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Cryopreservation of two species of the multicellular volvocine green algal genus *Astrephomene*

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Abstract

Background *Astrephomene* is an interesting green algal genus that, together with *Volvox*, shows convergent evolution of spheroidal multicellular bodies with somatic cells of the colonial or multicellular volvocine lineage. A recent whole-genome analysis of *A. gubernaculifera* resolved the molecular-genetic basis of such convergent evolution, and two species of *Astrephomene* were described. However, maintenance of culture strains of *Astrephomene* requires rapid inoculation of living cultures, and cryopreserved culture strains have not been established in public culture collections.

Results To establish cryopreserved culture strains of two species of *Astrephomene*, conditions for cryopreservation of the two species were investigated using immature and mature vegetative colonies and two cryoprotectants: N,N-dimethylformamide (DMF) and hydroxyacetone (HA). Rates of cell survival of the *A. gubernaculifera* or *A. perforata* strain after two-step cooling and freezing in liquid nitrogen were compared between different concentrations (3 and 6%) of DMF and HA and two types of colonies: immature colonies (small colonies newly released from the parent) and mature colonies (large colonies just before daughter colony formation). The highest rate of survival [11 ± 13% (0.36–33%) by the most probable number (MPN) method] of *A. gubernaculifera* strain NIES-4017 (established in 2014) was obtained when culture samples of immature colonies were subjected to cryogenic treatment with 6% DMF. In contrast, culture samples of mature colonies subjected to 3% HA cryogenic treatment showed the highest "MPN survival" [5.5 ± 5.9% (0.12–12%)] in *A. perforata*. Using the optimized cryopreservation conditions for each species, survival after freezing in liquid nitrogen was examined for six other strains of *A. gubernaculifera* (established from 1962 to 1981) and another *A. perforata* strain maintained in the Microbial Culture Collection at the National Institute for Environmental Studies (MCC-NIES). We obtained ≥0.1% MPN survival of the *A. perforata* strain. However, only two of the six strains of *A. gubernaculifera* showed ≥0.1% MPN survival. By using the optimal cryopreserved conditions obtained for each species, five cryopreserved strains of two species of *Astrephomene* were established and deposited in the MCC-NIES.

Conclusions The optimal cryopreservation conditions differed between the two species of *Astrephomene*. Cryopreservation of long-term-maintained strains of *A. gubernaculifera* may be difficult; further studies of cryopreservation of these strains are needed.

Keywords Asexual cycle, Astrephomene, Cryopreservation, Cryoprotectant, Culture collection, Volvocine green algae

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Background

The volvocine green algae are composed of the unicellular genus *Chlamydomonas* and multicellular genera such as *Gonium* and *Volvox* (Additional file 1: Fig. S1). Because these green algae represent a unique model lineage for experimental studies of the evolution of sex and multicellularity [1], multicellular volvocine algae have been investigated in molecular and genomics studies [2–4]. Among the volvocine green algae, two independent lineages, Volvocaceae (including *Volvox*) and *Astrephomene* (Fig. 1), show similar or convergent evolution of multicellular spheroidal bodies with germ-soma differentiation (Additional file 1: Fig. S1) [5–7]. Whole-genome sequencing of *A. gubernaculifera* provided insight into the molecular-genetic basis of such convergent evolution [8]. Thus, *Astrephomene* represents a hopeful key organism for studies of multicellularity and germ-soma differentiation.

The genus *Astrephomene* was originally described by Pocock [11] based on a single species: *A. gubernaculifera*. Using culture strains of *A. gubernaculifera* originating from the USA and Mexico, morphology, sexual isolation within the morphological species, and physiology

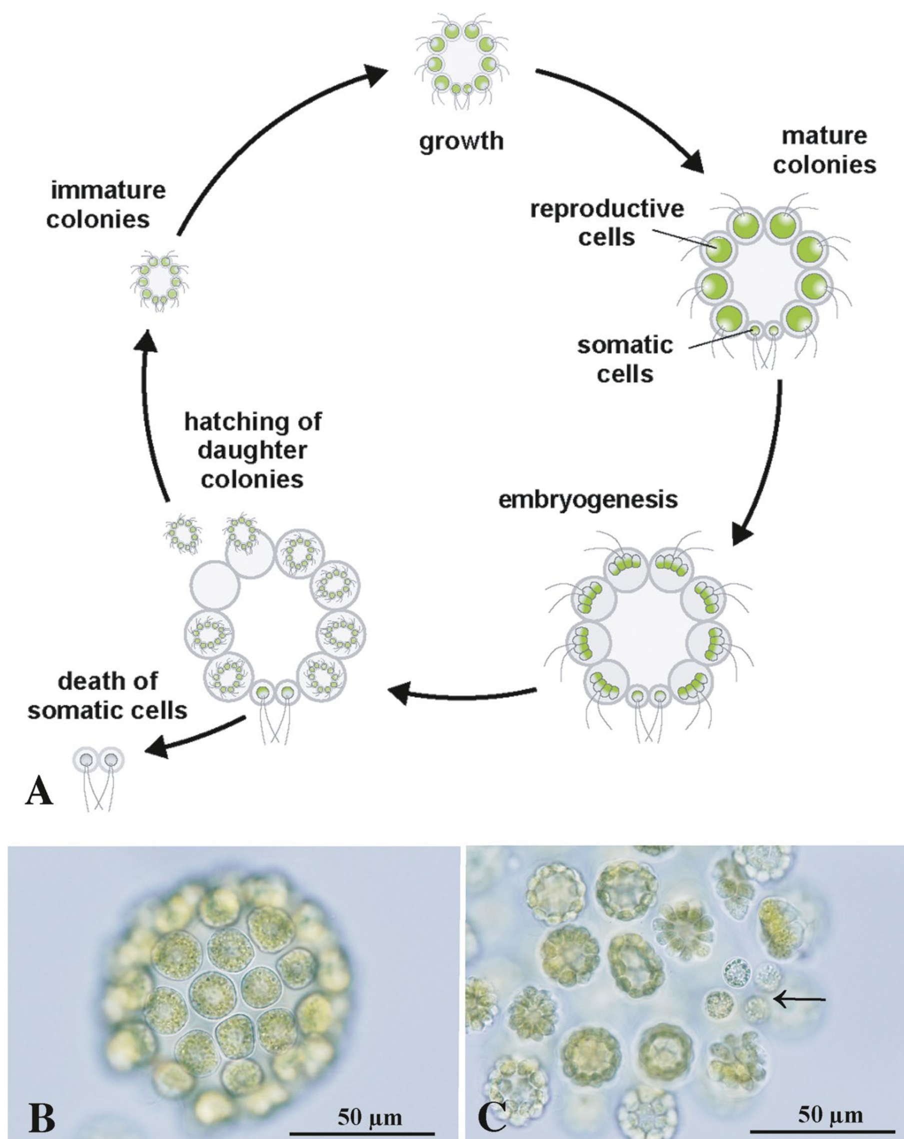

Fig. 1 Asexual life cycle of *Astrephomene*. **A** Diagram of asexual life cycle of *Astrephomene*. Based on Nozaki [9] and Yamashita et al. [10]. Colonies are generally 32- or 64-celled with two or four somatic cells in the posterior pole. Each reproductive cell performs cell divisions to produce a daughter colony. **B** Mature vegetative colony of *A. gubernaculifera* strain NIES-4017. **C** Posterior portion of parental colony of *A. gubernaculifera* strain NIES-4017 showing embryogenesis of reproductive cells and four undivided somatic cells (arrow)

were studied [12–14]. The second species, *A. perforata*, was described based on clonal cultured materials from Japan [9]. *A. perforata* differs from *A. gubernaculifera* in the morphology of the individual sheaths of cells in the spheroid and pyrenoids in the chloroplast [9]. Fifteen strains of the two species of *Astrephomene* established in these studies were deposited in the Culture Collection of Algae at the University of Texas at Austin (CCA-UTEX) [15]. Nine strains of the two *Astrephomene* species are available from the MCC-NIES (https://mcc.nies.go.jp/index_en.html [16]) and one strain of *A. gubernaculifera* is available from the Culture Collection of Algae at Goettingen University (SAG) (https://uni-goettingen.de/en/45175.html [17]). Since these culture strains are maintained by serial inoculations of living cells to new media, high costs are carried in the culture collections. However, cryopreserved culture strains of *Astrephomene* have not been established.

Although Mori et al. [18] examined cell survival after freezing in liquid nitrogen in six strains of two species of *Astrephomene* maintained in the MCC-NIES [16] (https://mcc.nies.go.jp/index_en.html) by using dimethyl sulfoxide (DMSO) as a cryoprotectant, none survived freezing. Later, Nakazawa & Nishii [19] demonstrated poor recovery (i.e., recovery of one or two of three replicates) of *A. gubernaculifera* strain NIES-418 after cryopreservation in liquid nitrogen when N,N-dimethylformamide (DMF) or hydroxyacetone (HA) was used as a cryoprotectant for two-step freezing. However, no other studies of the cryopreservation or establishment of cryopreserved strains of *Astrephomene* have been performed.

This study was undertaken to determine the optimal conditions for cryopreservation of culture strains of two species of *Astrephomene*. Optimal conditions for the cryopreservation of the two species were determined using immature and mature colonies from the asexual cycle of the two *Astrephomene* species (Fig. 1) and two cryoprotectants (DMF and HA). By using these conditions, cryopreserved strains of the two species were established.

Materials and methods

Culture strains

Nine culture strains of two species of *Astrephomene* maintained at the MCC-NIES [16] were used (Table 1). The cultures were grown in screw-cap tubes (18×150 mm) containing 10 mL of *Volvox* thiamin acetate (VTAC) medium or urea soil *Volvox* thiamin (USVT) medium [16] at 25 °C, with a 12 h:12 h light:dark schedule under cool-white fluorescent lamps at an intensity of 100–130 $\mu mol\, m^{-2} s^{-1}$. To maintain the cultures, USVT medium was used for *A. gubernaculifera* strain NIES-853, whereas the other six strains of *A. gubernaculifera* and two strains of *A. perforata* were cultured in VTAC medium.

To prepare cultures of immature colonies (newly released small daughter colonies with reproductive cells approximately 5 μm in diameter) (Fig. 2A, C), 0.2–0.3 mL of 4–5-day-old cultures (approximately 10^6 cells/mL) were inoculated into 10 mL of USVT medium in a screw-cap tube 4–6 h after the onset of the light period of the 12 h:12 h light:dark cycle. The inoculated cultures were incubated for 48 h at 25 °C with a 12 h:12 h light:dark cycle, as described above. Cultures of mature colonies (large colonies just before daughter colony formation, with reproductive cells approximately 15 μm in diameter) (Fig. 2B, D) were obtained as described above, except that the inoculum was diluted 30–50-fold with USVT medium.

Cryopreservation

The optimal cryopreservation conditions for two species of *Astrephomene* were assessed using DMF or HA as a

Table 1 List of strains of two species of *Astrephomene* used in this study

Species	Strain designation	Locality (Date of collection)[a]	Date of establishment of strain[a]	Growth medium
A. gubernaculifera	NIES-4017	Chiba, Japan (July 2014)	2014 October	VTAC
	NIES-418	Kanagawa, Japan (April 1981)	1981 May	VTAC
	NIES-419	Kanagawa, Japan (April 1981)	1981 May	VTAC
	NIES-628	Kanagawa, Japan (December 1980)	1981 July	VTAC
	NIES-853 (UTEX 1392)	Michigan, USA (July 1961)	1962 June	USVT
	NIES-854 (UTEX 1394)	Indiana, USA (October 1962)	1962 October	VTAC
	NIES-855 (UTEX 1398)	California, USA (August 1953)	1965 April	VTAC
A. perforata	NIES-564 (UTEX 2474)	Kanagawa, Japan (December 1980)	1981 June	VTAC
	NIES-565 (UTEX 2475)	Kanagawa, Japan (December 1980)	1981 June	VTAC

[a] From Nozaki [9], Starr & Zeikus [15], Kawachi et al. [16] and Yamashita et al. [10]

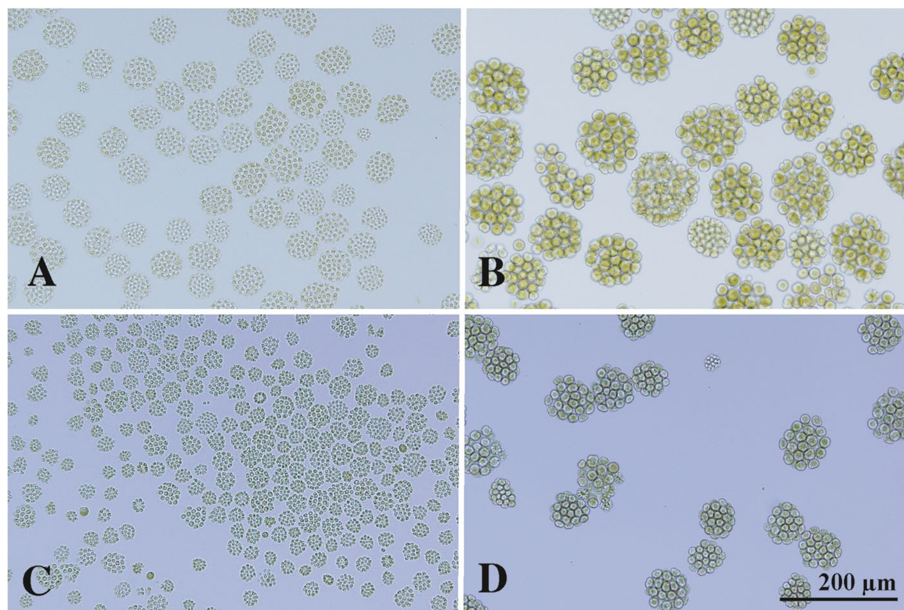

Fig. 2 Immature and mature colonies of two species of *Astrephomene* (Fig. 1A) that were used for cryogenic treatments (Table 2), shown at the same magnification throughout. **A** Immature colonies of *A. gubernaculifera* strain NIES-4017. **B** Mature colonies of *A. gubernaculifera* strain NIES-4017. **C** Immature colonies of *A. perforata* strain NIES-564. **D** Mature colonies of *A. perforata* strain NIES-564

cryoprotectant. Nakazawa and Nishii [19] demonstrated partial survival of *A. gubernaculifera* cells after freezing in liquid nitrogen with 3% DMF and 3% HA. Nakazawa and Nishii [19] studied the cryopreservation of multicellular volvocine algae using 0.25 mL PCR tubes as vials for two-step freezing. However, we recently demonstrated that use of 2 mL cryotubes (Cryo.s, 2 mL, Round Bottom, Starfoot Base; Greiner Bio-One, Kremsmünster, Austria) as vials resulted in a higher survival rate than achieved using 0.20 mL PCR tubes for cryopreservation of the multicellular volvocine alga *Gonium pectorale* [20]. Thus, we prepared 1.0 mL samples in 2 mL cryotubes for cryopreservation of two species of *Astrephomene*, and eight cryopreservation conditions were examined for *A. gubernaculifera* strain NIES-4017 and *A. perforata* strain NIES-564: immature colonies in 3% DMF, immature colonies in 6% DMF, immature colonies in 3% HA, immature colonies in 6% HA, mature colonies in 3% DMF, mature colonies in 6% DMF, mature colonies in 3% HA, and mature colonies in 6% HA. For cryopreservation, a 48-day-old culture of immature or mature colonies (see above) actively growing in USVT medium (2–4 mL) was mixed with an equal volume of USVT medium containing 6% or 12% DMF (or HA) to prepare a sample with 3% or 6% DMF (or HA), respectively. The cells were exposed to the cryoprotectant at room temperature (20–25 °C) for 15 min. Next, 1.0 mL of the culture sample with DMF (or HA) was transferred to a 2 mL cryotube. The sample cryotube was subjected to two-step cooling in liquid nitrogen [18, 20, 21]. Cell suspensions in tubes were frozen in vapor-phase liquid nitrogen at a rate of − 1 °C/min to − 40 °C using a programmable freezer (Controlled Rate Freezer, KRYO 560-16; Planer, Sunbury-on-Thames, UK). After 15 min of maintenance at − 40 °C, the cell suspensions were cooled rapidly to − 196 °C by immersion in liquid nitrogen, and finally stored at − 190 °C in vapor-phase liquid nitrogen. To assess the viability of cells frozen in liquid nitrogen, the frozen samples in tubes were thawed in a 40 °C or 60 °C water bath while the tube was shaken by hand until the ice crystals almost disappeared (approximately 120 or 75 s, respectively); then, 0.1 mL of the diluted sample was immediately subjected to analysis using the most probable number (MPN) method [18, 20–23]. For the MPN method, eight wells in each dilution series of a 48-well microplate (CellStar Cell Culture Multiwell Plate with Lid, Greiner Bio-One) were filled with 0.9 mL of USVT medium. Three replicates of eight 1/10th dilutions were performed for each cryotube of sample using a 6-channel pipette (Pipet-Lite Adjustable Spacer LA6-1200XLS; Mettler-Toledo, Greifensee, Switzerland). As a control, three replicates of eight 1/10th dilutions of cultures without cryogenic treatment and cryoprotectant were treated in the same manner. The plates were initially incubated in darkness at 25 °C for 2 days, then transferred to a 12 h:12 h light:dark schedule at 25 °C for 2 weeks [20]. Each well was scored for growth and MPN values (cell numbers) were estimated based on those scores using MPN Calculator 3.1 (https://softdeluxe.com/MPN-Calcu

lator-444229/) [24, 25]. The recovery rate of viable cells (%) was calculated relative to the viable cell count in the unfrozen control using the MPN method. For each of the four types of cryopreservation conditions, recovery rates were measured based on six tubes from two independent experiments (Table 2).

In addition, immediately after thawing of the three frozen cryotubes of each sample, 0.5 mL of the melted sample in each cryotube was inoculated into fresh growth medium (10 mL) in a six-well plate (PS with Lid; Greiner Bio-One) (first inoculation); subsequently, 0.5 mL of the first inoculation was transferred to 10 mL of fresh growth medium (second inoculation) in a six-well plate to confirm the recovery of frozen and thawed cells in the same volume of culture medium used in the MCC-NIES.

Results

"MPN survival" after the eight cryogenic treatments differed between the two species of *Astrephomene* (Table 2). For *A. gubernaculifera* strain NIES-4017, the highest recovery rate after freezing in liquid nitrogen and thawing was achieved when immature colonies were subjected to 6% DMF during two-step freezing (11 ± 13% MPN survival, Table 2). In addition, recovery of active growth was observed in the six 10 mL cultures after two successive inoculations of liquid nitrogen-frozen cultures of immature colonies of *A. gubernaculifera* strain NIES-4017 using 6% DMF (Table 2). However, 0% MPN survival and partial recovery of active growth in six 10 mL cultures after two successive inoculations were observed in samples of mature colonies of *A. gubernaculifera* strain NIES-4017 treated with DMF or HA (Table 2). In contrast, < 0.1% MPN survival was found in samples of *A. perforata* strain NIES-564 treated with 3% HA, with mature or immature colonies (Table 2). The highest rate of MPN survival among the eight cryogenic treatments in *A. perforata* strain NIES-564 was 5.5 ± 5.9% when mature colonies were mixed with 3% HA during two-step freezing (Table 2).

Because the effect of sample parameters (colony maturation and cryoprotectants) on recovery after cryopreservation were species-specific, recovery based on the MPN method and two successive inoculations in 10 mL of new medium after the cryopreservation of seven other strains of *Astrephomene* was examined using immature colonies of *A. gubernaculifera* with 6% DMF, or mature colonies of *A. perforata* with 3% HA. Based on these potentially optimized cryopreserved conditions for each

Table 2 Comparison of results of eight types of cryopreservation conditions for two species of *Astrephomene* based on most probable number (MPN) methods

Conditions for cryopreservation (species)	Total viability[a] (range) %	Experiment I MPN cell numbers in three cryotubes (/mL) (control); [Number of viable cultures by 1st (2nd) inoculation with 3 (3) 10 mL cultures]	Experiment II MPN cell numbers in three cryotubes (/mL) (control); [Number of viable cultures by 1st (2nd) inoculation with 3 (3) 10 mL cultures]
Immature colonies in 3% DMF (AG[b])	0.36 ± 0.38 (0.022-1.0)	92, 92, 230 (22000); [3(2)]	9.2, 23, 92 (42000); [3(0)]
Immature colonies in 6% DMF (AG)	11 ± 13 (0.36-33)	2300, 4200, 7300 (22000); [3(3)]	150, 230, 230 (42000); [3(3)]
Immature colonies in 3% HA (AG)	0.24 ± 0.20 (0.014-0.46)	3, 7.3, 74 (22000); [3(1)]	140, 420, 420 (92000); [3(3)]
Immature colonies in 6% HA (AG)	0 ± 0 (0-0)	0, 0, 0 (22000); [0(0)]	0, 0, 0 (92000); [0(0)]
Mature colonies in 3% DMF (AG)	0 ± 0 (0-0)	0, 0, 0 (22000); [0(0)]	0, 0, 0 (9200); [0(0)]
Mature colonies in 6% DMF (AG)	0 ± 0 (0-0)	0, 0, 0 (22000); [2(0)]	0, 0, 0 (9200); [0(0)]
Mature colonies in 3% HA (AG)	0 ± 0 (0-0)	0, 0, 0 (22000); [2(1)]	0, 0, 0 (14000); [3(0)]
Mature colonies in 6% HA (AG)	0 ± 0 (0-0)	0, 0, 0 (22000); [0(0)]	0, 0, 0 (14000); [0(0)]
Immature colonies in 3% DMF (AP[c])	0.053 ± 0.098 (0-0.25)	23, 42, 230 (92000); [3(2)]	0, 0, 0 (9200); [0(0)]
Immature colonies in 6% DMF (AP)	0.032 ± 0.037 (0-0.1)	9.2, 9.2, 92 (92000); [3(1)]	0, 3, 3.6 (9200); [3(1)]
Immature colonies in 3% HA (AP)	2.2 ± 1.9 (0.3-4.6)	920, 1500, 4200 (92000); [3(3)]	28, 92, 420 (9200); [3(3)]
Immature colonies in 6% HA (AP)	0.071 ± 0.077 (0-0.14)	0, 0, 0 (92000); [0(0)]	13, 13, 13 (9200); [3(3)]
Mature colonies in 3% DMF (AP)	0 ± 0 (0-0)	0, 0, 0 (7400); [1(0)]	0, 0, 0 (9200); [0(0)]
Mature colonies in 6% DMF (AP)	0.093 ± 0.12 (0-0.31)	9.2, 9.2, 23 (7400); [3(1)]	0, 0, 0 (9200); [3(0)]
Mature colonies in 3% HA (AP)	5.5 ± 5.9 (0.12-12)	740, 740, 920 (7400); [3(3)]	11,15, 21 (9200); [3(3)]
Mature colonies in 6% HA (AP)	0 ± 0 (0-0)	0, 0, 0 (7400); [0(0)]	0, 0, 0 (9200); [0(0)]

[a] Significant difference ($p < 0.01$) was detected in interactions between *Astrephomene* species and cryoprotectants, based on unweighted-mean ANOVA analyzed by js-STAR XR release 1.6.6j <http://www.kisnet.or.jp/nappa/software/star/index.htm>

[b] *A. gubernaculifera* strain NIES-4017

[c] *A. perforata* strain NIES-564

species (Table 2), we obtained ≥0.1% MPN viability rates and active growth based on two successive inoculations in 10 mL cultures of *A. gubernaculifera* strains NIES-418 and NIES-853, and *A. perforata* strain NIES-565 (Table 3). However, the other four strains of *A. gubernaculifera* did not recover after freezing in liquid nitrogen and thawing (≥ 0.1% MPN viability), and did not grow after one and two inoculations to 10 mL of medium (Table 3). Thus, five cryopreserved strains of *Astrephomene* were deposited in the MCC-NIES.

Discussion

Because *Astrephomene* requires organic compounds such as acetate for photoheterotrophy, and grows extremely rapidly under photoheterotrophic conditions [14] (Additional file 1: Fig. S2; Additional file 2: Text S1), serial inoculations of living cells to new media during short intervals are required for maintenance of living cultures [10, 16] (https://mcc.nies.go.jp/index_en.html). In addition, during the long-term maintenance of growing cultures by subculturing, the ability to perform normal morphogenesis gradually decreases in *Astrephomene* [10]. Thus, cryopreservation of culture strains of *Astrephomene* is needed.

In the present study, we determined the optimal liquid-nitrogen cryopreservation conditions for *A. gubernaculifera* strain NIES-4017 and *A. perforata* strain NIES-564 by selecting mature or immature colonies of *Astrephomene* and two types of cryoprotectants, DMF and HA (Table 2). Amidic and acetonic cryoprotectants, such as DMF and HA, enable cryopreservation of cells based on their ability to cross the cell membrane and cytotoxic effects [19]. We examined MPN survival of unfrozen cells of mature and immature colonies of two species of *Astrephomene* treated with 3% DMF, 6% DMF, 3% HA and 6% HA (Additional file 1: Table S1, Fig. S3). When immature colonies were treated with 6% DMF, unfrozen cells of *A. gubernaculifera* strain NIES-4017 exhibited a moderate survival rate (39%), but frozen NIES-4017 cells showed the highest survival rate (11%) among all frozen cell types. By contrast, a high survival rate (99%) for unfrozen cells and a low rate (0.032%) for frozen cells were observed with *A. perforata* strain NIES-564 (Table 2; Additional file 1: Table S1, Fig. S3). Using mature colonies treated with 3% HA, > 100% survival was detected for unfrozen cells of *A. gubernaculifera*, compared to 0% for frozen colonies (Additional file 1: Table S1, Fig. S3). By contrast, mature colonies of *A. perforata* treated with 3% HA had the highest survival rate (5.5%) among frozen cell types and a moderate survival rate (57%) relative to the other unfrozen cell types (Table 2; Additional file 1: Table S1, Fig. S3). Therefore, the ability of HA and DMF to cross the cell membrane, and their toxic effects on cells in immature and mature colonies, differ between *A. gubernaculifera* and *A. perforata*.

In *A. gubernaculifera* strain NIES-4017, mature colonies treated with 3% DMF, 6% DMF and 3% HA exhibited 0% MPN survival after freezing and thawing. By contrast, immature colonies showed a < 0.2% MPN survival rate when treated with 3% DMF, 6% DMF, or 3% HA (Table 2). The difference in survival between immature and mature colonies of *A. gubernaculifera* could be attributed to differences in cell volume. Mature colonies of *Astrephomene* contain larger cells than immature colonies (Fig. 2A, B). Cell size is a critical factor for cryopreservation; cryopreserving large algal cells is problematic [26, 27]. However, in *A. perforata* strain NIES-564, mature colonies treated with 3% HA showed the highest MPN survival rate (5%) after freezing and thawing, while immature colonies treated with 3% HA had a 2.2% MPN survival rate

Table 3 Comparison of recovery results of seven strains of two *Astrephomene* species (Table 1) after possible optimal cryogenic treatment (immature colonies of *A. gubernaculifera* [AG] with 6% DMF, or mature colonies of *A. perforata* [AP] with 3% HA; Table 2) in liquid nitrogen

Strain designation [species]	Total viability (range) %	Experiment I MPN cell numbers in three cryotubes (/mL) (control); [Number of viable cultures by 1st (2nd) inoculation with 3 (3) 10 mL cultures]	Experiment II MPN cell numbers in three cryotubes (/mL) (control); [Number of viable cultures by 1st (2nd) inoculation with 3 (3) 10 mL cultures]
NIES-418 [AG]	0.570 ± 0.88 (0.0026-2.2)	7.3, 15, 15 (280000); [3(3)]	92, 420, 920 (42000); [3(3)]
NIES-419[a] [AG]	0 (0-0)	0, 0, 0 (81000); [0(0)]	0, 0, 0 (15000); [0(0)]
NIES-628[a] [AG]	0.032 ± 0.0020 (0.011-0.065)	9.2, 11, 20 (81000); [3(3)]	9.2, 9.2, 15 (23000); [3(2)]
NIES-853 [AG]	0.54 ± 0.19 (0.41-0.91)	380, 420, 740 (81000); [3(3)]	38, 42, 42 (9200); [3(3)]
NIES-854[a] [AG]	0 (0-0)	0, 0, 0 (40000); [0(0)]	0, 0, 0 (9200); [1(0)]
NIES-855[a] [AG]	0 (0-0)	0, 0, 0 (180000); [0(0)]	0, 0, 0 (23000); [0(0)]
NIES-565 [AP]	9.6 ± 5.0 (3.8-18)	1500, 2300, 4200 (40000); 3 [3]	2100, 2300, 4200 (23000); [3(3)]

[a] Not used for cryopreserved strain in MCC-NIES because of < 0.1% MPN survivability

(Table 2). Therefore, cell size may not critically influence the survival of *A. perforata* cells.

A. gubernaculifera strain NIES-4017 showed 11% MPN survival when immature colonies were treated with 6% DMF (Table 2). However, the four other strains of *A. gubernaculifera* showed < 0.1% MPN survival when immature colonies were treated with 6% DMF (Table 3). These *A. gubernaculifera* strains have been maintained by serial inoculations in liquid cultures since their establishment [16] (https://mcc.nies.go.jp/index_en.html). *A. gubernaculifera* strain NIES-4017 was originally established in 2014 from a single colony in a re-wetted soil sample [10], whereas other strains of this species were established from 1962 to 1981 (Table 1). During the cryopreservation of vegetative colonies of *Gonium pectorale*, 6% DMF as a cryoprotectant in two-step freezing was effective for cryopreservation, with MPN survival rates of ≥0.1% being maintained in 10 strains from the MCC-NIES [20]. However, three other strains of *G. pectorale* did not exhibit MPN survival rates ≥0.1% under identical cryogenic conditions (6% DMF) [20]. These three strains (NIES-2261, 469 and 570) have been maintained as growing subcultures since establishment of the original cultures in the period 1979–1994 [20]. Therefore, long-term maintenance of algal strains as growing subcultures by serial inoculation could decrease the survival rates of some strains of *Gonium* and *Astrephomene*.

Conclusion

A. gubernaculifera colony maturation and cell volume are critical factors affecting survival after cryopreservation, possibly as a result of cryoprotectant permeability and/or toxicity (Additional file 1: Fig. S3). Large reproductive cells in mature colonies of *A. gubernaculifera* (Fig. 2B) do not survive 6% DMF treatment, which enables cryopreservation of small reproductive cells (Fig. 2A) (Table 2). Although this factor is not critical in *A. perforata* and may be species-specific (Table 2), the selection of cells of a suitable age or size may be important for successful cryopreservation in other colonial or multicellular volvocine genera.

Cryopreservation of some long-term-maintained strains of *A. gubernaculifera* (Table 3) and *G. pectorale* (NIES-2261, 469 and 570) is difficult [19]. However, strains established concomitantly are readily cryopreserved, particularly of complementary mating types of *G. pectorale* (NIES-2262, 468, and 569, respectively) [20]. Thus, during the long-term maintenance of cultures by subculturing, survival after cryopreservation may be decreased in certain strains of multicellular volvocine algae. Similarly, the inducibility of sexual reproduction and ability to perform normal morphogenesis gradually decrease during the long-term maintenance of cultures of multicellular volvocine species [10, 28]. Therefore, cryopreservation of newly established culture strains is important for future studies of multicellular volvocine algae.

The present study demonstrated that two species of *Astrephomene* can be cryopreserved using the optimal cryopreserved conditions for each species (Table 2). However, the survival rates are still low [11 ± 13% (0.36–33%) in *A. gubernaculifera* strain NIES-4017 and 5.5 ± 5.9% (0.12-12%) in *A. perforata* strain NIES-564 (Table 2)], which highlights that more effective conditions need to be standardized to obtain better survival.

Abbreviations

CCA-UTEX	Culture Collection of Algae at the University of Texas at Austin
DMF	N,N-dimethylformamide
DMSO	Dimethyl sulfoxide
HA	Hydroxyacetone
MPN	The most probable number
MCC-NIES	Microbial Culture Collection at the National Institute for Environmental Studies
SAG	The Culture Collection of Algae at Goettingen University
VTAC	Volvox thiamin acetate
USVT	Urea soil *Volvox* thiamin

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-023-02767-3.

Additional file 1: Table S1. Comparison of effects of eight types of cryopreservation conditions (Table 2) on viabilities of *Astrephomene gubernaculifera* strain NIES-4017 (AG) and *A. perforata* strain NIES-564 (AP) without freezing and thawing, based on most probable number (MPN) methods. **Fig. S1.** Diagrammatic representation of the evolution of *Astrephomene* within the volvocine green algae, showing convergent evolution of germ-soma differentiation in spheroidal bodies. **Fig. S2.** Comparison of autotrophic (left, mVT medium) and photoheterotrophic (right, mVTAC medium) growth in six-day-old cultures of four multicellular volvocine species (*Astrephomene gubernaculifera* strain NIES-4017, *Volvulina steinii* strain NIES-4471, *Gonium pectorale* strain NIES-2863 and *Eudorina* sp. strain NIES-3984), based on the quantitative measurement (Additional file 2: Text S1, Table S2). **Fig. S3.** Comparison of mean rates of MPN survivability between two species of *Astrephomene* under 16 different conditions (Table 2; Additional file 1: Table S1).

Additional file 2: Text S1. Growth measurement of four multicellular volvocine species. **Table S2.** Composition of modified VT (mVT, for autotrophic growth condition) and modified VTAC (mVTAC, for photoheterotrophic growth condition) media.

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Authors' contributions

HN, FM and SY prepared the manuscript. HN, FM, YT and SY performed experiments and analysis of the data. RM and HY discussed the methods and the data. MK supervised the whole study. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its additional file.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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