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Three sex phenotypes in a haploid algal species give insights into the evolutionary transition to a self-compatible mating system*

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Mating systems of haploid species such as fungi, algae, and bryophytes are either heterothallic (self-incompatible) with two sex phenotypes (male and female, or mating type *minus* and *plus* in isogamous species) or homothallic (self-compatible) with only a bisexual phenotype producing zygotes within a clone. The anisogamous volvocine green alga *Pleodorina starrii* is a haploid species previously reported to have a heterothallic mating system. Here, we found that two additional culture strains originating from the same water system of *P. starrii* were taxonomically identified as *P. starrii* and produced male and female gametes and zygotes within a clone (bisexual). Sequences of rapidly evolving plastid genome regions were identical between the bisexual and unisexual (male or female) *P. starrii* strains. Intercrossings between the bisexual and unisexual strains demonstrated normal thick-walled zygotes and high survivability of F1 strains. Thus, these strains belong to the same biological species. *Pleodorina starrii* has a new haploid mating system that is unique in having three sex phenotypes, namely, male, female, and bisexual. Genetic analyses suggested the existence of autosomal "bisexual factor" locus independent of volvocine male and female determining regions. The present findings increase our understanding of the initial evolutionary step of transition from heterothallism to homothallism.

KEY WORDS: **Haploid species, inbreeding, life-history evolution, mating systems, reproductive isolation, sex.**

Androgyny is found in certain gods in mythologies worldwide, and it is one of the three sex phenotypes of ancient humans (double creatures) according to Aristophanes' Speech from Plato's Symposium (Dover 1966). Co-existence of three sex phenotypes (males, females, and hermaphrodites) is called "trioecy," which is relatively common in flowering plants (Fleming et al. 1994), while it is rare in animals, recognized only in some invertebrates (Weeks 2012; Tandonnet et al. 2019). Mixed mating systems such as trioecy may represent intermediate states of evolutionary transitions between dioecious (with male and female) and monoecious (with only hermaphrodites) mating systems in diploid organisms (Weeks 2012). However, haploid mating

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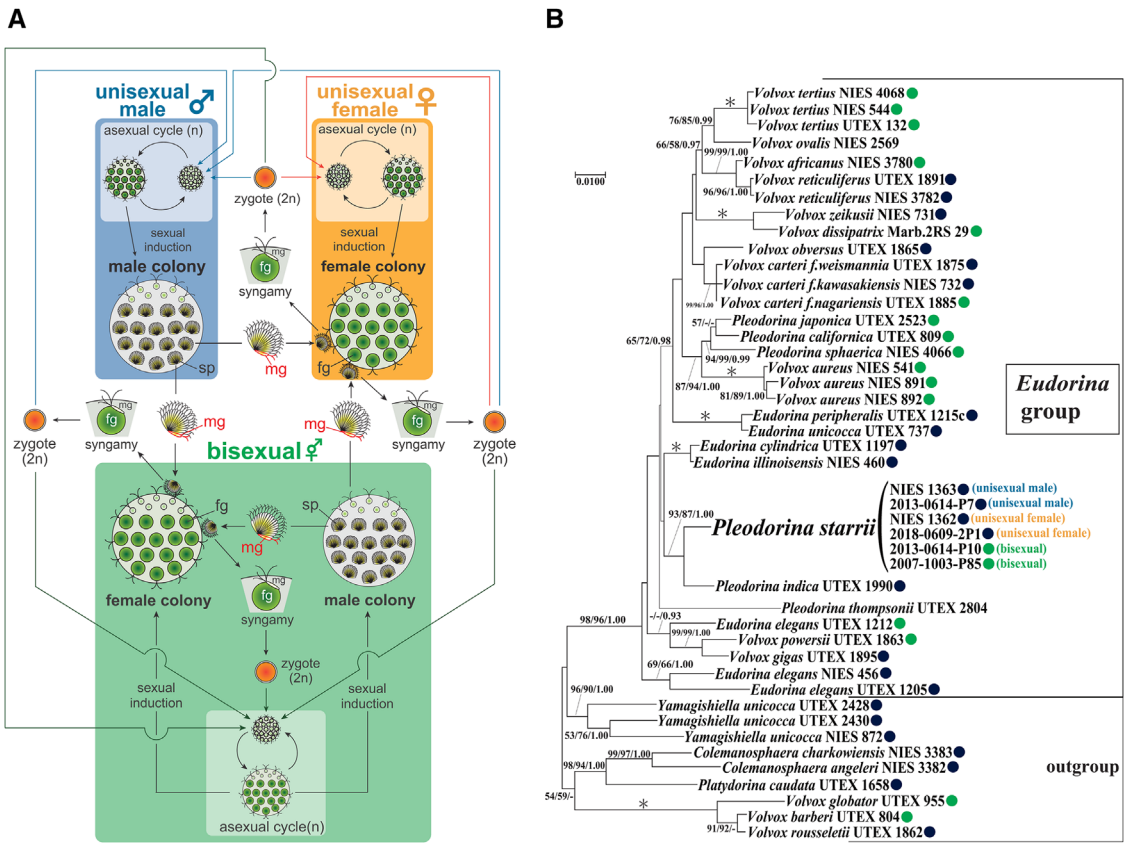

Figure 1. Schematic drawings of life cycle and phylogenetic position of *Pleodorina starrii* with three sex phenotypes. (A) When sexually induced, unisexual male and female strains produce male and female sexual colonies, respectively, whereas bisexual strain produces both male and female ones. Possible F1 sex phenotypes are shown by arrows from four types of zygotes. sp: sperm packet (bundle of male gametes), mg: male gamete, fg: female gamete. (B) Maximum-likelihood (ML) tree (based on the GTR + G + I model) based on coding regions of *rbcL* (1128 base pairs) from anisogamous/oogamous members of colonial volvocine species (*Eudorina* group, Nozaki et al. 2014) (Table S2). Branch lengths are proportional to the estimated nucleotide substitutions, which are indicated by the scale bar. Numbers at left, middle, and right above branches indicate bootstrap values of the ML (≥50%), maximum parsimony (≥50%), and posterior probabilities (PP) of Bayesian inference (≥0.90), respectively. Asterisks at the branches indicate 100% bootstrap values and 1.00 PP by the three methods. Navy circles and green circles represent unisexual and bisexual strains, respectively.

systems with three sex phenotypes within a single biological species have not been previously reported. It is generally considered that bryophytes, haploid algae, and fungi have only two types of mating systems, heterothallism and homothallism, which exhibit only two [unisexual males and females (mating type *minus* and *plus* in isogamous species)] and one (bisexual) genetically determined sex phenotype, respectively (Bold and Wynne 1985; Ni et al. 2011; Haig 2016; Coelho et al. 2018).

In 2006, a new male-specific gene "*PlestMID*" (*Pleodorina starrii* minus dominance gene) was found from male culture strains of the anisogamous colonial volvocine green alga *P. starrii* originating from the Sagami River water system, Japan (Nozaki et al. 2006a). The finding of this gene triggered subsequent extensive studies on the evolution of sex focusing on *MID* homologs and their harbored sex-determining regions (SDRs) in the colonial volvocine algae (e.g., Ferris et al. 2010; Hamaji et al. 2018). *Pleodorina starrii* has been believed to have a heterothallic mating system with males and females (Nozaki et al. 2006b; Nozaki 2008). However, during our long-term field surveys in the Sagami River water system, we encountered two bisexual *Pleodorina* strains producing zygotes within a clonal culture. The present study was undertaken to resolve the evolutionary relationships among the male, female, and bisexual culture strains of *Pleodorina* originating from the same water system. Our molecular and genetic data unambiguously demonstrated culture strains of all three sex phenotypes belong to the same biological species of *P. starrii* (Fig. 1).

Materials and Methods

STRAIN AND CULTURE CONDITIONS

Culture strains of *P. starrii* used in this study are shown in Table S1. The cultures were grown in screw-cap tubes containing 10 mL AF-6 medium (Kato 1982; Kawachi et al. 2013) at 20°C

on a 14-h light:10-h dark schedule under cool-white fluorescent lamps at an intensity of 55–80 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Sexual reproduction was induced as described previously (Nozaki et al. 2006b) with slight modifications (Supporting Information S1).

GENOMIC PCR AND SEQUENCING

To determine nucleotide sequences of exons and group I introns of *rbcL* (large subunit of Rubisco) (Fig. S1), plastid microsatellite region (Fig. S2), and *PlestMID* gene (Fig. S3), genomic PCR and DNA sequencing were performed (Supporting Information S1). To detect the presence of *PlestMID* gene in two bisexual strains (P10 and P85) (Fig. S3), genomic PCR of *PlestMID* was performed (Supporting Information S1).

CDNA SEQUENCING AND SEMIQUANTITATIVE RT-PCR

RNA was extracted from sexually induced cultures of unisexual male strain (P7) and bisexual strain (P10) with Dynabeads™ mRNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) after the cells had been homogenized with lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM EDTA, 2% SDS). Using the mRNA, amplification of cDNA, sequencing of *PlestMID* cDNA, and semiquantitative RT-PCR of *PlestMID* were carried out (Supporting Information S1).

PHYLOGENETIC ANALYSES OF *rbcL* GENES

For phylogenetic analyses, nucleotide sequences of *rbcL* coding regions (1128 base pairs) from 31 ingroup operational taxonomic units (OTUs) (representing 36 strains of anisogamous/oogamous members of the colonial Volvocales [*Eudorina* group; Nozaki et al. 2014] including four unisexual and two bisexual strains of *P. starrii*) and nine outgroup OTUs (Table S2) were aligned and subjected to maximum likelihood and maximum parsimony methods and Bayesian inference (Supporting Information S1). The alignment is available in TreeBASE (https://www.treebase.org/treebase-web/home.html; Study ID 28238).

MORPHOLOGICAL OBSERVATIONS

Vegetative colonies and sexual reproduction were observed as described in Supporting Information S1.

ESTABLISHMENT OF THE FIRST FILIAL GENERATION (F1) STRAINS OF THE *Pleodorina starrii* BISEXUAL STRAIN P10

To establish the F1 strains of *P. starrii* bisexual strain (P10), germination of the hypnozygotes (thick-walled zygotes) was induced as described in the previous study (Nozaki 2008), and F1 gone colonies were picked up by a micropipette and kept in screw-cap tubes containing 10 mL AF-6 medium (Supporting Information S1).

DAPI-STAINING FOR ESTIMATING GENOME SIZE

Methods for examining *Volvox* genome size (Yamamoto et al. 2017) were modified here (Supporting Information S1).

INTERCROSSING BETWEEN *Pleodorina starrii* UNISEXUAL AND BISEXUAL STRAINS

To investigate intercrossing between *P. starrii* unisexual strains (P7 male or 2P1 female) and bisexual strain (P10), 10 male colonies and 10 possible female colonies were collected from sexually induced cultures (see Supporting Information S1) by a micropipette and mixed in 0.25 mL new mating medium in a hole of a glass plate. Four types of experiments were performed: unisexual P7 male × bisexual P10 female; unisexual 2P1 female × bisexual P10 male; bisexual P10 male only (control); and bisexual P10 female only (control). The glass plates within Petri dishes (20 × 90 mm) were then placed at 25°C on a 12-h light:12-h dark schedule under cool-white fluorescent lamps at an intensity of 180–220 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

To establish F1 strains from intercrossed zygotes, zygote germination was induced as described above. F1 gone colonies were picked up by a micropipette and kept in screw-cap tubes containing 10 mL AF-6 medium. Isolated F1 strains were maintained at 20°C on a 14-h light:10-h dark schedule under cool-white fluorescent lamps at an intensity of 55–80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for one month and evaluated survival rates whether vegetative colonies proliferated normally (survive) or perished (Table S3). The sex phenotypes of F1 strains were evaluated by morphology of their sexual reproduction and genomic PCR of *PlestMID* (Table S4). Using χ^2 tests with Microsoft® Excel® 2016, we calculated the goodness-of-fit for each of the two alternating models of genotypes of three sex phenotypes (see below).

To demonstrate two genotypes of female strains, F1 unisexual female strains were obtained from unisexual female 2P1 × bisexual P10 (Table S1). Further genetic analysis between such a F1 unisexual female strain and unisexual male strain (P7) were performed by following procedure. Female and male strains cultured in 10 mL of AF-6 and grown at 20°C on a 14-h light:10-h dark schedule under cool-white fluorescent lamps at an intensity of 55–80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ were taken 0.25 mL each and mixed in 10 mL VTAC+soil medium (Supporting Information S1). The mixture was then grown at 25°C on a 12-h light:12-h dark schedule under cool-white fluorescent lamps at an intensity of 180–220 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After three days, the 10 mL mixed culture was mixed with 20 mL of mating medium in Petri dishes (90 × 20 mm) and grown at 25°C on a 12-h light:12-h dark schedule under cool-white fluorescent lamps at an intensity of 180–220 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Hypnozygotes were then formed and the establishment and evaluation of sex phenotypes of F2 strains were performed as described above.

Results and Discussion

BISEXUALITY IN CULTURE STRAINS OF *Pleodorina* ORIGINATING FROM THE TYPE LOCALITY OF *P. starrii*

Two *Pleodorina* strains (P10 and P85; Table S1) showed bisexuality but they were taxonomically identified as *P. starrii* based on morphological traits of vegetative colonies (Fig. 2A and B) and molecular phylogeny (Fig. 1B). When cultured in the nitrogen-deficient medium for induction of sex (Nozaki et al. 2006a, b), these two strains produced both male and female sexual colonies in each culture. Except for production of both types of sexual colonies within a clone, development of the male and female sexual colonies and formation of zygotes in strains P10 and P85 were essentially the same as those of the unisexual male and female culture strains of *P. starrii* (Nozaki et al. 2006a, b) as described below. Thus, strains of all three sexual types belong to the same species by the morphological species concept (Aldhebiani 2018).

All reproductive cells of the male colony in a bisexual strain differentiated into sperm packets (bundles of spindle-shaped male gametes) via successive cell divisions (Fig. 2C). Female colonies produced in the bisexual strain exhibited apparent differentiation of somatic and reproductive cells and could not be clearly distinguished from mature vegetative colonies in morphology (Fig. 2D), except that the female colonies were penetrated by male gametes to form zygotes inside the female colonial matrix. The sperm packets were released from the parental male colony and swam to the female colony (Fig. 2D) and dissociated into individual male gametes that penetrated the female colonies (Fig. 2E). When stained with 4',6-diamidino-2-phenylindol (DAPI), fluorescence of the nuclei of male gametes was strong (Fig. 2H) compared with that of female gamete nuclei (Fig. 2J). Newly formed zygotes with an apparently male gamete nucleus within the female gamete cytoplasm were observed in DAPI-stained cells (Fig. 3L). After about 10 days, reddish-brown mature zygotes were formed. They had a smooth heavy wall and were reddish brown in color and formed a clump originating from their parental female colony (Fig. 2N).

To examine the inheritance of the bisexuality, five F1 strains of P10 (strains P10-F1_I, II, III, IV, and V) were examined. Each F1 strain showed production of male and female gametes from sexual male and female colonies within the clonal culture, respectively, and formation of hypnozygotes within the female colony (Fig. S4D–H). Penetration of a male gamete nucleus within the female gametes was observed in P10-F1_IV after DAPI-staining (Fig. S4B). Thus, the bisexuality in *P. starrii* is vertically transmitted to the next generation.

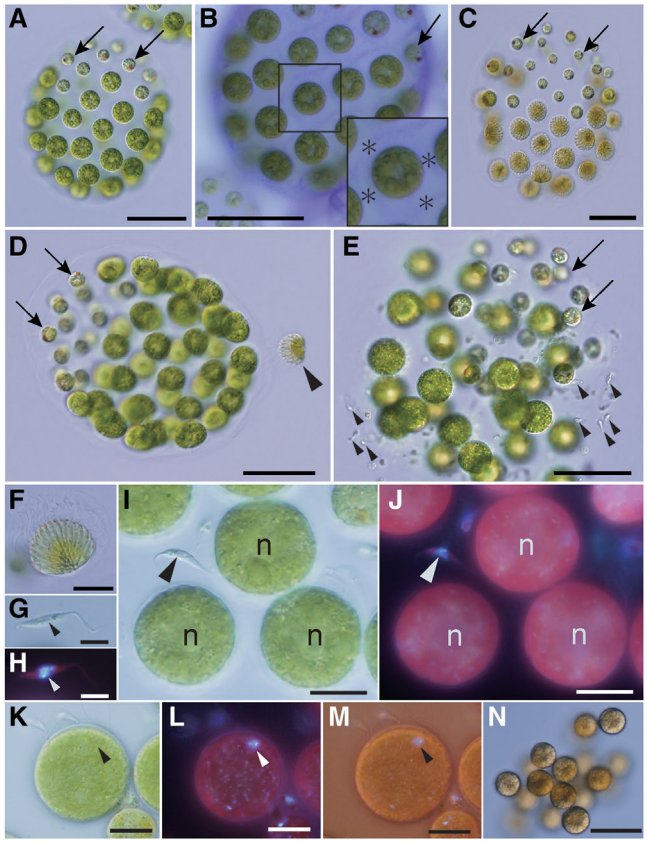

Figure 2. Morphology of the bisexual strain P10 of *Pleodorina starrii*. Arrows indicate somatic cells. (A) Sixty-four-celled vegetative colony. Scale bar = 50 μm. (B) Colony stained with methylene blue showing individual cellular sheaths (asterisks). Scale bar = 50 μm. (C) Sexually induced male colony. Note that all reproductive cells divide into sperm packets (bundles of male gametes). Scale bar = 50 μm. (D) Female colony with sperm packet (arrowhead). Scale bar = 50 μm. (E) Female colony with dissociated male gametes (arrowhead). Scale bar = 50 μm. (F) Sperm packet. Scale bar = 10 μm. (G, H) Fixed, DAPI-stained male gamete. Note that nucleus of male gamete (arrowhead) is strongly stained with DAPI (H). Scale bars = 5 μm. (G) DIC image. (H) Fluorescence image. (I, J) Fixed, DAPI-stained female gametes with a male gamete. Note that DAPI fluorescence of female gamete nuclei (n) is vague compared with that of male gamete (arrowhead). Scale bars = 10 μm. (I) DIC image. (J) Fluorescence image. (K–M) Fixed, DAPI-stained zygote with a male gamete nucleus (arrowhead) penetrating into the female gamete cytoplasm. Scale bars = 10 μm. (K) DIC image. (L) Fluorescence image. (M) DIC + fluorescence image. (N) Ten-day-old mature hypnozygotes. Scale bar = 50 μm.

COMPARISON OF PLASTID GENOME SEQUENCES BETWEEN UNISEXUAL AND BISEXUAL STRAINS OF *Pleodorina starrii*

Phylogenetic relationships within the colonial volvocine algae based on 1128 base pairs of *rbcL* coding regions demonstrated

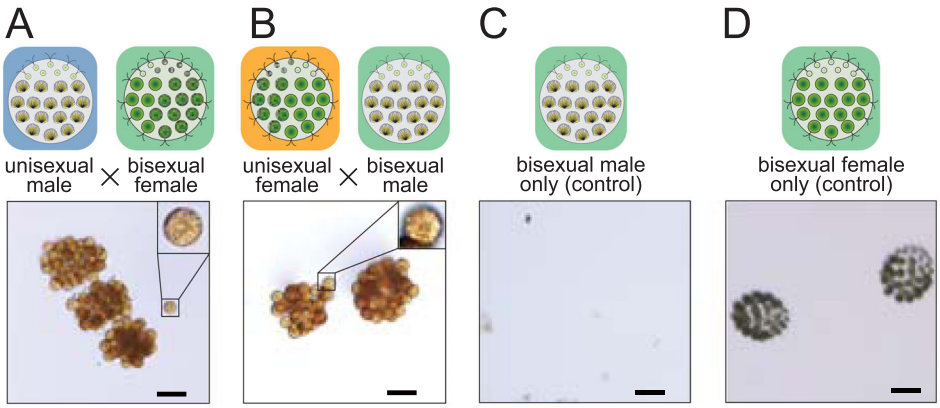

Figure 3. Results of intercrossings between unisexual and bisexual strains of *Pleodorina starrii*. Scale bars = 50 μm. (A) Five-day-old matured hypnozygotes from P7 (unisexual male strain) ×P10 (bisexual strain) female colonies. (B) Six-day-old matured hypnozygotes from 2P1 (unisexual female strain) ×P10 male colonies (bisexual strain). (C) Ten days after the isolation of sexually induced male colonies of bisexual strain P10 only (control). (D) Ten days after the isolation of sexually induced female colonies of bisexual strain P10 only (control).

that the bisexual strains belonged to *P. starrii* (Fig. 1B). In addition, the plastid genome sequences including more rapidly evolving regions, 1357 base pairs of two group I introns inserted in the *rbcL* gene (Fig. S1) and 1108 base pairs of an intergenic region harboring a microsatellite region (Fig. S2), were exactly the same between bisexual strains (P10 and P85) and unisexual male and female strains (NIES-1363, P7, NIES-1362, and 2P1) (Table S5).

In the genus *Volvox*, both heterothallism and homothallism were recognized within a single morphological species such as "*Volvox africanus*" (Starr 1971) and "*V. dissipatrix*" (Starr 1972; Starr and Zeikus 1993). However, recent studies delineated two different species, one heterothallic and the other homothallic: *Volvox reticuliferus* (heterothallic) and *Volvox africanus* (homothallic) in "*V. africanus*" (Nozaki et al. 2015) and *V. zeikusii* (heterothallic) and *V. dissipatrix* (homothallic) in "*V. dissipatrix*" (Nozaki et al. 2019), based on genetic and morphological differences between heterothallic and homothallic entities (Fig. 1B). In contrast, the present results demonstrated that the unisexual strains and the bisexual strains of *P. starrii* formed a very closely related group that could not be distinguished from each other even based on the rapidly evolving sequences in the plastid genome (Table S5). Thus, the unisexual and bisexual strains of *Pleodorina* originating from the Sagami River water system (Table S1) fall within the single species *P. starrii* by the phylogenetic species concept (Aldhebiani 2018).

INTERCROSSINGS BETWEEN UNISEXUAL AND BISEXUAL STRAINS OF *Pleodorina starrii*

Results of intercrossing are shown in Figure 3. After about five days from the intercrossing experiment between the unisexual strains (P7 male and 2P1 female) and a bisexual strain (P10), clumps of reddish brown hypnozygotes were observed in both of the two intercrossings: sexual male colonies of unisexual male strain (P7) × isolated female colonies of bisexual P10 strain, and sexual female colonies of unisexual female strain (2P1) × isolated male colonies of bisexual P10 strain (Fig. 3A and B). Almost all of the female gametes developed into mature hypnozygotes as in the sexual reproduction in the bisexual strain (Fig. 2N). However, hypnozygotes were not observed in two control cultures that included only sexual male or female colonies from the bisexual strain (Fig. 3C and D).

Survivability of F1 strains originating from such intercrossed zygotes (sexual male colonies of unisexual P7 × P10 sexual female colonies and sexual female colonies of unisexual 2P1 × P10 sexual male colonies) was 0.76–0.84, whereas that from the zygotes of unisexual parents (P7 × 2P1) was 0.84 (Table S3). Thus, no postzygotic isolation could be considered between the bisexual strain and unisexual strains of *P. starrii*. They all belong to the same biological species (Mayr 1942; Aldhebiani 2018).

PlestMID GENE IN BISEXUAL STRAINS

Based on the genomic PCR of *PlestMID*, the two bisexual strains (P10 and P85) showed presence of *PlestMID* as in the unisexual male strains (Fig. S5). Genome sequences of 1608 bp *PlestMID* exon–intron regions (Fig. S3) of the two bisexual strains were exactly identical to those of unisexual male strains (NIES-1363 and P7). A *PlestMID* cDNA sequence (818 bp) covering the entire coding regions (Fig. S3) obtained from a sexually induced culture of the bisexual strain (P10) was also identical to those of the unisexual male strain (P7) (Table S5). Our semiquantitative RT-PCR of *PlestMID*

demonstrated upregulation of this gene in sexually induced cultures of the bisexual strain as in unisexual male strain (Nozaki et al. 2006a; Fig. S6). Thus, *PlestMID* of the bisexual strain might be functionally identical to that of unisexual male strains of *P. starrii*.

ESTIMATION OF GENOME SIZES OF *P. starrii* UNISEXUAL AND BISEXUAL STRAINS

By using the fluorescence value of nuclei of *Volvox carteri* strain EVE (Ferris et al. 2010) somatic cells as control, genome sizes of *P. starrii* unisexual male strain (NIES-1363) and bisexual strain (P10) could be considered to be 0.7–0.9 times of the genome size of control (Fig. S7). Thus, no genome duplication could be considered in the origin of the bisexual strain in *P. starrii*.

GENETIC ANALYSIS OF THREE TYPES OF SEX

To examine how three phenotypes of sex are inherited in *P. starrii*, we examined F1 strains obtained from intercrossing between unisexual strains (P7 male and 2P1 female) and a bisexual strain (P10). F1 strains originating from P7 male and P10 bisexual (using isolated female sexual colonies) were either unisexual male (21 strains) or bisexual (17 strains) (Table 1). On the other hand, F1 strains from 2P1 female and bisexual P10 (using isolated male sexual colonies) exhibited three phenotypes, unisexual male (12 strains), unisexual female (21 strains), and bisexual (nine strains) (Table 1).

Two alternating hypotheses could be considered to explain the three sex phenotypes maintained in a single biological species, *P. starrii* (Fig. 4). Based on the presence and absence of *PlestMID* in the unisexual male and unisexual female *P. starrii* strains, respectively (Fig. S5), both hypotheses consider that the unisexual male and female strains harbor *MID*-containing and -lacking SDRs, respectively, as in other heterothallic volvocine species (Hamaji et al. 2018). One of the two models proposes a hypothesized bisexual factor (BF) localized on the autosomal region, and BF and male and female haplotypes of SDRs or mating type locus (*MT*) (*MTM* and *MTF*, respectively) are needed to maintain three sex phenotypes in *P. starrii* (Fig. 4A). Under this hypothesis ("autosomal BF model"), *MTM* determines the unisexual male or bisexual phenotype in the absence or presence of BF, respectively (Fig. 4A). *MTF* in the autosomal BF model determines the unisexual female phenotype, irrespective of the presence or absence of BF (Fig. 4A). The other model ("bisexual *MT* model") hypothesizes an additional type of SDR (bisexual *MT*) that is an allele of *MTM* and *MTF* (Fig. 4B). Thus, under the bisexual model, three types of SDR, namely, *MTM*, *MTF*, and bisexual *MT*, directly determine the unisexual male, unisexual female, and bisexual phenotypes, respectively (Fig. 4B).

As shown in Figure 4, the results of sex phenotypes of F1 strains of intercrossing between unisexual female and bisexual genotypes are different between these two hypotheses. In the autosomal BF model, F1 strains are male, female, and bisexual at the ratio of 1:2:1 when the parent female lacks BF (Fig. 4A). This ratio was consistent with our genetic results of F1 between unisexual and bisexual strains (Table 1). In contrast, the bisexual *MT* model suggests that the F1 strains show only two phenotypes of sex, namely, female and bisexual (1:1) (Fig. 4B). Therefore, autosomal BF model is preferred to explain three sex phenotypes of *P. starrii*.

To confirm the autosomal BF model by other genetic or crossing results, we focused on two genotypes of unisexual female strains in this model: the unisexual female strains may either have or lack BF (BF + *MTF* or BF – *MTF*, respectively) (Fig. 4A). In contrast, female strains in the bisexual *MT* model have only a single genotype (*MTF*) (Fig. 4A). To determine the presence of these two genotypes of the unisexual female strains, further crossing experiments were carried out by using F1 unisexual female strains from intercrossing between unisexual female and bisexual genotypes because these F1 female strains may have two genotypes, BF+*MTF* and BF – *MTF* (Fig. 4A). We found two F1 female strains that showed different genetic results when crossed with P7 male (Fig. S8). One F1 female strain (F1-1) produced only male and female F2 strains (14 and 20 strains, respectively) (Table S6). The other F1 female strain (F1-2) produced F2 strains of three sex phenotypes, male, female and bisexual (5, 13, and 6 strains, respectively) (Table S6). These results strongly indicate presence of two genotypes in the unisexual female strains of *P. starrii* and are consistent with the autosomal BF model (Fig. 4A).

Conclusion

THREE SEX PHENOTYPES IN A SINGLE BIOLOGICAL SPECIES

Intercrossing between closely related heterothallic and homothallic strains has previously been reported in two algal groups. In the zygnematalean *Closterium peracerosum-strigosum-littorale* complex, formation of hybrid zygospores was observed between heterothallic mating type *plus* and homothallic cells, but zygospores were not formed in intercrossing between heterothallic mating type *minus* and homothallic cells. In addition, formation of hybrid zygospores between heterothallic and homothallic cells was much less than that of the homothallic cells alone (Tsuchikane et al. 2012). Formation of possible hybrid zygotes based on intercrossing between female spheroids of *Volvox reticuliferus* (heterothallic) and male spheroids of *Volvox africanus* (homothallic) was recently reported (Nozaki et al. 2015).

Table 1. Results of intercrossings between *Pleodorina starrii* unisexual and bisexual strains.

Cross	Conditions	Numbers of F1 strains (ratio by model)			χ^2	*P*-value
		Unisexual male	Unisexual female	Bisexual		
male colonies (unisexual P7) × female colonies (bisexual P10)	Observed	21	0	17		
	Expected under autosomal BF model (Fig. 4A)	19 (1/2)	0 (0)	19 (1/2)	0.42	0.81
	Expected under bisexual *MT* model (Fig. 4B)	19 (1/2)	0 (0)	19 (1/2)	0.42	0.81
female colonies (unisexual 2P1) × male colonies (bisexual P10)	Observed	12	21	9		
	Expected under autosomal BF model with unisexual female lacking BF (BF-*MTF*) (Fig. 4A)	10.5 (1/4)	21 (2/4)	10.5 (1/4)	0.43	0.81
	Expected under autosomal BF model with unisexual female harboring BF (BF+*MTF*, Fig. 4A)	0 (1/2)	21 (1/2)	21 (1/2)	6.86	8.8×10^{-3}
	Expected under bisexual *MT* model (Fig. 4B)	0 (0)	21 (1/2)	21 (1/2)	6.86	8.8×10^{-3}

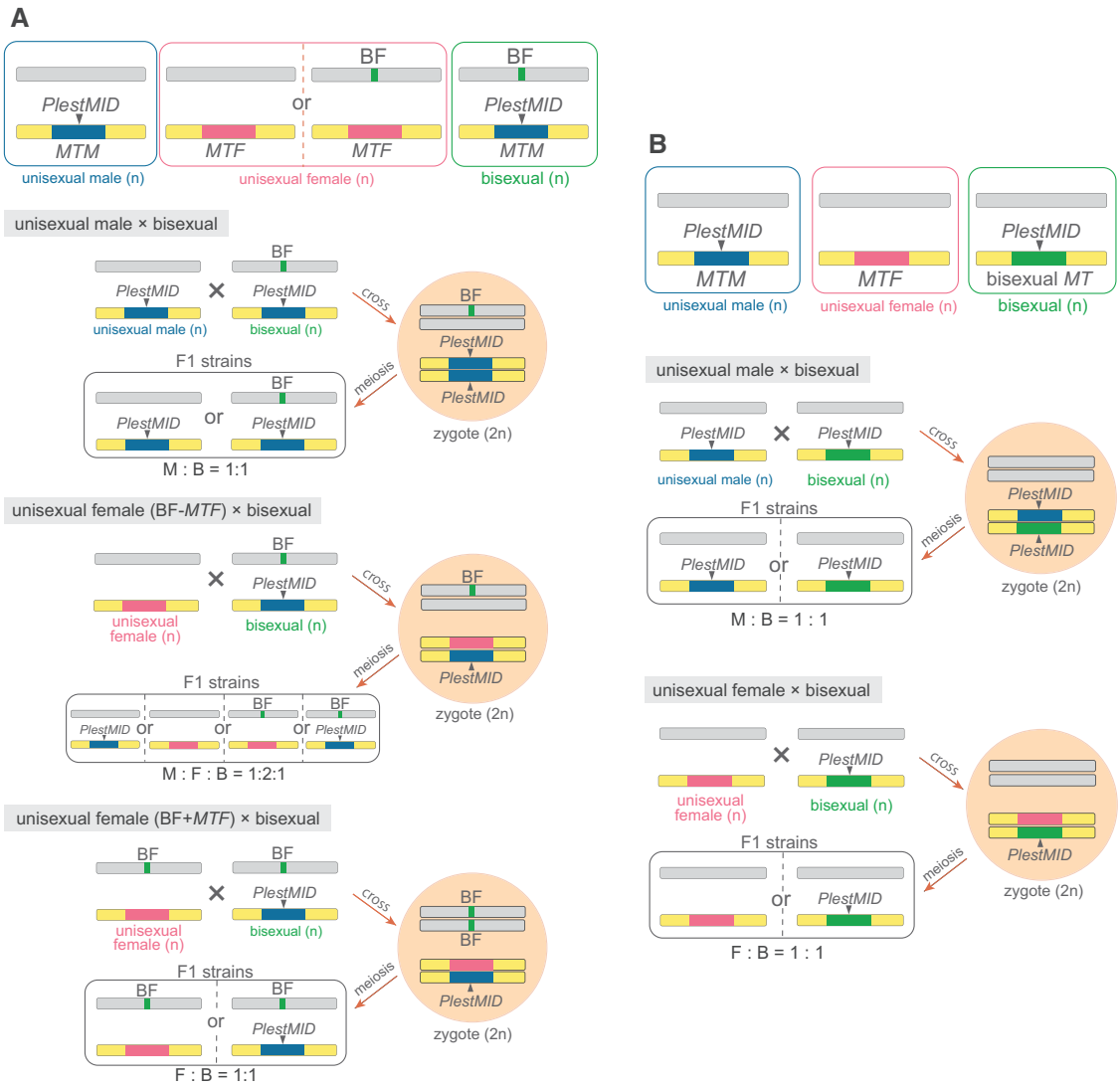

Figure 4. **Schematic drawings of possible genotypes and intercrossings of *Pleodorina starrii*, on the basis of two alternative hypotheses. Gray and yellow bars represent autosome and UV sex chromosome, respectively. Blue and red regions within UV chromosomes represent male sex-determining region (*MTM*), female sex-determining region (*MTF*), respectively. M: Unisexual male. F: Unisexual female. B: Bisexual. (A) Genotypes and intercrossings of three sex phenotypes in autosomal bisexual factor (BF) model. Short green region within autosome represents BF. Bisexual has both BF and *MTM* (BF + *MTM*), whereas unisexual male lacks BF (BF-*MTM*). Unisexual female has two possible genotypes: lacking BF (BF-*MTF*) and having BF (BF + *MTF*). (B) Genotypes and intercrossings of three sex phenotypes in bisexual *MT* model. Green region within UV chromosome represents bisexual sex-determining region (bisexual *MT*). Note that each of three sex phenotypes has sex-specific *MT*.**

However, such intercrossed zygotes become disintegrated within three weeks (Nozaki et al. 2015). Therefore, a genetic barrier exists between closely related heterothallic and homothallic entities of *Closterium* and *Volvox*, representing different biological species (Mayr 1942). In contrast, three sex phenotypes (male, female, and bisexual) are naturally present within the same biological species *P. starrii* (Fig. 1).

According to Goldstein (1964), "selfing male" strains were recorded in several populations of *Eudorina*; selfing male strains produced a small number of zygotes intraclonally, whereas a large number of zygotes were formed when they were paired with complementary female strains. However, detailed genetic or molecular studies have not been performed in such possible bisexual strains, and presence of three types of sex phenotypes within a single population was not described. Considering these studies and the present study, co-existence of three sex phenotypes in a single biological species may not be an unusual phenomenon in wild populations. The continued field-collection studies may reveal further existence of three sex phenotypes in other volvocine species.

EVOLUTIONARY TRANSITION FROM HETEROTHALLISM TO HOMOTHALLISM AMONG VOLVOCINE ALGAE

In the volvocine algae, transition from heterothallism to homothallism might have occurred independently and repeatedly (Hanschen et al. 2018; Nozaki et al. 2019), but the molecular and genetic bases for this evolutionary event have been poorly understood. In mosses and liverworts, polyploidy is associated with evolution of homothallism (monoecy) (Renner et al. 2017). However, the present comparison of genome sizes between *P. starrii* unisexual male and bisexual strains suggests that genome duplication did not occur within this species (Fig. S7). The present genetic analyses of the three sex phenotypes in *Pleodorina starrii* demonstrated a possible autosomal factor (BF) that determines the bisexuality in the presence of *MTM*. Thus, evolution of such an autosomal factor in the ancestral heterothallic species might have occurred in the initial stage of transition from heterothallism to homothallism in some ancestral volvocine algae.

AUTHOR CONTRIBUTIONS

KT, THig, and HN designed the project. KT, THam, and HN performed field work. KT, HKT, RO, THam, YT, and HS performed morphological and molecular analyses. KT and HN performed culture experiments. KT and HN wrote the manuscript. All authors provided edits and critical feedback on the manuscript and the revision.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA ARCHIVING

New gene sequence and alignment data were archived under the DDBJ/EMBL-EBI/NCBI accession numbers (MN606057-MN606071) and TreeBASE study ID (28238), respectively. All other data have been deposited in Dryad: https://doi.org/10.5061/dryad.f7m0cfxwc.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Diagram showing partial sequence of *rbcL* gene compared between unisexual and bisexual strains of *Pleodorina starrii* (Table S5).
Figure S2. Diagram of microsatellite region within the plastid genome of *Pleodorina starrii* strain NIES-1363 (JX977846.1; Smith et al. 2013).
Figure S3. Diagrams of exon–intron structure of PlestMID gene and PlestMID cDNA of *Pleodorina starrii*.
Figure S4. Zygotes of F1 strains obtained by selfing of *Pleodorina starrii* bisexual strain P10.
Figure S5. Results of genomic PCR of PlestMID for unisexual male (M), unisexual female (F), and bisexual (B) strains of *Pleodorina starrii*.
Figure S6. Semiquantitative RT-PCR of PlestMID in unisexual male strain (P7) and bisexual strain (P10) of *Pleodorina starrii*.
Figure S7. DAPI staining for estimating comparative genome size in *Pleodorina starrii* unisexual male strain (NIES-1363) and bisexual strain (P10).
Figure S8. Schematic drawings of possible results of intercrossings of *Pleodorina starrii* between unisexual male and two genotypes of female, on the basis of autosomal bisexual factor (BF) model (Fig. 4A).
Table S1. Strains of *Pleodorina starrii* used in the present study.
Table S2. List of Volvocales included in the phylogenetic analysis and DDBJ/EMBL-EBI/NCBI accession numbers of *rbcL* genes.
Table S3. Survival rates of F1strains obtained by intercrossings between *Pleodorina starrii* unisexual and bisexual strains.
Table S4. Evaluation items for determining sex phenotypes of *Pleodorina starrii* strains.
Table S5. List of genome sequences determined in the present study.
Table S6. Results of intercrossings between *Pleodorina starrii* unisexual male and two F1 unisexual females showing two possible genotypes of unisexual female phenotype.
Table S7. Specific primers of *Pleodorina starrii* used in the present study.
Table S8. Conditions for PCR cycles and primers used in semi-quantitative RTPCR analyses (Fig. S6).