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First successful production of adult corals derived from cryopreserved larvae

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Coral reefs worldwide are declining due to increasing concentrations of greenhouse gases, which, combined with local anthropogenic pressure, are exacerbating unprecedented mass coral bleaching. For corals to survive, restoring coral reefs through cryopreservation is crucial. The aim of this study was to vitrify and laser-warm *Stylophora pistillata* planulae to allow for feasible settlement, post-settlement survival, and the production of adult corals. The no-observed-effect concentrations were used to determine the best cryoprotective agents for *S. pistillata*. The larvae were then subjected to cooling and nanolaser warming (300 V, 10 ms pulse width, 2 mm beam diameter) by using two vitrification solutions (VSs; VS1: 2 M dimethyl sulfoxide and 1 M ethylene glycol [EG]; VS2: 2 M propylene glycol and 1 M EG) and gold nanoparticles. The results revealed that VS1-treated larvae had a higher vitrification rate (65%), swimming rate (23.1%), settlement rate (11.54%), and post settlement survival rate (11.54%) than those treated with VS2. Seasonal variations also affected the cryopreservation of the planulae; VS1 was more favorable for the planulae in spring than in fall. Although laser-warmed larvae developed slower morphologically than their controlled counterparts, the production of cryopreserved adult *S. pistillata* corals was achieved. The proposed technique can improve the cryopreservation of corals and advance efforts to protect endangered coral species.

KEYWORDS

***Stylophora pistillata*, cryopreservation, laser warming, larvae, season**

Introduction

Global warming poses a threat to the world's coral reefs. Unprecedented declines in coral reefs manifest at the regional level, where the composition of coral assemblages are drastically changing (Hughes et al., 2018). Most reefs cannot maintain positive net carbonate production because of ocean warming and acidification (Cornwall et al.,

2021). Ocean temperatures continue to rise, and corals cannot acclimate or adapt quickly. Thus, coral bleaching events continue to increase in frequency and intensity (Hoegh-Guldberg, 1999). The projected decline in coral reefs is being met with mitigation strategies that use new and radical interventions such as cryopreservation (Lin and Tsai, 2020). Cryopreservation is the process of preserving biological samples at extremely cold temperatures (Tsai and Lin, 2012). Cryobiology is a developing science that has a rudimentary theoretical framework (Gwo, 2011; Vanderzwalmen et al., 2020). Cryopreservation research is empirical and conducted through trial and error; thus, the protocol for cryopreservation continues to be optimized (Whaley et al., 2021).

The freezing protocols for coral samples such as sperm (Hagedorn et al., 2006; Hagedorn et al., 2017; Viyakarn et al., 2018; Zuchowicz et al., 2021), oocyte (Tsai et al., 2011; Tsai et al., 2015; Tsai et al., 2016a; Tsai et al., 2016b), tissue balls (Feuillassier et al., 2014), planula larvae (Daly et al., 2018; Cirino et al., 2019; Cirino et al., 2021a), and symbiotic algae of corals (Hagedorn et al., 2010; Chong et al., 2016; Lin et al., 2019) have been optimized to cryopreserve corals. The protocols involve a series of assessments of the suitability (Feuillassier et al., 2014), chilling sensitivity, cooling, and warming rates (Viyakarn et al., 2018; Zuchowicz et al., 2021) of cryoprotective agents (CPAs). These initial steps are crucial for developing cryobanks (Martínez-Páramo et al., 2017; di Genio et al., 2020; Toh et al., 2022) to mitigate the loss of genetic and species diversity (Mayfield et al., 2019; Lin CC et al., 2022) in coral reefs.

Cryopreservation techniques have evolved beyond the slow, fast, and ultrafast freezing techniques that use temperature control equipment (Narida et al., 2022b). Vitrification is gaining popularity in cryopreservation because it prevents ice crystal formation when the speed of temperature conduction is increased, which significantly increases cooling rates (Liebermann et al., 2002). Vitrification is the glass-like solidification of a solution (from approximately −80°C to −130°C) and differs from conventional freezing techniques (Bojic et al., 2021). In vitrification, the sample solidifies without ice crystals, and multiple (Tsai et al., 2016b; Jang et al., 2017; Magnotti et al., 2018) or no CPAs (Isachenko et al., 2003; Spis et al., 2019) are used for biological samples that require extremely high cooling rates (Bojic et al., 2021). Vitrification was first used in mouse (*Mus musculus*) embryos (Rall and Fahy, 1985) and then plants (Stiles, 1930), erythrocytes (Rapatz and Luyet, 1968), mammalian embryos (Rall and Fahy, 1985; Massip et al., 1986; Yuswiati and Holtz, 1990; Rall, 1993; Schiewe and Anderson, 2017), fish (Cuevas-Uribe et al., 2013; Figueroa et al., 2013; Godoy et al., 2013), invertebrates (Guo and Weng, 2020; Heres et al., 2021), and now corals (Tsai et al., 2015) and their unicellular dinoflagellate symbionts (di Genio et al., 2020; Kihika et al., 2022a; Kihika et al., 2022b). Researchers are striving to cryobank genetic materials from endangered coral species and save regional populations in decline.

Contemporary vitrification techniques are coupled with the use of lasers to rewarm the samples rather than applying conventional warming rates of 40°C/s to 200°C/s (Figueroa et al., 2013; Figueroa et al., 2015). Mouse oocytes and embryos were among the first samples that were successfully laser warmed (Jin et al., 2014; Jin and Mazur, 2015). One study applied droplet vitrification and laser warming to *Danio rerio* (zebra fish) embryos (>2mm) (Khosla et al., 2020). Vitrification and ultrarapid laser warming were also tested on *Saccharomyces cerevisiae* (Paredes, 2021). Another study used the gonadal tissues and oocytes from a cat to explore the effects of laser warming versus those of conventional thawing methods (Rowlison et al., 2022). The effectiveness of this technique has led to its popularity in coral cryopreservation. To date, the planula larvae from three species of corals, namely *Lobactis* [*Fungia*] *scutaria* (Daly et al., 2018), *Pocillopora verrucosa*, and *Seriatopora caliendrum* (Cirino et al., 2019), have successfully survived cryopreservation and resumed swimming after vitrification and nanolaser warming. Two of these species (*P. verrucosa* and *S. caliendrum*) (Cirino et al., 2019) were successfully settled.

Cryopreservation of coral oocytes and larvae poses many challenges due to their large sizes, high-fat content (Lin et al., 2013), high chilling sensitivities (Lin and Tsai, 2012; Cirino et al., 2022), and high lipid phase transition temperatures (Lin et al., 2014), Two ecologically distinct groups of corals exist from which biomaterials can be collected for cryopreservation. The first group, spawners, simultaneously release eggs and sperm, and fertilization occurs externally. Brooders, the second group, fertilize internally and release complex planulae that can metamorphose and settle within hours (Harrigan, 1972; Hughes et al., 1999). Spawners comprise 80% of scleractinian corals, whereas brooders only comprise 20%. Although brooder corals settle planulae quickly, they are not as prevalent in reefs. Moreover, only brooders can acquire gonochorism, which is 100 times more likely to be lost than gained (Kerr et al., 2011). These characteristics make coral planula larvae excellent candidates for cryobanking. With the advent of cryopreservation techniques, novel devices for vitrification that enable long-term storage and cryobanking can be used to laser-warm coral samples; thus, planulae are a suitable coral biomaterial for cryopreservation (Narida et al., 2022a; Lin et al., 2023). The aim of this study was to vitrify and laser-warm *S. pistillata* planulae to allow for feasible settlement, post-settlement survival and the production of adult corals.

Materials and methods

Target coral species

S. pistillata (Esper, 1797) is a key scleractinian coral commonly found in reef fronts and submarine terraces of protected reefs around Taiwan and offshore islets (Dai, 1990). *S. pistillata* is an opportunistic and pioneering coral that can colonize new and undisturbed environments (Loya, 1976; Baird and Morse, 2004). It is a thermally intolerant (Cacciapaglia and van Woesik, 2016) and bleaching-sensitive (Loya et al., 2001; Marshall and Schuttenberg, 2006; van Woesik et al., 2011) species. A brooder that reproduces year-round (Atoda, 1947) and exhibits lunar periodicity (Zakai et al., 2006) does not require chemical cues to induce metamorphosis (Baird, 2001), which makes *S. pistillata* a good candidate for cryopreservation.

Coral collection

Colonies of *S. pistillata* (10–15 cm in diameter) were collected at a depth of 10 m from Houbihu Reef, Kenting National Park (KNP), Nanwan, Taiwan (GPS coordinates: N 21.931867°, E 120.744683°). An approved collection permit from KNP was received (permit number: 1090009719). The coral colonies were transported in a 60-L plastic crate with seawater. The individual colony was transferred and reared in 5-L flow-through tanks at the National Museum of Marine Biology. Handmade traps with a mesh size of 250 μm were deployed before sunset, and the planulae were collected at least 2 h after sunrise. The planulae were transported to the laboratory and washed thrice with filtered seawater (FSW) by using a 47-mm glass fiber filter with a mesh size of 0.2 μm (Pall, New York, NY, USA) attached to a vacuum pump (Rocker, Kaohsiung, Taiwan). Only planulae that were actively swimming and recently released were selected for the experiments.

Species identification and DNA analyses

The *S. pistillata* colonies were morphologically identified using Veron (2000) as a reference, and DNA analyses were performed to validate the identification of the species. The collected *S. pistillata* planulae were circular, approximately 640 μm in diameter, and had an elongated length of approximately 1000 μm. The planulae contained Symbiodiniaceae that were well distributed with a distinct dark-brown tail. Tissue samples were collected to verify the species of *S. pistillata*, and the DNA was extracted using a DNasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA samples were amplified using a 10× AccuPrime polymerase chain reaction (PCR) buffer, 200 nM of each primer, and 5 U of AccuPrime Pfx (Invitrogen, Carlsbad, CA, USA) in a 50 μL reaction. The forward primer for coral nuclear ribosomal DNA was 5′-TAAAAGTCGTAACAAGGTTTCC-3′, and the reverse primer was 5′- CCTCCGCTTATTGATATGCTTAAAT-3′ (Forsman et al., 2009). The PCR and other sequencing procedures were described in a previous study (Wang et al., 2015).

Effect of cryoprotectants

The collected planulae (five planulae per treatment) were exposed to five CPAs that varied in concentration (0.5, 1, and 2 M) and exposure time (5, 10, and 15 min). The five CPAs were dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA), methanol (MeOH; Merck, Darmstadt, Germany), glycerol (GLY; Merck, Darmstadt, Germany), propylene glycol (PG; JT Baker, Phillipsburg, NJ, USA), and ethylene glycol (EG; JT Baker). After CPA exposure, the planulae were washed thrice with FSW and kept in six-well petri dishes (Alpha Plus, Taipei, Taiwan) to observe viability (i.e., moving and swimming) and settlement. The number of planulae that moved and settled was used to calculate the no-observed-effect concentration (NOEC) and determine the highest concentration at which no effect on *S. pistillata* larvae was observed over a given time. Three replicates were performed for each CPA (Figure 1).

Gold nanoparticle synthesis

The synthesis of gold nanoparticles (AuNPs) in the absence of surfactants was based on the methods of Turkevich et al. (1951) and Frens (1973). All the solutions of $HAuCl_4$ (Alfa Aeser, Haverhill, MA, USA) and Na_3-citrate (Merck) were prepared using deionized water. First, 20 mL of 1 mM HAuCl was heated to boiling. Then, 0.6 mL of 10 mM Na3-citrate was added to the boiling solution. After approximately 30 s, the solution first turned dark blue (i.e., nucleation) and then changed to wine red, which indicated the formation of AuNPs. Digital images of the AuNPs were captured using a transmission electron microscope (TEM; JEM-2100, JEOL, Tokyo, Japan) connected to a camera system (Gatan digital micrograph; Gatan, Pleasanton, CA, USA) at an accelerating voltage of 200 kV (filament, 56 μA). Before being analyzed under the TEM, the AuNP colloid was dripped onto a carbon-coated copper grid and air-dried at room temperature.

Vitrification and laser warming

The vitrification solutions (VSs) were prepared according to the results of the CPA experiments. Two VSs were created. VS1 was composed of 2 M EG and 1 M PG, and VS2 was composed of 2 M PG and 1 M EG. Both VSs were mixed with 40% (w/v) Ficoll and 10% (v/v) AuNPs in FSW, the final concentration of which was 1.2 × 10^{18} particles/m^3 with an optimized emission wavelength of 535 nm. Equilibration solutions (ESs) were prepared by serially diluting VS1 and VS2 at ratios of 1:3 (ES1) and 1:1 (ES2) to create quarter- and half-strength VSs. Each coral larva was initially exposed to a 10-μL drop of ES1 for 4 min and then a 10-μL drop of ES2 for 2 min. Finally, the coral larva was submerged in 10-μL VS for 1 min. After each solution was added to the planula, signs of irregularities and damage were recorded. Planulae that were damaged were discarded

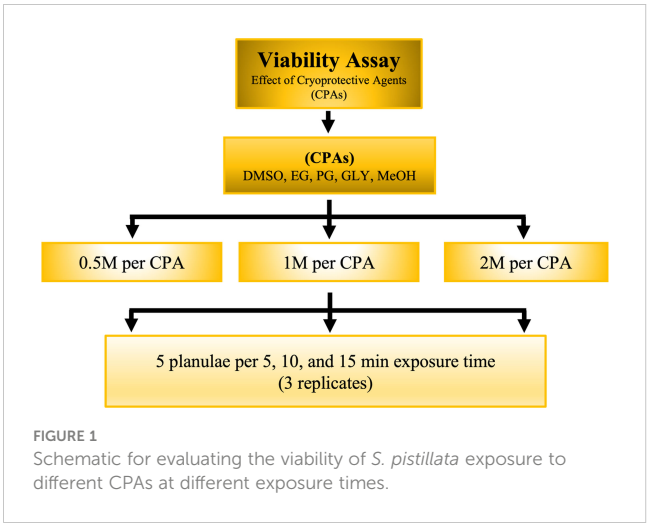

FIGURE 1
Schematic for evaluating the viability of *S. pistillata* exposure to different CPAs at different exposure times.

to preserve the larval quality before cryopreservation. After a series of solution exposures, a 0.80-µL drop (VS with planula) was placed on a self-made cryostick for vitrification; the cryostick was made using a bamboo stick (approximately 8 cm long) and a laminated thin sheet of acetate (160 µm; width: 1 mm; length: 4 cm). The planula was lowered into LN_2 for at least 1 min by using a custom device and placed under a benchtop iWeld infrared Nd : YAG laser welder (980 Series; LaserStar Technologies, Riverside, RI, USA) at 60 joules for warming.

Assessment of larval vitrification, viability, settlement, and post settlement rates

A planula was successfully vitrified when it remained clear of crystals after being hit by a laser beam (as verified using a laser microscope). Vitrified planulae were rehydrated at 25°C by using 10 µL of ES2 for 2 min, followed by 10 µL of ES1 for 4 min. The planulae were then washed in FSW. Vitrification rates were computed using the following formula:

$$Vitrification\ rate\ (\%) = \frac{Total\ \#\ of\ vitrified\ planulae}{Total\ \#\ of\ planulae} \times 100$$

The *S. pistillata* planulae that were successfully laser-warmed were washed thrice with FSW and observed under a stereo-zoom microscope (Olympus, Tokyo, Japan). The planulae were transferred to a petri dish and left for 12 h. The untreated planulae were also kept inside a petri dish for comparisons between vitrified *S. pistillata*. Comparisons were made by dividing the number of settled laser-warmed planulae by the total number of settled untreated planulae and multiplying the result by 100. Successfully settled planulae were reared in 600-L fiberglass tanks with wavemakers (IceCap, Slidell, LA, USA) to maintain water circulation and a seawater temperature of 26°C. The petri dishes were cleaned at least thrice per week to avoid algal overgrowth. Digital images of *S. pistillata* spats were taken daily by using a SteREO Discovery V8 (Zeiss, White Plains, NY, USA) stereomicroscope. The swimming, settlement, and post settlement rates were computed using the following formulas:

$$swimming\ rate\ (\%) = \frac{Total\ \#\ of\ swimming\ planulae}{Total\ \#\ of\ vitrified\ planulae} \times 100$$

$$settlementrate\ (\%) = \frac{Total\ \#\ of\ settled\ planulae}{Total\ \#\ of\ vitrified\ planulae} \times 100$$

$$post-settlementrate\ (\%) = \frac{Total\ \#\ of\ post-settled\ planulae}{Total\ \#\ of\ vitrified\ planulae} \times 100$$

Statistical analyses

Data analyses were performed using SPSS (version 17.0; Chicago, IL, USA). A one-sample Kolmogorov–Smirnov test was performed to test for normality, and Levene's test was performed to test the equality of variance. A one-way analysis of variance (ANOVA) was conducted to determine the differences in NOEC within each CPA between the control and treatment groups.

Results

Species verification

The colonies were verified using 18s and internal transcribed spacer (ITS) sequencing to ensure that the planulae were taken from the same species regardless of differences in planula size or morphology (Figure 2A). The results of the 18s sequencing revealed 100% similarity, whereas the ITS sequences showed three base-pair differences between the colonies (see Figure S1 in the Supporting Information). Upon release, the planulae had a rod-shaped appearance (Figure 2B) and transitioned into a flat ball shape before settlement (Figure 2C). The planulae were released with Symbiodiniacea through direct inheritance from the parent (i.e., vertical transmission). According to the genetic analysis, the *S. pistillata* in this study was dominated by *Cladocopium*.

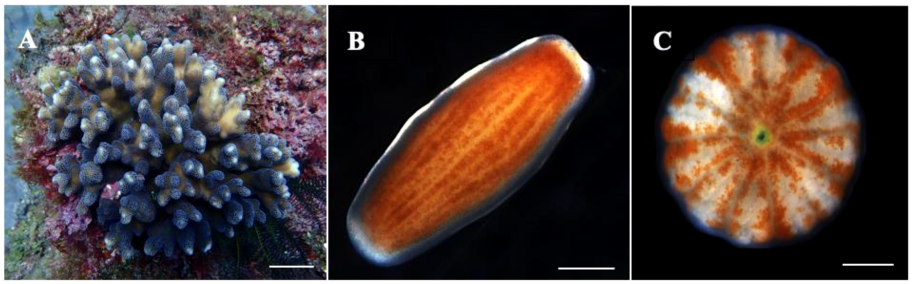

FIGURE 2
Sample photo of an *S. pistillata* colony **(A)**. A rod-shaped swimming planula **(B)** was released from the colony and morphed into a circular flat planula shortly before settling **(C)**. Scale bar: **(A)** 2.5 cm, **(B)** 250 µm, and **(C)** 250 µm.

Effects of cryoprotectants

The effects of CPAs on *S. pistillata* were assessed before the experiment to identify the most suitable CPA for vitrification and laser warming. The results in Figure 3 and Table 1 show the NOECs of the CPAs at exposure times of 5, 10, and 15 min. As previously mentioned, the CPAs used in this study were DMSO, EG, PG, GLY, and MeOH, and the highest concentration was 2 M. The effects of the CPAs increased in the following order: EG< DMSO = MeOH< PG< GLY. Therefore, the most suitable CPA for *S. pistillata* was EG. The planulae could withstand EG at a concentration of 2 M for up to 10 min. According to the results of the NOECs, the planulae could be exposed to DMSO and MeOH at a concentration greater than 2 M for 5 min. GLY was the most toxic among the CPAs, even at concentrations of less than 0.5 M for 5 min. PG was the second most harmful, and the planulae could tolerate a concentration of up to 1 M regardless of exposure time. Therefore, the viability of the *S. pistillata* planula larvae in this study was not affected when treated with 2 M EG for 10 min and 2 M DMSO and MeOH for 5 min. The viabilities of the planulae after exposure to 2 M DMSO, EG, and MeOH for 5 min were 93% for DMSO and EG and 80% for MeOH. However, the viabilities when exposed to 1 M PG for 5 min and 0.5 M GLY for 5 min were 100% and 53%, respectively.

Vitrification and nanolaser warming

The vitrification rates of *S. pistillata* were tested against two VSs after cooling and laser warming. The results in Figure 4 reveal that the vitrification rates after cooling (i.e., after VSs were applied to planulae and planulae were directly submerged into LN_2) were 100% for both VSs. Experiments were halted when samples were devitrified after cooling. Thus, a 100% vitrification rate was required to laser warm the samples. After laser warming, the vitrification rates dropped significantly for both VSs; VS1 was associated with a slightly higher vitrification rate (65%) than VS2 (56.2%).

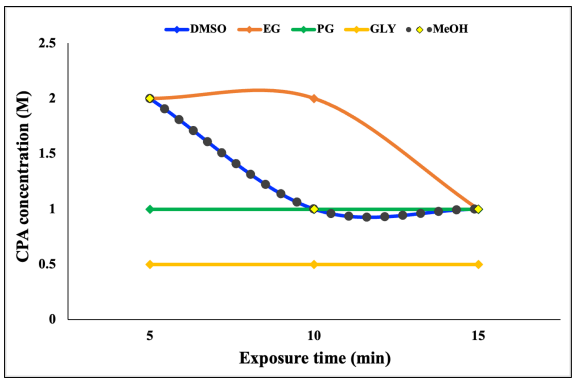

FIGURE 3
No-observed-effect concentrations of five cryoprotective agents in *Stylophora pistillata* planula larvae. Data are presented as the average triplicates; each replicate contained five coral planulae.

Post warming viability

The viability of *S. pistillata* planulae after laser warming is presented in Figure 5. A total of 333 pooled planulae were used in this study. The viabilities were based on the planulae's ability to swim, settle, and survive post settlement when using two VSs. The results revealed that the planula swimming rate after laser warming was generally higher than the settlement rate and post settlement survival rate. Additionally, larval cryopreservation with VS1 led to a significantly higher swimming rate among planulae (14%) than treatment with VS2 (3.2%). After swimming, the planulae were monitored for calcification and settlement, and not all planulae that swam settled. Thus, the settlement rate and post settlement survival rate for VS1 were 2%, whereas those for VS2 were 1.6%.

Seasonal variability

The graphs in Figure 6 demonstrate the seasonal variations between fall and spring when using the two VSs. The results indicate that *S. pistillata* planulae had a higher swimming rate, settlement rate, and post settlement survival rate during the fall than the spring (Figure 6A). When using VS1, the swimming rate during the fall was 23.08%, which was higher than that during the spring. The high swimming rate also led to a significantly high settlement rate and post settlement survival rate (both 11.54%). The swimming rate remained at 11.54% during the spring, but no settlement or post settlement survival was observed when using VS1. When using VS2 during the fall, no swimming, settlement, or post settlement survival of planulae was observed; however, a relatively low rate of swimming, settlement, and post settlement survival (all 3.1%) was observed when using VS2 during the spring (Figure 6B).

Developmental differences between untreated and laser-warmed *S. pistillata* spat

The *S. pistillata* planulae (approximately 1000 μm in diameter) were spherical upon shedding and changed to pyriform, disk-like, and rod-like shapes during the presettlement stage. The planulae exhibited a light-brown color due to the presence of Symbiodiniacea. Upon release, the ball-shaped planulae could elongate to a 1-to-2-mm-long rod-like shape with smooth margins. After laser warming and cryopreservation, the ball-shaped planulae moved, swam, and gradually transitioned into a rod-like shape. During the presettlement stage, the planulae sank to the bottom of the tank and flattened into a disk-like shape. The basal plate started to form and attached to the polystyrene substrate on day 1. The laser-warmed planulae developed similarly to untreated planulae, except for a delay in the emergence of the secondary corallite (Figure 7), which produced buds as early as the first week (Figure 7A). For the laser-warmed planulae, the corallite emerged 3 weeks after settlement (Figure 7B). No physical

TABLE 1 No-observed-effect concentrations of cryoprotective agents used for *Stylophora pistillata* planulae.

	Exposure time (min)	NOEC	ANOVA
DMSO	5	> 2M	$F_{(9,20)} = 10.889$ $p< 0.001$
	10	1M	
	15	1M	
EG	5	> 2M	$F_{(9,20)} = 1.910$ $p = 0.109$
	10	> 2M	
	15	1M	
PG	5	1M	$F_{(9,20)} = 7.9480$ $p< 0.001$
	10	1M	
	15	1M	
GLY	5	< 0.5M	$F_{(9,20)} = 6.667$ $p< 0.001$
	10	< 0.5M	
	15	< 0.5M	
MeOH	5	>2M	$F_{(9,20)} = 6.667$ $p = 0.328$
	10	1M	
	15	1M	

differences or defects were observed in the skeletal development of the laser-warmed coral spats.

Discussion

Cryopreservation in coral reef conservation is both challenging and promising. This proof-of-concept experiment verified that coral planulae can be cryopreserved, nanolaser warmed, and settled and ultimately survive under husbandry conditions. For aquatic species, sperm is the most common sample for cryopreservation when breeding and managing fish such as salmonids, cyprids, silurids, and fish of the family Acipesiridae (Tsvetkova et al., 1996; Barun, 2015). Most sperm research has focused on optimizing protocols for managing oyster sperm to improve hatchery operations, even in the absence of broodstock (Hassan et al., 2015). However, scaling up aquaculture operations from research to commercial production remains challenging. Studies in larval cryopreservation were not common until the early 1990s. More current studies on aquatic larval cryopreservation have been limited to shrimp (Alfaro et al., 2000; Diwan and Kandasami, 1997; Dong et al., 2004; Subramoniam and Arun, 1999; Subramoniam and Newton, 1993), clams (Anjos et al., 2022; Chao et al., 1997; Choi et al., 2008; Heres et al., 2021), mussels (Heres et al., 2019; Liu et al., 2020; Paredes et al., 2013, Paredes et al., 2012; Rusk et al., 2020; Wang et al., 2011), oysters (Anjos et al., 2022; Chao et al., 1997; Choi et al., 2007; Choi and Nam, 2014; Paniagua-Chávez and Tiersch, 2001; Paniagua-Chávez et al., 2011; Paniagua-Chávez et al., 1998; Paredes et al., 2013; Suneja et al., 2014; Usuki et al., 2002), barnacles (Khin Maung et al., 1998; Piazza et al., 2022), worms (Olive and Wang, 2002), and scleractinian corals (Daly et al., 2018; Cirino et al., 2019; Cirino et al., 2021a). Nonetheless, studies have only performed slow freezing until

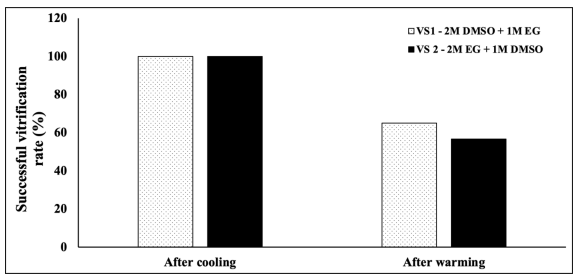

FIGURE 4
Vitrification rates of two vitrification solutions after cooling and laser warming (n = 333).

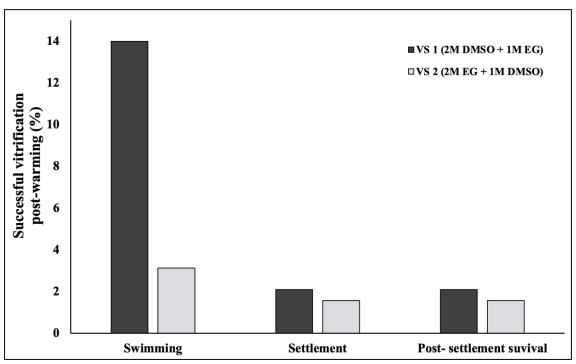

FIGURE 5
Swimming rate, settlement rate, and post settlement survival rate for two vitrification solutions.

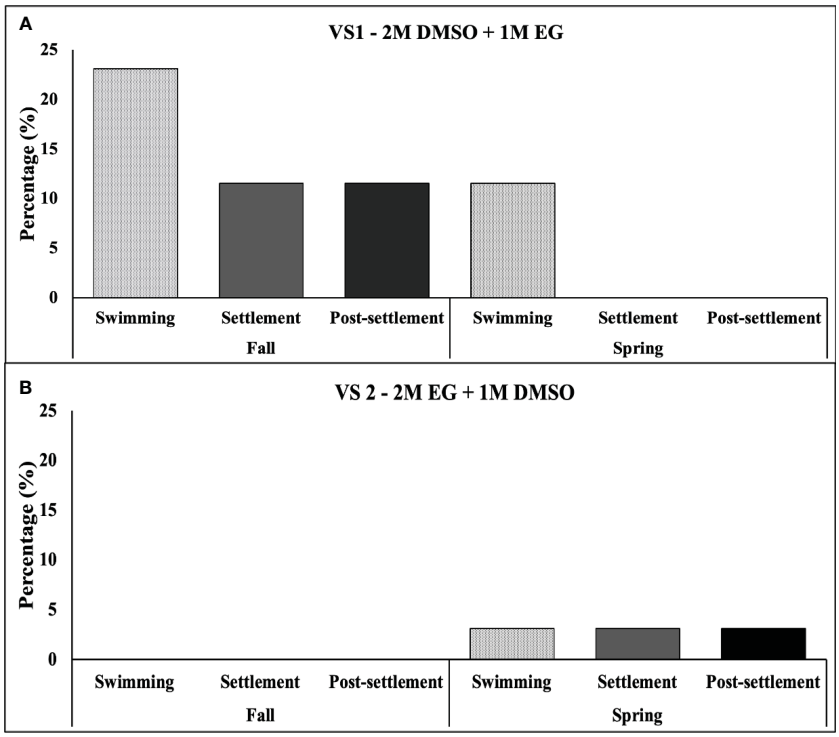

FIGURE 6
Rates (in %) of swimming, settlement, and post settlement survival of *S. pistillata* planulae during spring and fall when using VS1 **(A)** and VS2 **(B)**. (*n* = 333).

more recent studies on scleractinian coral. The advent of such technology has successfully been used to successfully cryopreserve coral larvae.

S. pistillata is a brooder; thus, the planulae develop within the coelenteron of the adult coral (Veron and Stafford-Smith, 2000). Our study demonstrated that the cryopreserved and laser-warmed planulae of *S. pistillata* could grow into adult corals when using a VS composed of DMSO and EG. One study reported that *L. scutaria* could survive laser warming and the use of DMSO, PG, and trehalose as a CPA; however, no settlement was observed (Daly et al., 2018). Another study found that *S. caliendrum* could be vitrified and warmed with EG and PG as VSs, settled, and then survive for 1 week (Cirino et al., 2019). Supplementing linoleic acid and phosphatidylethanolamine with both DMSO and EG as the VSs resulted in a higher settlement rate for *P. verrucosa* (Cirino et al., 2021a). Thus, the choice of VS is species-specific and an essential factor for achieving successful cryopreservation and laser warming.

Our findings revealed that larval seasonality was a key component in effective cryopreservation. We hypothesized that the variation in lipid content and the composition of *S. pistillata* planulae affect the swimming rate, settlement rate, and post settlement survival rate more during the fall than during the spring. According to Kim et al. (2001) and Seidel (2006), lipids provide essential functions during freezing and can alter cell

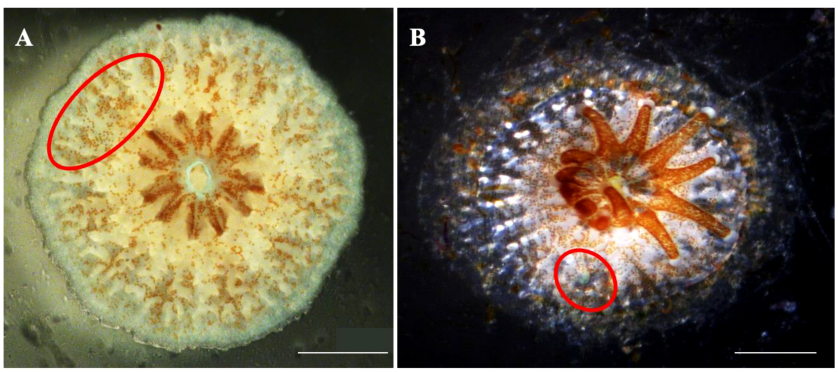

FIGURE 7
Differences between the untreated **(A)** and laser-warmed **(B)** *Stylophora pistillata* planulae. The secondary corallite (red circle) budded 1 week after settlement for the untreated planulae but 3 weeks after settlement for the laser-warmed planulae (B; red circle). Scale bar: 500 µm.

viability. However, the lipid concentration and the composition of coral change with the seasons (Oku et al., 2003). The planula larvae are released from the parent colony and are comprised of 70% total lipid (TL) content, aiding in positive buoyancy and dispersal (Richmond, 1987; Harii et al., 2007). Some studies have investigated the TL content of scleractinian (Harii et al., 2007; Cirino et al., 2021b; Harii et al., 2002; Rivest et al., 2017) and nonscleractinian (Harii et al., 2002) corals. Harii et al. (2007) examined the TL content of newly released larvae from two brooder scleractinian corals, *A. brueggemanni* and *P. damicornis*, which demonstrated a TL content of 58% and 68%, respectively. Another scleractinian coral, namely the spawner coral, *A. tenuis*, and its 5-day-old planulae were examined, and they exhibited a TL content of 61%. Additionally, the nonscleractinian coral *Heliopora coerulea* had a TL content of 41%. The TL content from these corals contained wax esters (WEs), triacylglycerol (TG) (except for *A. tenuis*), and phospholipids (PLs). Another study on *P. damicornis* from Taiwan (Rivest et al., 2017) revealed that the planulae contained an average TL content of 20.47 µg. The TL content included WEs (39%), TG (18%), and PLs (6%). Cirino et al. (2021b) also examined the TL content of *S. caliendrum* and *P. verrucosa* to determine how seasonality affects the lipid content of planulae. Analogous to the reported lipid content of coral planulae, intracellular WEs and TG were the predominant lipids of both *S. caliendrum* and *P. verrucosa*, primarily during fall. Higher TL concentrations can also lead to higher cellular membrane fluidity at low temperatures (Lin et al., 2013). Although the TL content and composition were not examined in the present study, the lipid composition of corals in the same genera or family may be similar and have similar metabolic patterns (Harii et al., 2007). *S caliendrum* and *P. verrucosa* are from the same family as *S. pistillata* and presumably share similar seasonal lipid patterns. Additionally, the planulae have specific lipid phase transition (LPT) temperatures—specific low temperatures—at which the physical state of lipids changes from a gel to a disordered liquid crystalline (Lin et al., 2014). Thus, the significant seasonal viability after vitrification and laser warming may be caused by the lipid content and composition, changes in the lipid profile, and the LPT of the planulae, all of which lead to changes in the lipid profile with the season and temperature (Oku et al., 2003). Such lipid storage patterns in coral from the same family may be worth exploring to improve vitrification for *S. pistillata*. Under the same circumstances, modifying the VS can increase planula vitality after laser warming.

Studies on coral skeletogenesis from settlement to post settlement have employed scanning electron microscopy (Vandermeulen and Watabe, 1973; Jell, 1979; Wallace, 1999; Stolarski, 2003; Sun et al., 2020) and microcomputed tomography systems (Janiszewska et al., 2013; Perrin et al., 2015) and involved killing (i.e., fixing) the sample to describe the complex processes. This study observed the skeletal ontogeny and biomineralization from coral settlement to adulthood after successfully cooling and laser-warming *S. pistillata* planulae. The skeletogenesis of the live broadcast spawning corals *Galaxea fascicularis* (12 months) and *Mycedium elephantotus* (14 months), the brooder coral *S. caliendrum* (2.5 months), and *P. verrucosa* (approximately 1 month) were described by Lin C. et al. (2022). At the macro level, the skeleton of the laser-warmed *S. pistillata* is composed of corallites (i.e., the building unit of the skeleton) with basic skeletal elements such as the septa, costae, septocostae, coenosteum, and the wall. The skeletal development was verified using the studies of Baird (2001) and Baird and Babcock (2000). No physical deformation was observed when compared with the control. *S. pistillata* possesses a tissue-over-skeleton growth mechanism, a typical characteristic for brooding Pocilloporid corals such as *P. verrucosa* and *S. caliendrum* (Lin C. et al., 2022). The development of *S. pistillata* was initially described by Atoda (1947) in Japan, Rinkevich and Loya (1979) in the Red Sea, and Baird and Babcock (2000) in Australia. The differences in the onset of development can be attributed to the location and environmental conditions of the species. However, the skeletal development of the laser-warmed planulae was similar to that of typical coral. Nonetheless, a delay in the emergence of the secondary corallite and a possible stagnation in growth were observed. Rinkevich and Loya (1979) found that a 2-year-old *S. pistillata* had an average size of 4–5 cm. However, due to the weedy nature of *S. pistillata* (Loya, 1976), the 8-month-old laser-warmed coral was smaller in diameter and possessed 16 fully developed corallites. Further experiments can be conducted to better understand the effects of cooling and warming on *S. pistillata* planula larvae. Nonetheless, no physical injuries were incurred from cooling or warming. Coral planulae have been studied to understand the skeletogenesis in scleractinians (le Tissier, 1988) and the effects of factors such as pollution (Rinkevich and Loya, 1979). Studies have also tested new biotechnology for coral reef research and restoration (Randall et al., 2019).

Successful cooling and warming are crucial for the post settlement survival of planulae. More research is necessary on open flow-through systems to ensure the survival of coral spats. The water conditions in the rearing tank post settlement were maintained at 26 °C, and the settlement plates were cleaned 3 times per week or as needed to prevent algal overgrowth. The algae compete for space and can even outgrow coral recruitment during husbandry setup, leading to coral mortality (Diaz-Pulido et al., 2009). Slow growth and the demise of juvenile coral in the present study were not directly related to possible damage during cryopreservation but to the postrearing state. The noncryopreserved larvae developed similarly to the cryopreserved planula larvae. Thus, the husbandry conditions should be closely monitored during the early stage of development to successfully produce viable coral recruits from the cryopreserved and laser-warmed planulae. Cryopreservation can be successful when the planulae swim and settle after laser warming. Growing and maintaining coral under husbandry conditions is a significant achievement.

In this study, successful cooling and laser warming were affected by various factors, namely the CPA, droplet volume, supplementation of GNP, and mastery of the technique. DMSO, EG, Ficoll, and GNP were the primary components of the VSs. The use of multiple CPAs necessitates lowering the CPA concentration and making it less toxic than when using a single CPA (Kader et al., 2009); therefore, this was performed in our VSs. Incorporating macromolecules such as Ficoll into the VS also reduces the osmotic shock of the sample (Liebermann et al., 2002). Studies have shown that adding Ficoll to a VS protected the samples from cryoinjury (Dumoulin et al., 1994), lowered the

toxicity of the VS, and increased the effectiveness of the VS in the vitrification of mouse embryos (Kasai et al., 1990). In our study, the media or droplet's volume and shape were carefully controlled when the planulae with VS were loaded onto the cryostick. The smaller the CPA droplet is, the less likely that ice crystals form. To increase the survival of vitrified embryos, this technique follows the minimum volume cooling concept, which involves reducing the volume of the VS with the sample (Hamawaki et al., 1999). The addition of GNP into the VS evens the distribution of heat when the laser hits the droplet to lessen or eliminate membrane damage to the planula (Daly et al., 2018; Cirino et al., 2019). The high VS concentrations of glass-promoting solutes during cooling allows for planulae to be suspended. A high cooling rate is crucial for achieving a high degree of vitrification and survival (Liebermann et al., 2002); therefore, direct-contact vitrification was applied in our study. The survival of vitrified samples depends on the warming conditions (Paredes, 2021). Ultrafast laser warming is gaining popularity as an alternative to slow, convective warming. Ultrafast warming can block ice recrystallization and retain cells (Seki and Mazur, 2009; Seki and Mazur, 2012), and the results of ultrafast warming are 100 times higher than conventional warming (Kleinhans and Mazur, 2015). Laser warming was used to prevent ice crystal formation and rewarm the *S. pistillata* planulae rapidly and uniformly. Finally, successful vitrification is dependent on the technician, who must decide on the CPAs, sample loading, and machine settings. Thus, technicians must possess a particular skillset that requires training and mastery.

Conclusion

This study was the first proof-of-concept experiment on cryopreserved and laser-warmed planulae, which successfully settled and grew into adult corals. Coral planulae can be cryopreserved, stored, warmed, and grown ex situ by optimizing the nanolaser warming technology and the CPAs used in vitrification. Moreover, raising coral spats under controlled and well-monitored husbandry conditions can enhance the survival of laser-warmed planula larvae. This study was also the first attempt to describe coral skeletogenesis in successfully settled planulae through coral spat development after cooling and laser warming. Additionally, the sample was not fixed. This technological breakthrough can revolutionize the study, banking, and conservation of coral species. The technology can also address the need to preserve the genetic materials of threatened and endangered coral species.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

Conceptualization, CL and ST. methodology, WCH, LHW, CLH and AN. Validation, CL and ST. Formal analysis, WCH, CLH and AN. Resources, CL, ZHW and ST. Writing—original draft preparation, CL and ST. Writing—review and editing, CL and ST. Visualization, CL and ST. Supervision, CL, ZHW and ST. Funding acquisition, CL and ZHW. All authors contributed to the article and approved the submitted version.

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Conceptualization, CL and ST. methodology, WCH, LHW, CLH, and AN. Validation, CL and ST. Formal analyses, WCH, CLH, and AN. Resources, CL, ZHW and ST. Writing—original draft preparation, AN. Writing—review and editing, CL, ST and AN. Visualization, CL and ST. Supervision, CL, ZHW and ST. Funding acquisition, CL and ZHW.

Conflict of interest

Author WCH is the owner of He Wei Precision Company Limited.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1172102/full#supplementary-material

SUPPLEMENTARY FIGURE 1
18s **(A)** and internal transcribed spacer (ITS) **(B)** sequencing results for *Stylophora pistillata* showing no base-pair differences for 18s and three base-pair differences (highlighted in yellow) by using ITS.

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