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The environmental adaptability and reproductive properties of invasive green alga *Codium fragile* from the Nan'ao Island, South China Sea

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Abstract

It has been widely recognized that biological invasion has become one of the greatest threats to the ecosystem. *Codium fragile* is an invasive species which exhibits a variety of attributes like parthenogenesis, winter fragment, and vegetative reproduction; and therefore, it has become a successful invader, colonizing most subtropical regions. In China's southeast coastal aquaculture waters, the green algal bloom caused by *C. fragile* will probably become a serious problem. In order to understand more details about the species, an experiment focused on its reproductive characteristics was conducted using culture established from a sample collected in the aquaculture raft of the Nan'ao Island in the South China Sea. The results showed that there were two types of gametes resembling aplanospores and zoospores respectively, both of which were able to germinate. During the gametes liberation, a long mucilage tube was formed out of the mouth of the gametangium assisting dispersal of gametes away from the parent plant. This tube was adapted not only to its surrounding flowing water environment but also to its parent plant's outer gelatinous structure. In general, the optimum temperature for gametes release and germination was 15–20°C and 15°C, respectively, which corresponded to the local offshore marine water. The plant was observed to produce vegetative buds under favourable reproductive conditions which were called propagules. They were capable of developing into filamentous thalli. The results will provide some scientific evidences for revealing the biological mechanism of bloom and control strategies of invasive green algae.

Key words: *Codium fragile*, gametes, propagules, mucilage tube, temperature, environmental adaptation

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1 Introduction

As one of the most invasive species, now the geographical expansion of *Codium fragile,* which originated in Japan, has colonized in the northeast and northwest Atlantic coast, southern England, the Mediterranean Sea, Australia, New Zealand, and Chile (Bird et al., 1993; Carlton and Scanlon, 1985; Chapman, 1998; Churchill and Moeller, 1972; Dromgoole, 1975; Neill et al., 2006; Silva, 1955; Trowbridge, 1995). Invasive species have become widely recognized as one of the greatest threats to the ecosystem (Vitousek et al., 1997), and coastal marine eco-systems are among the most impacted aquatic systems (Grosholz, 2002; Ruiz and Hewitt, 2002).

What are the probable reasons for the invasive success of *C. fragile*? Many researchers have investigated this phenomenon. Several attributes have been proposed for potentially successful invaders of this genus: high growth rates over the summer and early fall (Malinowski and Ramus, 1973), broad physiological tolerance (Benson et al., 1983; Hanisak, 1979a; Hanisak and Harlin, 1978; Yang et al., 1997), potyphagia (Hanisak, 1979b), and minimal grazing (Trowbridge, 1995). Though seaweeds grow generally by attaching to hard substrates such as rocky shores or fouling communities, they also survive by inhabiting soft-bottom environments (Williams, 2007). All of the above attributes are beneficial for the survival of *C. fragile*.

From the perspective of vegetative propagation, the filamentous thalli of *C. fragile* could be formed from isolated utricles, medullary filaments, propagules, or parthenogenetic female gametes, as spongy thallus of *C. fragile* formed from filamentous thallus had been observed in the field (Arasaki et al., 1955; Yotsui and Migita, 1989). In the northwest Atlantic, *C. fragile* reproduces parthenogenetically, the swarmers settle, and could develop into a dichotomously branching adult thallus (Churchill and Moeller, 1972; Malinowski and Ramus, 1973). In addition, Fralick and Mathieson (1972) reported that maximum fragmentation occurred predominately in winter in New England, Scheibling and Melady (2008) provided evidence that the producing segments were capable of dispersal and reattachment to the substratum. Mature thalli also produced lateral, vegetative "buds" (~1 cm to 10 cm

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in length), defined as unbranched, elongated structures, constricted at their base, that readily detach from the parent thallus, and were capable of attaching to the substratum (Scheibling and Melady, 2008). Dislodgment of entire plants also occurred, particularly towards the end of the growing season when wave activity increased (D'Amours and Scheibling, 2007), with the basal holdfast persisting to regenerate in the spring. The detached buds, drifting thalli, and fragments might also greatly enhance the dispersal potential of *C. fragile*.

Thus, we can see that both diverse modes of reproduction of *C. fragile* and its tolerance to environmental stress allow *C. fragile* to adapt to newly colonized territory. However, the environmental adaptation of micro-stage of *C. fragile* has seldom been investigated in combination with the description and ultrastructure of male or female gametes from several *Codium* species (Miravalles et al., 2011, 2012; Prince and Trowbridge, 2004). This work illustrates the environmental adaptation of *C. fragile* from the perspective of its reproductive process, types of female gametes with their germination and development, the regularity of gametes liberation response to temperature, and the appearance of propagules, and provides more evidence of reproductive properties that account for the invasive success of *C. fragile*.

2 Materials and methods

2.1 *Materials preparation*

The mature spongy thalli of *C. fragile* were collected on the scallop culture raft from the Shen'ao Bay, Nan'ao Island, China in March 2012. The collected specimens were transported in a sample box to the laboratory. Fine paintbrushes were used to clean the surface of the plant, and the plants were rinsed several times with sterile seawater to eliminate protozoa or diatoms. Then, the apical part of materials were removed by needle and scalpel to expose the utricles bearing gametangium and checked under the dissecting microscope (Olympus LG-PS 2, Japan) to make sure that most gametangium were mature. Mature gametangia were characterized by division of their contents into individual cells (Borden and Stein, 1969). The apical tips of the plants (5 cm long) were cut off using a scalpel, weighed one by one, and each tip randomly placed in a 90 mm-diameter petri dish filled with 50 mL sterile natural seawater (salinity, 30).

2.2 *Laboratory culture*

With four culture temperature designs, which were 15°C, 20°C, 25°C, and 30°C respectively, all the cultures were maintained in climate-controlled culture chambers (GXZ-380 B, China) and were illuminated by daylight-type, white fluorescent tubes with 30 μmol/(m^2·s) (according to photons), photo period 12:12 (L:D). The medium was replaced every day.

2.3 *Pure culture and time-course experiment*

Culture plates were prepared by selecting some prepared mature thalli and dissecting them into fragments, picking up some utricles bearing mature gametangium by needles and scalpel, then putting them one by one into every hole of the six-well culture plate which was filled with sterilized seawater. The culture plates were placed in the culture chambers（GXZ-380 B, China）under the conditions of 20°C, 30 μmol/(m^2·s) (according to photons), photo period 12:12 (L:D). In order to capture the moment of gametes dispersal, the cultured utricles were checked every hour from 7:00 am to 4:00 pm under an inverted light microscope (Leica DMI 3000B, Germany), and continuous time-course experiments were conducted to determine the process of gametes germination and development.

2.4 *Cell counting*

The practical aim was to tally all of the gametes in a known volume that were released by thalli in the culture. A counting slide was applied in our experiment. The volume of the center hole was about 0.5 mL with a diameter of 18 mm and a depth of 2 mm. The liquid from the gametes filled the hole under the cover slide which was then placed on the platform of the inverted microscope (Leica DMI 3000B, Germany) under a 100× magnitude field. Thus, the computational formula for counting cells (cells/mL) was as follows:

$$N = 2\pi nDr_2^4/r_1^2,$$

where, n is the number of cells counted under 100× magnified field; D, the depth of the hole of counting slide (D=2 mm); r_2, the radius of the hole of counting slide (r_2=9 mm); r_1, the radius of field under 100× (r_1=0.9 mm) and r_1 equals to field number/objective multiple.

2.5 *Statistical analysis*

Univariate one-way analysis of variance (ANOVA) was done followed by Turkey's multiple comparison test, or t test, to compare the significance among data sets. All statistical analyses were carried out using GraphPad prism 5.0 with the level of significance set at p<0.05 or p<0.01. All data were expressed as mean±SD.

3 Results

3.1 *Dispersal processes of swarmers and aplanospores*

We observed that *C. fragile* produced two types of gametes, including swarmers (motile spores) and aplanospores (non-motile spores), both of which were released from the enclosed sporangial mass (Figs 1c, d). The swarmers (Fig. 2a) bore two flagella and revolved freely, whilst the aplanospores (Fig. 2b) bore no flagellum and were immersed in mucilage "strips". The mean long diameter of a swarmer was 22.27 μm with a short diameter of 18.08 μm, but the mean diameter of aplanospore was 21.17 μm (Fig. 3). We also captured the first moment of the discharge process of gametes (Fig. 1b). The first two gametes were suddenly

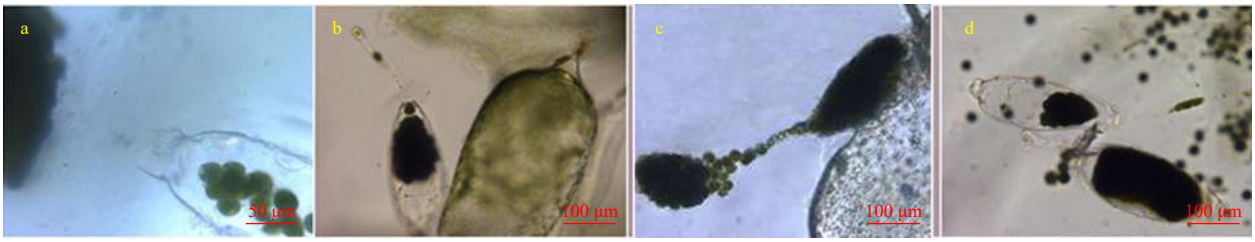

Fig. 1. Dispersal process of swarmers and aplanospores. a. The tip of gametangia ruptures into two flaps; b. the first moment of gamete dispersal; c. dispersal process of aplanospores; d. dispersal process of swarmers.

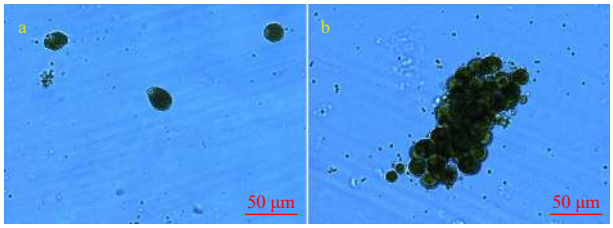

Fig. 2. Swarmers (a) and aplanospores (b).

ejected from the gametagium, but the remaining gametes got stuck in the gametagium. Fortunately, we observed another gamete dispersal. The gamete shape was transformed in order to go through ostiole on the top of the gametangia (Fig. 1d), and the diameter of the ostiole was 3–4 μm. Subsequently the gametes slipped away singly, emerging as spindular bodies through a mucilage canal or just regarded as a mucilage tube to the surrounding water, looking like a stream (Fig. 1c).

3.2 *Some unusual behaviors of swarmers*

We collected some swarmers that had just been released by capillary pipet and placed them into the counting slide to observe their movement, we unexpectedly observed some gametes whose diameter exceeded 40 μm after about 7 h, and we believe that the big ones were formed by the fusion of two or more normal swarmers. More surprisingly, the big ones generated one or more transparent vesicles and the protoplasts transferred into it. The vesicles became bigger and this process continued until the vesicles became detached from the mass forming a new body of protoplast (Fig. 4).

3.3 *The process of germination and early development of swarmers and aplanospores*

We picked up the mature female gametangia to separate them individually to the cell of the culture vessel in order to perform a time-course experiment. One single gametangium had a shedding of 128 swarmers (Fig. 5a), some of which attached to the plastic wall and began to germinate after 16 h from release (Fig. 5b), more sporelings appeared after 28 h (Fig. 5c), and the sporelings grew longer 48 h later (Fig. 5d). Finally, the sporelings

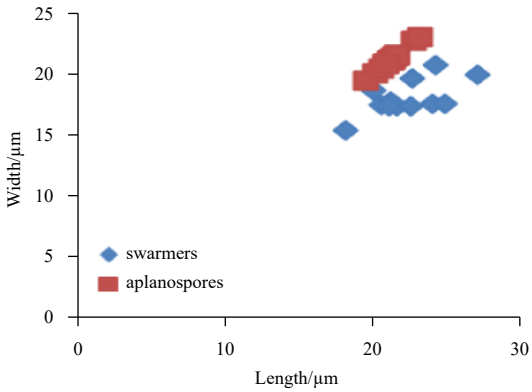

Fig. 3. Size comparison between swarmers and aplanospores.

grew into filamentous thalli 20 d later (Fig. 5g). In the case of aplanospores, they gathered into a mass (Fig. 5e), germinated (Fig. 5f) and grew into a free-floating cluster of filamentous thalli as well after 20 d (Fig. 5g).

3.4 *Gametes release and germination response to temperature*

Both the release and germination of swarmers were sensitive (p<0.001) to temperature. The maximum number of swarmers released per gram wet weight of thallus observed at 15°C and 20°C, was higher than those observed at 25°C or 30°C (Fig. 6a). The number of swarmers was 78.9×10^3 g^{-1} wet weight of thallus at 15°C with its germination of 54.82%, and but it was 87.8×10^3 g^{-1} wet weight of thallus at 20°C with its germination of 18.88% (Figs 6a and 7a). In the case of the release and germination of aplanospores, temperature also had a highly significant (p<0.001) effect (p<0.001). It was 40.3×10^3 g^{-1} wet weight of thallus at 15°C with its germination of 87.37% (Fig. 6b) and 44.6×10^3 g^{-1} wet weight of thallus at 20°C with its germination of 50.9% (Fig. 7b). In general, the optimum temperature for gametes release was at 15°C and 20°C; however, the optimum temperature for germination was 15°C.

3.5 *The generation of propagules*

Many more gametangia-like bodies were generated by the side of the utricles of mature thalli when cultured at 25°C which

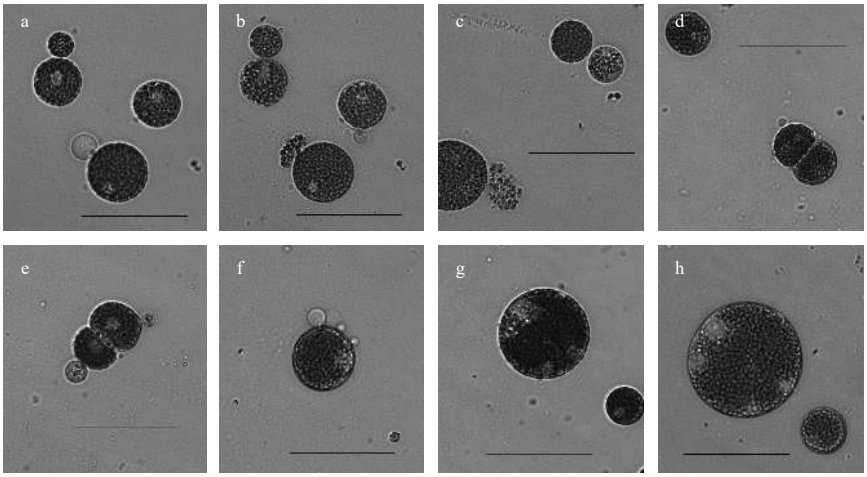

Fig. 4. Some unusual behaviors of swarmers. a. Transparent vesicle formed upon the surface of swarmer; b. protoplast transferred into transparent vesicle; c. transparent vesicle which contained protoplast detached the swarmer, forming a new protoplast body; d. possible confusion between swarmers; e. transparent vesicles occurred once again upon the confused swarmers; f. three transparent vesicles occurred on the surface of a protoplast body, whose diameter was about 35 μm; g. a protoplast body, whose diameter was about 44 μm; h. a protoplast body, whose diameter was about 56 μm (scale bar: 50 μm).

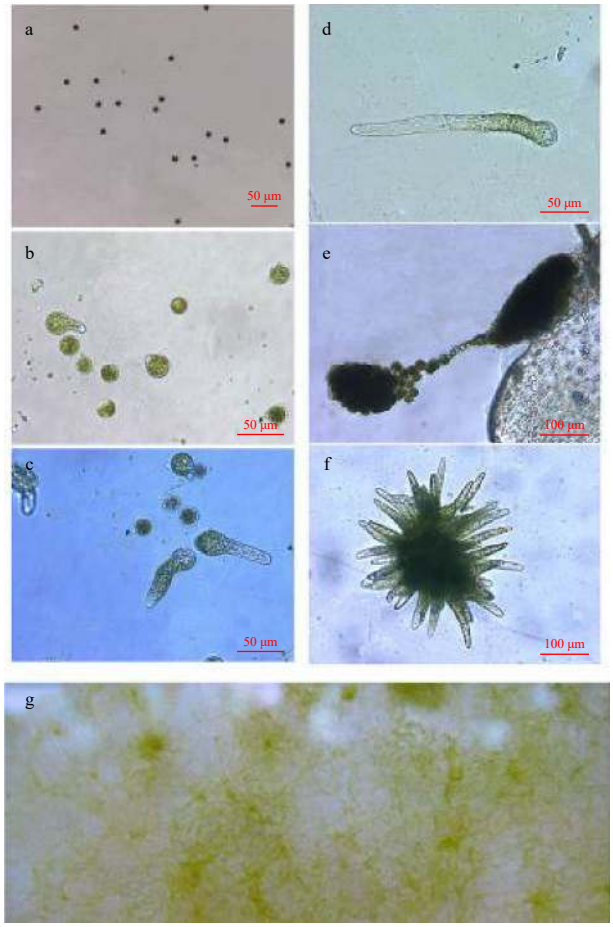

Fig. 5. Germination and early development of swarmers and aplanospores. a. The initially released swarmers; b. some swarmers began to germinate after 22 h; c. germination tube continued to elongate after 48 h; d. germination tube continued to elongate after 72 h; e. the initially released aplanospores; f. the body of aplanospores germinated; g. both swarmers and aplanospores developed into filamentous thalli after 7 d which were visible by eye.

were called propagules (Figs 8a, b). We picked the propagules up for mariculture, and observed that they generated one, two or three germination bodies which grew into branched filamentous thalli 40 d later.

4 Discussion

Hurd (1916) believed that the female gametes escape when the gametangium tip splits into two "flaps". Borden and Stein (1969) stated that the tip may be pushed aside as a whole rather than split during the appearance of discharging gametangia, at least in some gametangia. We observed both ways (Figs 1a, b). The process of discharge of gametes had been previously reported for several *Codium* species (Arasaki et al., 1955; Borden and Stein, 1969; Gibson and Auld, 1900; Oltmanns, 1922), but it was unclear whether the gametes were in or accompanied by a slimy substance or "slime canal". Oltmanns (1922) stated that the slime sheath in *C. elongatum* came from slime accumulated in the tip of the gametangium just prior to discharge. We were still uncertain about whether this was also true for *C. fragile* and about the composition of the slime sheath. Actually, the slimy canal does exist in the species of *Bolbocoleon piliferum*, as O'kelly et al. (2004) described a membranous tubular structure that was attached to the exit aperture of the zoosporangial/gametangial apex. The gametangia were embedded within the surrounding utricles as they were nearly attached to the middle part of the utricles. Thus the appearance of the mucilage tube appeared to be of benefit in assisting dispersal of gametes away from the parent plant. We consider this good evidence of environmental adaptation.

The gametes of *C. fragile* were anisomorphic, and the conjugation took place between a macro (female) and a micro (male) gamete (Arasaki et al., 1955). Borden and Stein (1969) also found that zygotes and germlings appeared only in cultures with both male and female branches. Churchill and Moeller (1972) found a large seasonal variation in the diameter of gametes released by *C. fragile* but little variation in the diameter of gametes released from the same gametangium. Prince (1988) recorded that two different-sized gametes were discharged from the same gametangium, both types of which were biflagellate, and fusion occurred between the large and small cells. A general description for male and female gametes of five species of the siphonaceous green macroalga *Codium* from Atlantic and Pacific shores was provided, which said both types of gametes were biflagellate (Prince and Trowbridge, 2004). But, we discovered another type of female gametes of *C. fragile* that were collected from the Nan'ao Island of China which we called aplanospores, and these aplanospores were immersed in mucilage "strips". The physical properties of the mucilage strips enabled them to withstand the mechanical stress imposed by water movement without breakage. Large strips which remained in one piece tended to sink, and this would again be advantageous in ensuring spore dispersal in the thin boundary layer of slowly moving water against a substratum (Boney, 1978).

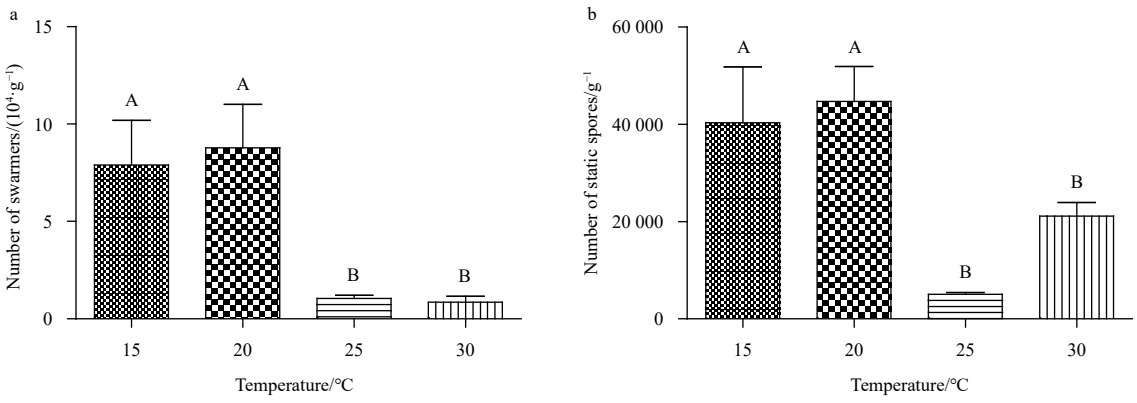

Fig. 6. The effects of temperature on the release of swarmers (a) and aplanospores (b). The letters (A, B) represent significant differences among temperatures (p<0.05, ANOVA, followed by Tukey's multiple comparison test).

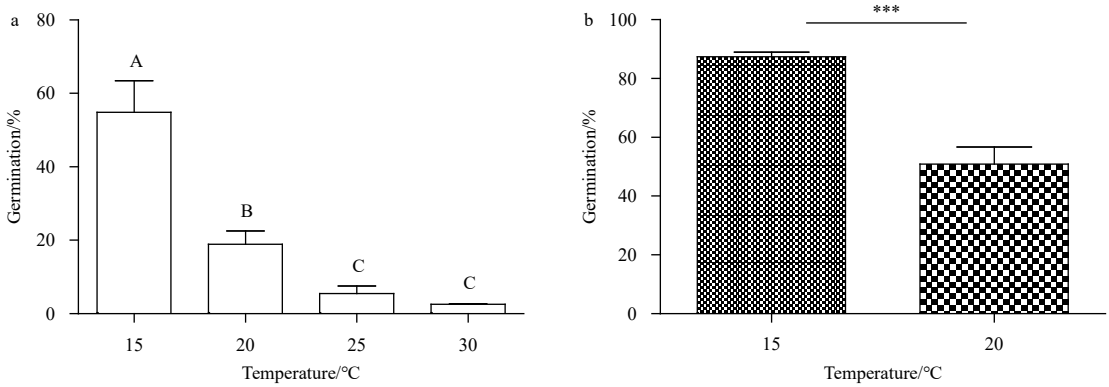

Fig. 7. The effects of temperature on the germination of swarmers (a) and aplanospores (b). In (a), letters (A, B and C) represent significant differences among temperatures (p<0.05, ANOVA, followed by Tukey's multiple comparison test); in (b), *** represents significant differences between 15°C and 20°C (p<0.01, unpaired t test).

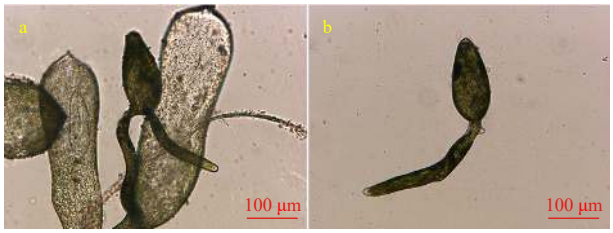

Fig. 8. Propagule (a) and propagule that detached from utricle (b).

Swimming zoospores were both strongly thigmotactic and phototactic, resulting in uneven distribution of adhered spores (Evans and Christie, 1970), gregarious settlement behavior was frequently observed leading to the formation of rafts of cells in the species *Enteromorpha* popagule (Callow et al., 1997). However, in our study, the swarmers of *C. fragile* could not only get conjunction, but also could give birth to novel protoplast mass, which has not been reported. Why could the conjunction happen between the same female gametes? Several brown alga had been discovered in which one gamete released a volatile attractant for the other (Müller et al., 1982), and the process of recognition and fusion had been studied in some Fucales (Evans et al., 1982), the eggs initially lack a wall and the membrane appeared lumpy due to protrusion of cytoplasmic vesicles, the spermatozoid probed the surface of the egg with the tip of its anterior flagellum. Attachment took place first by the flagellum tip. Later the body of the cell, after fertilization of the zygote, rapidly secreted a smooth membrane. We speculated that the swarmers of *C. fragile* were also able to secrete a kind of attractant that led to the fusion of themselves. Another unusual phenomenon was described by Miravalles et al. (2012) which provided the first description of gametogenesis and characteristics of female gametes of *C. fragile* subsp. *novae-zelandiae* illustrating that in the final stages of the formation of *C. fragile* female gametes, transparent vesicles were protruded into the plasmalemma and the periplasmatic. They assumed that these vesicles were involved in the settlement process, since they strongly resembled dictyosomic vesicles in spores of different alga general such as *Chondrus*, *Gigartina*, *Ceramium*, *Palmaria*, *Laminaria*, *Nereocystis*, *Enteromorpha*, *Ulva* and *Bulbochaete* (Clayton, 1992), which contain polysaccharides and proteins involved in the adhesion to substrates. Hence, we believe that the transparent bubbles were just from the abundant vesicles inside the membrane of the gametes. The protoplasts were able to transfer to the bubble, and finally formed a new spherical cell which might be a particular function of *C. fragile*.

Codium fragile was affinitive with warm water (Neill et al., 2006), and how temperature affects the formation, growth and seasonal growth patterns of *Codium* species in various areas has been investigated (Hanisak, 1979a; Park and Sohn, 1992; Malinowski and Ramus, 1973). The reproductive properties of *C. fragile* were concentrated on the description of gametes, germination and early development. We performed an experiment on gametes release and germination response to temperature in order to explore the influences of temperature on the reproductive properties of *C. fragile*. The annual average water temperature of the Shen'ao Bay was 22.2°C. During the fastigium period for reproduction of *C. fragile*, it was about 17–21°C from March to April. Our results suggest that the gametes release and germination were suitable to the seasonal surrounding water. While from May to July, the temperature had gone up to 24–27°C, during this time, we investigated the juveniles of *C. fragile* on the ropes of aquaculture raft. In our laboratory culture, we found that 25°C was not suitable for the gametes release and germination, subsequently we dissected the thalli in culture that bore mature gametangia initially, and discovered the appearance of gametangium-like buds attached to the utricles, the similar sites of gametangia. We called them propagules, which has been described in *C. edule* by Chang et al. (2003). We isolated some propagules, cultured them for two months, and the buds grew into filamentous thalli but no utricles appeared. While the propagules of *C. edule* could grow utricles after several months of culture (Chang et al., 2003), *C. fragile* did not. Anyhow, the filamentous thalli were also one of the important methods for vegetative proliferation.

Based on stationary culture, this work describes the details of the dispersal process of gametes as they are released through a mucilage tube, which is better for their successive dispersal and describes the discovery of another type of female gametes which probably are favorable for settlement. Also discussed are two unusual behaviors of swarmers, the release and germination of two types of female gametes in response to temperature which was adjusted to the local environment, as well as the appearance of propagules and their function. All the aspects above are related to the reproductive properties of *C. fragile* and reflect the environmental adaptation of *C. fragile*.

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