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AN IMPROVED STRIPPING TECHNIQUE FOR LIGHTLY ARMORED DINOFLAGELLATES[1]

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Identification of armored heterotrophic dinoflagellates relies, in part, on plate tabulations obtained by SEM. Currently, two methods are used to visualize plate morphology and develop plate tabulations: swelling the sutures between the cellulose plates of intact organisms or stripping off the outer membranes with ethanol to expose the underlying cellulose plates. Both approaches are problematic with lightly armored dinoflagellates because sutures do not consistently swell to enable visualization, and the outer membranes are not consistently stripped. Further, generic and species differences necessitate frequent modification of these protocols to obtain reliable results. We describe an improved membrane stripping technique using the detergent Triton X-100. Our method provides a more consistent standardized approach to removing the outer membranes of lightly armored dinoflagellates, including *Pfiesteria shumwayae* Glasgow & Burkholder, a taxon that has, until now, proven very difficult to strip with currently published methods. This method allows visualization of the sulcus, a region previously difficult to observe, and will greatly facilitate taxonomic studies of the lightly armored forms.

Key index words: **method; *Pfiesteria piscicida*; *Pfiesteria shumwayae*; scanning electron microscopy; taxonomy; Triton X-100**

Heterotrophic dinoflagellates belonging to the toxic *Pfiesteria* complex have been implicated in fish kills, fish lesion events, and human disease in mid-Atlantic U.S. estuaries (Burkholder et al. 1992, 1995, Burkholder and Glasgow 1997). Recently, increased concern about these dinoflagellates, and morphologically similar cells whose toxicity is not documented (i.e. *Pfiesteria*-like organisms), has resulted in the development of methods for their definitive identification with SEM (Steidinger et al. 1996, 2001, Truby 1997). Currently, identification of *Pfiesteria* and *Pfiesteria*-like dinoflagellates involves removal of the outer membranes to expose the underlying cellulose plates. These plates are arranged in specific patterns (Kofoidian series) that are currently regarded as one of the most important morphological criteria for taxonomic classification of the small lightly armored dinoflagellates (Fensome et al. 1993, Steidinger et al. 2001). Further, molecular analyses support the taxonomy based on plate morphology and tabulation (Litaker et al. 1999, Taylor 1999). The standard protocol (Steidinger et al. 1996, Truby 1997) for stripping the plasmalemma and outer layers of the amphiesmal vesicles to expose these cellulose plates has proven unsatisfactory for members of the toxic *Pfiesteria* complex and related species. Frequently, numerous samples must be prepared due to species-specific responses to the membrane stripping protocol.

Unfortunately, *Pfiesteria shumwayae* (and, to a lesser extent, *Pfiesteria piscicida* Steidinger & Burkholder) has consistently proven extremely difficult to strip by conventional methods (K. A. Steidinger and R. W. Litaker, personal communication, and our unpublished data). A suture-swelling technique has recently been developed in which the sutures between the plates are rendered visible beneath the outer membranes in osmotically swollen cells (Glasgow et al. 2001). However, this method is also inconsistent and fails to provide a complete tabulation because the sulcal plate structure cannot be adequately visualized. SEM of cells with intact membranes does not permit complete plate tabulation due to the retention of the flagella and the furrowed nature of the sulcus (Steidinger et al. 1996). Attempts to expose the cellulose plates of *P. shumwayae* using sonication in conjunction with ethanol treatment, alone or in combination with other organic solvents (of various polarities), acids, and enzymes, have all proven ineffective in removing the membranes (our unpublished data). We report an improved more reliable method for stripping the outer membranes from various species of lightly armored dinoflagellates, including *P. shumwayae* and *P. piscicida*, that leaves the cells sufficiently intact for critical SEM analysis of plate tabulation.

MATERIALS AND METHODS

Cultures of *P. shumwayae* and *P. piscicida* were grown using previously described methods (Vogelbein et al. 2001). *Pfiesteria shumwayae* (VIMS-1049) was obtained from a water sample collected in the Pamlico River, North Carolina on 12 November 1999. It has been deposited with the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP no. 2089). *Pfiesteria piscicida* (VIMS-P11) was isolated as a contaminant from a *Rhodomonas* sp. culture in 1998. Both cultures were tentatively identified by SEM plate tabulations using suture swelling and membrane stripping techniques and verified using species-specific PCR primers and probes (Vogelbein et al. 2001). An undescribed "Shepherd's crook" (VIMS-P314) isolate was obtained from a water sample collected in Balls Creek, Great Wicomico River, Virginia on 1 June 1999. In addition, two cultures of undescribed lightly armored dinoflagellates, a cryptoperidiniopsoid (VIMS-P28) and a "Lucy" (VIMS-P27) isolate, were kindly provided to us by Dr. Patricia A. Tester (NMPS-NOAA, Beaufort, NC, USA). (The names used for the dinoflagellate isolates "Lucy" and "Shepherd's crook" are terms created by investigators. The "Lucy" isolate has two dia-

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mond-shaped apical intercalary plates, and the name "Shepherd's crook" is in reference to the crook-shaped apical pore complex, comprised of a pore plate, a cover plate, and a canal or "X" plate.)

The primary reagent used in the stripping method was the detergent Triton X-100 (Sigma, St. Louis, MO, USA). Triton X-100 was diluted in either HPLC grade water or 12‰ seawater to concentrations ranging from 0.5% to 20% and 0.22 μm filtered to prevent mold growth. These dilutions were combined 1:1 with equal amounts of live zoospores of various species in 12‰ seawater, initially augmented with 2500 units of penicillin and 2.5 mg of streptomycin L^{-1} (no. P0781, Sigma) to reduce bacterial growth until the culture was established and sonicated at 35.5–40.5 kHz in a VWR brand Aquasonic ultrasonic bath (model 50T, VWR, Charlotte, NC, USA) for 30 min to 2 h. The temperature of the bath rose to approximately 29° C during the sonication process. Cells were collected on polycarbonate filters (3 μm, 13 mm diameter, Whatman Nuclepore, VWR) smooth surface up, followed by treatment with a diluted (40%, or 4.8‰ final concentration) seawater swelling step for 30 min at room temperature, and then fixed using the post-stripping protocol described by Steidinger et al. (1996). Briefly, cells were fixed in 4% paraformaldehyde buffered with 0.1 M sodium cacodylate (pH 7.2) for 30 min at room temperature and then buffer washed and dehydrated through a graded ethanol series. The filters, containing zoospores, were then mechanically stabilized in modified Beem embedding capsules (size 00, Electron Microscopy Sciences, Fort Washington, PA, USA), critical point dried in a Polaron E3100, mounted on specimen stubs with colloidal graphite, sputter coated with gold-palladium alloy in an Anatech Hummer VII (Anatech, Ltd., Alexandria, VA, USA), and analyzed on a scanning electron microscope (model 435VP, LEO Electron Microscopy, Ltd., Cambridge, England).

RESULTS

Treatment for 1 h with Triton X-100 diluted in HPLC grade water to a concentration of 10% to 20%, mixed 1:1 with live cells (i.e. final concentration of 5%–10%), was most effective in stripping the outer membranes of live *P. shumwayae* zoospores (Fig. 1, A and B). Larger vegetative cells were more readily stripped of their outer membranes than smaller recently excysted cells, as has been the case with all the techniques we used on this species. Most importantly, the sulcal plates were readily visualized with this method (Fig. 1B) due to swelling of the cell and flattening of the sulcus. The pores in the plates and the morphology of the apical pore complex were also more easily visualized. Results of this method contrasted sharply with those obtained by standard methods using 60% ethanol as the stripping agent (Fig. 1C). The treatment of dinoflagellate cells in Triton X-100 diluted in seawater of the same salinity as the original culture material resulted in stripping, but the plates were too wrinkled to see sutures even with the application of the osmotic swelling step (Fig. 1D).

A post-stripping swelling treatment of the zoospores with seawater at a concentration of 40% that of the original culture media (i.e. 4.8‰ vs. the original 12‰) proved valuable in overcoming possible collapse due to osmotic changes during the exposure to Triton X-100. We introduced this swelling step during the ethanol stripping method to overcome the plate wrinkling caused by exposure to the solvent. This post-stripping swelling step proved beneficial with the Triton protocol because the zoospores were much less wrinkled and the additional swelling enhanced visualization of the morphology of the sulcus (Fig. 1B).

Regardless of the stripping method, the plate sutures of *P. shumwayae* and of *P. piscicida* remained difficult to visualize due to the thinness of the plates relative to the other species examined. Sutures in these two species were less prominent and frequently could not be resolved, especially in areas of critical importance such as the location of the apical intercalary plate. In both *P. shumwayae* and *P. piscicida* isolates, the larger vegetative cells had more prominent sutures that aided in confirming the plate tabulations of these two species.

Treatment of *P. piscicida* and the other species tested with 10% (final concentration) Triton X-100 gave virtually identical results to that of *P. shumwayae*: cell membranes were usually completely removed, allowing visualization of the underlying plates (Fig. 2, A and B). However, wrinkling of the plates or incomplete stripping of the outer membranes was more frequent in recently excysted zoospores of both *P. shumwayae* and *P. piscicida*, suggesting that cellular physiological state, possibly related to population dynamics, could influence the results of this and other stripping protocols. In all species processed, some replicates demonstrated cellular disruption, with percentages varying from approximately 10% (the cryptoperidiniopsoid) to as high as approximately 30% (*P. shumwayae*). However, in all cases sufficient numbers of intact cells remained, allowing determination of plate tabulations.

Triton X-100 consistently and cleanly removed the outer membranes of all species tested, with far less structural artifacts, than observed with the ethanol protocol. The cell membranes of the cryptoperidiniopsoid isolate were completely removed (Fig. 2, C and D), even with many of the smaller zoospores (Fig. 2C). A low number of cells in each preparation collapsed, but the yield of clean intact cells usable for analysis was large (>80%). The "Lucy" isolate (Fig. 3, A and B) in comparison fell between the *Pfiesteria* species and the cryptoperidiniopsoid isolate in terms of the overall condition and number of cells available for analysis: wrinkling of the cellulose plates (Fig. 3A) and cellular collapse, when they occurred, were not as severe as in the *Pfiesteria* species, yet the cells were not in as good a condition as those in the cryptoperidiniopsoid isolate. The stripping method also worked very well for the "Shepherd's crook" isolate with the membranes of the zoospores stripping off cleanly (Fig. 3, C and D).

DISCUSSION

We report a highly effective method for stripping off the outer membranes of lightly armored dinoflagellates. This improved method is consistent in its ability to remove the membranes of all five species tested so far and allows clear critical visualization of important taxonomic characters of the underlying cellulose plates. It also facilitates the collection of new morphological and taxonomic information due to the

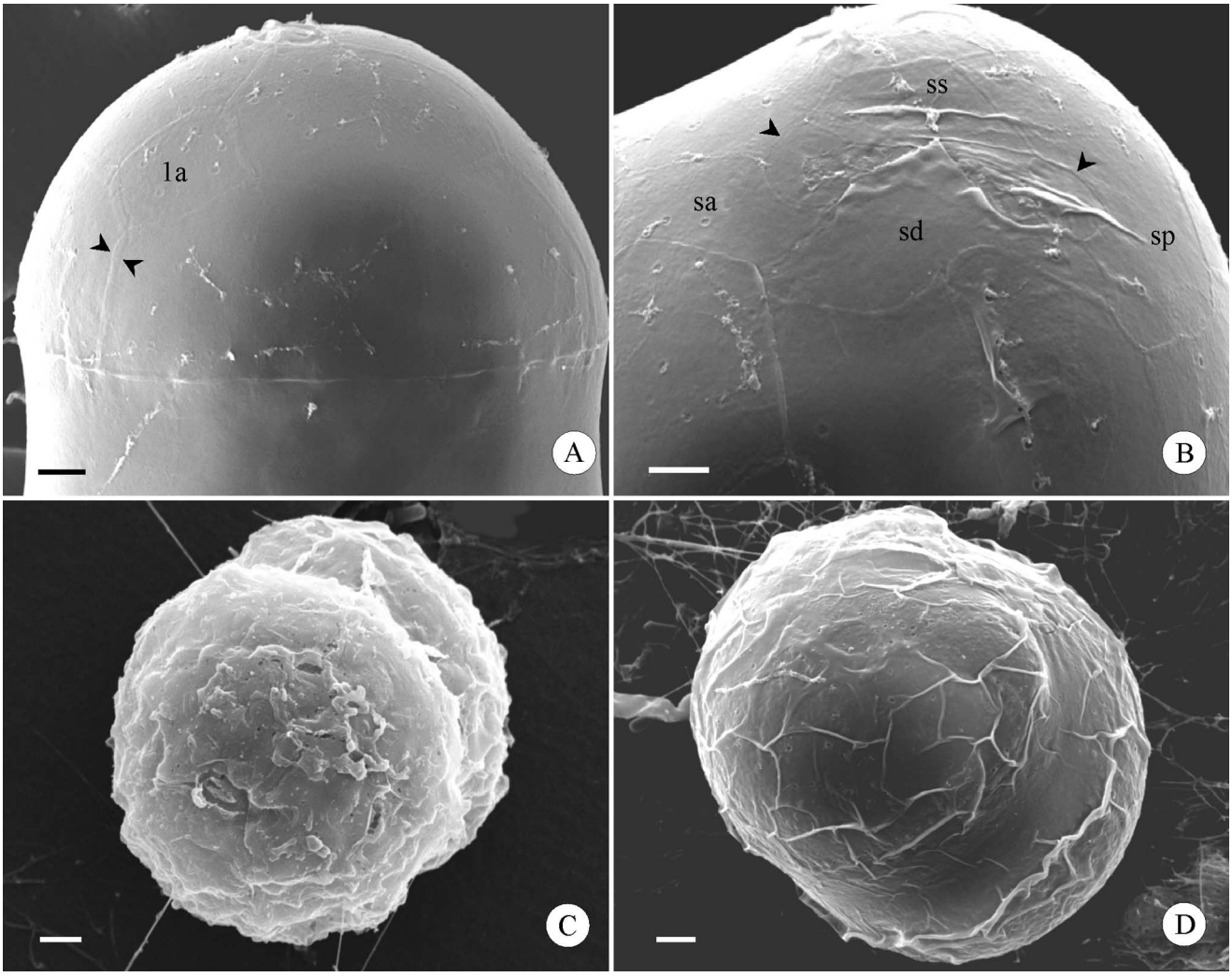

FIG. 1. SEM of Triton X-100 and ethanol-stripped *Pfiesteria shumwayae* zoospores. Scale bars, 1 μm. (A) Dorsal view of epitheca demonstrating characteristic diamond-shaped first intercalary plate (1a) and sutures with plate overlap (arrowheads). Note discernible pores. (B) Enlarged ventral view demonstrating six sulcal plates, including two new plates (arrowheads). (C) Apical view of postethanol-treated zoospore with outer membranes intact. (D) Antapical view of zoospore treated with Triton X-100 in seawater showing visible pores in plates; sutures are obscured by wrinkles.

enhanced visualization of the sulcus and the morphology and patterns of thecal pores, an advantage that the suture swelling methods have failed to provide (Glasgow et al. 2001).

Complete plate tabulations have so far been impossible to obtain for *P. shumwayae* because the outer membranes of the cells resist currently available stripping techniques (Glasgow et al. 2001, our unpublished data). Suture swelling methods have proven inadequate as well (Glasgow et al. 2001). *Pfiesteria shumwayae* has exceptionally thin thecal plates relative to the other small heterotrophic dinoflagellates examined, and repeated attempts to remove the outer membranes with ethanol treatments (20%–30%, final concentration) resulted in incomplete stripping and excessive damage to the cells as indicated by moderate to severe wrinkling of the plates. Lower ethanol concentrations provided better zoospore morphology but did not remove the outer membranes (our unpublished data). As a result, visualization of the sutures and generation of complete plate tabulations using ethanol as the stripping agent to remove the cell membranes has proven difficult. The use of nonethanol stripping protocols on *P. shumwayae* including enzymes (pronase, phospholipase A_2, trypsin, pepsin) or acids (hydrochloric and sulfuric) resulted either in encystment or excessive damage of the zoospores.

Triton X-100, along with Tween 20 and other nonionic commercial detergents, were considered as possible candidates for solubilizing the outer membranes of these dinoflagellates because these compounds remove membrane-bound proteins (Helenius and Simons 1975). Tween 20, or polyoxyethylenesorbitan monolaurate, successfully stripped zoospores of their

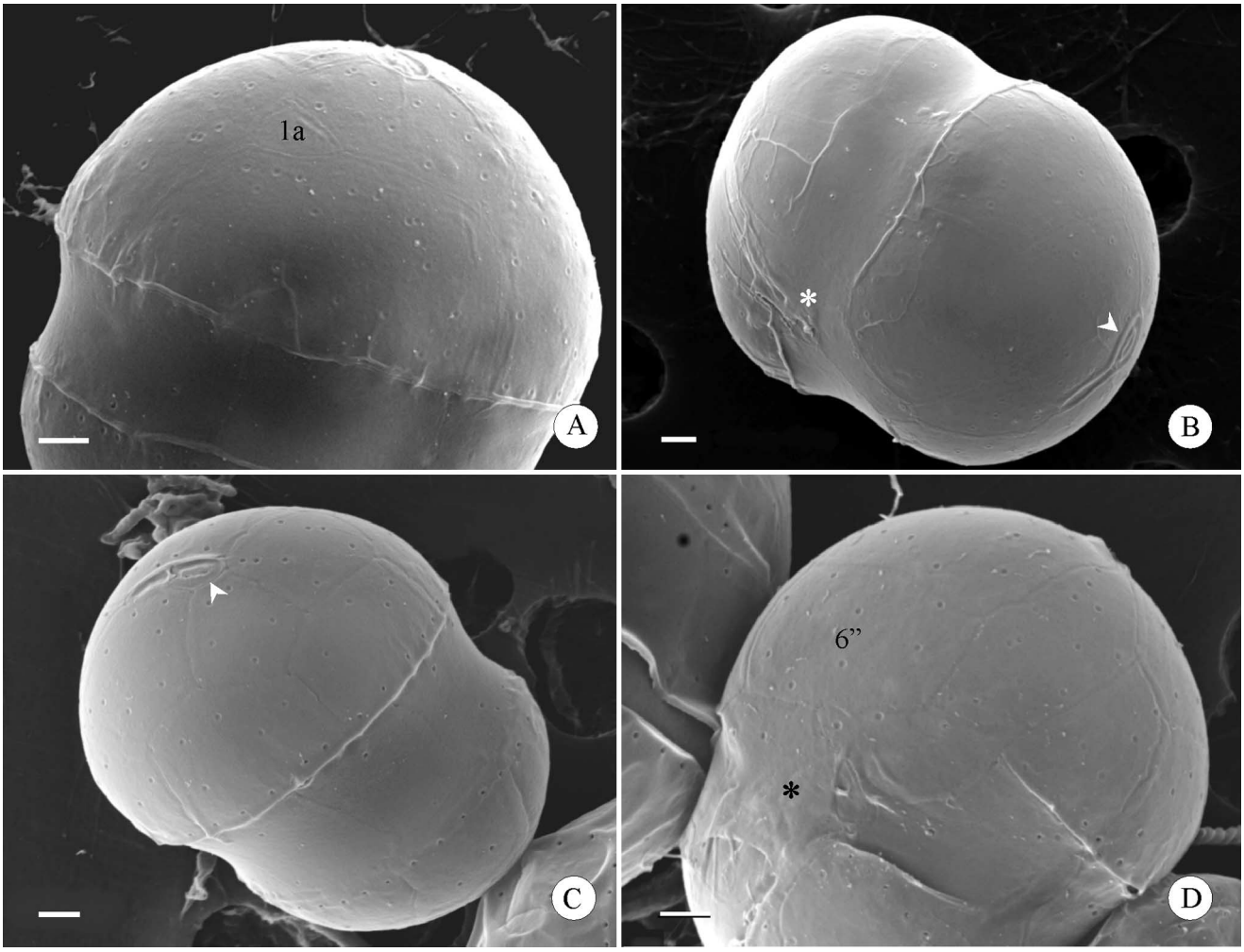

FIG. 2. SEM of Triton X-100 treated *Pfiesteria piscicida* (A, B) and a cryptoperidiniopsoid isolate (C, D). Scale bars, 1 μm. (A) Dorsal-apical view showing characteristic triangular intercalary (1a), plate sutures, and pore patterns. (B) Dextral-ventral view of swollen cell with flattened sulcus (*) and demonstrating plates of apical pore complex (arrowhead). (C) Sinister-dorsal view displaying plate sutures, prominent pore patterns, and apical pore complex (arrowhead). (D) Flattened sulcal region (*) demonstrating large degree of sulcal torsion, prominent sixth precingular plate (6"), and discernible sutures and pore patterns.

outer membranes, but the underlying plates were badly damaged making tabulation impossible. Triton X-100, or t-octylphenoxypolyethanol, is a gentle nonionic detergent also routinely used for various applications where it is necessary to break down cell membranes (Hayat 1981) and, in combination with other compounds, as a permeabilizer for immunofluorescent staining of cellular components in certain species of dinoflagellates and other phytoplankton (Lin and Carpenter 1996). Triton X-100, diluted with HPLC grade water, cleanly and consistently stripped the plasmalemma and outer layers of the amphiesmal vesicles off *P. shumwayae* and four other small heterotrophic dinoflagellates without inducing encystment. Cellulose plates remained intact and retained pore patterns, demonstrating that plates, and not suture remnants from the encystment process, were being visualized. Cells within a sample varied in condition and orientation and still required hours of analysis to collect all the micrographs necessary to generate a complete plate tabulation. However, the need for repeated modification of the stripping protocol was eliminated, and the process yielded large numbers of cells suitable for analysis, providing another great advantage over the ethanol protocol. The only constraint was that of zoospore size in that the more recently excysted zoospores resisted stripping or, in some species, the plates tended to wrinkle.

As a result of its superior stripping qualities, this Triton X-100 protocol has demonstrated its effectiveness by revealing one or more new plates within the sulcus of *P. shumwayae*, rather than the 4s plate tabulation previously reported (Glasgow et al. 2001, Fig. 1b). Thus, for *P. shumwayae*, and potentially for other species, this method allows elucidation of significant new taxonomic information and will be of great value in future taxonomic studies of the lightly armored dinoflagellates.

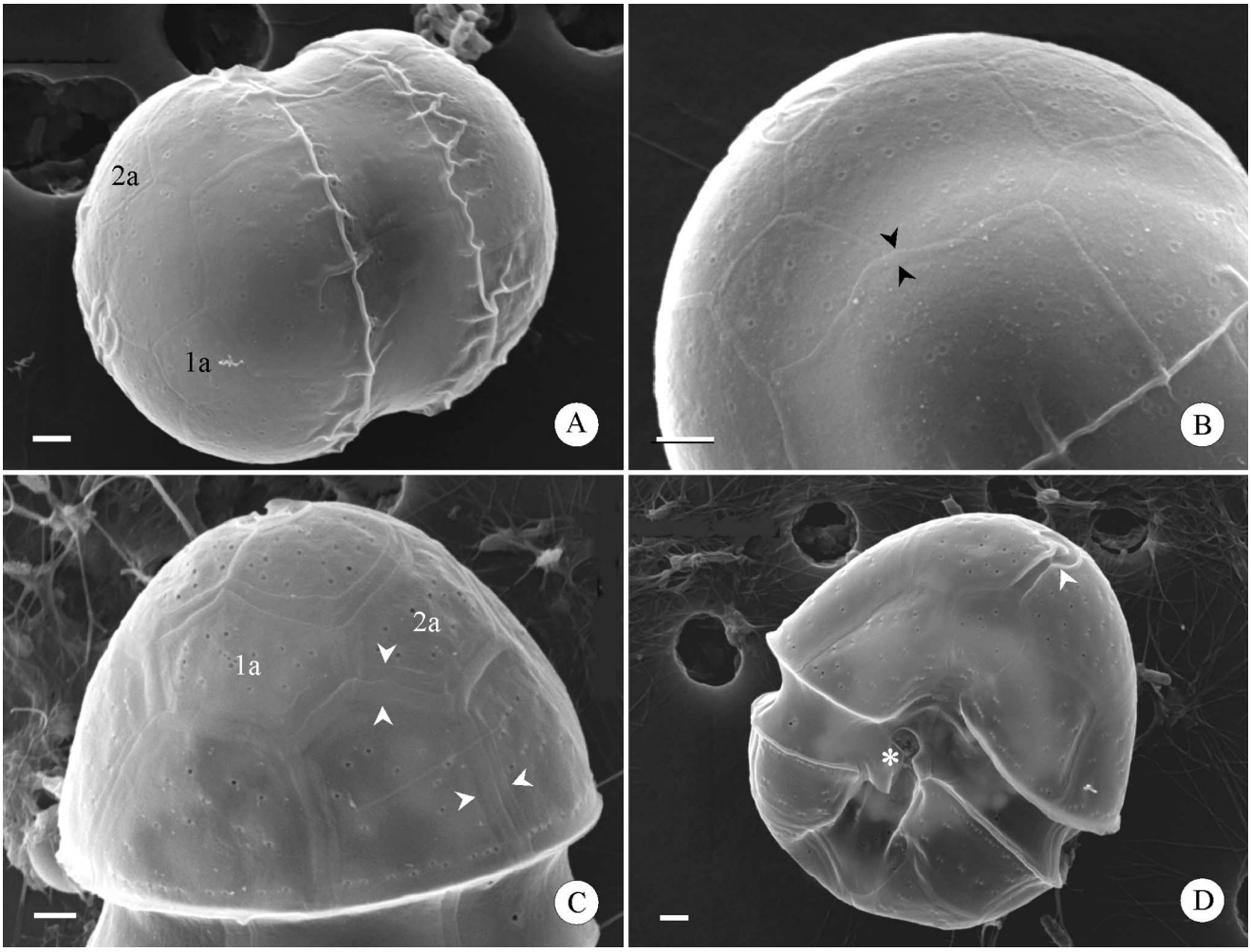

FIG. 3. SEM of Triton X-100 treated "Lucy" isolate (A, B) and "Shepherd's crook" isolate (C, D). Scale bars, 1 μm. (A) Sinister-dorsal view showing characteristic first and second diamond-shaped apical intercalaries (1a, 2a), plate sutures, and pore patterns. (B) Dorsal view demonstrating prominent sutures and plate growth patterns associated with large vegetative cells (arrowheads). (C) Dorsal view of older zoospore displaying prominent sutures and plate overlap (arrowheads) and characteristic "bow-tie" configuration of first and second apical intercalaries (1a, 2a). (D) Ventral view showing sulcus (*) and demonstrating distinctive "Shepherd's crook"-shaped apical pore complex (arrowhead).

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