually 7–10	1–16, usually 6–10
Diameter of gonidia	10.2–11.0 μm	12–14 μm	Irregular	13–15 μm
Arrangement of gonidia	Irregular	Irregular	Irregular	3–5 equatorial, others posterior
Shape of asexual colony	Ovoid	Ellipsoidal	Spherical-subsppherical	Subglobose to ovoid
Shape of somatic cell	Pear-shaped, stellate in posterior view	Stellate (posterior view)	Pear-shaped	Pear-shaped to ellipsoidal
Size of somatic cell	6.0–7.9 μm, average 7.0 μm wide 6.8–9.2 μm, average 8.0 μm long			
Size of female spheroid	433–866 × 432–807 μm, average 687 × 618 μm	340–500 × 485–535 μm	400–1090 μm	646–1292 μm
Number of somatic cells in female spheroid	2200–8100, average 5900	8500–12,900	8500–12,900	
Number of eggs in sexual spheroid	18–86, average 53	48–121, usually 50–100	80–500, usually 80–200	60–227, usually 160–170
Diameter of eggs	21–29 μm, average 25.5 μm			
Parthenospore diameter	20–29 μm, average 25 μm		24.5–26.4 μm	

Size of male colony	379–739 × 330–618 μm, average 521 × 431 μm	320–440 μm	315–420 μm	700–770 μm
Number of cells in male spheroid	4600–8900, average 6300	5300–9200	5300–9200	
Number of sperm packets in male spheroid	48–73, average 64	60–80	25–55, but up to 300	108–214
Size of sperm packets	34.2–46.3 × 21.4–37.3 μm, average 42.3 × 28.8 μm	25–34 μm	30–40 μm	35–43 μm
Diameter of zygotes (without spines)	21–31 μm, average 27 μm	30–34 μm	30–36 μm	35–44 μm
Shape of spines of zygote	Straight, apices rounded	Straight, apices rounded	Straight, apices rounded	Curved, apices acute
Length of zygote spines	2.5–5.4 μm, average 4.0 μm	4 μm	5–8 μm	4.5–12 μm
Number of embryos ceasing to develop	0–3, average 0.33 in good conditions 0–4, average 1.18 in older cultures			
Diameter of embryos ceasing to develop	18.2–38.8 μm, average 25.9 μm			
Geography	USA (Tucson, AZ)	USA (Nebraska, Kansas, California)	India (Madras, Gujarat)	Egypt, Zimbabwe, South Africa
Other notes	Embryos rarely cease to develop, more often when in poor conditions	Embryos rarely cease to develop between 16- and 64-cell stage		
References	This study	Powers (1908) Smith (1944) Dillard (1989)	Iyengar (1933) Smith (1944) Patel (1978)	West (1910, 1918) Rich and Poccock (1933) Poccock (1933) Smith (1944)

two flagella and an eyespot (stigma, Fig. 1E) and were broadly arranged in a hexagonal grid and connected by cytoplasmic bridges (Fig. 1F).

Sexual reproduction of *V. perglobator* did not occur in nitrogen deprivation media or after addition of acetate [some species of *Volvox* section *Volvox* sexually differentiate in acetate media (Isaka *et al.*, 2012)]. In media supplemented with acetate, *V. perglobator* produced 11–35 offspring (Fig. 1G). Sexual reproduction of *V. perglobator* instead occurred after approximately 7 days of growth in 2 mL of SVM. The frequency of sexual differentiation was low, approximately 10 colonies per 2 mL of SVM. Every isolate produced either sperm packets (3 isolates) or eggs (4 isolates), never both in the same isolate or strain, indicating dioecious heterothallism (Powers, 1908; Smith, 1944). Male colonies were 379–739 μm (length) \times 330–618 μm (width), with 4600–8900 somatic cells and 48–73 sperm packets (Table 1). Female colonies were 433–866 μm (length) \times 432–807 μm (width), with 2200–8100 somatic cells and 18–86 eggs (Table 1). When male and female colonies were mixed, sex was oogamous (flagellated sperm and unflagellated eggs; Fig. 1K,M) with internal fertilization. When fertilized, eggs developed into zygotes, turning red and producing straight, rounded-tip spines (Fig. 1N). Zygotes were 21–31 μm in diameter with 2.4–5.4 μm spines (Table 1). When not fertilized, eggs formed reddish-brown, smooth-walled parthenospheres (Fig. 1L). We were unable to germinate zygotes or parthenospheres.

Occasionally, *V. perglobator* contains embryos that cease to develop between the 16- and 64-cell stage and disintegrate (Smith, 1944). The rediscovery of *V. perglobator* allows investigation into this poorly understood and seemingly deleterious trait. The embryos that cease to develop are 18.2–38.8 μm in diameter [which is larger than somatic cells (6.0–9.2 μm in diameter) and smaller than reproductive daughter colonies in the same colony (55.9–180.4 μm in diameter)] and much darker (Fig. 1P, Table 1). Even when transferred to fresh SVM media, these embryos do not reproduce, instead they die (Fig. 1Q). We counted the number of embryos that produce reproductive daughter colonies ($n = 100$) and embryos that cease to develop ($n = 100$) in two conditions – fresh SVM media and older SVM media (see Methods and materials). The number of reproductive embryos and embryos that ceased to develop in our two treatments was significantly different [Hotelling's T^2 -test, $T^2 = 1057.1$, $P = 0$ (Hotelling, 1931)]. Colonies in older cultures have significantly fewer embryos producing daughter colonies [two-sided Mann-Whitney U -test, $W = 9999$, $P = 2.2 \times 10^{-16}$ (Mann and Whitney, 1947)] and have significantly more embryos that cease to develop (two-sided Mann-Whitney U -test, $W = 2658.5$, $P = 3.2 \times 10^{-10}$). As older cultures have fewer nutrients (consumed by previous generations) and more waste products (produced by previous generations), embryos that cease development may be used to ensure sufficient resources for fewer offspring when colonies develop in deteriorating environments.

Phylogenetic analysis with both multi-gene chloroplast (Figs. 2A, S2) and nuclear ITS (Fig. 2B) demonstrated that *V. perglobator* is a member of *Volvox* section *Volvox*, as previously predicted (Smith, 1944). *Volvox merrillii*, *V. amboensis*, and *V. prolificus* are also predicted to be members of *Volvox* section *Volvox* (Smith, 1944). The phylogenetic placement of *V. perglobator* in *Volvox* section *Volvox* may be used as support for treating other lost species of *Volvox* as indeed belonging to this clade. Importantly, this phylogenetic analysis allows ancestral-state reconstruction of outcrossing and self-fertilization in *Volvox* section *Volvox*.

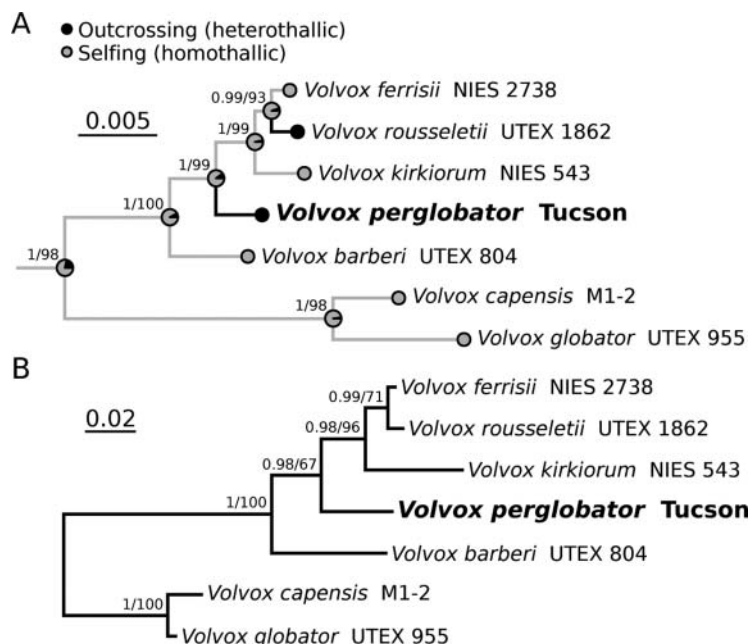


Fig. 2. (A) Rooted volvocine tree based on five chloroplast genes with ancestral-state reconstruction of outcrossing and self-fertilization. Branch colour – outcrossing (black, heterothallism) and selfing (grey, homothallism) – refers to the most likely state inferred by maximum likelihood reconstruction. Pie charts at nodes represent scaled marginal likelihoods, calculated using all available volvocine algae (69 taxa) and an ultrametric tree. See Hanschen *et al.* (2018b) and Fig. S2 for ancestral-state reconstruction of self-fertilization across all volvocine algae. (B) An unrooted maximum likelihood tree of ITS region of nuclear ribosomal DNA (ITS-1, 5.8S rDNA, and ITS-2). For both trees, independent Bayesian and maximum likelihood analyses were performed, resulting in the same topology (branch lengths for (A) are from the Bayesian analysis, branch lengths from (B) are from the maximum likelihood analysis). For both trees, numbers indicate Bayesian posterior probabilities and ML bootstrap values, respectively.

Evolution of mating systems

Based on ancestral-state reconstruction (see [Appendix](#)), self-fertilization has arisen eleven times independently in the volvocine algae (Fig. S2) (Hanschen *et al.*, 2018b), including in *Gonium pectorale* Russia (Fig. S2). Outcrossing has arisen from a self-fertilizing ancestor on two independent occasions in *Volvox* section *Volvox*: in *V. perglobator* and *V. rousseletii* (Figs. 2A, S2) (Hanschen *et al.*, 2018b). However, this analysis does not include several lost species of *Volvox* section *Volvox*, including outcrossing *V. prolificus*, selfing *V. amboensis*, and selfing *V. merrillii*. The phylogenetic placement of lost *Volvox* species has important effects on ancestral-state reconstruction (Fig. S3). Depending on phylogenetic relationships, ancestral-state reconstruction infers different evolutionary histories of self-fertilization in *Volvox* section *Volvox*, from two reversions to outcrossing to seven origins of selfing and no reversions to outcrossing (Fig. S3). Furthermore, morphological traits do not inform the phylogenetic placement within *Volvox* section *Volvox* of these species (Fig. S4). Therefore, we used phylogenetic simulations to account for these lost species (Fig. S5).

Focusing on the evolutionary history of selfing within *Volvox* section *Volvox*, simulations ranged from a largely outcrossing evolutionary history in *Volvox* section *Volvox* (with 5–7 independent origins of selfing and no reversions) to a largely selfing evolutionary history in *Volvox* section *Volvox* (with 1–2 origins of selfing and 2–3 reversions). This latter

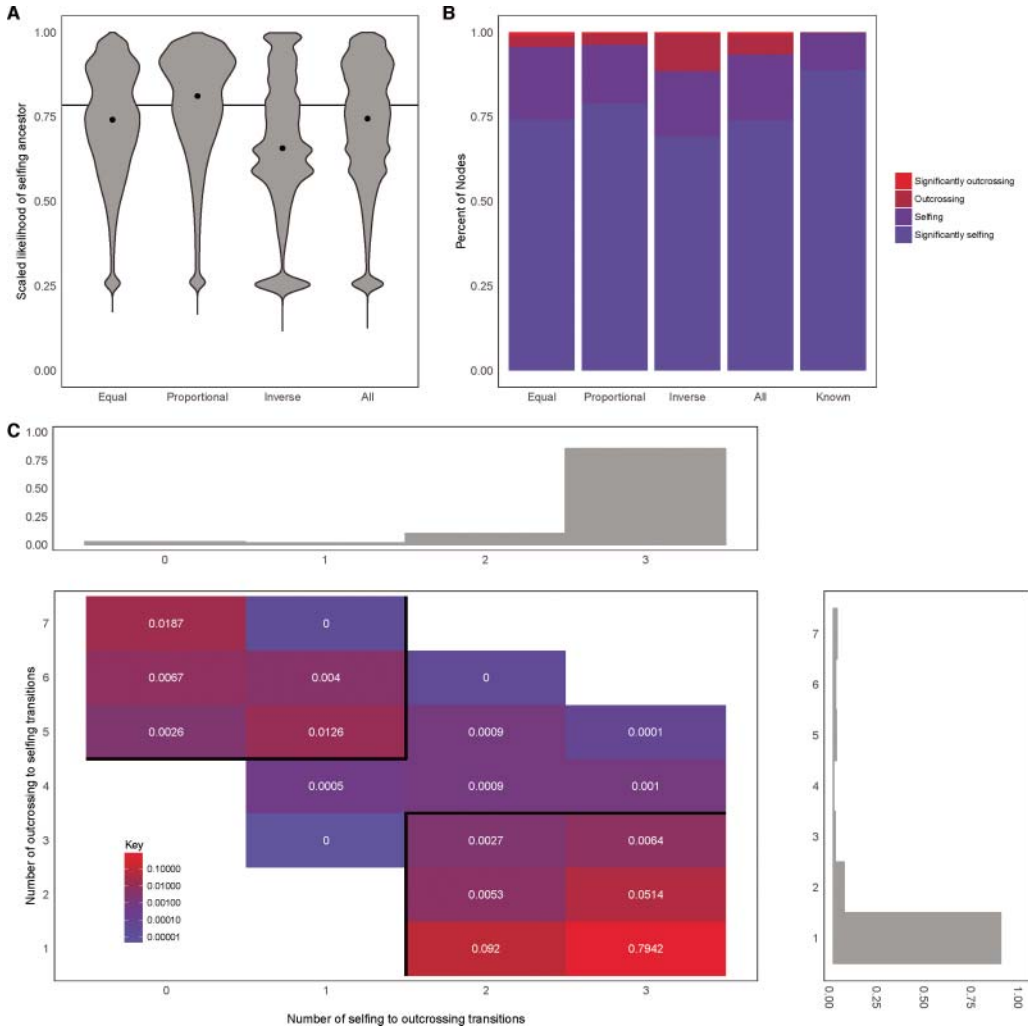


Fig. 3. Summary of phylogenetic simulations including lost *Volvox* section *Volvox* species. (A) Distribution of scaled marginal likelihoods for the ancestral state of *Volvox* section *Volvox* being selfing for equal, proportional, inverse proportional, and pooled (All) simulation data. The median of each treatment is indicated by a dot. The marginal likelihood when including only known species is denoted by a horizontal line. (B) The percentage of statistically significant and non-significant nodes inferred to be selfing or outcrossing for all simulations; the distribution of nodes when including only known species is indicated (Known). (C) Heat map of the number of transitions from selfing to outcrossing and outcrossing to selfing with univariate distributions plotted as histograms (top, right). The frequency of each cell is indicated in white. Including lost species in analyses does not substantially change the ancestral-state reconstructions, selfing is ancestral and predominant in *Volvox* section *Volvox*, with multiple reversions to outcrossing.

reconstruction occurred in 95.2% of all simulations (Fig. 3C). Across all simulations, the ancestor of all *Volvox* section *Volvox* was selfing (Fig. 3A) and 94.1% of internal nodes in *Volvox* section *Volvox* were selfing (Fig. 3B). Furthermore, the existence of at least one transition from self-fertilization to outcrossing was strongly supported (simulation *P*-value, $P = 0.0279$) and the existence of multiple independent transitions from self-fertilization to outcrossing was strongly supported (simulation *P*-value, $P = 0.0451$).

Genetic mechanism underlying transitions between selfing and outcrossing

To understand the genetic mechanism underlying transitions between selfing and outcrossing in the volvocine algae, we investigated sex genes in an independent origin of selfing (*Gonium pectorale* Russia) and a reversion to outcrossing (*V. perglobator*; see Appendix for methods). Given the restriction of sex-determining *MID* to (–) and males and the restriction of *FUS1* to (+) and females in all studied heterothallic species (Nozaki *et al.*, 2006; Ferris *et al.*, 2010; Hamaji *et al.*, 2016, 2018), we hypothesize that *FUS1* and *MID* loci recombine into the same homothallic genotype during the evolution of selfing, and re-segregate during reversals to outcrossing. We sequenced the *FUS1* and *MID* genes in single-cell isolates of selfing *G. pectorale* Russia (Figs. 4A,B, S6), demonstrating these genes have recombined into the same genotype (Fig. 4B). In contrast, in the closely related but phylogenetically divergent (Hanschen *et al.*, 2018b) heterothallic outcrossing *Gonium pectorale* NIES-1711 (Fig. S2), the *FUS1* and *MID* genes are restricted to genetically differentiated mating type loci (Hamaji *et al.*, 2016). Thus, recombination resulting in *FUS1* and *MID* present in the same genotype correlates with the evolution of homothallic self-fertilization.

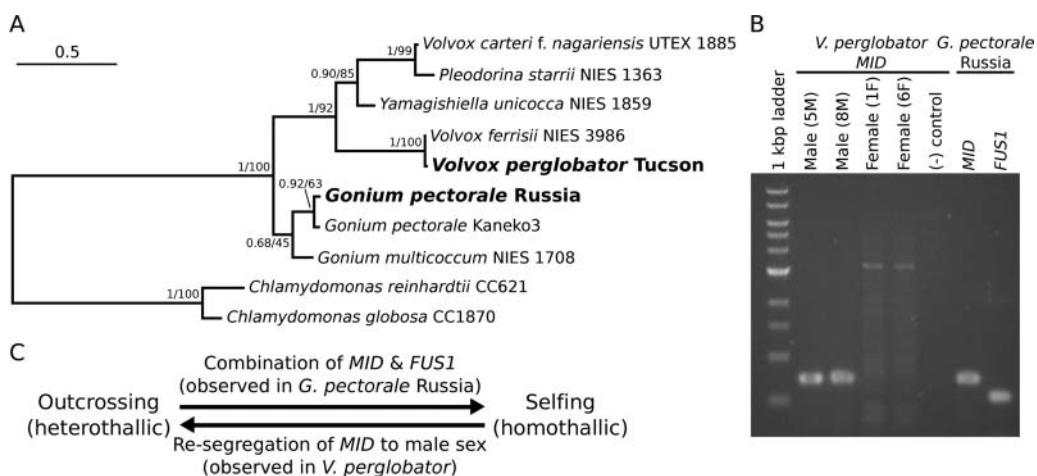


Fig. 4. Phylogeny and sex distribution of the mating type- and sex-determining *MID* gene. (A) An unrooted maximum likelihood tree of *MID* genes. Independent Bayesian and maximum likelihood estimations produced the same topology; branch lengths shown here are from the maximum likelihood analysis. Numbers indicate Bayesian posterior probabilities and ML bootstrap values, respectively. (B) Gel showing sex-restriction of *MID* to males in outcrossing *Volvox perglobator* and recombination of *MID* and *FUS1* into the same genome of selfing *Gonium pectorale* Russia. (C) Summary of the genetic changes underlying transitions between outcrossing and selfing in the volvocine green algae.

Furthermore, the *MID* gene in *V. perglobator* (Fig. 4A) is restricted to male individuals and is not found in females (Fig. 4B). Thus, recombination resulting in re-segregation of *FUS1* and *MID* into the separate genotypes correlates with the evolution of heterothallic outcrossing. As predicted, the *FUS1* and *MID* genes combine during the evolution of selfing (Fig. 4C) and *MID* re-segregates to the male sex during the evolution of outcrossing (Fig. 4C). To our knowledge, this is the first demonstration of such recombination underlying mating type transitions in the volvocine algae.

DISCUSSION

The rediscovery of outcrossing *Volvox perglobator* (Fig. 1) allows investigation into the evolution of self-fertilization, informing both the evolutionary history of self-fertilization and the genetics that underlie transitions between selfing and outcrossing. Phylogenetic analysis and ancestral-state reconstructions demonstrate that *V. perglobator* belongs to *Volvox* section *Volvox* (lending support to the inclusion of other lost species in *Volvox* section *Volvox*) and represents a rare reversion from selfing to outcrossing (Figs. 2, S2). However, given the profound effect of lost *Volvox* species on the evolutionary history of self-fertilization (Fig. S3), comprehensively taking lost *Volvox* species into account is critical. Our results when including other lost species of *Volvox* in phylogenetic simulations (Fig. 3) are consistent with the conclusion of reversions to outcrossing. We show that recombination resulting in the combination and re-segregation of sex-determining loci underlies the evolutionary transitions between self-fertilization and outcrossing (Figs. 4, S6).

Mating system evolution in the volvocine green algae

Similar to multiple origins of self-fertilization across eukaryotes, we have previously shown that self-fertilization repeatedly evolved in the volvocine green algae (Fig. S2) (Hanschen *et al.*, 2018b). We inferred two unexpected reversions from self-fertilization to outcrossing (Figs. 2A, S2) (Hanschen *et al.*, 2018b), including *V. rousseletii* and the rediscovered *V. perglobator*. However, previous analyses did not take into account other lost species of *Volvox* (in *Volvox* section *Volvox*). Including these lost species may contradict the inferred evolutionary history of mating systems (Fig. S3). Our phylogenetic simulations take three described yet lost species of *Volvox* section *Volvox* into account. Based on these simulations, we reject the possibility of no transitions from self-fertilization to outcrossing (simulation *P*-value, $P = 0.0279$) and the possibility of only a single transition from self-fertilization to outcrossing (simulation *P*-value, $P = 0.0451$). Although a future rediscovery of *V. prolificus*, or other *Volvox* section *Volvox* species, would inform phylogenetic inference, our simulations strongly suggest that multiple transitions from self-fertilization to outcrossing occurred in *Volvox* section *Volvox* (Fig. 3).

Why might these unexpected transitions from self-fertilization to outcrossing occur? We speculate the haploid-dominant life cycle of the volvocine green algae may negate major factors influencing transitions between selfing and outcrossing: inbreeding depression and the purging of deleterious mutations. In the haploid-dominant life cycles of volvocine algae (Coleman, 2012), inbreeding depression due to increased homozygosity is not possible. Inbreeding depression may accumulate through deleterious alleles only expressed in the dormant, zygotic phase; unfortunately, little is known about gene expression in the zygote.

Therefore, little inbreeding depression is expected in volvocine algae. On one hand, this means an advantage of outcrossing (the masking of deleterious alleles) is not applicable and fixation of selfing is more likely (Lande and Schemske, 1985). On the other hand, this means purging, thought to be primarily preventing the transition to outcrossing (Lande and Schemske, 1985), is also not applicable. In the absence of both inbreeding depression and purging of inbreeding depression, other evolutionary factors may drive the transition from selfing to outcrossing.

Other evolutionary factors inhibiting transitions from selfing to outcrossing likely include the genetic transmission advantage and reproductive assurance of self-fertilizing individuals (Fisher, 1941; Baker, 1955). Reproductive assurance may be particularly important to the evolution of selfing during colonization of a new pond by a single individual. In this scenario, while a homothallic individual would be mating with genetically identical individuals, a heterothallic individual would be extirpated upon failure to mate after sexual differentiation. Colonization facilitated by selfing would also strongly select for local adaptation (Lenormand, 2012). However, self-fertilization has the significant evolutionary disadvantage of reduced rates of adaptation to environmental, including parasitic, pressures compared with outcrossing (Fisher, 1930; Stebbins, 1957; van Valen, 1973; Felsenstein, 1974; Barton and Otto, 2005; Wright *et al.*, 2013). Similarly, local adaptation may reduce genetic variation, thereby reducing evolutionary potential. Outcrossing may also have the benefit of decreased fixation of deleterious mutations (Muller, 1964; Heller and Maynard Smith, 1979; Kondrashov, 1984, 1985, 1988; Morran *et al.*, 2009). The advantage of faster rates of adaptation and fewer fixed deleterious mutations in *V. perglobator* and *V. rousseletii* lineages may have outweighed the reduced genetic transmission and reproductive assurance, resulting in transitions from self-fertilization to outcrossing.

Alternatively, non-adaptive processes may explain the repeated transitions between outcrossing and self-fertilization. Adaptive explanations for the advantage of self-fertilization (leading to the fixation of selfing) or the advantage of outcrossing (leading to the fixation of outcrossing) would be caused by ecological differences among specific lineages. What unique ecology would have selected for outcrossing in *V. perglobator* and *V. rousseletii* but not in *V. capensis* or *V. globator*? In the absence of known ecological differences, especially in species which are highly morphologically similar (see Table 1; although, of course, important ecological differences may exist) (Isaka *et al.*, 2012), non-adaptive processes such as mutational recombination may explain these transitions. Perhaps heterothallic mutants drifted to fixation in the *V. perglobator* and *V. rousseletii* lineages but were lost in other homothallic lineages.

A caveat when inferring multiple reversions from selfing to outcrossing is the possibility that the chloroplast tree does not accurately estimate the species tree (Hanschen *et al.*, 2018b). A multi-locus nuclear gene dataset would be preferable; however, such a dataset is currently unavailable for the volvocine algae. Nonetheless, two nuclear gene datasets support the chloroplast tree included here. First, a phylogenomic analysis including eight outgroup green algae, *Chlamydomonas reinhardtii*, *Gonium pectorale*, and *Volvox carteri* (Hanschen *et al.*, 2016) found a species tree congruent with the chloroplast tree used here. Second, the nuclear ITS tree (Fig. 2B) is congruent with the chloroplast tree (Fig. 2A), suggesting both the ITS and chloroplast trees accurately estimate the species tree topology.

An additional caveat when inferring transitions between selfing and outcrossing is the possibility of mixed-mating strategies. While mixed-mating strategies, a combination of selfing and outcrossing, are predicted to be evolutionarily unstable (Lande and Schemske, 1985),

natural populations commonly display mixed mating (Vogler and Kalisz, 2001; Ivey and Carr, 2005). Mixed mating is hypothesized to have implications for inbreeding depression and the transmission advantage of selfing individuals (Goodwillie *et al.*, 2005; Karron *et al.*, 2012). Given how little is known about self-fertilization rates in natural volvocine populations, we are unable to determine if homothallic volvocine species deploy mixed-mating strategies, although homothallic *Volvox* section *Volvox* species may be predominantly selfing (Dr. Hisayoshi Nozaki, personal communication). Furthermore, we cannot speculate on how a volvocine mixed-mating strategy would impact inbreeding depression in zygotes and individual fitness.

Genetic mechanisms of mating system evolution

The repeated evolution of self-fertilization in both volvocine algae and fungi is mechanistically caused by the recombination of sex-determining genes. Recombination and fusion of *MAT* genes is the genetic mechanism underlying the evolution of homothallic self-fertilization in fungi (Glass *et al.*, 1990; Randall and Metzenberg, 1995; Pöggeler *et al.*, 1997; Yun *et al.*, 1999; Lee *et al.*, 2010). Similarly, we have shown that recombination of *MID* and *FUS1* genes into the same genotype occurs on the same branch as the evolution of self-fertilization in the volvocine algae (Figs. 4, S2, S6). This result is consistent with previous experiments in *Chlamydomonas reinhardtii* and *Volvox carteri* where *MID* was expressed at low levels in the (+) or female genotype, respectively. Both experiments resulted in self-fertile, hermaphroditic colonies (Ferris and Goodenough, 1997; Geng *et al.*, 2014), suggesting recombination underlies the evolutionary origin of self-fertilization across the volvocine algae.

The reverse transition, from self-fertilization to outcrossing, may involve chromosome breaks and segregation of *MAT* genes in fungi (Lee *et al.*, 2010). Similarly, we have shown re-segregation of the (-)/male-determining *MID* gene to males correlates with the origin of outcrossing (Fig. 4B,C). This suggests a similar genetic mechanism, recombination causing combination and re-segregation, underlies transitions in mating systems in these lineages. While further investigation into the genomic structure and genomic consequences of transitions between self-fertilization and outcrossing is warranted, the genetics investigated here (Figs. 4, S6) represent a step towards understanding the relatively simple genetics underlying mating system evolution in the volvocine green algae.

A caveat of these results is that we have not demonstrated recombination of *MID* and *FUS1* into the same genotype of homothallic *Volvox* section *Volvox* species, only homothallic *Gonium pectorale* Russia. Future genomic studies in *Volvox* section *Volvox* will further elucidate the correlation of mating-type genes with mating system evolution.

CONCLUSION

Self-fertilization has evolved numerous times from outcrossing, though the reverse transition, from self-fertilization to outcrossing, is thought to be rare. Selfing individuals are predicted to have the advantages of purging, reproductive assurance, and increased genetic transmission, making it unlikely that outcrossing individuals invade a selfing population. The rediscovery of *Volvox perglobator* allows investigation into transitions from selfing to outcrossing by combining phylogenetic analysis and genetics. In this study, we: (1) characterize recent isolates of *V. perglobator* and use ancestral-state reconstruction to demonstrate *V. perglobator* represents a reverse transition from selfing to outcrossing; (2) account for

other described but lost species of *Volvox*, supporting the main conclusion of multiple reverse transitions; and (3) investigate the combination of sex-determining genes during the origin of self-fertilization and the re-segregation of these genes during the transition back to outcrossing. The haploid-dominant life cycle of volvocine algae may reduce the effects of inbreeding depression, leaving other evolutionary factors to have greater influence on transitions between self-fertilization and outcrossing. Together, these results illuminate the transitions between self-fertilization and outcrossing and the genetic mechanism underlying these transitions in the volvocine algae.

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