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COMPARATIVE POLLEN MORPHOLOGY AND ULTRASTRUCTURE OF THE CALLITRICHACEAE[1]

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The Callitrichaceae are an aquatic family of dicots that include the single, geographically cosmopolitan genus *Callitriche*. *Callitriche* contains 40–50 terrestrial, amphibious, and obligately submersed species, and it is the only known genus in the plant kingdom with co-occurring aerial and hydrophilous pollination syndromes. Pollen morphology and ultrastructure were described for 13 *Callitriche* species using scanning electron and transmission electron microscopy. Representative taxa of each growth form were examined; these included three terrestrial species (*C. deflexa, C. peploides,* and *C. nuttallii*), nine amphibious species (*C. brutia, C. cophocarpa, C. cophocarpa-stagnalis* hybrid, *C. cribrosa, C. hamulata, C. heterophylla* var. *heterophylla, C. lusitanica, C. marginata,* and *C. trochlearis*), and one obligately submersed species (*C. truncata*). Of the amphibious taxa, *C. heterophylla* var. *heterophylla* and *C. trochlearis* had internal geitonogamy, a type of internal self-fertilization. Pollen from all taxa was spheroidal, small, intectate, and lacked well-defined apertures. Taxa primarily differed with respect to exine thickness, surface ornamentation, and the presence or absence of aperture-like regions. The pollen of terrestrial species, as well as that of *C. marginata,* had well-developed exines with thick sculptured and basal layers. In general, amphibious taxa produced pollen with distinct, but thinner, exines than that of terrestrial taxa. Pollen of the amphibious taxa with internal geitonogamy had a thicker basal layer than species without internal geitonogamy, whereas the overall exine was reduced in *C. hamulata* and absent in *C. brutia* and *C. lusitanica.* Pollen of the obligately submersed *C. truncata* also lacked an exine. These palynological data were correlated with growth habits and related pollination biologies, as well as with phylogenetic interpretations of Callitrichaceae. Exine reduction or loss has evolved at least twice in the family, and it is associated with aneuploid reduction in chromosome number.

Key words: aquatic plants; Callitrichaceae; *Callitriche*; hydrophily; morphology; pollen; pollination; ultrastructure.

The Callitrichaceae (water starworts) are an aquatic family of dicots that include the single genus *Callitriche*. *Callitriche* contains 40–50 terrestrial, amphibious, and obligately submersed species (Philbrick and Jansen, 1991). Plants of all species of *Callitriche* are small, herbaceous, and limp-stemmed. Terrestrial species occur in seasonally wet areas, have prostrate stems that often grow in dense mats, and produce aerial flowers. Plants of amphibious species can grow either terrestrially, with submersed stems that reach to the water surface and produce floating rosettes of leaves, or as completely submersed stems that lack floating leaves. Several amphibious species lack the terrestrial growth form. Some amphibious species can produce flowers on both aerial and submersed stems, whereas others produce only aerial flowers. Obligately submersed species grow and flower entirely underwater (Philbrick and Osborn, 1994).

Species of *Callitriche* are monoecious and have reduced flowers that lack petals and sepals (Philbrick and Anderson, 1992). Pollination systems are diverse in *Callitriche* (Philbrick and Anderson, 1992, and references therein), although the details of pollination remain unknown for most species. Both aerial (anemophilous) and aquatic (hydrophilous) pollination occur, and the type of pollination is often associated with growth habit. Anemophily is the most likely pollination system of both terrestrial species and terrestrial growth forms of amphibious species. Two types of water pollination have been reported in the genus: epihydrophily (pollination at the water surface) and hypohydrophily (pollination below the water surface). Epihydrophily has been reported for various amphibious species, although supporting evidence is usually anecdotal (see discussion in Philbrick and Anderson, 1992). Hypohydrophily occurs in the obligately submersed species, *C. hermaphroditica* L. (Philbrick, 1993) and *C. truncata* Gussone.

Geitonogamy (pollination among flowers on one plant) characterizes many species of all three growth habits. Furthermore, seven amphibious species have internal geitonogamy, a type of internal self-fertilization. Here, pollen grains germinate within indehisced anthers and the pollen tubes grow through vegetative tissues to nearby pistillate flowers where fertilization occurs (Philbrick, 1984; Philbrick and Anderson, 1992; Philbrick and Bernardello, 1992). Internal geitonogamy occurs in both aerial and submersed flowers of these species.

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TABLE 1. Collection information for the 13 *Callitriche* taxa studied.

Taxon	Sampling locality	Voucher data[a]	Growth information
Terrestrial taxa (including *C. marginata*)			
C. deflexa A. Br. ex Hegelm.	Alabama	CTP 3223	terrestrial form
C. nuttallii Torrey	Alabama	CTP 2128	terrestrial form
	Alabama	CTP 2138A	terrestrial form
C. peploides Nutt.	Alabama	CTP 2127	terrestrial form
	Alabama	CTP 2137	terrestrial form
	Alabama	CTP 2138	terrestrial form
C. marginata Torrey	California	CTP 2200	floating rosettes (no flowers when submersed)
	California	CTP 2204	floating rosettes (no flowers when submersed)
	California	CTP 2210	floating rosettes (no flowers when submersed)
Amphibious taxa with internal geitonogamy			
C. heterophylla Pursh v. *heterophylla*	Connecticut	CTP 4611	terrestrial form
C. trochlearis Fassett	California	CTP 2043	amphibious form
Amphibious taxa without internal geitonogamy			
C. brutia Petagna	Spain	CTP 4568	submersed stems
	Spain	CTP 4603	submersed and floating rosette stems
C. cophocarpa Sendter	Sweden	CTP 4561	floating rosettes (no flowers when submersed)
	Sweden	CTP 4563	floating rosettes (no flowers when submersed)
C. cophocarpa-stagnalis hybrid	Sweden	CTP 4562	terrestrial form
C. cribrosa Schotsman	Spain	CTP 4588	amphibious form
C. hamulata Kütz.	England	CTP 4539	floating rosettes (aerial flowers)
	England	CTP 4550	submersed plants
	England	CTP 4553	submersed plants
	England	CTP 4556	submersed and floating rosettes
Obligately Submersed taxa (including *C. lusitanica*)			
C. lusitanica Schotsman	Spain	CTP 4597A	submersed plants (some floating rosettes)
C. truncata Gussone	Spain	CTP 4595	submersed plants
	Spain	CTP 4601	submersed plants

[a] C. Thomas Philbrick (CTP) collection numbers.

Uniformity in overall floral structure among species of *Callitriche* is interesting given the range of pollination systems that occurs in the genus. Many authors have discussed the high degree of floral diversity that occurs among closely related aerial- and underwater-flowering species in other angiosperm groups (Arber, 1920; Sculthorpe, 1967; Tomlinson, 1982; Dahlgren and Rasmussen, 1983; Philbrick, 1991). Perhaps the most dramatic difference between aerial-flowering and hydrophilous species pertains to the pollen. In particular, pollen of many hydrophilous taxa has an elongate shape (e.g., Pettitt, 1981; Cox, 1988) and reduced exine thickness (e.g., Osborn and Philbrick, 1994; Philbrick and Les, 1996). However, only limited data about pollen structure, especially ultrastructure, are known for *Callitriche.*

Most information about *Callitriche* pollen has been gained from studies with light microscopy (e.g., Erdtman, 1952; Moar, 1960; Díez, Talavera, and Garcia-Murillo, 1988) and scanning electron microscopy (SEM; Díez, Talavera, and Garcia-Murillo, 1988). Only three published studies used transmission electron microscopy (TEM) to describe pollen wall structure in the family (Martinsson, 1993; Osborn and Philbrick, 1994; Philbrick and Osborn, 1994). These investigations demonstrated that pollen of terrestrial and amphibious taxa of *Callitriche* had a well-defined exine, whereas that of the obligately submersed species *C. hermaphroditica* lacked an exine. However, *C. hermaphroditica* has two growth forms. Exineless pollen characterized the perennial form, whereas a rudimentary exine was present in pollen of the annual form (Osborn and Philbrick, 1994). A well-developed, two-layered intine is present in both growth forms.

Despite the observations of Martinsson 1993, Osborn and Philbrick 1994, and Philbrick and Osborn 1994, these authors collectively examined the pollen from only nine taxa, including one terrestrial, seven amphibious, and one obligately submersed species. In the current study, pollen morphology and ultrastructure of 13 *Callitriche* species were investigated using SEM and TEM. Representative taxa of each growth form were examined, including three terrestrial species (*C. deflexa* A. Br. ex Hegelm., *C. nuttallii* Torrey, and *C. peploides* Nutt.), nine amphibious species (*C. brutia* Petagna, *C. cophocarpa* Sendter, *C. cophocarpa-stagnalis* hybrid, *C. cribrosa* Schotsman, *C.*

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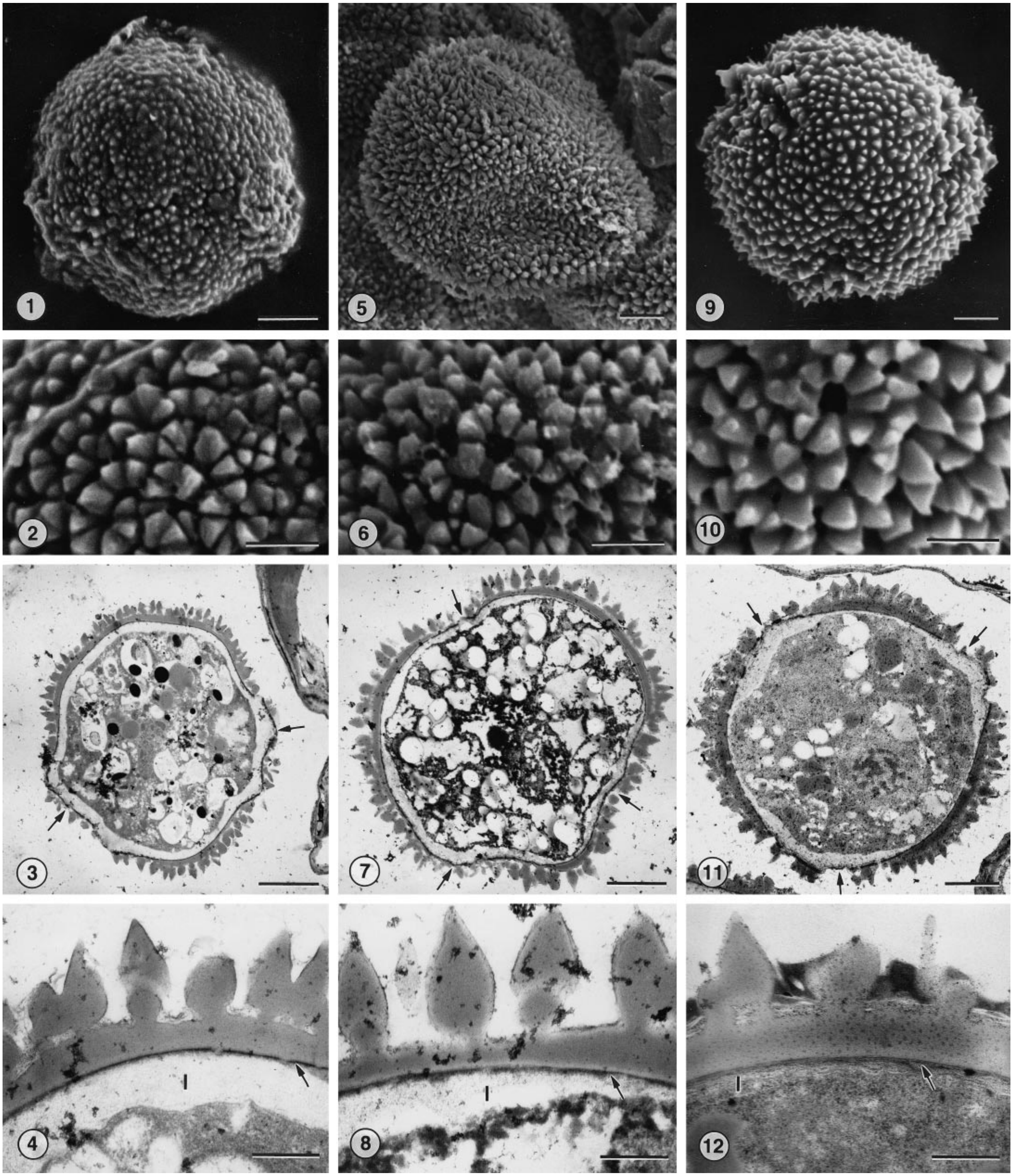
Figs. 1–12. Terrestrial taxa. *Callitriche deflexa* (Figs. 1–4). **1.** Pollen grain showing spheroidal shape, microgemmate surface ornamentation, and three aperture-like regions. Bar = 2 μm. **2.** Detail of pollen surface with pyramidal-shaped microgemmae in polygonal patterns. Bar = 1 μm. **3.** Transverse section through pollen grain showing thin exinous basal layer in two aperture-like regions (arrows). Bar = 3 μm. **4.** Transverse section of pollen wall showing sexinous microgemmae, thick basal layer, electron-dense line (arrow), and intine (I). Bar = 0.5 μm. *Callitriche nuttallii* (Figs. 5–8). **5.** Pollen grain showing spheroidal shape and microgemmate surface ornamentation. Bar = 2 μm. **6.** Detail of pollen surface showing pointed microgemmae in polygonal patterns. Bar = 1 μm. **7.** Transverse section through pollen grain showing thin exinous basal layer and thicker



intine in the three aperture-like regions (arrows). Bar = 3 μm. **8.** Transverse section of pollen wall showing sexinous microgemmae, thick basal layer, electron-dense line (arrow), and intine (I). Bar = 0.5 μm. *Callitriche peploides* (Figs. 9–12). **9.** Pollen grain showing spheroidal shape and microgemmate surface ornamentation with three aperture-like regions. Bar = 2 μm. **10.** Detail of pollen surface showing pointed microgemmae in polygonal patterns. Bar = 1 μm. **11.** Transverse section through pollen grain showing thin exinous basal layer and thicker intine in the three aperture-like regions (arrows). Bar = 2 μm. **12.** Transverse section of pollen wall showing sexinous microgemmae, thick basal layer, electron-dense line (arrow), and thin intine (I). Bar = 0.5 μm.

TABLE 2. Selected palynological characters for the 18 *Callitriche* taxa studied with combined SEM and TEM. Sample sizes are given in parentheses.

Taxon	Grain diameter [X ± SD (μm)]	Exine thickness [X ± SD (μm)]	Sexine thickness [X ± SD (μm)]	Nexine thickness [X ± SD (μm)]	Intine thickness [X ± SD (μm)]	Microgemmae width [X ± SD (μm)]	Microgemmae shape	Apertural condition
Terrestrial taxa (including *C. marginata*)								
C. deflexa	12.9 ± 0.9 (42)	1.06 ± 0.09 (9)	0.71 ± 0.07 (9)	0.34 ± 0.05 (9)	0.45 ± 0.05 (10)	0.38 ± 0.04 (10)	pointed	aperture-like
C. nuttallii	16.8 ± 1.5 (31)	1.25 ± 0.08 (9)	0.97 ± 0.05 (9)	0.41 ± 0.03 (7)	0.35 ± 0.06 (10)	0.48 ± 0.04 (9)	pointed	aperture-like
C. peploides	15.6 ± 1.6 (40)	1.11 ± 0.11 (18)	0.74 ± 0.12 (18)	0.37 ± 0.09 (18)	0.15 ± 0.04 (13)	0.42 ± 0.05 (23)	pointed	aperture-like
C. marginata	16.4 ± 1.4 (45)	1.18 ± 0.12 (16)	0.90 ± 0.13 (16)	0.27 ± 0.07 (16)	0.56 ± 0.09 (22)	0.51 ± 0.07 (20)	pointed	aperture-like
Amphibious taxa with internal geitonogamy								
C. heterophylla v. *heterophylla*	16.0 ± 1.0 (57)	0.73 ± 0.14 (6)	0.47 ± 0.09 (6)	0.26 ± 0.12 (6)	0.27 ± 0.03 (6)	0.50 ± 0.07 (15)	round	aperture-like
C. heterophylla v. *bolanderi*[a]	21.2	0.76	0.61	0.15	0.14	0.54	round	aperture-like
C. trochlearis	22.3 ± 1.8 (54)	0.74 ± 0.12 (12)	0.60 ± 0.09 (12)	0.18 ± 0.07 (12)	0.55 ± 0.06 (13)	0.55 ± 0.07 (12)	round	aperture-like
C. verna (= *C. palustris*)[b]	19.2	0.51	0.38	0.13	0.21	0.42	round	aperture-like
Amphibious taxa without internal geitonogamy								
C. cophocarpa	23.0 ± 2.1 (92)	0.90 ± 0.09 (4)	0.72 ± 0.08 (4)	0.19 ± 0.06 (4)	0.56 ± 0.06 (6)	0.61 ± 0.08 (10)	round	inaperturate
C. cophocarpa-stagnalis hybrid	23.3 ± 2.7 (50)	0.51 ± 0.10 (11)	0.42 ± 0.02 (5)	0.10 ± 0.04 (5)	0.52 ± 0.13 (12)	0.44 ± 0.03 (8)	round	inaperturate
C. cribrosa	13.9 ± 1.2 (7)	0.80 ± 0.08 (8)	0.69 ± 0.13 (8)	0.12 ± 0.03 (8)	0.13 ± 0.05 (9)	0.54 ± 0.08 (6)	round w/ spines	inaperturate
C. platycarpa[b]	25.3	0.83	0.63	0.20	0.65	0.38	round	aperture-like
C. stagnalis[a]	19.7	0.83	0.71	0.12	0.30	0.51	pointed	inaperturate
C. hamulata (submersed)	21.5 ± 1.9* (44)	0.19 ± 0.04* (16)	0.13 ± 0.02* (13)	0.07 ± 0.03* (13)	0.67 ± 0.09* (16)	0.15 ± 0.02* (8)	round	inaperturate
C. hamulata (aerial)	16.1 ± 1.5 (41)	0.33 ± 0.06 (18)	0.20 ± 0.03 (18)	0.14 ± 0.05 (18)	0.42 ± 0.10 (18)	0.22 ± 0.04 (24)	round	inaperturate
C. brutia	24.9 ± 3.4 (38)	0.00 (26)	0.00 (26)	0.00 (26)	0.35 ± 0.09 (26)	0.00 (26)	absent	inaperturate
Obligately submersed taxa (including *C. lusitanica*)								
C. lusitanica	17.3 ± 1.9 (44)	0.00 (31)	0.00 (31)	0.00 (31)	0.12 ± 0.02 (31)	0.00 (31)	absent	inaperturate
C. truncata	17.9 ± 1.7 (47)	0.00 (45)	0.00 (45)	0.00 (45)	0.10 ± 0.05 (45)	0.00 (45)	absent	inaperturate
C. hermaphroditica (perennial)[a]	20.1	0.00	0.00	0.00	0.32	0.00	absent	inaperturate
C. hermaphroditica (annual)[a]	—	0.05–0.16	0.05–0.16	0.00	0.32	0.00	absent	inaperturate

* Indicates statistically significant differences between palynological characters of submersed vs. aerial flowers of *C. hamulata* ($\alpha = 0.05$; *t* test).

[a] Data from Osborn and Philbrick (1994) and Philbrick and Osborn (1994); sample sizes not reported.

[b] Data from Martinsson (1993); sample sizes not reported.

hamulata Kütz., *C. heterophylla* Pursh var. *heterophylla*, *C. lusitanica* Schotsman, *C. marginata* Torrey, and *C. trochlearis* Fassett), and one obligately submersed species (*C. truncata*). Of the nine amphibious taxa, *C. heterophylla* var. *heterophylla* and *C. trochlearis* have internal geitonogamy.

The primary objectives of this paper were to describe pollen structure from the 13 species of *Callitriche* and to correlate new palynological data with growth habits and related pollination biologies of the taxa. Furthermore, these data, particularly information on exine thickness, were interpreted in a phylogenetic context.

MATERIALS AND METHODS

Plants of the 13 *Callitriche* species were collected from sites in Europe and North America (Table 1). Two growth forms of the amphibious *C. hamulata* were collected, those with aerial and those with submersed flowers. Voucher specimens were deposited at the Western Connecticut State University herbarium.

Whole plants were fixed in 3% glutaraldehyde (in 0.2 mol/L phosphate buffer; pH 7.4) for 24 h and washed in buffer at least three times. Plants of *Callitriche trochlearis* were initially fixed in formalin-acetic acid-ethanol and then transferred to glutaraldehyde. Anthers were dissected from the plants, postfixed in 1% buffered osmium tetroxide for 3–6 h, washed in buffer at least three times, and then dehydrated in a graded ethanol series.

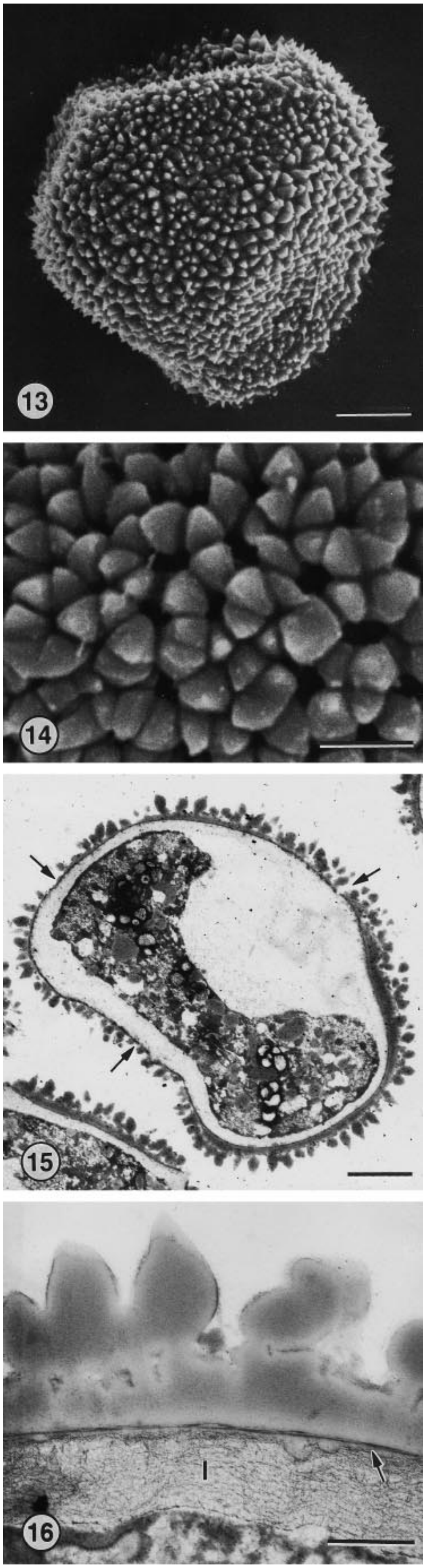
Three to five dehydrated anthers from different plants of each species were gradually infiltrated and embedded in Spurr epoxy resin. To identify appropriate specimens for ultrastructural studies (e.g., those with mature pollen), individual anthers were thick-sectioned on an ultramicrotome with glass or diamond knives. Thick sections (850 nm) were stained with either 10% toluidine blue O or Richardson's stain (50% methylene blue/50% Azure II) and examined on an Olympus BHS Compound Light Microscope.

To study pollen wall ultrastructure, anthers were thin-sectioned with glass or diamond knives. Thin sections (90–100 nm) were collected on 1 × 2 mm copper slot grids and dried onto formvar support films (Rowley and Moran, 1975). Grids were stained with 1% potassium permanganate (1 min), 1% uranyl acetate (15 min), and lead citrate (8 min; Venable and Coggeshall, 1965) and then examined and imaged using a JEOL JEM-100SX transmission electron microscope at 60–80 kV.

To study pollen surface morphology, dehydrated anthers were critical point dried. To isolate individual pollen grains, dried anthers were macerated using a syringe needle and mounted onto aluminum stubs with double-sided adhesive tape, which had been secured to the stubs with colloidal graphite. Stubs were sputter-coated with gold-palladium, and pollen was examined and imaged using a JEOL JSM-6100 scanning electron microscope at 5 kV.

RESULTS

Palynological data are presented with the individual taxa grouped according to growth habit. *Callitriche mar-*



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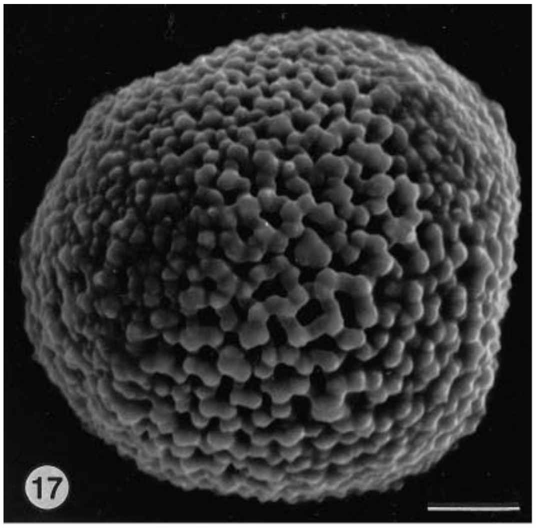
Figs. 13–16. *Callitriche marginata*. **13.** Pollen grain showing spheroidal to subtriangular shape, microgemmate surface ornamentation, and three aperture-like regions. Bar = 3 μm. **14.** Detail of pollen surface showing pyramidal-shaped microgemmae in polygonal patterns. Bar = 1 μm. **15.** Transverse section through pollen grain showing thin exinous basal layer in the three aperture-like regions (arrows); note that the protoplasmic contents are partially plasmolyzed. Bar = 3 μm. **16.** Transverse section of pollen wall showing sexinous microgemmae, thick basal layer, electron-dense line (arrow), and intine (I). Bar = 0.5 μm.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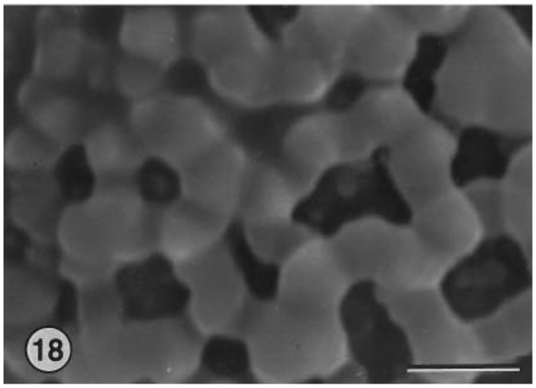
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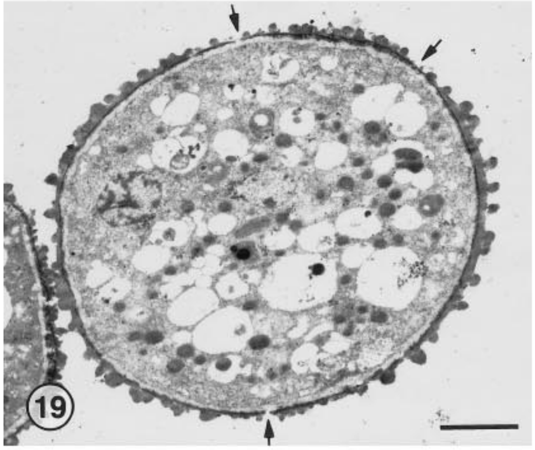
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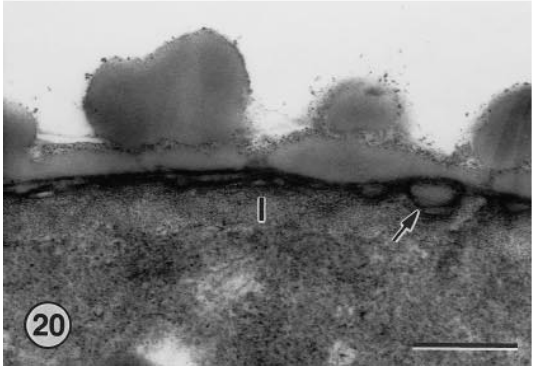
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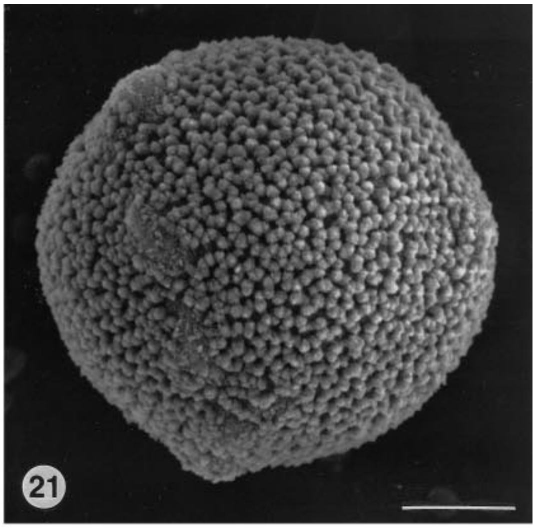
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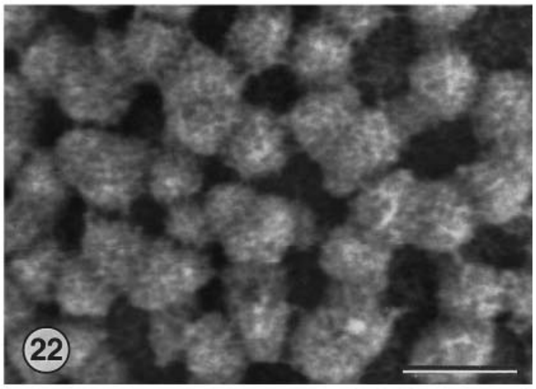
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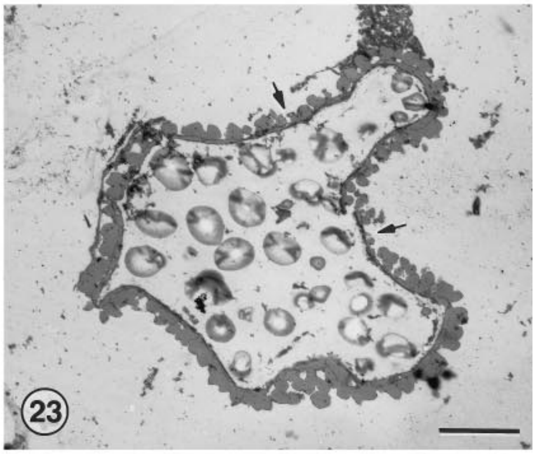
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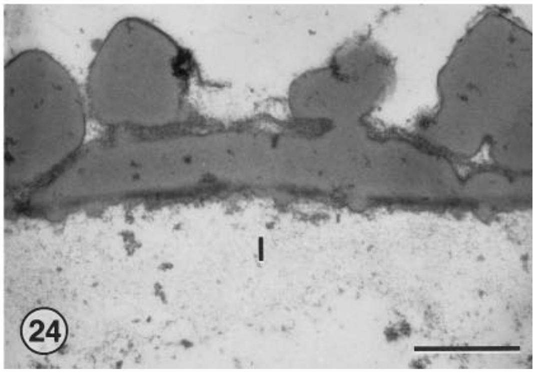
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ginata is grouped with the terrestrial taxa and *C. lusitanica* is grouped with the obligately submersed taxa, because of phylogenetic interpretations of these two amphibious species (see below; Philbrick and Jansen, 1991; Philbrick and Les, 1995). The palynological features for each species are described below; these, as well as additional characters, and statistical information are summarized in Table 2.

Terrestrial taxa (including Callitriche marginata)—*Callitriche deflexa*—Pollen spheroidal; 12.9 μm in diameter with three aperture-like areas (Fig. 1); surface ornamentation microgemmate with sculptural elements organized in polygonal patterns (Figs. 1–2); individual microgemmae pyramidal with pointed tips (Fig. 2); exine intectate and 1.06 μm thick; sculptural elements with some lateral fusion (Figs. 3–4); nexine 0.34 μm thick and separated from intine by thin, electron-dense line (Fig. 4); nexine thinner and intine thicker in aperture-like regions (Fig. 3).

Callitriche nuttallii—Pollen spheroidal; 16.8 μm in diameter with three aperture-like areas (Fig. 5); surface ornamentation microgemmate with sculptural elements in polygonal patterns (Figs. 5–6); individual microgemmae pyramidal-shaped with pointed tips (Fig. 6); exine intectate and 1.25 μm thick; sexinous microgemmae with lateral fusion near the bases (Figs. 7–8); nexine 0.41 μm thick and separated from intine by thin, electron-dense line (Fig. 8); nexine thinner in aperture-like regions (Fig. 7).

Callitriche peploides—Pollen spheroidal; 15.6 μm in diameter with three aperture-like areas (Fig. 9); surface ornamentation microgemmate with sculptural elements in polygonal patterns (Figs. 9–10); individual microgemmae pyramidal-shaped with pointed tips (Fig. 10); exine intectate and 1.11 μm thick; sexinous microgemmae with some lateral fusion near the bases (Figs. 11–12); nexine 0.37 μm thick and separated from intine by thin, electron-dense line (Fig. 12); nexine thinner and intine thicker in aperture-like regions (Fig. 11).

Callitriche marginata—Pollen spheroidal; 16.4 μm in diameter with three aperture-like areas (Fig. 13); surface ornamentation microgemmate with sculptural elements in polygonal patterns (Figs. 13–14); individual microgemmae mostly pyramidal-shaped and pointed (Fig. 14); exine intectate and 1.18 μm thick; sexinous microgemmae with lateral fusion near the bases (Figs. 15–16); nexine 0.27 μm thick and separated from intine by thin, electron-dense line (Fig. 16); some exine material extends into intine as globular protrusions (Fig. 16); nexine thinner in aperture-like regions (Fig. 15).

Amphibious taxa with internal geitonogamy—*Callitriche heterophylla var. heterophylla*—Pollen spheroidal; 16.0 μm in diameter with three aperture-like areas (Fig. 17); surface ornamentation microgemmate with sculptural elements in polygonal to reticulate patterns (Figs. 17–18); individual microgemmae typically round (Figs. 17–18); exine intectate and 0.73 μm thick; many sexinous sculptural elements laterally fused (Figs. 19–20); nexine 0.26 μm thick and separated from intine by thin, electron-dense line (Fig. 20); some globular exinous elements protrude into intine (Fig. 20); nexine thinner in aperture-like regions (Fig. 19).

Callitriche trochlearis—Pollen spheroidal; 22.3 μm in diameter with three aperture-like areas (Fig. 21); surface ornamentation microgemmate with sculptural elements in polygonal patterns (Fig. 22); individual microgemmae typically round and covered with a thread-like material (Figs. 22, 24); exine intectate and 0.74 μm thick; many sexinous sculptural elements laterally fused (Figs. 23–24); nexine unevenly thick, averaging 0.18 μm, and separated from intine by thin, electron-dense line (Fig. 24); some globular exinous elements protrude into intine (Fig. 24); nexine thinner in aperture-like regions (Fig. 23).

Amphibious taxa without internal geitonogamy—*Taxa with distinct exines: Callitriche cophocarpa*—Pollen spheroidal; 23.0 μm in diameter; inaperturate (Fig. 25); surface ornamentation microgemmate with sculptural elements in polygonal patterns (Figs. 25–26); individual microgemmae mostly round, some pyramidal-shaped (Figs. 25–26); exine intectate and 0.90 μm thick; many sexinous sculptural elements laterally fused (Figs. 27–28); nexine unevenly thick, averaging 0.19 μm, and slightly thicker under sculptural elements (Fig. 28); electron-dense line separating nexine from intine present, but less distinct than in other taxa (Fig. 28).

Callitriche cophocarpa-stagnalis hybrid—Pollen spheroidal; 23.3 μm in diameter; inaperturate (Fig. 29); surface ornamentation microgemmate with sculptural elements in slight polygonal patterns; individual microgemmae round (Figs. 29–30); exine intectate and 0.51 μm thick; many sexinous sculptural elements laterally fused at bases (Figs. 31–32); nexine unevenly thick, averaging 0.10 μm, and slightly thicker under sculptural elements (Fig. 32); electron-dense line separating nexine from intine not distinct (Fig. 32).

Callitriche cribrosa—Pollen spheroidal; 13.9 μm in diameter; inaperturate (Fig. 33); surface ornamentation microgemmate with sculptural elements in polygonal pat-

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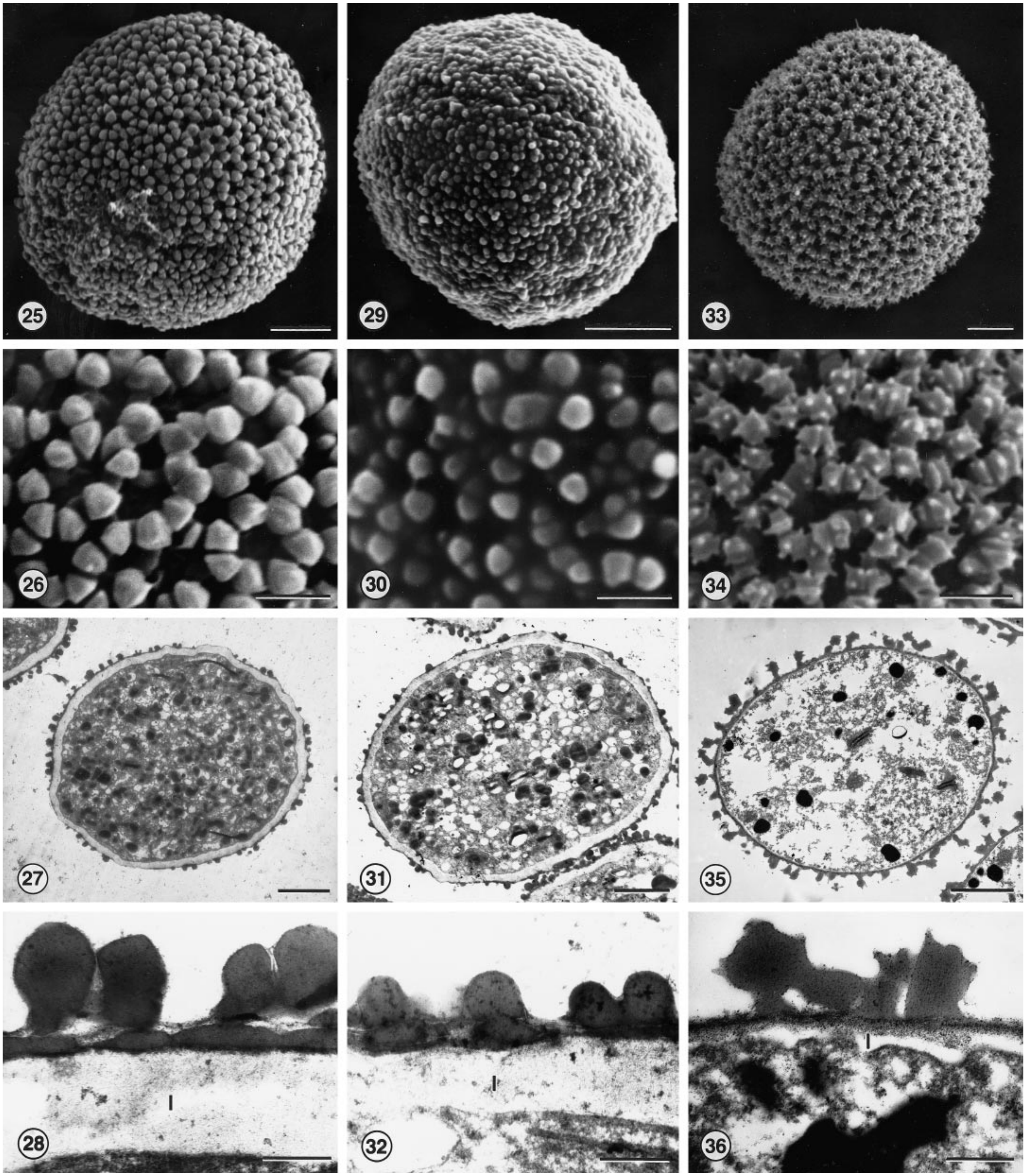
Figs. 17–24. Amphibious taxa with internal geitonogamy. *Callitriche heterophylla* var. *heterophylla* (Figs. 17–20). **17.** Pollen grain showing spheroidal shape and microgemmate surface ornamentation. Bar = 3 μm. **18.** Detail of pollen surface showing fused microgemmae in polygonal to reticulate patterns. Bar = 1 μm. **19.** Transverse section through pollen grain showing thin exinous basal layer and thicker intine in the three aperture-like regions (arrows). Bar = 3 μm. **20.** Transverse section of pollen wall showing sexinous microgemmae and thick basal layer; note the globular exine protrusions (arrow) that penetrate the intine (I). Bar = 0.5 μm. *Callitriche trochlearis* (Figs. 21–24). **21.** Pollen grain showing spheroidal shape and microgemmate surface ornamentation. Bar = 5 μm. **22.** Detail of pollen surface showing round microgemmae covered by a thread-like material. Bar = 1 μm. **23.** Transverse section through pollen grain showing thin exinous basal layer in two aperture-like regions (arrows). Protoplasmic contents and intine are not well-preserved in this grain due to initial fixation in formalin-acetic acid-ethanol rather than glutaraldehyde. Bar = 3 μm. **24.** Transverse section of pollen wall showing sexinous microgemmae, thick basal layer, and intine (I). Bar = 0.5 μm.



Figs. 25–36. Amphibious taxa without internal geitonogamy. *Callitriche cophocarpa* (Figs. 25–28). **25.** Pollen grain showing spheroidal shape and microgemmate surface ornamentation. Bar = 3 μm. **26.** Detail of pollen surface showing round microgemmae in polygonal patterns. Bar = 1 μm. **27.** Transverse section through pollen grain showing uniformly thin basal layer. Bar = 5 μm. **28.** Transverse section of pollen wall showing sexinous microgemmae, reduced basal layer, and intine (I). Bar = 0.5 μm. *Callitriche cophocarpa-stagnalis* hybrid (Figs. 29–32). **29.** Pollen grain showing spheroidal shape and microgemmate surface ornamentation. Bar = 5 μm. **30.** Detail of pollen surface showing round microgemmae in polygonal patterns. Bar = 1 μm. **31.** Transverse section through pollen grain showing uniformly thin basal layer. Bar = 4 μm. **32.** Transverse section of pollen wall showing sexinous microgemmae, reduced basal layer, and intine (I). Bar = 0.5 μm. *Callitriche cribrosa* (Figs. 33–36).

terns; individual sculptural elements rounded with apical microechinae (Figs. 33–34); exine intectate and 0.80 μm thick; sexinous sculptural elements laterally fused; nexine unevenly thick, averaging 0.12 μm (Figs. 35–36); electron-dense line separating nexine from intine not distinct (Fig. 36).

Taxa with reduced or no exines—Callitriche hamulata—Pollen of both aerial and submersed flowers spheroidal and inaperturate (Fig. 37); aerial pollen 16.1 μm in diameter; submersed pollen significantly larger, 21.5 μm in diameter (Table 2); surface ornamentation of both pollen types consisting of small, granular microgemmae (Figs. 37–38); individual microgemmae distinct and with some lateral fusion; exine thin and intectate in both pollen types; exine significantly thicker in aerial pollen, 0.33 μm thick, and 0.19 μm thick in submersed pollen (Table 2); nexine uniformly thin in both pollen types (Figs. 39–40); nexine significantly thicker in aerial pollen, 0.14 μm thick, and 0.07 μm thick in submersed pollen (Table 2); intine well-developed, thick, and fibrillar in both pollen types (Figs. 39–40); intine 0.42 μm thick in aerial pollen and significantly thicker in submersed pollen, 0.67 μm thick (Table 2).

Callitriche brutia—Pollen spheroidal; 24.9 μm in diameter; inaperturate (Fig. 41); surface ornamentation smooth to slightly ‘‘wrinkled’’ (Figs. 41–42); exine absent (Figs. 43–44); intine well-developed, unevenly thick, averaging 0.35 μm, and fibrillar (Fig. 44).

Obligately submersed taxa (including Callitriche lusitanica)—*Callitriche lusitanica*—Pollen spheroidal; 17.3 μm in diameter; inaperturate (Fig. 45); surface ornamentation smooth to ‘‘wrinkled,’’ due to slight desiccation of the grains during SEM preparation (Figs. 45–46); exine absent (Figs. 47–48); intine well-developed, unevenly thick, averaging 0.12 μm, and two-layered (Fig. 48); outer intine layer more densely fibrillar (Fig. 48).

Callitriche truncata—Pollen spheroidal; 17.9 μm in diameter; inaperturate (Fig. 49); surface smooth, without ornamentation (Figs. 49–50); exine absent (Figs. 51–52); intine well-developed, unevenly thick, averaging 0.10 μm, and fibrillar (Fig. 52).

DISCUSSION

Palynological characters—Pollen from all 13 *Callitriche* taxa in the present study was spheroidal, small, intectate, and lacked well-defined apertures. Taxa primarily differed with respect to surface ornamentation, exine thickness, and the presence or absence of aperture-like regions.

Although *Callitriche* pollen has been characterized as having true apertures (e.g., Erdtman, 1952; Moar, 1960; Díez, Talavera, and Garcia-Murillo, 1988), these have been described as either weakly defined or difficult to detect in transmitted light. In the present study, pollen from all 13 species lacked bona fide apertures, although aperture-like areas were identified in six species (*C. deflexa, C. nuttallii, C. peploides, C. marginata, C. trochlearis,* and *C. heterophylla* var. *heterophylla*). In addition, aperture-like areas have previously been described in four other species, including *C. heterophylla* var. *bolanderi* (Hegelm.) Fassett (Osborn and Philbrick, 1994), *C. verna* L. (= *C. palustris*), *C. cophocarpa,* and *C. platycarpa* Kütz. (Martinsson, 1993). Although Martinsson (1993) reported ‘‘aperturoid areas,’’ or leptomata, in the pollen of *C. cophocarpa* using SEM and light microscopy, this species was determined to be inaperturate in the present study. Characterization of the apertural condition in *Callitriche* is difficult even with electron microscopy. Using SEM, aperture-like areas can be distinguished by irregular and less dense spacing of the sculptural elements; however, this character is not consistent. In our opinion, TEM is more revealing, as the aperture-like areas can be identified by thin regions in the basal layer (nexine).

Correlations with growth habit and pollination biology—Anemophily and ‘‘typical’’ geitonogamy occur in terrestrial species (Arber, 1920; Moar, 1960; Sculthorpe, 1967; Philbrick and Anderson, 1992). All three terrestrial species (*C. deflexa, C. nuttallii,* and *C. peploides*) had pollen grains with thick exines. The thick exine may play a role in preventing desiccation of the aerial-dispersed pollen in this growth habit. Furthermore, the pollen of terrestrial species had aperture-like areas, characterized by a reduction in the otherwise thickened basal layer. These thin exine regions may facilitate pollen tube germination. It is also noteworthy that the terrestrial taxa collectively had the smallest pollen diameters. This trend is correlated with the smallest overall anther size (Cooper et al., unpublished data) and the fewest number of pollen grains produced per anther (Philbrick and Anderson, 1992) in comparison with amphibious and obligately submersed species.

Pollen of the amphibious taxa generally had a thinner overall exine compared to that of the terrestrial taxa. Exine reduction in amphibious species was a result of both thinner sexine and nexine layers in most cases. *Callitriche marginata,* which does not produce flowers when plants are submersed, was the only exception. This species had the thickest exine of all amphibious taxa, and it is allied phylogenetically with the terrestrial taxa. Furthermore, surface ornamentation, patterning of sculptural elements, exine thickness and ultrastructure, and the presence of three aperture-like areas in the pollen of *C. marginata* are similar to that of the terrestrial taxa.

Two amphibious taxa in the present study had internal geitonogamy, *C. heterophylla* var. *heterophylla* and *C. trochlearis,* as did two other previously studied amphib-

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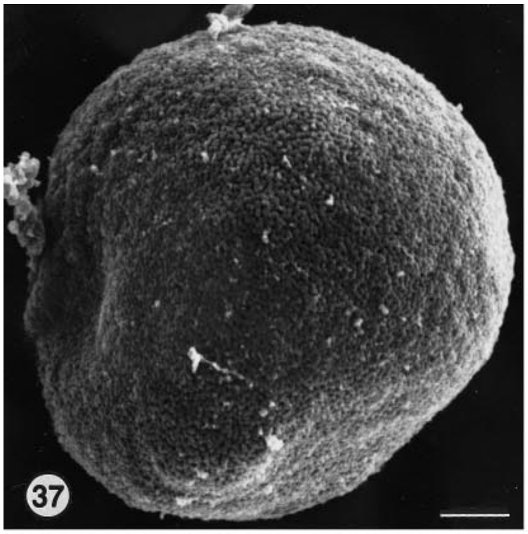
33. Pollen grain showing spheroidal shape and microgemmate surface ornamentation. Bar = 2 μm. **34.** Detail of pollen surface showing sculptural elements in polygonal patterns; note apical microechinae on individual rounded elements. Bar = 1 μm. **35.** Transverse section through pollen grain showing uniformly thin basal layer. Bar = 3 μm. **36.** Transverse section of pollen wall showing sexinous microgemmae, reduced basal layer, and thin intine (I). Bar = 0.5 μm.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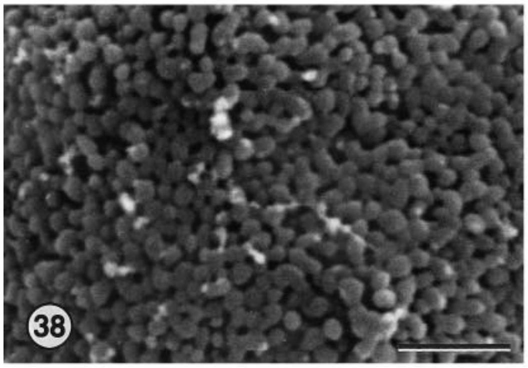
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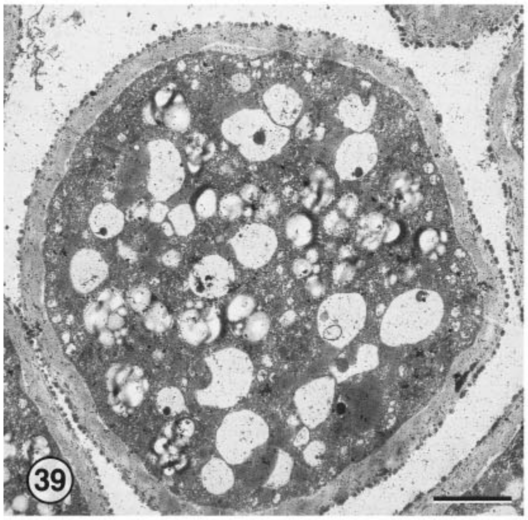
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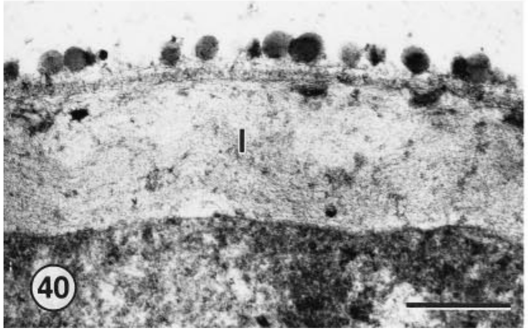
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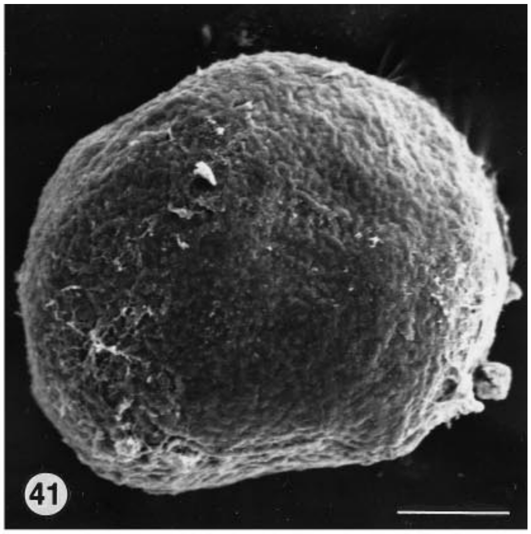
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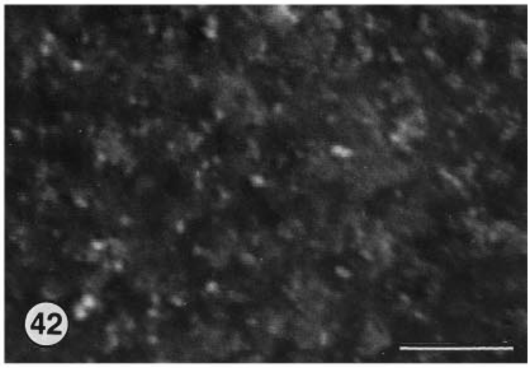
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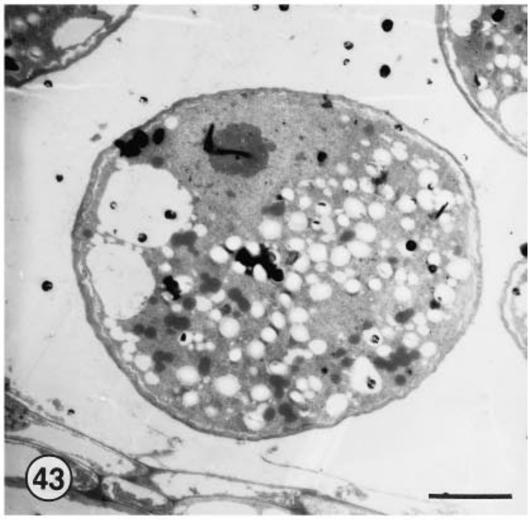
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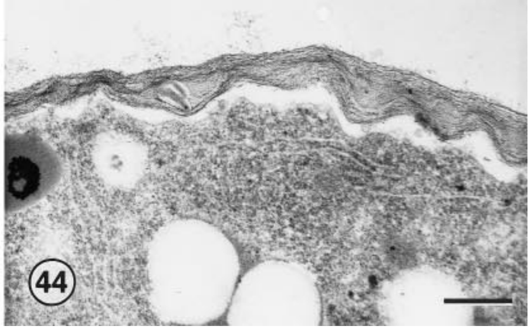
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ious species: *C. heterophylla* var. *bolanderi* (Osborn and Philbrick, 1994; Philbrick and Osborn, 1994) and *C. verna* (= *C. palustris*; Martinsson, 1993). Although overall exine thickness was reduced in these taxa, the basal layer (nexine) was thicker than that of amphibious taxa without internal geitonogamy, and the pollen had aperture-like areas. Thinning of the exine in the aperture-like areas may facilitate pollen tube germination as in terrestrial taxa. In this case, however, pollen tube germination occurs precociously within indehiscent anthers (Philbrick and Anderson, 1992).

Taxa without internal geitonogamy, *Callitriche cophocarpa, C. cophocarpa-stagnalis* hybrid, and *C. cribrosa*, had a thinner exine overall, with the basal layer primarily being reduced. Pollen of these taxa was also inaperturate. Martinsson (1993), however, reported aperture-like areas in *C. cophocarpa* and *C. platycarpa.* Although *C. cophocarpa* was determined to be inaperturate in the current study, it is noteworthy that these two species had slightly thicker basal layers than those of the other amphibious taxa without internal geitonogamy.

Amphibious taxa without internal geitonogamy can exhibit ''typical'' geitonogamy and/or anemophily if flowers are produced aerially; epihydrophily has also been reported when flowers are produced at the water level or when pollen is shed onto the water surface by aerial flowers (Schotsman, 1954, 1982; Sculthorpe, 1967; Philbrick and Anderson, 1992). The thinner exine and the absence of aperture-like regions in these taxa (except for *C. platycarpa*) may be correlated with epihydrophilous pollination. As such, the lack of aperture-like areas may decrease the likelihood of water uptake and thereby enable the pollen to be more buoyant for surface dispersal. Martinsson (1993) suggested that the ''reticulate'' surface ornamentation of *C. cophocarpa, C. platycarpa,* and *C. stagnalis* may enhance flotation for pollen transport at the water surface. Martinsson's (1993) hypothesis was based on a comparison with underwater-flowering *Ruppia,* in which air bubbles are released within submersed flowers, become trapped between the sculptural elements of the exine, and allow the pollen grains to float upward in the water column (Schwanitz, 1967; Pettitt and Jermy, 1975; Verhoeven, 1979). However, this hypothesis is not as relevant in *Callitriche,* because *C. cophocarpa, C. platycarpa,* and *C. stagnalis* produce flowers only in floating rosettes, but not when plants grow in submersed forms.

Callitriche hamulata, depending on the type of amphibious growth form, may produce aerial flowers exclusively, submersed flowers exclusively, or both on the same plant. When mature pollen from aerial and submersed flowers of *C. hamulata* was compared, many characters varied statistically (Table 2). Grain diameter and intine thickness were significantly greater in pollen of submersed flowers. By contrast, overall exine thickness, sexine thickness, and nexine thickness were significantly thicker in pollen of aerial flowers. As the exine is important for the prevention of water loss and maintenance of pollen viability, it might be expected that pollen from aerial flowers would have a thicker exine than that of submersed flowers. Nevertheless, exine thickness in aerial pollen of *C. hamulata* was extremely reduced when compared to other aerial-flowering *Callitriche* species. The smaller size of the aerial pollen of *C. hamulata* may minimize dehydration and thereby compensate for the overall exine reduction.

Two amphibious taxa lacked exines: *C. brutia* (the closest relative of *C. hamulata*) and *C. lusitanica.* These findings provide the first documentation of exine loss in the amphibious growth habit. However, pollen of *C. brutia* has previously been reported to have a reticulate surface ornamentation and an ellipsoidal shape (Díez, Talavera, and Garcia-Murillo, 1988). It is possible that Díez, Talavera, and Garcia-Murillo (1988) studied pollen from an aerial flower of *C. brutia,* whereas pollen from submersed flowers was examined in the current study.

New data on the obligately submersed *C. truncata* confirm the lack of an exine in this growth habit. Pollen of *C. hermaphroditica* has previously been shown to be exineless (Osborn and Philbrick, 1994; Philbrick and Osborn, 1994).

Evolution of hypohydrophily—Underwater pollination systems in angiosperms are derived from aerial floral systems (e.g., Faegri and van der Pijl, 1979; Philbrick and Les, 1996). Exine reduction or complete exine loss appear to be an essential component in the evolution of hydrophily (e.g., Philbrick, 1991, and references therein). This has been documented in hydrophilous members of the Najadaceae (e.g., Pettitt and Jermy, 1975), Hydrocharitaceae (e.g., Pettitt, 1981), Cymodoceaceae (e.g., Pettitt, Ducker, and Knox, 1978; Pettitt, 1980, 1981), Zannichelliaceae (e.g., McConchie, Knox, and Ducker, 1982), and Ceratophyllaceae (e.g., Pettitt and Jermy, 1975; Les, 1988; Takahashi, 1995). The Callitrichaceae are unique in being the only known angiosperm family in which aerial, epihydrophilous, and hypohydrophilous pollination systems co-occur (Philbrick and Anderson, 1992). Therefore, the family provides the opportunity to evaluate questions concerning the evolution of underwater pollination.

The presence of an exine appears to be less critical when pollination is water-mediated. This is evident in Callitrichaceae, where exine structure and thickness are correlated with growth habit and pollination type. Pollen of the two obligately submersed taxa studied either lacked an exine (*C. truncata* and C. *hermaphroditica*—

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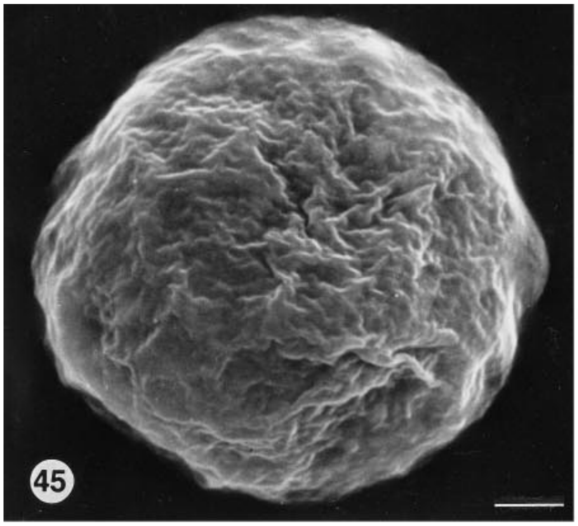
Figs. 37–44. Amphibious taxa without internal geitonogamy. *Callitriche hamulata*—submersed (Figs. 37–40). **37.** Pollen grain showing spheroidal shape and granular surface ornamentation. Bar = 2 μm. **38.** Detail of pollen surface showing granular microgemmae. Bar = 1 μm. **39.** Transverse section through pollen grain showing reduced exine and thick intine. Bar = 3 μm. **40.** Transverse section of pollen wall showing small, sexinous sculptural elements, thin basal layer, and thick intine (I). Bar = 0.5 μm. *Callitriche brutia* (Figs. 41–44)**. 41.** Pollen grain showing spheroidal shape and slightly wrinkled surface. Bar = 5 μm. **42.** Detail of pollen surface showing reduced surface ornamentation. Bar = 1 μm. **43.** Transverse section through pollen grain showing lack of an electron-dense exine. Bar = 4 μm. **44.** Transverse section of pollen wall showing fibrillar intine and lack of exine. Bar = 0.5 μm.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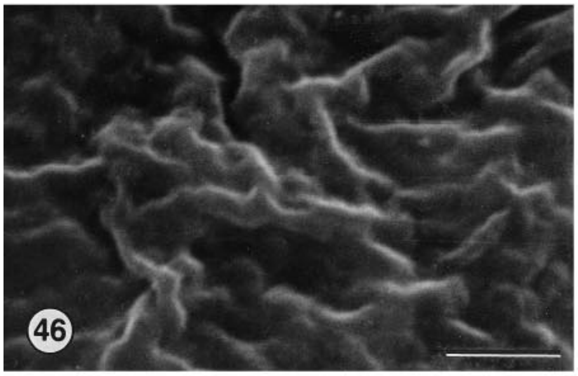
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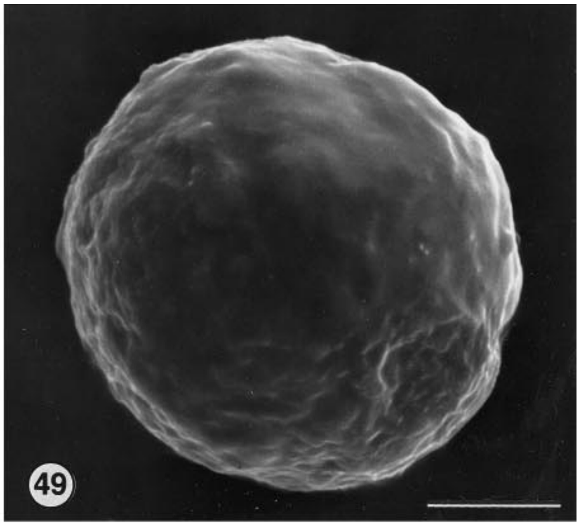
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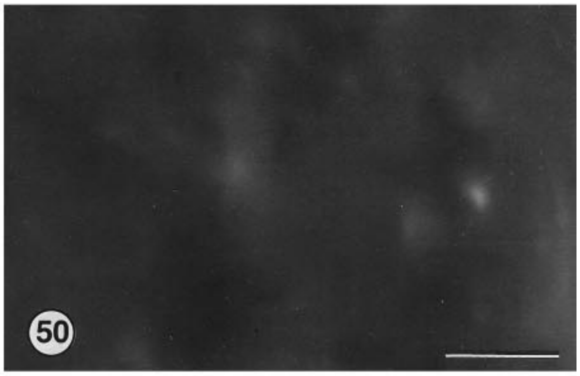
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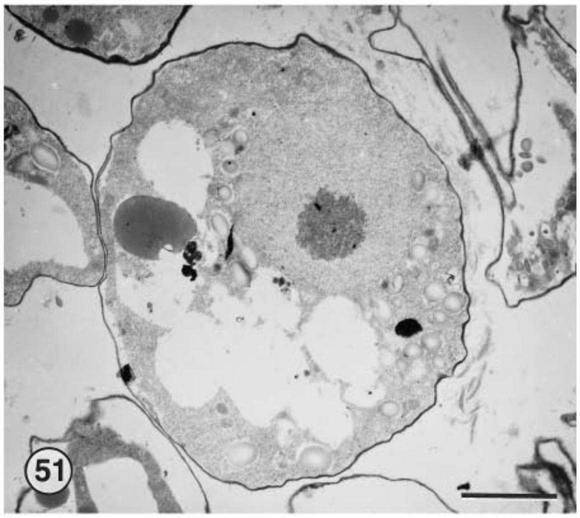
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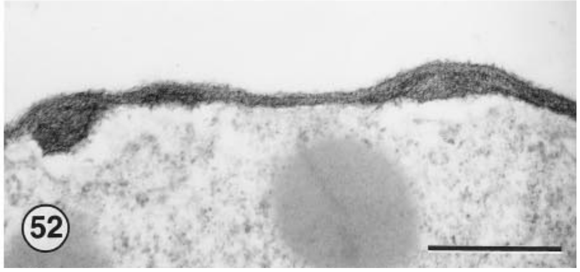
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perennial form) or had a highly reduced exine (*C. hermaphroditica*—annual form); nevertheless, these plants pollinate successfully underwater. By contrast, pollen of aerial-flowering terrestrial species had well-defined exines that were considerably thicker than that of most amphibious taxa. Exine thickness was quite variable among amphibious species, especially with respect to the basal layer (nexine) in taxa with vs. without internal geitonogamy.

A similar correlation between pollen wall ultrastructure (i.e., exine reduction) and growth habit was evident within the single amphibious taxon *C. hamulata.* In *C. hamulata,* the statistically significant variation in exine thickness (and several other characters; Table 2) between fully mature pollen from aerial vs. submersed flowers may indicate that pollen in this species exhibits developmental plasticity. Such significant intraspecific variation in exine structure is not common. This ontogenetic hypothesis may also explain the variation in pollen of *C. brutia* observed in the present paper (exineless from submersed flowers) with that studied by Díez, Talavera, and Garcia-Murillo (1988; with a reticulate exine).

The palynological data indicate that underwater pollination in *Callitriche* may have had a recent origin. For example, the degree of exine loss is dissimilar between the two obligately submersed taxa studied. The pollen of *C. truncata* was completely exineless, and that of *C. hermaphroditica* was variable. Pollen of the perennial growth form of *C. hermaphroditica* lacked an exine, whereas pollen of the annual growth form had a rudimentary exine (Osborn and Philbrick, 1994; Philbrick and Osborn, 1994). Martinsson (1993) also identified extreme exine reduction in *C. hermaphroditica,* but not complete loss.

Furthermore, a shift from aerial pollination to hypohydrophily within the amphibious growth habit may be occurring within the *C. hamulata*/*C. brutia* clade. Although hydrophily has not been documented in this clade, these taxa exhibit exine reduction and exine loss (Fig. 53, clade H). Possible ontogenetic plasticity in the pollen wall of *C. hamulata* (i.e., from aerial vs. submersed flowers) provides further support for this hypothesis. Additionally, *C. hamulata* and *C. brutia* are unusual among amphibious *Callitriche* species in that they are able to produce seed in submersed flowers. However, seed set in these two taxa may not be related to hydrophily, but rather the result of agamospermy. Additional studies of the reproductive biology and systematics of these species are needed and may clarify these questions.

Phylogenetic considerations—Philbrick and Jansen (1991) and Philbrick and Les (1995) have proposed hypotheses of evolutionary relationships among species of *Callitriche*. Philbrick and Jansen (1991) included 11 species, primarily from North America, whereas Philbrick and Les (1995) incorporated 18 species, including all North American and most western European taxa. These species represent terrestrial, amphibious, and obligately submersed growth habits, as well as aerial and hydrophilous pollination systems. The general topology proposed by Philbrick and Les (1995), a phylogeny based on parsimony analyses of both *rbcL* and morphological characters, is illustrated in Fig. 53. In the present paper, details of exine structure for 17 *Callitriche* species have been character-mapped onto the proposed phylogeny.

Consideration of palynological characters relative to the proposed phylogeny provides support for several hypotheses concerning pollen structural evolution. Pollen of most *Callitriche* species has a well-developed exine. In contrast, exine reduction or absence occurs in five species: *C. hermaphroditica, C. truncata, C. lusitanica, C. brutia*, and *C. hamulata.* It is evident that exine reduction has evolved at least twice in *Callitriche* (Fig. 53, clades A and H). Exine reduction is associated with aneuploid reduction in chromosome number (except for *C. cribrosa,* see below). The three species in clade A have $2n = 6$ or 8 and represent aneuploid reduction from $2n = 10$. *Callitriche brutia* ($2n = 28$) and *C. hamulata* ($2n = 38$) seem to represent aneuploid reductions from polyploids ($2n = 30$ and 40, respectively), although alternative derivations of their chromosome numbers cannot be ruled out. Sexine reduction is not apparent in the aneuploid *C. cribrosa* ($2n = 8$), but the nexine (basal layer) is reduced. Exine reduction in clade A is also associated with hypohydrophilous pollination, although the submersed form of the amphibious *C. lusitanica* also lacks an exine. Possible relationships between pollen structure and pollination in this species remain to be elucidated.

Two apertural conditions occur in *Callitriche*: aperture-like and inaperturate. Aperture-like areas do not occur in species that either have reduced or absent exines; predictions about the evolution of this feature correspond to the evolution of exine reduction noted above. Inaperturate grains also characterize three species with well-developed exines: *C. cophocarpa, C. cribrosa,* and *C. stagnalis.* It is not possible to determine whether the inaperturate condition is ancestral in *Callitriche* given the current phylogenetic insight. However, pollen with true apertures is present in the probable outgroups (Scrophulariaceae and Hippuridaceae; e.g., Erdtman, 1952).

The present study is the first to incorporate data on pollen morphology and ultrastructure in a phylogenetic interpretation of the Callitrichaceae. Although these data do not fully answer all of the systematic queries associated with the family, the exine appears to be an excellent character to incorporate into cladistic analyses. Moreover, exine architecture correlates well with the various growth

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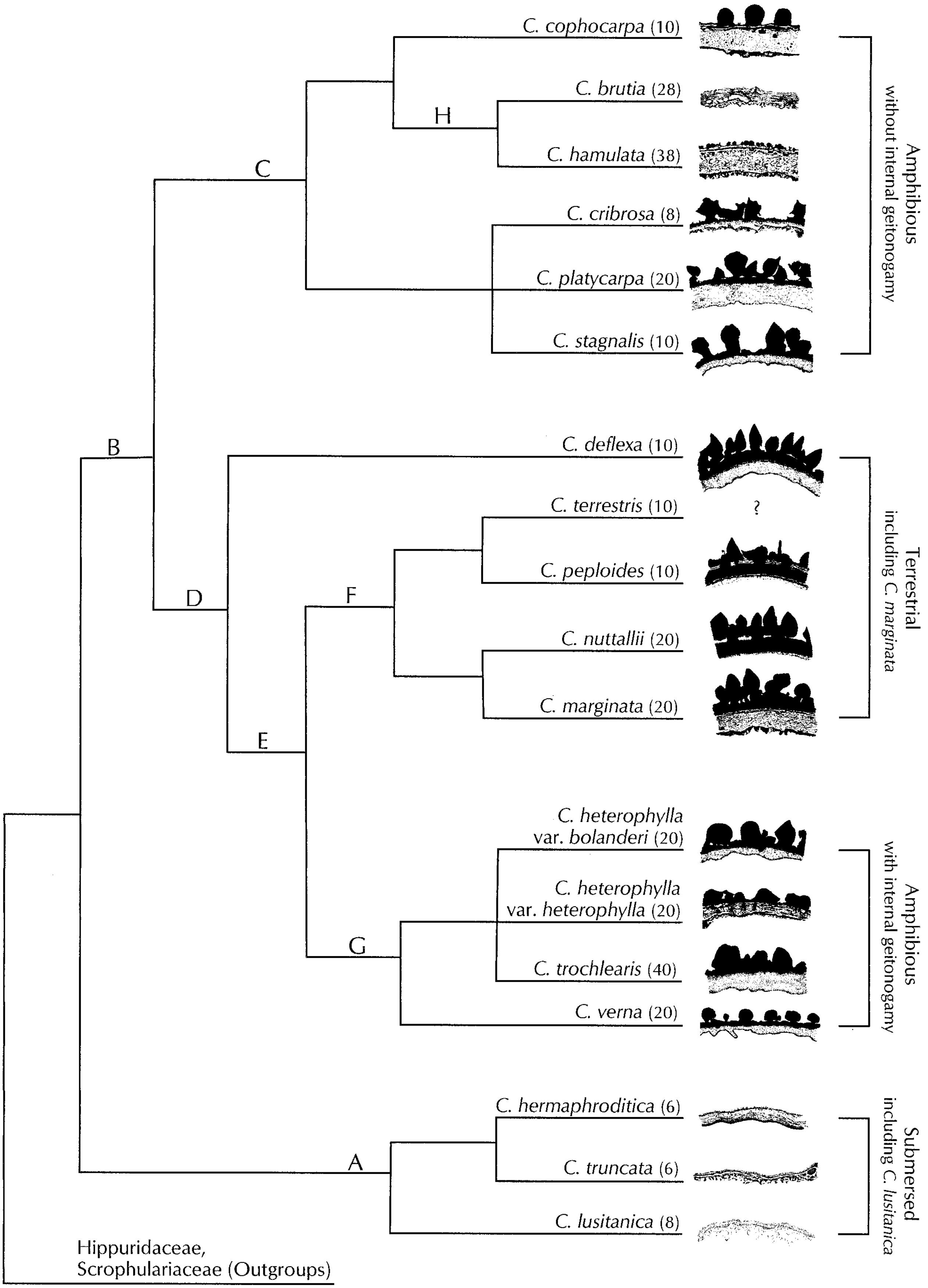
Figs. 45–52. Obligately submersed taxa (including *C. lusitanica*). *Callitriche lusitanica* (Figs. 45–48). **45.** Pollen grain showing spheroidal shape and wrinkled surface. Bar = 2 μm. **46.** Detail of pollen surface showing wrinkled morphology. Bar = 1 μm. **47.** Transverse section through pollen grain showing lack of an electron-dense exine. Bar = 3 μm. **48.** Transverse section of pollen wall showing fibrillar intine and lack of exine. Bar = 0.5 μm. *Callitriche truncata* (Figs. 49–52). **49.** Pollen grain showing spheroidal shape and smooth surface. Bar = 5 μm. **50.** Detail of pollen surface showing lack of sculptural elements. Bar = 1 μm. **51.** Transverse section through pollen grain showing lack of an electron-dense exine. Bar = 3 μm. **52.** Transverse section of pollen wall showing unevenly thick, fibrillar intine and lack of exine. Bar = 0.5 μm.


C. cophocarpa (10)
C. brutia (28)
C. hamulata (38)
C. cribrosa (8)
C. platycarpa (20)
C. stagnalis (10)
C. deflexa (10)
C. terrestris (10)
?
C. peploides (10)
C. nuttallii (20)
C. marginata (20)
C. heterophylla var. bolanderi (20)
C. heterophylla var. heterophylla (20)
C. trochlearis (40)
C. verna (20)
C. hermaphroditica (6)
C. truncata (6)
C. lusitanica (8)
Hippuridaceae, Scrophulariaceae (Outgroups)
A
B
C
D
E
F
G
H
Amphibious without internal geitonogamy
Terrestrial including C. marginata
Amphibious with internal geitonogamy
Submersed including C. lusitanica

habits and associated pollination biologies found within this unique aquatic family.

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Fig. 53. Cladogram of 18 *Callitriche* species showing the distribution of pollen wall characters, particularly exine thickness and structure (solid black), as well as intine thickness (stippled). All pollen wall drawings have been reproduced at the same scale. See text for discussion of letter-indicated nodes and pollen wall correlations with chromosome number (2*n*; indicated in brackets following each species name). The cladogram has been modified from Philbrick and Les (1995) and is based on analyses of both *rbcL* and morphological characters. Pollen wall data for *C. stagnalis*, *C. heterophylla* var. *bolanderi*, and *C. hermaphroditica* are taken from Philbrick and Osborn (1994), and those for *C. platycarpa* and *C. verna* (= *C. palustris*) are derived from Martinsson (1993).