Supporting Information

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

The following text, tables, figures and references are described in this document.

**Supplementary Text S1: Detailed methods**

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Supplementary References
Supplementary Text S1: Detailed methods

Induction of sexual reproduction

About 0.25 mL of the growing cultures of *Pleodorina starrii* (Table S1) were transferred into 10 mL of new 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 μmol·m⁻²·s⁻¹. After about 5 ~ 10 days, 0.25 mL of the actively growing cultures was transferred into 10 mL VTAC+soil medium (USVT medium (Nozaki et al. 2015a) excluding urea) 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 μmol·m⁻²·s⁻¹. After three or four days, the 10 mL culture grown in VTAC+soil medium was mixed with 20 mL of mating medium (Nozaki et al. 1989) 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 μmol·m⁻²·s⁻¹. Sexual colonies developed within a day (male strains), or within 2 days (female strains and bisexual strains).

Genomic PCR and sequencing

Genomic PCR was performed using KOD One PCR Master Mix (TOYOBO, Osaka, Japan) with specific-primer pairs (Table S7). PCR templates were prepared by disruption of concentrated culture grown in AF6 medium with ceramic beads by Retsch Mixer Mill MM300 (F. Kurt Retsch GmbH & Co.KG, Haan, RP, Germany). PCR cycles were 2 min at 94°C, followed by 45 cycles of 98°C, 10 sec, 50°C, 30 sec, 68°C, 30 sec (*rbcL*), 40 cycles of 98°C, 10 sec, 68°C, 1 min (plastid microsatellite region), or 35 cycles of 98°C, 10 sec, 66°C, 30 sec, 68°C, 30 sec (*PlestMID*). PCR products were sequenced directly using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with a BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems), as described previously (Nozaki et al. 2000).

To detect the presence of *PlestMID* gene in two bisexual strains (P10 and P85) (Fig. S3), genomic PCR of *PlestMID* was performed with KOD One PCR Master Mix
with *PlestMID* gene-specific primer pair (Nozaki et al. 2006a, PlMT_F71 and PlMT_F73, Table S7). For control, we used elongation factor 1 like gene (*EF-1 like*)-specific primer pair designed based on sequence of *EF1-like* mRNA sequence (Nozaki et al. 2006a, PlestEFL_F1 and PlestEFL_R1, Table S7). PCR cycles were 2 min at 94°C, followed by 30 cycles of 98°C, 10 sec, 60°C, 30 sec, 68°C, 30 sec (for *PlestMID* and *EF-1 like*). The amplified products were electrophoresed on 2% (w/v) agarose gels and stained with ethidium bromide. The gel images were captured using a ChemiDoc XRS system with Quantity One Software (Bio-Rad, Hercules, CA, USA).

**cDNA sequencing and semi-quantitative RT-PCR**

Amplification of cDNA was carried out by Superscript 3 reverse transcriptase (Invitrogen, Carlsbad, CA, USA). To determine the sequence of *PlestMID* cDNA of P7 and P10, PCR amplification was carried out at the condition (2 min at 94°C, followed by 35 cycles of 98°C, 10 sec, 66°C, 30 sec, 68°C, 30 sec) using KOD One PCR Master Mix with *PlestMID* gene specific primer pair PlMT_F1 and PlMT_R73 or PlMT_F1 and PlMT_R9 (Table S7). PCR products were sequenced directly as described above.

For examining the expression of *PlestMID* and *EF-1 like* genes, semi-quantitative RT-PCR amplification was performed using full-length cDNA constructed from various cultures with two specific primer pairs (PlMT_RT_F1 and PlMT_RT_R2 for *PlestMID*, Table S7; PlestEFL_F1 and PlestEFL_R1 for *EF-1 like*, Table S7) and KOD One PCR Master Mix as described in Table S8. The 24 amplified products were electrophoresed on 2% (w/v) agarose gels and stained with ethidium bromide. The gel images were captured using a ChemiDoc XRS system with Quantity One Software, level adjusted and gradation inverted with Adobe Photoshop CC 2019 (Adobe Systems Inc., San Jose, CA).

**Phylogenetic analyses of *rbcL* genes**
Sequences of the *rbcL* coding regions from 40 operational taxonomic units (OTUs) were aligned using Clustal W (Thompson et al. 1994). Maximum likelihood (ML) tree was constructed by MEGA7 (Kumar et al. 2016), based on the best-fitted model (GTR+G+I) selected by MEGA7, with initial trees constructed by BioNJ (Gascuel 1997). Maximum parsimony (MP) method using tree bisection reconnection search method by MEGA 7.0 was also used to construct trees. Bootstrap values (Felsenstein 1985) based on 1,000 replications were calculated in ML and MP analyses. In addition, Bayesian inference was carried out. Each data set for Bayesian inference was divided into three partitions: first, second, and third codon positions of the *rbcL* gene and then an evolutionary model of each partition was selected using MrModeltest 2.2 (Nylander 2004): GTR+G+I (first), JC+G+I (second), GTR+G (third) model, respectively. The partition models were unlinked in each analysis. Bayesian phylogenetic analyses were performed using MrBayes 3.2 (Ronquist et al. 2012). Convergences of Markov chain Monte Carlo iterations were evaluated based on the average standard deviation of split frequencies for every 1,000,000 generations, discarding the first 25% as burn-in, and the iterations were automatically stopped when the average standard deviations were below 0.01, indicating convergence. Consequently, 1,000,000 generations of iterations were performed for each data set.

**Morphological observations**

Vegetative colonies of two bisexual strains (P10 and P85) in an actively growing culture in AF-6 medium at 20°C on a 14-h light: 10-h dark schedule under cool-white fluorescent lamps at an intensity of 55-80 μmol·m⁻²·s⁻¹ were used. Light microscopy was carried out using a BX-60 microscope (Olympus, Tokyo, Japan) equipped with Nomarski optics. In order to examine the structure of individual sheaths of the P10 colonies, methylene blue staining of vegetative colonies was performed as described previously in *Pleodorina* (Nozaki et al. 1989; Nozaki et al. 2006b). About 5 μL of P10
vegetative colonies in an actively growing culture in AF-6 medium were mixed with 2-5 µL of 0.002% (w/v in distilled water) methylene blue on slides and observed by a BX-60 microscope equipped with Nomarski optics.

To observe sexual reproduction of P10 and its F1 strains obtained by selfing of P10, male and female colonies induced as described above were isolated by a micropipette from sexually induced culture. The isolated colonies were put in an angular depression or hole made in an adhesive tape (Scotch® OPP acrylic adhesive tape for box sealing tape 313, 3M Japan, Shinagawa, Tokyo, Japan) on a slide glass. Then cover slip was placed on the depression and the samples were observed by a BX-60 microscope equipped with Nomarski optics.

For 4',6-diamidino-2-phenylindole (DAPI) -staining of gamete nuclei, 5 µL sexually induced culture, 5 µL 2.0% glutaraldehyde in NS-buffer (Sando et al. 1981, 20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM MgCl₂, 0.1 mM ZnCl₂, 0.1 mM CaCl₂, 0.4 mM phenylmethylsulfonylfluoride (PMSF), 7 mM 2-mercaptoethanol), and 5 µL DAPI in NS-buffer were mixed in a slide glass. Then, a cover slip was flipped on the mixed sample, and the cells were examined by a BX-60 microscope equipped with Nomarski and epifluorescence optics.

Establishment of the first filial generation (F1) strains of the *Pleodorina starrii* bisexual strain P10

Approximately 10-day-old mature hypnozygotes of P10 were transferred to a 1% agar (in distilled water) plate, and put into darkness for about one month at 20°C. After the dark treatment, the zygotes were transferred to the liquid AF-6 medium under 12-h light: 12-h dark schedule at 25°C. Zygote germination occurred and gone colonies developed after one to three days, and F1 strains were established by isolating a single gone colony into a screw-cup tube containing 10 mL AF-6 medium, and then placed under 14-h light: 10-h dark schedule at 20°C. Sex phenotypes of F1 strains are
evaluated by four items (Table S4).

**DAPI-staining for estimating genome size**

To estimate comparative genome size of *P. starrii* male and bisexual strains, DAPI staining was performed using somatic cells of *P. starrii* unisexual male strain (NIES-1363) and bisexual strain (P10) and *Volvox carteri* strain EVE for control (Yamamoto et al. 2017). Colonies in 10 mL AF-6 culture were centrifuged for 2000 rpm, 1 min and fixed with ethyl acetate (C$_2$H$_5$OH : CH$_3$COOH = 3:1) for 30 rpm, RT, 1 hour. After fixation, samples were centrifuged for 5,000 rpm, 5 min and supernatant was disposed before 100% EtOH was added (this control was repeated twice). Then 900 µL NS-buffer was added before 5000 rpm, 10 min centrifugation and supernatant disposal (this control was repeated twice). 1 mL NS buffer + 1 µL DAPI (100 µg/mL) staining (final concentration = 0.1 µg/mL) was added and rotated for 30 rpm, 4°C, 1 hour. DAPI-stained somatic cells of *P. starrii* unisexual male or bisexual strain were mixed with EVE control and mounted in same slide with inclusion compound (12.5 µL 1 × glycerol, 0.025 µL 100 µg/mL DAPI, 2.5 µL 10 × NS-buffer, 1.25 µL ProLong Gold (Thermo Fisher Scientific, Waltham, MA, USA) (inclusion compound : samples = 3:1). The camera was mounted on an Olympus™ BX-60 microscope with fluorescence microscopy. The image analyses were performed using Image J, measuring mean gray value of 10 nucleus (Fig. S7C) with each exposure time (0.5, 0.67, 1.0, 1.5, 2.0 and 2.5 s).
Figure S1. Diagram showing partial sequence of rbcL gene compared between unisexual and bisexual strains of Pleodorina starrii (Table S5). Numbers at the bar represent nucleotide positions of the plastid genome of P. starrii strain NIES-1363 (JX977846.1; Smith et al. 2013). Double-headed arrow indicates the region determined in the present study (2,695 base pairs; Table S5).
Figure S2. Diagram of microsatellite region within the plastid genome of *Pleodorina starrii* strain NIES-1363 (JX977846.1; Smith et al. 2013). Numbers at the bar represent nucleotide positions of the plastid genome. Note that the TA repeat is not an inverted repeat. Double-headed arrow indicates the region compared between unisexual and bisexual strains of *P. starrii* (1,108 base pairs; Table S5).
Figure S3. Diagrams of exon-intron structure of *PlestMID* gene and *PlestMID* cDNA of *Pleodorina starrii*. Based on *PlestMID* gene and *PlestMID* cDNA of *P. starrii* strain NIES-1363 (AB272616 and AB272612, respectively; Nozaki et al. 2006a). Double-headed arrows indicate the regions determined in the present study (1,680 bp of *PlestMID* and 818 bp of *PlestMID* cDNA, Table S5).
Figure S4. Zygotes of F1 strains obtained by selfing of Pleodorina starrii bisexual strain P10. (A-C) Fixed, DAPI-stained newly formed zygote with a male gamete nucleus (arrowhead) penetrating into the female gamete cytoplasm. Strain P10-F1_IV. Scale bars = 10 μm. (A) DIC image. (B) fluorescence image. (C) DIC+fluorescence image. (D-H) Matured, 10-day-old hypnozygotes. Scale bars = 50 μm. (D) Strain P10-F1_I. (E) Strain P10-F1_II. (F) Strain P10-F1_III. (G) Strain P10-F1_IV. (H) Strain P10-F1_V.
Figure S5. Results of genomic PCR of *PlestMID* for unisexual male (M), unisexual female (F) and bisexual (B) strains of *Pleodorina starrii*.
| strain | P7 | P10 | P7 | P10 |
|--------|----|-----|----|-----|
| sex    | unisexual male | bisexual | unisexual male | bisexual |
| condition | sexually induced | uninduced | |
| cycles | 26 | 28 | 30 | 26 | 28 | 30 | 26 | 28 | 30 |

Figure S6. Semi-quantitative RT-PCR of *PlestMID* in unisexual male strain (P7) and bisexual strain (P10) of *Pleodorina starrii*. The loading volume for each lane was normalized to the quantity of *EF-1* like (internal control) product.
Figure S7. DAPI staining for estimating comparative genome size in *Pleodorina starrii* unisexual male strain (NIES-1363) and bisexual strain (P10). (A), (B) Mean gray value of ten nuclei with image J at 0.5, 0.67, 1.0, 1.5, 2.0, 2.5 s exposure time. Bars show means and standard error. (A) NIES-1363 and *Volvox carteri* EVE, (B) P10 and *V. carteri* EVE. (C) DAPI stained somatic cell and gray scale image of *V. carteri* EVE and *P. starrii* P10. Arrowheads show location of nucleus. Yellow rings show the region of measurement in image J. Scale bar = 10 μm (*V. carteri* EVE), 5 μm (*P. starrii*). (D) Fluorescence of stained somatic cell nuclei in *P. starrii* unisexual male strain and bisexual strain relative to *V. carteri* EVE strain (control) at 1.5 s exposure time. Bars show means and standard error of 10 biological replicates.
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). Gray and yellow bars represent autosome and UV sex chromosome, respectively. Blue and red regions within UV chromosomes represent male sex-determining region (*MTM*) and female sex-determining region (*MTF*), respectively. Short green region within autosome represents BF. M: unisexual male. F: unisexual female. B: bisexual. (A) Intercrossing between unisexual male and F1 unisexual female (BF-*MTF*). (B) Intercrossing between unisexual male and F1 unisexual female (BF+*MTF*).
Table S1. Strains of *Pleodorina starrii* used in the present study.

| Strain designation [abbreviation] | Origin                                                                 | Sex      | Reference          |
|----------------------------------|------------------------------------------------------------------------|----------|--------------------|
| 2000-602-P15 (=NIES-1363)         | Lake Sagami of Sagami River water system, Japan                        | Male     | Nozaki et al. (2006a) |
| 2000-602-P14 (=NIES-1362)         | Lake Sagami of Sagami River water system, Japan                        | Female   | Nozaki et al. (2006a) |
| 2013-0614-P7 [P7] (=NIES-4480)    | Water sample collected from Lake Sagami of Sagami River water system, Japan (23.0°C, pH 8.4, 35°36'43.08”N 139°11'19.5”E) in June 14th, 2013 | Male*    | The present study    |
| 2018-0609-2P1 [2P1] (=NIES-4481)  | Water sample (22.0°C, pH 8.85) collected from Lake Sagami of Sagami River water system, Japan (35°36'36.5"N 139°11'13.2"E) in June 9th, 2018 | Female*  | The present study    |
| 2013-0614-P10 [P10] (=NIES-4482)  | Water sample (23.0°C, pH 8.4) collected from Lake Sagami of Sagami River water system, Japan (35°36'43.08”N 139°11'19.5”E) in June 14th, 2013 | Bisexual*| The present study    |
| 2007-1003-P85 [P85] (=NIES-4479)  | Water sample (21.0°C, pH 7.2) collected from Lake Tsukui of Sagami River water system, Japan (35°35'19.5”N 139°16'18.6”E) in October 3rd, 2007 | Bisexual*| The present study    |
| 2019-0427-F1-1 [F1-1] (=NIES4483) | F1 progeny strain of 2018-0609-2P1 × 2013-0614-P10                   | Female*  | The present study    |
| 2019-0427-F1-2 [F1-2] (=NIES-4484) | F1 progeny strain of 2018-0609-2P1 × 2013-0614-P10                   | Female*  | The present study    |

* Determined in the present study based on four items (Table S4).
Table S2. List of Volvocales included in the phylogenetic analysis and DDBJ/EMBL-EBI/NCBI accession numbers of \textit{rbcL} genes.

| Species                        | Strain designation | Accession number | Reference                  |
|--------------------------------|--------------------|------------------|----------------------------|
| \textit{Yamagishiella unicocca} | UTEX 2428          | D86823           | Nozaki et al. (2014)       |
| \textit{Yamagishiella unicocca} | UTEX 2430          | D86825f          | Nozaki et al. (2014)       |
| \textit{Yamagishiella unicocca} | NIES-872           | AB044168         | Nozaki et al. (2014)       |
| \textit{Platydorina caudata}   | UTEX 1658          | D86828           | Nozaki et al. (2014)       |
| \textit{Colemanosphaera}       |                    |                  |                            |
| \textit{charkowiensis}         | NIES-3383          | AB905591         | Nozaki et al. (2014)       |
| \textit{Colemanosphaera}       |                    |                  |                            |
| \textit{angeleri}              | NIES-3382          | AB905592         | Nozaki et al. (2014)       |
| \textit{Eudorina cylindrica}   | UTEX 1197          | D86833           | Nozaki et al. (2014)       |
| \textit{Eudorina peripheralis} | UTEX 1215          | D63434           | Nozaki et al. (2014)       |
| \textit{Eudorina unicocca}     | UTEX 737           | D86829           | Nozaki et al. (2014)       |
| \textit{Eudorina elegans}      | NIES-456           | D63432           | Nozaki et al. (2014)       |
| \textit{Eudorina elegans}      | UTEX 1205          | D88805           | Nozaki et al. (2014)       |
| \textit{Eudorina elegans}      | UTEX 1212          | D88806           | Nozaki et al. (2014)       |
| \textit{Eudorina illinoensis}  | NIES-460           | D63433           | Nozaki et al. (2014)       |
| \textit{Pleodorina thompsonii} | UTEX 2804          | AB214408         | Nozaki et al. (2014)       |
| \textit{Pleodorina starrii}    | NIES-1362          | AB214427         | Nozaki et al. (2014)       |
| \textit{Pleodorina starrii}    | NIES-1363          | JX977846         | Smith et al. (2013)        |
| \textit{Pleodorina starrii}    | 2007-1003-P85      | MN606057         | The present study          |
| \textit{Pleodorina starrii}    | 2018-0609-2P1      | MN606058         | The present study          |
| \textit{Pleodorina starrii}    | 2013-0614-P7       | MN606059         | The present study          |
| \textit{Pleodorina starrii}    | 2013-0614-P10      | MN606060         | The present study          |
| \textit{Pleodorina indica}     | UTEX 1990          | D86834           | Nozaki et al. (2014)       |
| \textit{Pleodorina japonica}   | UTEX 2523          | D63440           | Nozaki et al. (2014)       |
| \textit{Pleodorina californica} | UTEX 809          | D63439           | Nozaki et al. (2014)       |
| \textit{Pleodorina sphaerica}  | NIES-4066          | LC215634         | Nozaki et al. (2017)       |
| \textit{Volvox gigas}          | UTEX 1895          | AB076084         | Nozaki et al. (2014)       |
| \textit{Volvox ovalis}         | NIES-2569          | AB592342         | Nozaki et al. (2014)       |
| \textit{Volvox obversus}       | UTEX 1865          | AB076085         | Nozaki et al. (2014)       |
Table S2 continued.

| Species               | Strain designation | Accession number | Reference                      |
|-----------------------|--------------------|------------------|--------------------------------|
| *Volvox africanus*    | NIES 3780          | LC090149         | Nozaki et al. (2015b)          |
| *Volvox reticuliferus*| UTEX 1891          | AB076101         | Nozaki et al. (2002), Nozaki et al. (2015b) |
| *Volvox reticuliferus*| NIES-3782          | LC090154         | Nozaki et al. (2015b)          |
| *Volvox tertius*      | UTEX 132           | AB076098         | Nozaki et al. (2014)           |
| *Volvox tertius*      | NIES-544           | AB086174         | Nozaki et al. (2014)           |
| *Volvox tertius*      | NIES-4068          | LC215631         | Nozaki et al. (2017)           |
| *Volvox powersii*     | UTEX 1863          | AB214415         | Nozaki et al. (2014)           |
| *Volvox carteri f.*   | NIES-732           | D63446           | Nozaki et al. (2014)           |
| kawasakiensis         |                    |                  |                                |
| *Volvox carteri f.*   | UTEX 1885          | AB076099         | Nozaki et al. (2014)           |
| nagariensis           |                    |                  |                                |
| *Volvox carteri f.*   | UTEX 1875          | AB076100         | Nozaki et al. (2014)           |
| weismannia            |                    |                  |                                |
| *Volvox aureus*       | NIES-541           | D63445           | Nozaki et al. (2014)           |
| *Volvox aureus*       | NIES-891           | AB076096         | Nozaki et al. (2014)           |
| *Volvox aureus*       | NIES-892           | AB076086         | Nozaki et al. (2014)           |
| *Volvox zeikusii*     | NIES-731           | D63447           | Nozaki et al. (2014), Nozaki et al. (2019) |
| *Volvox dissipatrix*  | Marb.2RS 29        | AB214420         | Nozaki et al. (2014)           |
| *Volvox rousseletii*  | UTEX 1862          | D63448           | Nozaki et al. (2014)           |
| *Volvox barberi*      | UTEX 804           | D86835           | Nozaki et al. (2014)           |
| *Volvox globator*     | UTEX 955           | D86836           | Nozaki et al. (2014)           |
Table S3. Survival rates of F1 strains obtained by intercrossings between *Pleodorina starrii* unisexual and bisexual strains. As control, unisexual pair (unisexual male × unisexual female) was also examined.

| Cross                                                                 | Number of isolated F1 strains | Number of survival F1 strains | Survival rate |
|-----------------------------------------------------------------------|-------------------------------|-------------------------------|---------------|
| Male colonies (unisexual P7) × female colonies (bisexual P10)         | 50                            | 38                            | 0.76          |
| Female colonies (unisexual 2P1) × male colonies (bisexual P10)        | 50                            | 42                            | 0.84          |
| Male colonies (unisexual P7) × female colonies (unisexual 2P1) (control) | 50                            | 42                            | 0.84          |
Table S4. Evaluation items for determining sex phenotypes of *Pleodorina starrii* strains. Presence or absence of item is shown by + or -, respectively. Checkmark represents essential evaluation item for each phenotype.

| Sex phenotype                                      | Unisexual |           | Bisexual |
|----------------------------------------------------|-----------|-----------|----------|
|                                                    | Male      | Female    |          |
| Formation of sperm packets                         | + ✓       | - ✓       | + ✓      |
| Formation of hypnozygotes within a single clonal culture | - ✓       | - ✓       | + ✓      |
| PlestMID                                           | +         | - ✓       | +        |
| Formation of hypnozygotes crossed with unisexual male | -         | +         | +        |
Table S5. List of genome sequences determined in the present study.

| Strain               | DNA region*                        | Sequence length [bp]\(^a\) | Accession number |
|----------------------|------------------------------------|----------------------------|------------------|
| 2000-602-P14 (NIES-1362) | rbcL                                | 2,695                      | MN606061         |
|                      | microsatellite region               | 1,108                      | MN606066         |
| 2013-0614-P7 [P7]    | rbcL                                | 2,695                      | MN606059         |
|                      | microsatellite region               | 1,108                      | MN606064         |
|                      | Ples MID                            | 1,680                      | MN606069         |
|                      | Ples MID (cDNA)                     | 818                        | MN606070         |
| 2018-0609-2P1 [2P1]  | rbcL                                | 2,695                      | MN606058         |
|                      | microsatellite region               | 1,108                      | MN606063         |
| 2013-0614-P10 [P10]  | rbcL                                | 2,695                      | MN606060         |
|                      | microsatellite region               | 1,108                      | MN606065         |
|                      | Ples MID                            | 1,680                      | MN606067         |
|                      | Ples MID (cDNA)                     | 818                        | MN606071         |
| 2007-1003-P85 [P85]  | rbcL                                | 2,695                      | MN606057         |
|                      | microsatellite region               | 1,108                      | MN606062         |
|                      | Ples MID                            | 1,680                      | MN606068         |

\(^a\)See Figs. S1-S3.
Table S6. Results of intercrossings between *Pleodorina starrii* unisexual male and two F1 unisexual females showing two possible genotypes of unisexual female phenotype. Two types of F1 unisexual female strains (F1-1 and F1-2) were obtained by intercrossing between unisexual female 2P1 and bisexual P10 strains (Fig. 4A). Expected results of three sex phenotypes of F2 strains by autosomal bisexual factor (BF) model are also shown. Chi-square tests were performed to evaluate the model.

| Cross | Conditions | Sex phenotypes of F2 strains (ratio by model) | $\chi^2$ | P-value |
|-------|------------|---------------------------------------------|--------|---------|
|       |            | Unisexual male | Unisexual female | Bisexual |
| Unisexual male (P7) × F1-1 (2P1×P10) unisexual female | Observed | 14 | 20 | 0 | 1.06 | 0.3 |
|       | Expected under the autosomal BF model (Fig. S8A) | 17 (1/2) | 17 (1/2) | 0 (0) | |
| Unisexual male (P7) × F1-2 (2P1×P10) unisexual female | Observed | 5 | 13 | 6 | |
|       | Expected under the autosomal BF model (Fig. S8B) | 6 (1/4) | 12 (2/4) | 6 (1/4) | 0.25 | 0.88 |
Table S7. Specific primers of *Pleodorina starrii* used in the present study.

| Primer name      | Sequence (5’ to 3’)                        | Forward (F) or reverse (R) | Gene/DNA region |
|------------------|---------------------------------------------|----------------------------|-----------------|
| rbcL_F1<sup>a</sup> | ATGGTTCCACAAACAGAAAC                       | F                          | *rbcL*          |
| rbcL_F4<sup>a</sup> | TATTCGAAAGGGTTCAGTAAC                      | F                          |                 |
| rbcL_F7<sup>a</sup> | GTTTCTTTTCTGTAGCTGAAGC                      | F                          |                 |
| rbcL_R2<sup>a</sup> | GCACTAAAGCTTGGAAAC                        | R                          |                 |
| rbcL_R3<sup>a</sup> | TTGCTCAAATGTATCAAATTG                      | R                          |                 |
| rbcL_R5<sup>a</sup> | TTAGCTGTGAACACACCTGTGTA                    | R                          |                 |
| rbcL_R8<sup>a</sup> | AAGATTGAACTAAAGCTGGCA                      | R                          |                 |
| rbcL_F9<sup>b</sup> | GTGACAAACTAAACAAATATGG                     | F                          |                 |
| rbcL_R10<sup>b</sup> | TGTGCTTTGTAATAGCTTCAG                     | R                          |                 |
| psbH-psaA_F1<sup>d</sup> | CGTCCGAAGGGTTAGTGGCATGCAAGCAA             | F                          | microsatellite region (between *psbH* and *psaA*) |
| psbH-psaA_F2<sup>d</sup> | GCGTTGTAAGCGGCACCCCTTCGGGGGC             | F                          |                 |
| psbH-psaA_R3<sup>d</sup> | CGGCCGTTCCTCTTAATCCCGCTCTACGAAC          | R                          |                 |
| psbH-psaA_R4<sup>d</sup> | GCCGCTTACGCAGCAGAGGCCCTAAACAC          | R                          |                 |
| PIMT_F71<sup>c</sup> | GAACATTACAGAGGGCTATGGGAGATT               | F                          | *PlestMID*      |
| PIMT_R73<sup>c</sup> | CAAGCACGCAACACCGTGTTGGG                  | R                          |                 |
| PIMT_F1<sup>d</sup> | ACTGGCAATTCGGGCCAGGC                   | F                          |                 |
| PIMT_F13<sup>d</sup> | CTCCATAAATAATGCTTCCGATG                 | F                          |                 |
| PIMT_F101<sup>d</sup> | GGAACCTTGGGCTCTCCTACTACGTATTTAAA      | F                          |                 |
| PIMT_SF1<sup>d</sup> | TTATTGCTTGAGGCAAGTTACGA              | F                          |                 |
| PIMT_R9<sup>d</sup> | GCAATTAACACGCGCCTTGAACAC               | R                          |                 |
| PIMT_R15<sup>d</sup> | CGGCCGTTCCTGGCCTGGATGCTGCC              | R                          |                 |
| PIMT_R104<sup>d</sup> | AGCTGGTAGAAACTAAGGTAATCTCCTCAT        | R                          |                 |
| PIMT_SR2<sup>d</sup> | ACACCTGGCTTCGTTAGCTGGAAAG           | R                          |                 |
| PlestEFL_F1<sup>d</sup> | TGTCATTGTGCGGCTATGGTACTC             | F                          | *EF-1 like*    |
| PlestEFL_R1<sup>d</sup> | CGGTCGATGATGTTGTAATGCCAC           | R                          |                 |

<sup>a</sup>Nozaki et al. (1995)
<sup>b</sup>Nozaki et al. (1997)
<sup>c</sup>Nozaki et al. (2006a)
<sup>d</sup>Designed in the present study.
Table S8. Conditions for PCR cycles and primers used in semi-quantitative RT-PCR analyses (Fig. S6).

| Gene      | PCR cycles                                                                 | Forward (F) or reverse (R) | Primer (Table S7)       |
|-----------|----------------------------------------------------------------------------|-----------------------------|--------------------------|
| PlestMID  | 2 min at 94°C, followed by 26, 28, 30 cycles of 98°C, 10 sec, 66°C, 30 sec, 68°C, 30 sec. | F                           | PIMT_RT_F1               |
|           |                                                                            | R                           | PIMT_RT_R2               |
| EF-1 like | 2 min at 94°C, followed by 24, 26, 28 cycles of 98°C, 10 sec, 60°C, 30 sec, 68°C, 30 sec. | F                           | PlestEFL_F1              |
|           |                                                                            | R                           | PlestEFL_R1              |
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