

SEXUAL REPRODUCTION AND TWO DIFFERENT ENCYSTMENT STRATEGIES OF *LINGULODINIUM POLYEDRUM* (DINOPHYCEAE) IN CULTURE¹

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Unreported aspects in the sexual cycle of the marine dinoflagellate *Lingulodinium polyedrum* (Stein) Dodge were described. Our observations included the description of two types of hypnozygote formation, because culture planozygotes were observed to encyst in two different ways: an ecdysal sexual stage or a spiny resting cyst. Phosphate deficiency was the main nutritional condition required for fusing gamete pairs to form resting cysts, whereas replete conditions prevented their appearance and favored the formation of ecdysal sexual forms. Mating experiments revealed the existence of two sexual types (+/–), which were enough to explain resting cyst appearance (simple heterothallism). Morphological aspects and timing of gamete mating, fusion, and the efficiency of encystment under different external levels of nitrate and phosphate were analyzed after isolating and monitoring individual pairs of fusing gametes. The staining of sexual stages showed that nuclear fusion was completed at the same time as the cytoplasmic fusion. After 1 to 2 h, the planozygotes presented one quadrolobulated nucleus. Germination of ecdysal sexual stages occurred after <24–72 h, whereas excystment of resting cysts was dependent on the studied parental cross and took place after 2–4 months. Newly germinated cells from both types of cysts had a similar, big, U-shaped nucleus. Twenty-four to 48 h after excystment, the germlings divided by desmoschisis, a process before which enlargement of the nucleus was observed.

Key index words: Dinophyceae; encystment; excystment; gametes; life cycle; *Lingulodinium polyedrum*; mating type; reproduction

Lingulodinium polyedrum (Stein) Dodge is a bloom-forming and widely distributed dinoflagellate recently related to the production of homoyessotoxin (Draisci et al. 1999). Three main processes have been shown to constitute its life cycle: vegetative reproduction, formation of ecdysal stages, and sexual reproduction (Lewis and Hallet 1997). Nevertheless, important gaps remain in the knowledge of its sexual cycle, despite a broad range of laboratory and field studies focusing on

this species. Previous works have reported aspects of asexual reproduction (Kofoid 1911, Dürr and Netzel 1974, Dürr 1979) and of isolated steps of the sexual cycle, such as the development of resting cysts (Kokinov and Anderson 1995) or the morphology of the newly excysted cells (Lewis and Burton 1988). Nevertheless, a detailed description has not been made of sexual reproduction, in which single cells are followed through the process, nor has there been a rigorous study of mating type, two aspects of great importance. The shift between sexual and asexual reproduction is presumed to play an important role in the regulation of bloom dynamics (Anderson et al. 1983, Ishikawa and Taniguchi 1996), but it is still unclear how this process is undertaken and regulated during the *L. polyedrum* life cycle. The production of dormant resting stages during the sexual life cycle of many phytoplankton species is a strategy that confers various ecological advantages such as genetic recombination, dispersal, and seeding or regulation of the seasonal succession of dinoflagellates (Wall 1971, Anderson and Wall 1978, Anderson et al. 1984). Apart from sexual recombination, the significance of these important functions is dependent on the germination characteristics of each species, mainly on the duration of the dormancy period and on the germination pattern. Cyst germination is considered to be regulated by both internal and external factors, although the species-specific requirements are poorly understood. Cysts need a minimum time for internal maturation, the mandatory dormancy period, which is therefore not influenced by external conditions. When cysts are out of dormancy and have entered into quiescence, they are in a phase in which environmental factors determine germination. Some investigations have analyzed the requirements for the germination of *L. polyedrum* cysts that were out of dormancy. Excystment is inhibited by anaerobiosis, darkness (Anderson et al. 1987, Blanco 1990), salinity, and low temperatures (Blanco 1990). Nevertheless, the mandatory dormancy period of these cysts has never been determined.

The purpose of this study was to investigate three unknown aspects in the sexual reproduction of *L. polyedrum* under culture conditions: 1) behavior, timing, and development of sexual stages followed individually; 2) the determination of mating type by means of several clonal crosses; and 3) the establishment of dormancy period duration, monitoring for this purpose, cyst formation, and germination in cultures.

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MATERIALS AND METHODS

Culture maintenance. Experiments were conducted with eight clonal strains of *L. polyedrum* from the culture collection of the Centro Oceanográfico in Vigo (Table 1). Cultures were grown at 24°C, approximately 90 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and a photoperiod of 10:14-h light:dark cycle. Culture stocks were maintained in Erlenmeyer vessels filled with 50 mL of L1 medium (Guillard and Hargraves 1993) without silica addition, prepared with Atlantic seawater adjusted to a salinity of 31 psu by the addition of sterile double-distilled water.

Sexual stages development and nutrient effects. The crosses 7V \times 4V and 9V \times 3A were conducted in 10 mL of medium with no phosphate added (L-P) in duplicate sterile polystyrene Petri dishes (35-mm diam, Iwaki, Tokyo, Japan) inoculated with exponentially growing cells (4000–6000 cells $\cdot\text{mL}^{-1}$) to a final concentration of 700 cells $\cdot\text{mL}^{-1}$ (350 cells $\cdot\text{mL}^{-1}$ from each compatible strain). Ninety-seven fusing gamete pairs of these crosses, all joined with the girdles perpendicular to each other, were individually isolated and separately transferred to wells of tissue culture plates (6.4-mm diam, Iwaki) filled with four different media: replete medium (L1), medium with no nitrate added (L-N), L-P medium, and medium with no nitrate or phosphate added (-N-P). They were then placed under the same conditions as those previously described for culture maintenance. The evolution of the isolated pairs and planozygotes formed was tested at least daily and photographed with an EOS digital camera (Canon Inc., Lake Success, NY, USA) and measured at 630 \times magnification using an inverted microscope (Axiovert 135, Zeiss, Jena, Germany) and an Image IPplus analyser (Media Cybernetics, San Diego, CA, USA). The planozygotes obtained took two different cyst forms, each type defined as follows:

- Resting cyst: cyst with spiny morphology and long dormancy period (>1 month).
- Ecdysal sexual cyst: ecdysal planozygote stage with no spiny morphology and short dormancy period.

Staining of sexual stages nuclei. Sexual stages in different phases of development ($n \geq 5$) were stained for nuclei observation. Fusing gamete pairs from the cross 7V \times 4V, formed in L-P medium, were individually isolated to sterile polystyrene Petri dishes (6.4-mm diam, Iwaki) filled with L-P or L-N medium. The evolution of the fusing gamete pairs was tested each 10 min to determine the moment of total cytoplasmic fusion. Germlings from sexual ecdysal cysts were obtained from those planozygotes that underwent ecdysis. Germlings from resting cysts were obtained from spiny cysts of the cross 7V \times 4V that were individually isolated to L1

TABLE 1. Isolation and history of the *Lingulodinium polyedrum* strains.

Strain name	Source	Year of isolation	Culture history	Clonal
4V	Lorbé (Spain)	2000	Bloom sample	Yes
5V	Lorbé (Spain)	2000	Bloom sample	Yes
7V	Ría de Ares (Spain)	2001	Bloom sample	Yes
8V	Ría de Ares (Spain)	2001	Wild cyst germination	Yes
9V	Ría de Ares (Spain)	2002	Bloom sample	Yes
10V	Ría de Ares (Spain)	2002	Bloom sample	Yes
2B	Gullmar (Sweden)	2003	Wild cyst germination	Yes
3A	Gullmar (Sweden)	2003	Wild cyst germination	Yes

TABLE 2. Number criteria for the *Lingulodinium polyedrum* parental crosses.

Clonal strains	4V	5V	9V	10V	7V	8V	3A	2B
4V					1	2	3	4
5V					5	6	7	8
9V					9	10	11	12
10V					13	14	15	16
7V	1	5	9	13				
8V	2	6	10	14				
3A	3	7	11	15				
2B	4	8	12	16				

medium and followed for excystment daily. Individual cells were fixed for 10–15 min in 2% paraformaldehyde in 0.01 M PBS buffer, pH 7.4. The fixed cells were washed in several drops of PBS buffer and stained with 1:100 Sybr green (Molecular Probes, Eugene, OR, USA) in 0.01 M at PBS pH 7.4 during 30 min, washed again, and observed in a fluorescence microscope (Leica DMLA, Heidelberg, Germany) at 365 nm. Photographs were taken with a digital EOS-D60 camera (Canon Inc.).

Mating type and resting cyst production. Intercrosses and intracrosses (self-crosses) were conducted in duplicate sterile polystyrene Petri dishes (16-mm diam, Iwaki) filled with 3 mL of two different modified media, L1 medium with no phosphates added (L-P) and L1 medium with no nitrate added (L-N), and inoculated with exponentially growing cells (4000–6000 cells $\cdot\text{mL}^{-1}$) to a final concentration of 700 cells $\cdot\text{mL}^{-1}$ (350 cells $\cdot\text{mL}^{-1}$ from each compatible strain). Cyst counting was performed at days 15, 20, and 30 after crossing, and the highest cyst production achieved was recorded for each cross. Thirty-five cysts of each encysting media, formed by the cross 9V \times 3A, were photographed and measured at 630 \times magnification using an inverted microscope (Axiovert, Zeiss) and an Image IPplus analyzer (Media Cybernetics).

Dormancy period and excystment of resting cysts. For brevity in the text and figures, we assigned one number to each clonal cross performed (Table 2). Crosses 4, 9, 11, and 16 were conducted in 10 mL L-P medium in duplicate sterile polystyrene Petri dishes inoculated with exponentially growing cells (4000–6000 cells $\cdot\text{mL}^{-1}$) to a final concentration of 700 cells $\cdot\text{mL}^{-1}$ (350 cells $\cdot\text{mL}^{-1}$ from each compatible strain). One month after crossing, resting cysts were transferred to seawater and maintained in darkness at 24°C. This moment was considered the initial time of formation for all cysts isolated. From that moment, 30–35 cysts from each cross were isolated monthly to sterile polystyrene Petri dishes and checked for excystment every 2 days. The excystment was defined as the complete emergence of the protoplast from the cyst even if the germling remained nonmotile (Anderson and Wall 1978). The number of germinated cysts divided by the number of ungerminated cysts was used to determine the germination percentage of cysts isolated on a given date. The evolution of the germinated cells (planomeiocytes) was tested at least daily and they were photographed with a Canon EOS digital camera and measured at 630 \times magnification using an inverted microscope (Axiovert Zeiss) and an Image IPplus analyzer (Media cybernetics).

RESULTS

Asexual stages. A brief description of the asexual reproduction of *L. polyedrum* follows to make a comparison of vegetative division and sexual stages, which appears later. Asexual reproduction in the

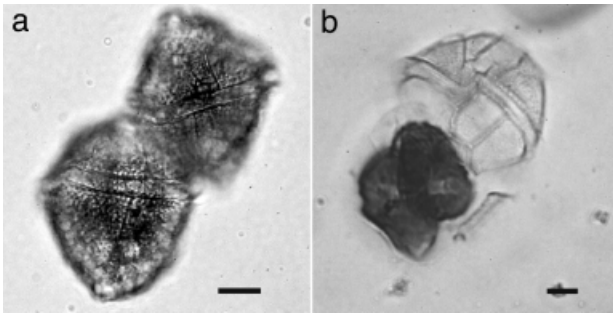


FIG. 1. *Lingulodinium polyedrum* asexual stages. (a) Desmoschisis, (b) Eleutheroschisis. Scale bars, 10 μ m.

mobile stage takes place mainly by sharing the thecal plates (Fig. 1a), a process known as desmoschisis. Under unfavorable conditions, the vegetative cells of *L. polyedrum* can undergo ecdysis (asexual cyst). The protoplast is released through the epitheca and subsequently undergoes division to form two naked cells (eleutheroschisis) (Fig. 1b).

Lingulodinium polyedrum sexual cycle. In Figure 2 we schematized the sexual cycle of *L. polyedrum*. The routes described were established after monitoring the fusion and subsequent development of 97 fusing gamete pairs. The next description analyzes each step of this cycle.

Fusing gametes morphology and planozygote formation: Sexual induction occurred 2–4 days after inoculating exponentially growing cells into P-deficient L1 medium. Two different mechanisms of cell attachments were observed: wall to wall adhesions or mating through both cells via transversal flagella and sulcal region attachment. The former happened only by means of three cell locations: cingular regions of

both cells (Fig. 3a); through the antapical regions of both cells (Fig. 3b); and cingular–apical region attachment, the cells forming a perpendicular angle (Fig. 3c). Apical–apical attachments were also observed in culture, though in very low proportions. It should be noted that neither of these described cases can be confused with asexual division stages (compare Fig. 3, a–c, with Fig. 1, a and b). Among these cases, only cell pairs that first coupled through their flagella and subsequently attached the sulcal regions (Fig. 3, d, f, and h) successfully formed a planozygote stage, and only those pairs were therefore considered gametes. Fusing pairs (gametes: 22–40 μ m long, 18–35 μ m wide) were armored and mainly of unequal size, although isolated pairs with a high degree of anisogamy were unviable. Gametes usually showed one or more red to orange accumulation bodies, a pale color, and a high metabolic activity, shown by fast cytoplasmic movements. Gametes in early stages of fusion had a U-shaped and bilobulated nucleus, in which chromosomes were slightly unwound (Fig. 3e). Gamete pairs in an advanced stage of fusion formed perpendicular angles to each other (Fig. 3f) and lacked some thecal plates (Fig. 3g). At this stage, their complete fusion could be very fast. The gamete pair in Figure 3h formed the young planozygote in Figure 3i in less than 10 min. Cytoplasmic fusion progressed before nuclear fusion started. Figure 3j shows the nuclei of a pair in a similar state of fusion than Figure 3f or 3h. However, both processes were completed nearly at the same time. Nuclear fusion began in late stages of fusion (Fig. 3, k and l) and it was very fast (<5 min), being always completed in newly formed planozygotes. In this stage (0–15 min after total cytoplasmic fusion),

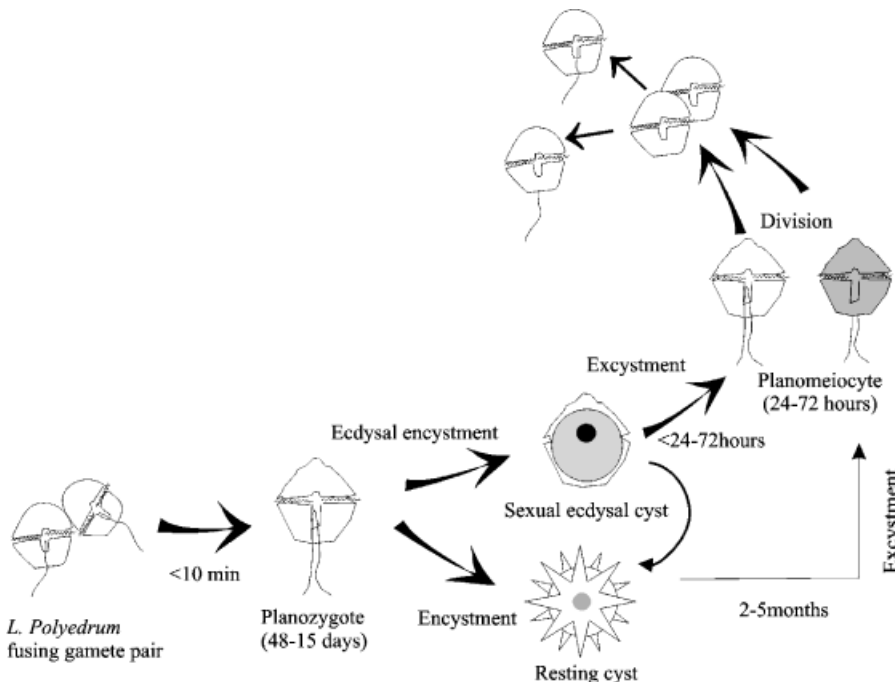


FIG. 2. *Lingulodinium polyedrum* sexual cycle.

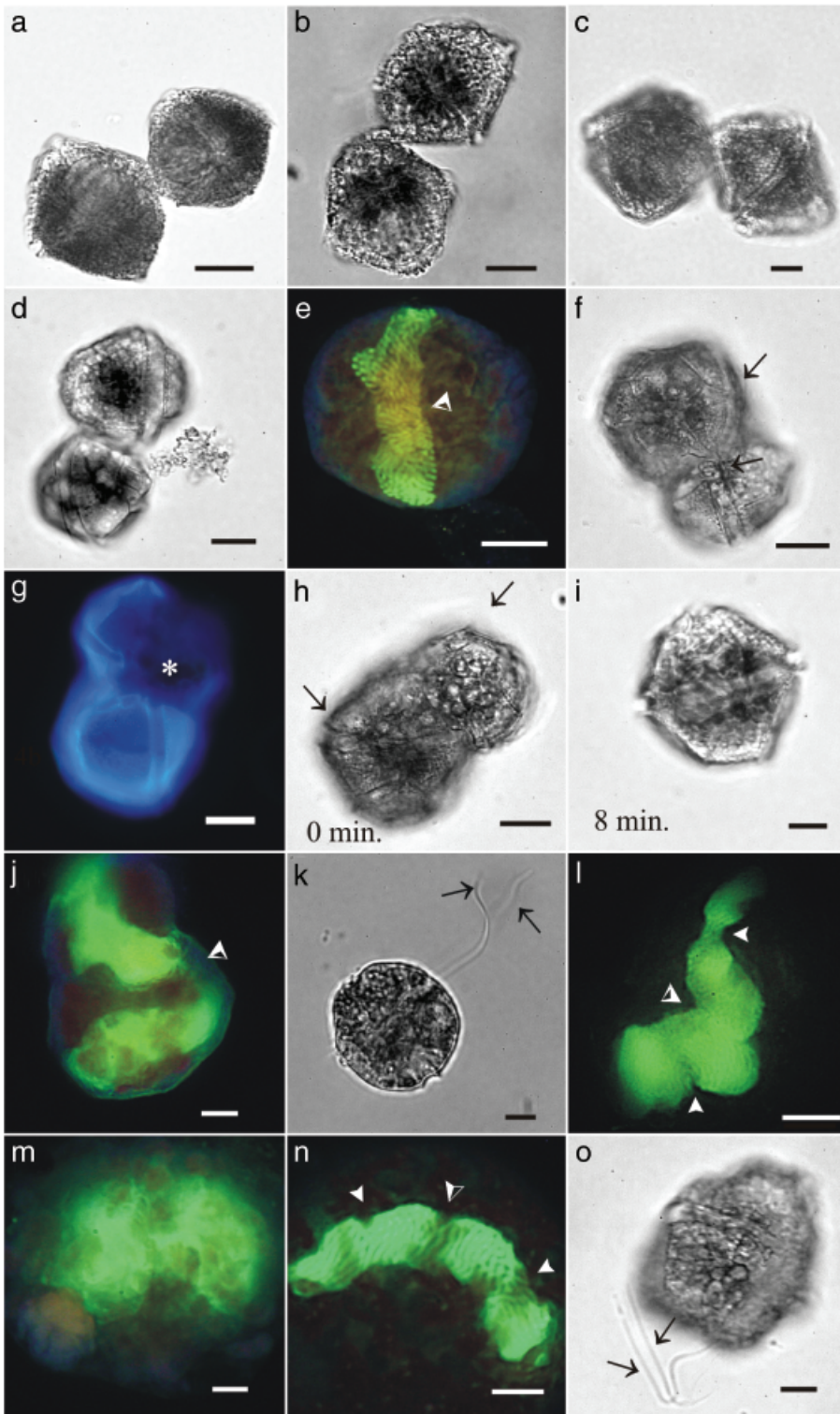


FIG. 3. *Lingulodinium polyedrum* coupling pairs. (a–c) Paired cells attached through wall-to-wall contact, at the cingular regions (a), through basal regions (b), and with cingular-apical joint (c). (d) Isogamous gamete pair with flagellar attachment. (e) Bilobulated nucleus from a gamete of a pair joined by flagellar attachment. The arrowhead indicates the point of connection between nuclear lobules. (f) Gamete pair in an advanced fusion stage. Arrows indicate the position of the cingular regions, the cells forming a perpendicular angle between each other. (g) Thecal plates of the gamete pair in f after isolation and calcofluor staining. The fusing area lacks some thecal plates (*). (h) Fusing pair forming a perpendicular angle between the cingular regions (indicated by arrow) and the young planozygote formed by them in less than 10 min. (i). (j) Nuclei disposition of a fusing pair in a similar stage than f and h. The arrowhead indicates the initial point of nuclear fusion. (k) Almost complete cytoplasmic fusion and partial nuclear fusion (l). (m) Nucleus of a newly formed planozygote. (n) Nucleus of a planozygote 1–2 h after its formation showing a quadrilobulated structure (arrowheads). (o) Old planozygote from culture showing two long and thick flagella. Scale bars: a–l, 10 μ m; m and n, 5 μ m.

the nucleus constituted a diffused area that occupied most of the cell (Fig. 3m). After 1–2 h, the chromosomes rewound and appeared in four lobular units (Fig. 3n). Older planozygotes (48–53.4 μ m long, 42–44.6 μ m wide) (Fig. 3o) were darker and had two noticeable long and thick longitudinal flagella.

Resting cyst formation and germination. Six to 15 days after the isolation of the gamete pairs, the typical spiny *L. polyedrum* resting cysts (35–41.8 μ m diam) were formed. The development of the spines was a very fast process (5–15 min), and a graded length of spines was observed in cysts formed by the same

clonal cross and under the same culture conditions, albeit at different times during the encystment process (Fig. 4a). Resting cyst germination (Fig. 4b) released dark planomeiocytes (46.4–47.5 μm long, 41–45.2 μm wide) with one orange to red basal spot and two long trailing flagella (Fig. 4, c and e), which divided by desmoschisis at between 24 and 72 h after cyst germination. Zero to 24-h germling cells (Fig. 4c) had one thick, homogenous, U-shaped nucleus (Fig. 4d). In older germlings (Fig. 4e), the nucleus suffered enlargement, and the chromosomes were further condensed before division (Fig. 4f).

Sexual ecdysal cyst formation and germination. *Lingulodinium polyedrum* planozygotes also formed a short-living form of cyst instead of the typical spiny and long-living resting cyst already described for this species. This fact means that there was a different and shorter route during its sexual cycle (Fig. 2). Figure 5 shows the ecdysal encystment and germination of a concrete fusing gamete pair by time-lapse photography, which can be generally described as follows: 48–96 h after gamete pair isolation (Fig. 5a), some planozygotes (Figs. 5b) ceased their movement, retracted cell contents from the theca, and underwent ecdysis (Fig. 5c). After <24–72 h, the protoplast was released through the epitheca (Fig. 5d), leaving the planozygote theca (Fig. 5e). The germling cell was naked, biflagellated, and with a basal red spot (Fig. 5, f and g). Newly germinated cells from sexual ecdysal cysts possessed, as in germlings from resting cysts, one thick U-shaped nucleus (Fig. 5h). As described previously, this nucleus enlarged in older germlings (Fig. 5i). Several hours after germination, the germling cell was observed to be biflagellated and thecated (Fig. 5j), and in 24–96 h they divided by desmoschisis. During this process, one of the daughter cells retained the two longitudinal flagella of the germling,

and a certain angle was formed between the dividing cells (Fig. 5k). Two viable cells were formed by this process. Except for their paler color, these germlings were observed to be similar to those formed after resting cyst germination. This route through ecdysal sexual stages presented specific characteristics that distinguished this process from both ecdysal asexual stages and spiny resting cyst formation. The main differences are shown in Table 3.

Nutrient effects on gamete pair encystment. Fusing gamete pairs formed in L-P medium were individually isolated in different media and monitored daily to establish the timing and efficiency of the encystment. Gamete pairs isolated in LI and L-N media formed resting cysts in percentages significantly lower (Kruskal-Wallis, $P < 0.05$) than those isolated to media with no phosphate added (Fig. 6a). Nevertheless, no differences were observed in the period of time (6–15 days) required for the fusing pairs to encyst. The percentage of ecdysal sexual cysts was inversely related to nutrient levels with cyst formation favored in a media replete in phosphate levels (Fig. 6b). The two percentages together do not add up to 100% because some planozygotes were not viable.

Mating type and resting cyst production. Eight clonal strains of *L. polyedrum* were intercrossed and self-crossed to study mating behavior. Production of the resting cyst, checked in L-N and L-P media, is shown in Table 4. These results indicate that clonal strains were always incompatible (discarding homothallism) and that there were only two sexual types (+/-), which corresponds to simple heterothallic mating behavior. Resting cyst production for all compatible crosses is shown in Figure 7. Three main facts related to cyst formation were observed: 1) cyst production in medium lacking in nitrate (Fig. 7a) was between 4 times (crosses 11 and 13) and 35 times

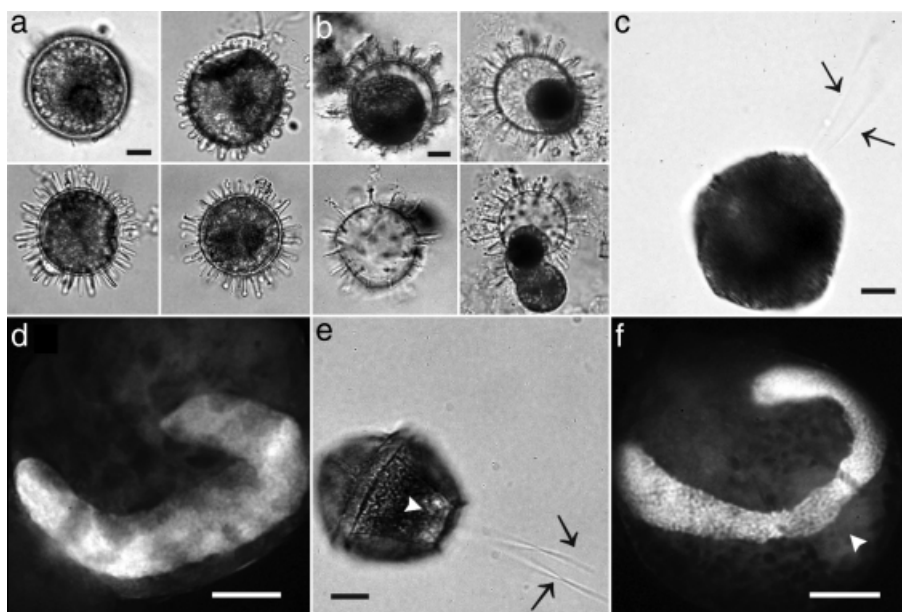


FIG. 4. Resting cyst formation and germination. (a) Resting cysts formed by the same parental cross showing different spine development. (b) Excystment progress of the resting stage. (c) 0–24 h deep dark germling showing two longitudinal flagella (arrows). (d) Nucleus of the cell showed in d. (e) 24–48 h germling showing two longitudinal flagella (arrows) and a basal red spot (arrowhead). (f) Nucleus of the cell in e. The arrowhead points to the red spot. Scale bars, 10 μm .

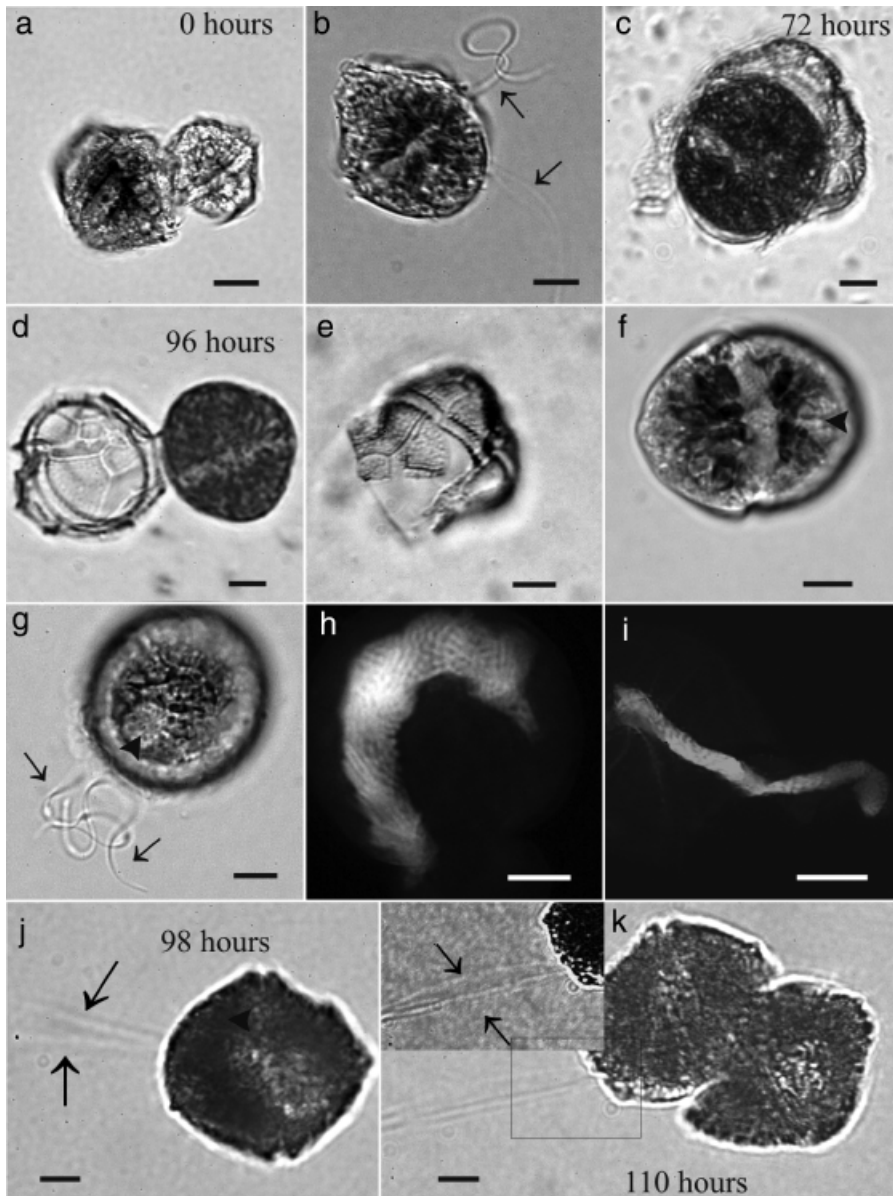


FIG. 5. Time-lapse photography of an ecdysal sexual cyst formation and germination. (a) Isolated fusing gamete pair. (b) Planozygote formed by the gamete pair fusion showing the two longitudinal flagella. (c) Ecdysal sexual cyst. (d) Excystment of the ecdysal sexual stage. (e) Shell left after germination. (f) Naked germling in frontal view and with the two trailing flagella in focus (g). (h) Nucleus of a 1–2 h germling from a sexual ecdysal cyst. (i) Nucleus of a 12–24 h germling. (j) Thecated and biflagellated (arrows) 2 h germling. (k) Germling division by desmoschisis. The basal region of the anterior cell has been treated with enhanced contrast to show the position of the two longitudinal flagella during the division process. Scale bars, 10 μm .

(cross 6) smaller than in a medium deficient in phosphate levels (Fig. 7b); 2) compatible crosses studied showed important differences in mean values of cyst production, which ranged from <10 to >200 cysts $\cdot \text{mL}^{-1}$ in L-N medium and from <50 cysts $\cdot \text{mL}^{-1}$ to >800 cysts $\cdot \text{mL}^{-1}$ in L-P medium; and 3) sig-

TABLE 3. Main differences between asexual and sexual cysts of *Lingulodinium polyedrum*.

<i>L. polyedrum</i> cysts	Encysting cell	Cyst shell	Division of the germinated cell
Asexual ecdysal cyst (temporary cyst)	Haploid	Vegetative cell theca	Eleutheroschisis
Sexual ecdysal cyst	Diploid	Planozygote theca	Desmoschisis
Spiny resting cyst	Diploid	Spiny shell	Desmoschisis

nificant differences (ANOVA, $P < 0.05$) were found in cyst sizes depending on the formation medium, with cysts formed in L-P medium growing longer ($39.3 \pm 1.5 \mu\text{m}$) than those in L-N medium ($36.8 \pm 1.8 \mu\text{m}$).

Dormancy period of resting cysts. An overall time range of 2 to 4 months was established for the mandatory dormancy period of *L. polyedrum* resting cysts formed by four parental crosses (Fig. 8). The length of this period and the germination percentage on each given date were related to the original parental cross. Crosses 4 and 16 had the fastest germination, because 60%–80% of these cysts germinated after 2–3 months. On the other hand, 40% of the cysts from cross 9 germinated after 3–4 months, and almost 100% reached this value after 4–5 months. Very low germination percentages were recorded for cysts

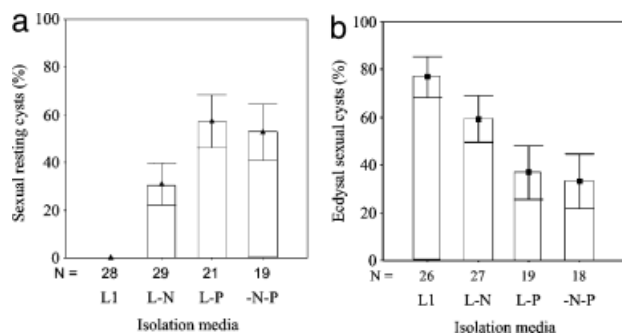


FIG. 6. Gamete pair encystment (%) after being isolated from L-P medium to replete medium (L1), media lacking nitrates (L-N), phosphates (L-P), or both (-N-P). (a) Resting cyst formation. (b) Ecdysal sexual cyst formation. Mean values and SD.

from cross 11. These cysts began to germinate after 4–5 months, though less than 20% of them underwent excystment.

DISCUSSION

Sexual stages development. After inducing sexuality, we have seen pairs of *L. polyedrum* cells joined in several ways. Because only those cells that coupled with their longitudinal flagella and subsequently attached their cingular regions successfully underwent fusion and encystment, only those pairs were considered gametes. Nevertheless, the remaining cases were quite common in culture, characterized by wall-to-wall contact and a kind of filamentous nexus between the joined cells. This fact recalls the case of *Chlamydomonas reinhardtii* Dangeard, in which the sexual agglutinin is a fibrous glycoprotein, and it was proposed that if sexual agglutinins recognize each other by the same mechanisms used by wall proteins, the interaction during the mating reaction could form an interconnecting meshwork of filaments in addition to those found in the wall layers (Adair et al. 1983). Because these pairs were not viable, they might constitute a memory of an ancestral wall-to-wall adhesion, such as in *Chlamydomonas* species and as occurs in fungi, the flagellar location being a secondary development designed to improve the chances of contact (Goodenough 1985). Though isogamy was possible, anisogamous pairings were more commonly observed, as happens for example in the *Gym-*

TABLE 4. Cyst appearance in the intercrossing experiment.

Clonal strains	4V	5V	9V	10V	7V	8V	3A	2B
4V	—	—	—	—	+	+	+	+
5V	—	—	—	—	+	+	+	+
9V	—	—	—	—	+	+	+	+
10V	—	—	—	—	+	+	+	+
7V	+	+	+	+	—	—	—	—
8V	+	+	+	+	—	—	—	—
3A	+	+	+	+	—	—	—	—
2B	+	+	+	+	—	—	—	—

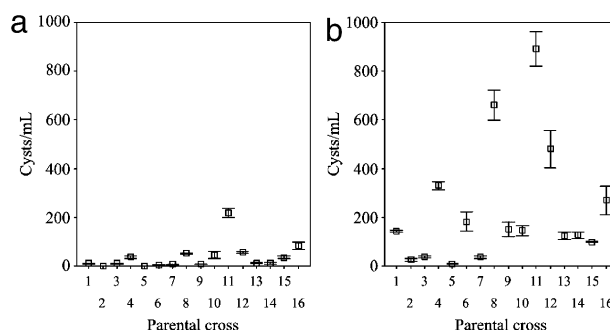


FIG. 7. Cyst production (cysts · mL⁻¹) in L-N (a) and L-P (b) media for the studied *Lingulodinium polyedrum* parental crosses. Mean ± SD ($n = 2$). See Table 2 for the number criteria for each parental cross.

nodinium catenatum Graham sexual cycle (Blackburn et al. 1989). *Lingulodinium polyedrum* gamete pairs individually isolated on culture plates could split and rejoin to form planozygotes that encysted in high percentages in poor media. This gamete pair behavior might be explained by sexual chemotaxis, as in yeast, where the mating locus type determines the production of and reaction to pheromones (Hoekstra 1987). Because the characteristic two flagella of the planozygote were noticeably longer and thicker than those usually present in vegetative cells, they may originate by complementation (new genetic information), as in the biflagellate planozygote of *Cryptohocodinium cohnii* Biecheler (Beam and Himes 1980), instead of representing a gametic character. Gamete pairs in an advanced state of fusion always formed a

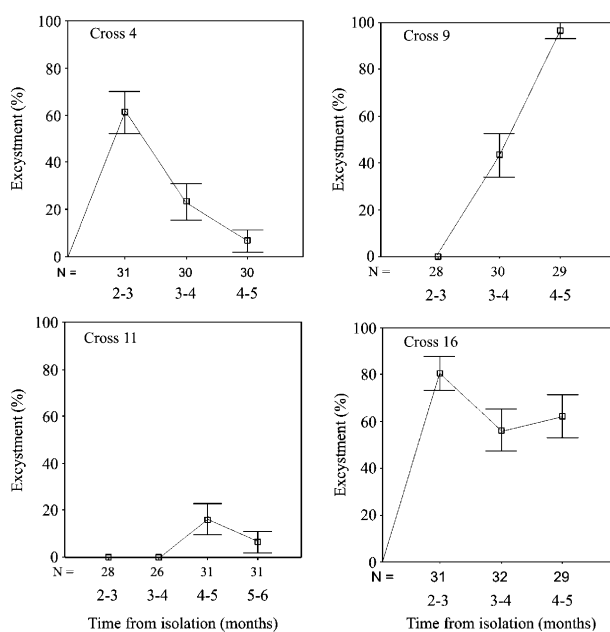


FIG. 8. Dormancy period for *Lingulodinium polyedrum* resting cysts formed by different parental crosses. Mean ± SD ($n = 2$). See Table 2 for the number criteria for each parental cross.

perpendicular angle between one other. At this stage, total fusion is a fast process that may last less than 10 min and that never takes over 24 h. On the other hand, planozygote encystment may need up to 15 days. This period is in agreement with previous reports of 1 to 2 weeks for *Alexandrium tamarense* (Lebour) Balech (Anderson et al. 1983) and *Peridinium cunningtonii* (Lemmermann) Lemmermann (Sako et al. 1984), though periods of as long as 4 weeks have also been reported in *Peridinium bipes* f. *oculatum* (Lindem.) Lef. (Park and Hayashi 1992). For *L. polyedrum*, this long period may serve for the planozygote to decide the encystment strategy, because planozygotes underwent two different encystment processes, as we are now going to discuss.

Nuclear status of sexual stages. In dinoflagellates, nuclear fusion has been reported to occur before cytoplasmic fusion is complete (Pfiester 1989). In *L. polyedrum*, nuclear fusion began in late states of the cytoplasmic fusion, though it was so fast that both processes ended nearly together. In newly formed zygotes, the nucleus was represented by a diffused area. One to 2 h later, the planozygotes had one well-developed quadrolobulated nucleus. In postzygotic cells of both types of cysts, the diploid nucleus was observed to enlarge and the chromosomes became more condense. Meiosis has to take place to restore the haplontic vegetative stage, so this nuclear stage may correspond to the eukaryotic prophase I, as was proposed for *Peridinium inconspicuum* Lemmermann (Pfiester 1989). In dinoflagellates, the meiotic divisions may be either consecutive or delayed in time (Elbrächter 2003). We have not studied whether *L. polyedrum* has a one- or two-step meiosis, but our observations indicated that two different forms of zygotes were unequivocally formed.

Encystment strategies of Lingulodinium polyedrum. There is a basic pattern in the haploid-diploid life cycle of the dinoflagellates, such as *L. polyedrum*, that form sexual resting stages: Haploid gametes fuse and form a diploid planozygote that undergoes encystment. After a mandatory dormancy period excystment occurs, and the diploid planomeiocyte divides to reestablish the haploid vegetative phase. The existence of asexual cysts, however, has also been proved for several species (e.g. *A. tamarense*, Anderson and Wall 1978; *L. polyedrum*, Hardeland 1994; *A. taylori* Balech, Garcés et al. 1998; *A. catenella* [Whedon and Kofoid] Taylor, Delgado 1999). These cysts, found in the literature with different names, such as temporary cysts (*A. catenella*, *L. polyedrum*, *A. taylori*), pellicle cysts (*A. tamarense*), or ecdysal stages (*L. polyedrum*), are formed by nonmotile vegetative cells exposed to unfavorable conditions. The possible role of sexual and asexual cysts has not been clearly established. The asexual cyst has often been associated with the response to a changing environment, because its faster germination would allow for a rapid shift between benthic and planktonic stages (Garcés 2002). Nevertheless, the triggers for

their formation may be species specific and also constitute a division stage during the life cycle of some species, such as in *Alexandrium pseudogonyaulax* (Biecheler) Horiguchi (Kita et al. 1985, Montresor 1995) and *Alexandrium taylori* (Garcés et al. 1998). On the other hand, resting stages are considered a survival mechanism for disturbances occurring on time scales of months to years (Dale 1983). Nevertheless, the asexual cyst can also function as a long-term resistant strategy and the sexual hypnozygote as a short-living stage.

In the genus *Chattonella* (Raphidophyceae), the haploid cyst stage needs a genetically regulated dormancy period of several months to enter in quiescence, a period mainly regulated by water temperature (Imai et al. 1998). On the other hand, short mandatory periods that do not fit a true dormancy condition have been observed for sexual cysts of several dinoflagellate species. Less than 15 days were reported for *Alexandrium taylori* by Giacobbe and Yang (1999), germination within 2 weeks or less were observed in *Gymnodinium catenatum* (Bravo and Anderson 1994), and *Peridinium gatunense* Nygaard cysts can even germinate within 12 h of their formation (Pfiester 1977). The present study gives another example of a complex life cycle in which the role of each type of cyst is not related to a short or long-term period of resistance. Instead of a functional division between sexual and asexual cysts, in *L. polyedrum* a faster or slower response to external challenging factors might be made by different sexual cyst forms, because we have observed that *L. polyedrum* planozygotes formed two kinds of sexual hypnozygotes. Marasovic (1993) proposed a possible connection between vegetative cells and sexual stages through temporary cysts, though detail was lacking in these observations (Lewis and Hallet 1997). We reported a short-living form that we named ecdysal sexual cyst because of its resemblance to the asexual ecdysal stage in both its morphological aspects and the brief time needed for excystment. Because cysts often germinated in <24 h (time of monitoring), a mandatory dormancy period could not be determined. After a few hours, the excysted cells had a morphology similar to that observed in vegetative cells, except for the presence of the two trailing flagella. These facts are comparable with the fast development (1–2 h) reported by Lewis and Burton (1988) for germlings from *L. polyedrum* resting stages, though the biflagellated condition was not mentioned. Sharing a characteristically fast germination with asexual cysts, sexual recombination in these stages would provide an extra advantage. The isolated gamete pairs in our experiments also formed the typical spiny resting cysts of *L. polyedrum*. This dormant stage needed a minimum mandatory period of 2–4 months to germinate. This period together with the percentage of cysts that could successfully undergo excystment was, however, dependent on the selected parental cross.

These different sexual routes may correspond to two encystment strategies, to be chosen as a function of

external factors. *Lingulodinium polyedrum* planozygotes formed ecdysal sexual cysts mainly in enriched media, whereas the formation of a spiny resting cyst was dependent on low environmental phosphate levels. There was no resting cyst formation in replete L1 medium. This fact indicates that sexual ecdysal cyst formation was highly dependent on the nutritional status of the external medium and that it was not due to a manipulation artifact. Nutritional influence over the planozygote behavior leading to a shorter sexual route has also been recorded for *Gymnodinium nolleri* (Ellgaard and Moestrup) (Figueroa and Bravo 2005). As we observed in *A. catenella* (Figueroa et al. 2005), sexual induction in a phosphate-deficient medium made cysts larger than those formed in a medium with low nitrate levels. Because the sexual induction trigger was the phosphate deficiency, the change in the external nutritional status seemed to be detected by *L. polyedrum* planozygotes, which formed short-living cysts instead of the long-time resistant form when conditions were no longer adverse. This encystment route would allow for a rapid transition between diploid and haploid cells, implying a saving of energy and an ecological advantage in challenging conditions in comparison with other species in which this fast switch is not possible.

We established that *L. polyedrum* sexuality can be explained by a simple heterothallic system formed by two sexual types (+/-), though different parental crosses presented significant differences in cyst production. This sexual pattern has previously been reported in several species, such as *Alexandrium catenella* (Yoshimatsu 1981), *Glenodinium lubieniense* (Diwald 1938), or *Ceratium cornutum* Claparede and Lachmann (von Stosch 1973). Because no resistant spiny cysts have ever been observed inside clonal cultures, we can state that a transition between ecdysal and spiny stages should only be performed by a planozygote ecdysal state, as we indicate in Figure 2.

Conclusions. This work focuses on previously unreported aspects of the sexual reproduction of the dinoflagellate *L. polyedrum*. After the step-by-step monitoring of the sexual stages development, we found the sexual cycle of this species to be a more complex process than was previously thought. The present results highlight the following implications in the ecological role of cyst formation by *L. polyedrum*. First, *L. polyedrum* planozygotes can form two different types of sexual cysts, which correspond to two different encystment strategies. In one of them, which we named ecdysal sexual encystment, short-living cysts were formed that germinated after 24–72 h. In other cases, the planozygotes formed long-living and spiny cysts, which did not germinate in <2–4 months. Second, each encystment route may be determined by environmental factors, because the percentage of planozygotes that “chose” each kind of encystment was dependent on the phosphate levels of the isolation media. Finally, by the inter- and intracrossing of several clonal cultures, we established

that the formation of resting cysts can be explained by the presence of only two different sexual types of gametes (+/-), mating behavior known as simple heterothallism. Nevertheless, different parental crosses presented important differences in cyst production and the dormancy period of the resting cysts formed.

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