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Morphology and ultrastructure of megaspores and microspores of *Isoetes savatieri* Franchet (Lycophyta)

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Abstract

The general morphology, organization, structure and ornamentation of the sporoderm of megaspores and microspores of *Isoetes savatieri* Franchet have been studied with a stereoscopic microscope, light microscopy, transmission and scanning electron microscopy. The megaspores are trilete, 420–580 µm in equatorial diameter, subtriangular to globose in polar view with an evident equatorial flange (zone). The ornamentation is rugulate. A siliceous cover with a three-dimensional network overlies an exospore sporopolleninous apparently laminar with an equatorial–distal separation of laminae. Each exospore lamina is fused to those of other planes delimiting lacunae. The lacunae located in the outer part of the exospore are filled with silica. The endospore is thick, its structure is fibrillar. In section it is apparently composed of two zones. The microspores are monolete, 35–40 µm long and 20–25 µm wide and elliptic in polar view. A supra-laesural expansion is present. The sculpture is microechinate. A contrasted perispore composed of thin joined threads that form a lacunose structure can be distinguished in the microspore sporoderm. Two parts of the underlying exospore structurally different are evident: a laminar outer part and an inner part, mostly compact. An equatorial–distal separation between both parts of the exospore is evident. The endospore is fibrillar. The ultrastructural similarity of the spores of *I. savatieri* with those previously studied in *Isoetes* can be regarded as indicative of the homogeneity of this group of living lycopsids.

Keywords: Lycophyta; megaspores; microspores; morphology; ultrastructure; Argentina; *Isoetes*

1. Introduction

The Isoetaceae constitute a family with a world wide distribution. They live in temperate-warm regions of all continents, from sea level to 4200 m, most frequently above 2000 m (Tryon and Tryon, 1982). A number of *Isoetes* are terrestrial while others are living submerged in the lake margins, ponds or streams. When the water is lacking, usually only plant elements exposed to the air, like the narrow part of the leaves, get dry. The plants may form dense lawns. They can reproduce also vegetatively by means of gemmae. Seven species (cf. Ponce, 1996) were reported to grow in Argen-

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tina. They are distributed in the provinces of Buenos Aires, Formosa, Córdoba, Tucumán and in the Patagonian region.

Isoetes savatieri Franchet grows in Argentina in the provinces of Río Negro and Neuquén and in the southern area of the Province of Tierra del Fuego (Marticorena and Rodríguez, 1995). In Chile, it grows from Bío-Bío to Magallanes (Looser, 1961). This species grows at elevations of 200–1300 m, as a submerged aquatic at 1–3 m below the water surface, in the shallows of streams and lakes margins.

Systematic (Pastore, 1936; Capurro, 1969) and floristic (de la Sota et al., 1998) aspects of *Isoetes* in Southern South America have received some attention but little information can be found on the palynological characteristics. The spores of *Isoetes savatieri* were considered in the papers by Morbelli (1980) and Hickey (1985, 1986), where the spores were analyzed with light microscopy (LM) and scanning electron microscopy (SEM).

The spores of other species of *Isoetes* were examined with transmission electron microscopy (TEM) by Lugardon (1973, 1986), Robert et al. (1973), Prada Moral and Sáenz de Rivas (1978), Tryon and Lugardon (1991), Taylor (1992, 1993) and Uehara et al. (1991).

The purpose of this work is to study with electron microscopy, the morphology and ultrastructure of megaspores and microspores of *Isoetes savatieri*.

Palynological analysis with electron microscopy (SEM and TEM) will contribute to a better knowledge of the general organization of the spores.

2. Materials and methods

This study was carried out with herbarium and fresh material fixed with FAA. The consulted herbaria are indicated with the initials corresponding to those used by Holmgren et al. (1990): Instituto-Fundación 'Miguel Lillo' (LIL), Instituto de Botánica Darwinion (SI) and Instituto de Botánica del Nordeste (CTES).

2.1. Material studied

ARGENTINA. Neuquén, *Lago Huechulafquen*, Fontana s/n (LP); ídem, *Parque Nacional Nahuel Huapi*, Burkart 6499 (SI); ídem, *Lag. Varvarco Campos*, Boelcke 14337 (SI); ídem, *Lago Nahuel Huapi*, Rincón, Sota 2812 (LP); ídem, *Lago Lácar*, Burkart y Troncoso 26447 (SI); ídem, Lagunas Epu-Lauquén, Boelcke et al., 10871 (SI); ídem, *Nahuel Huapi*, *Laguna Frías*, Burkart 6335 (SI); ídem, *Quetrihué*, Diem 646 (SI); ídem, Península Quetrihué, Diem 674 (CTES). Río Negro, *Laguna Frías*, Eskuche 380 (LP); ídem, *Lago Hess*, Meyer 8077 (SI).

The studies were done with a stereoscopic microscope, LM, SEM and TEM. Nevertheless we selected the records with electron microscopy (SEM and TEM) for illustration in this paper. To carry out the study with TEM the megaspores were fixed with 1% osmium tetroxide (OsO_4) for 2 h and then dehydrated in 100% acetone. The microspores were prefixed with 1% glutaraldehyde with 0.01% ruthenium red (RR) in 0.1 N phosphate buffer. After two 15-min rinses in 0.01% RR in 0.1 M phosphate buffer, they were postfixed for 1 h with a mixture of 0.01% of RR and 1% OsO_4 in water solution. They were dehydrated in an acetone series.

Then the material was embedded in Spurr resin. Thin and semi-thin sections of microspores and megaspores were cut. Semi-thin sections were stained with toluidine blue for study with LM. The thin sections collected on grids were stained with uranyl acetate and lead citrate. They were examined with an EM 109-Turbo Zeiss at the Laboratorio Nacional de Investigación y Servicios (LANAIS/CONICET) of the Facultad de Ciencias Médicas (Universidad Nacional de Buenos Aires).

For observation with SEM, megaspores and microspores were handled with fine moist brushes without any chemical treatment and placed on double stick tape on bronze stubs. The samples were coated with gold-palladium, and examined with a Jeol JSM-35 CF microscope at the SEM Laboratory of the Facultad de Ciencias Naturales y Museo (Universidad Nacional de La Plata).

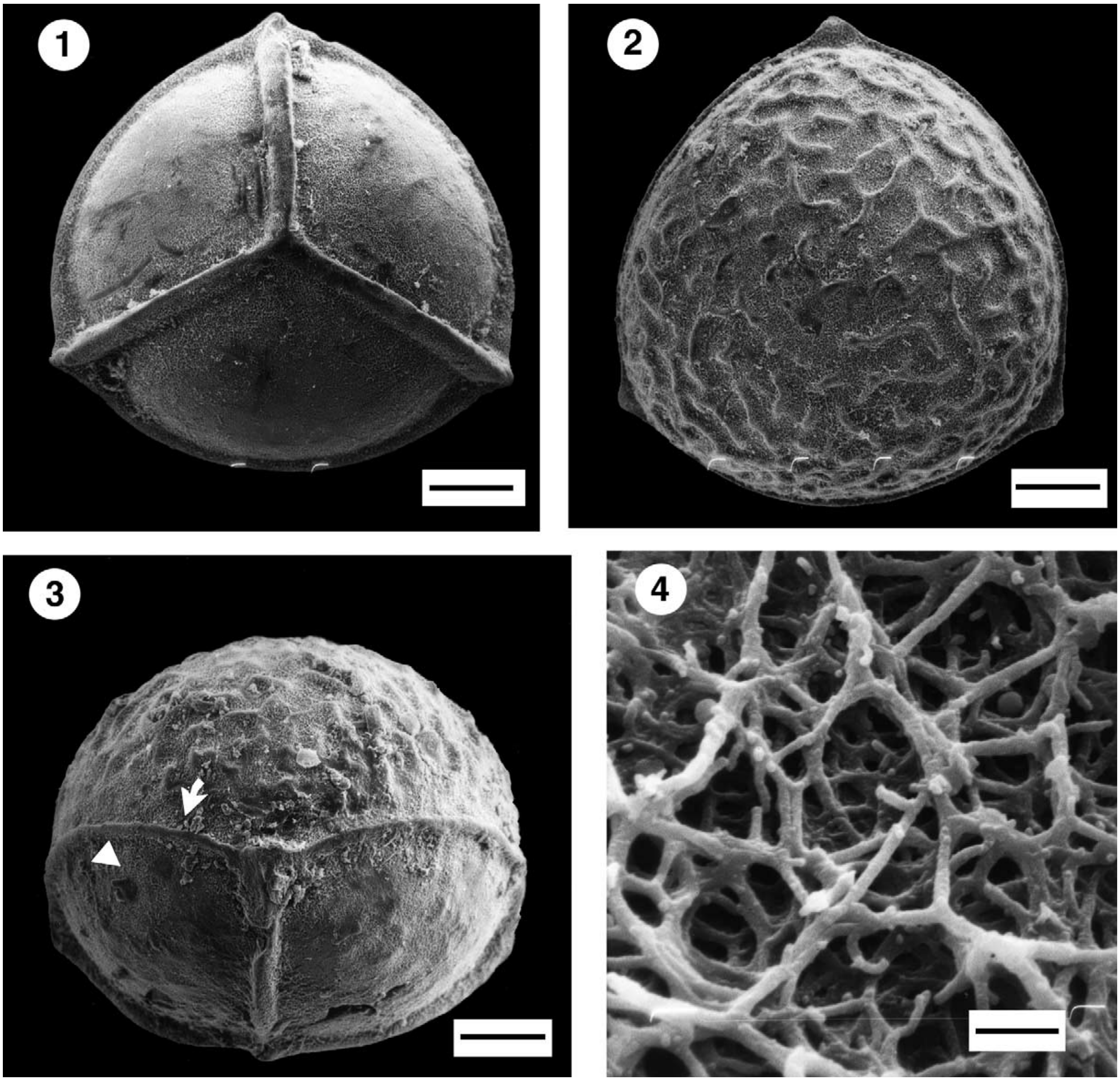
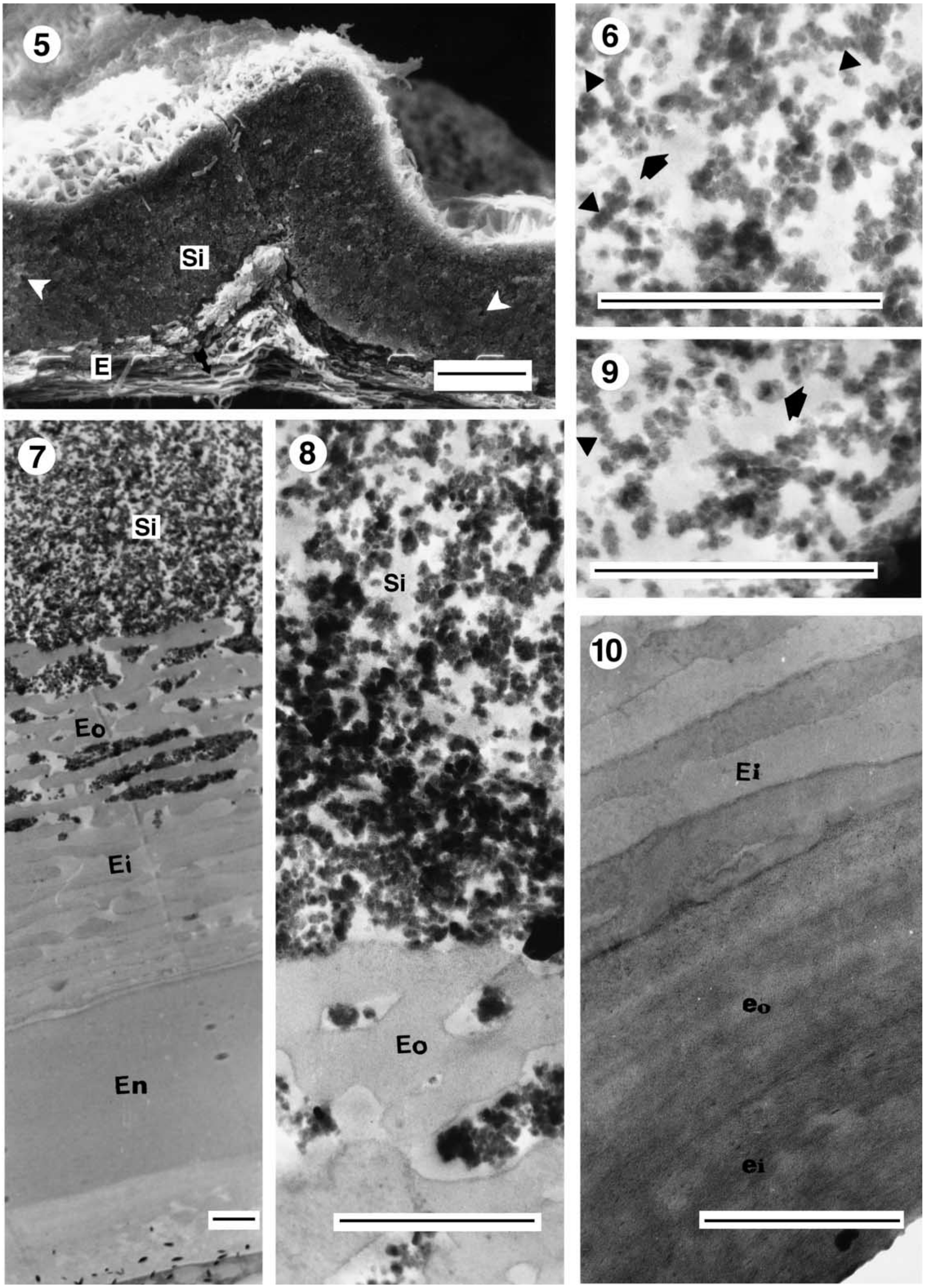


Plate I. Megaspores as seen with SEM

1. Proximal view. The sculpture of the silica cover in each facet is composed by a few slender ridges. The laesurae are high. The zone is prominent at the equator and the corners project outward in the places where the laesurae and the zone meet. Scale bar: 100 μm.
2. Distal view. The sculpture of the silica cover is ridged. The ridges are more marked and robust, single or partially fused on this face. Scale bar: 100 μm.
3. Equatorial view. The distal pole is at the top. The zone (arrowhead) marks the equator. The difference between the proximal and distal sculpture of the silica cover is evident. The girdle (arrow) has the same sculpture as the rest of the distal face. Scale bar: 100 μm.
4. Detail of the distal surface between ridges. The background is composed of a three-dimensional network of fused rodlets forming heterogeneous spaces. The surface is essentially open (discontinuous). Scale bar: 2 μm.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3. Results

3.1. Megaspores

The megaspores (Plate I, 1–3) are trilete, and 420–580 μm in equatorial diameter. Their shape is subtriangular to globose in polar view. The proximal face is convex and the distal one is hemispheric in equatorial view.

The laesurae are 36 μm high and blunt in transverse section. In some cases these laesurae can be slight and with undulated margins. Each arm is 250 μm long and as long as the total radius of the spore. Each laesura is fused to the equatorial zone or cingulum.

The equatorial zone (also named collar, arista or cingulum) is 21 μm wide and perpendicular to the laesurae. On the distal face adjacent to the equatorial zone, a band can be observed (= girdle, cf. Tryon and Lugardon, 1991; Taylor et al., 1993). It has an ornamentation that, in this case, is the same as the rest of the spore.

The ornamentation on the surface of the silica cover (Si) consists of ridges 14 μm high and 21 μm thick, single or partially fused (Plate I, 2).

The ridges are more marked on the distal face (Plate I, 2, 3). A three-dimensional reticulum of fused rods constitutes the background (Plate I, 4).

A siliceous cover, a sporopolleninous exospore and a well developed endospore can be distinguished in the sections of mature megaspores, from the outside to the inside, respectively.

When observed in section with the TEM and SEM, the silica cover (Si) (Plate II, 5, 7, 8) is strongly contrasted and 13–22 μm thick. It is single-layered with a uniform structure. The structural elements are short rods, circular in section, 35 nm in diameter. The rodlets have a less contrasted central core and in lateral view they have knobs (Plate II, 6, 8, 9). These are apparently associated in groups of four or five units with a central space (Plate II, 9).

These rodlets join together forming a network characterized by wide spaces between groups of rodlets. The groups of units are arranged radially through almost the whole layer, while they are mainly arranged tangentially and leave narrow spaces near the outer surface. The spaces between units are interconnected in the bulk of this layer and they are connected to the outside through discontinuities on the outer surface.

The exospore is less contrasted than the silica cover with the TEM and has a laminar structure (Plate II, 7, 8). The structural elements are laminae that are thinner inwards (Plate III, 11a). An equatorial–distal separation between laminae (Plate III, 11b) is evident. The outer part is 2.8–5.5 μm thick. The laminae of the outer part of the exospore (Eo) are anastomosed, forming lacunae. Silica is present in a variable amount in the out-

Plate II. The megaspore wall ultrastructure

5. SEM. Sporoderm in section at the equatorial zone. The silica cover (Si) is thicker and more contrasted than the exospore (E). The exospore has a laminar structure. Each lamina is fused in several places to others of different levels (arrowhead). Many small gaps (arrow) are evident in the bulk of the silica cover. Scale bar: 10 μm.

6–10. Sections of the megaspore wall with TEM. Figs. 6 and 9. Details of the complex structure of the silica cover. It is essentially an 'open' layer built with short rodlets, circular in section, joined in groups of four or five units around a central one (arrows). Each rodlet has a less contrasted central core and shows knobs in lateral view (arrowheads). Scale bar: 1 μm.

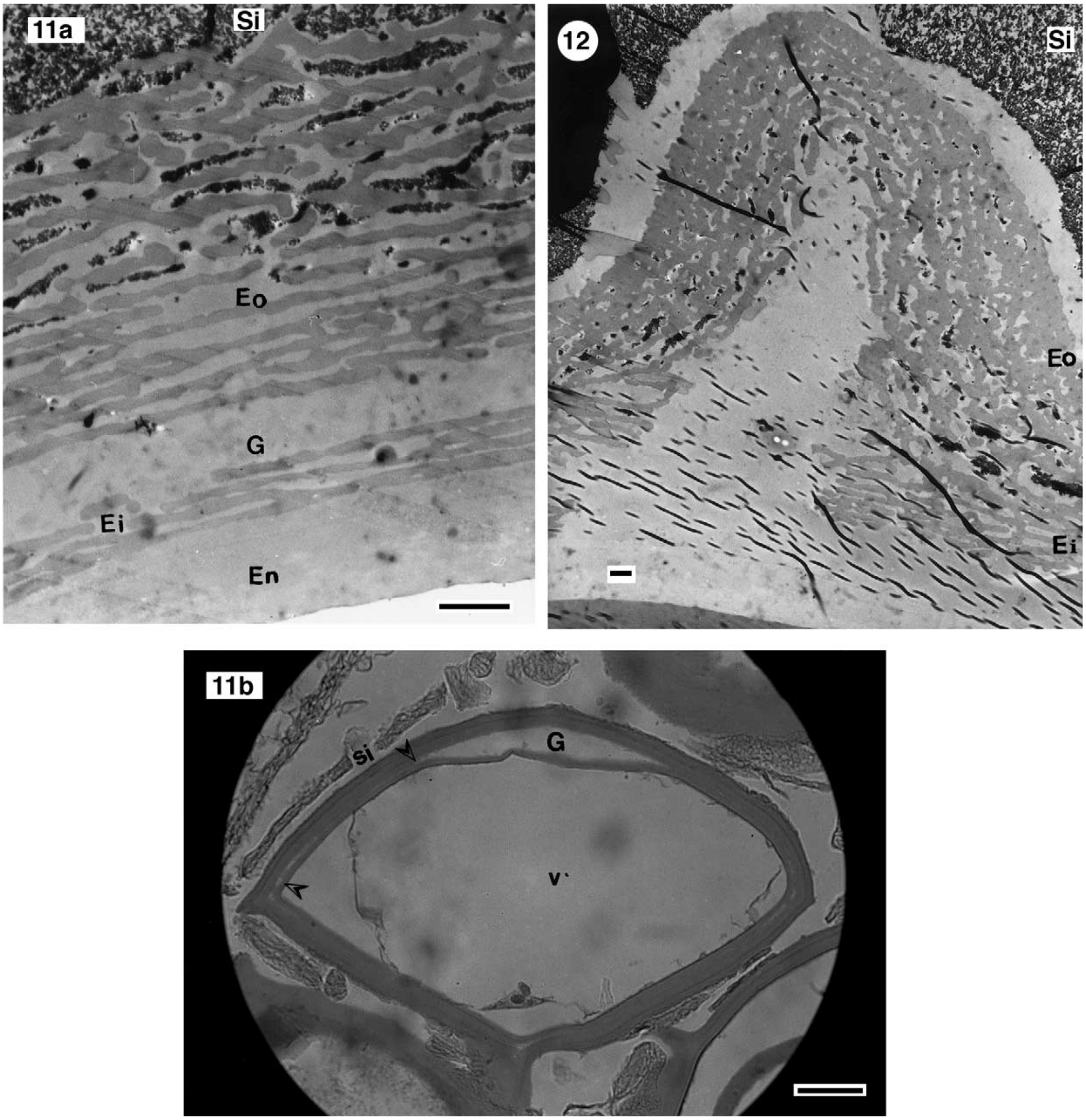
7. Sporoderm in section. The silica cover (Si) has a homogeneous structure. The exospore (E) is composed of parallel laminae that fuse to form lacunae. The lacunae of the outer zone of the exospore (Eo) are largely filled with silica. The inner zone of the exospore (Ei) is composed of thinner laminae and the spaces between laminae are narrower. The endospore (En) is next to the inner zone of the exospore. Scale bar: 1 μm.

8. Sporoderm in section. Detail showing the silica cover (Si) and the outer zone of the exospore (Eo). The exospore lacunae are filled with silica. Scale bar: 1 μm.

10. Detail of the endospore, below the innermost elements of the exospore (Ei). The endospore has a fibrillar structure and shows two distinct parts (e_o, e_i). The outer zone (e_o), adjoining the exospore inner surface, appears to have a laxer structure. Scale bar: 1 μm.

ermost cavities of Eo (Plate II, 7, 8 and Plate III, 11a). The inner part of the exospore (Ei) is 3.3–5.5 µm thick (Plate II, 7 and Plate III, 11a). The laminae arrangement at this level is tighter and the laminae are fused forming irregular lacunae usually devoid of silica. Only the outer part of the exospore is involved in the apertural differentiation at the proximal face (Plate III, 12).

The endospore (En) is 2–7 µm thick. Its structure is fibrillar (Plate II, 10) with bands of different density. Within it two zones are clearly seen, indicating that they could have been formed in



two sequential steps. The outer zone next to the exospore shows a looser structure.

3.2. Microspores

The microspores (Plate IV, 13–18) are monolete, elliptic in polar view, 35–40 μm long and 20–25 μm wide. In transverse equatorial view the proximal face is convex and the distal one hemispheric. A supra-laesural expansion, 7.0 μm high, is present proximally (Plate IV, 14). The laesura is well marked on almost the whole length of the proximal face (Plate IV, 15). The perforated background and the micro-elements of the ornamentation (0.4–0.7 μm high) are similar in the proximal and distal faces (Plate IV, 16, 18). The elements correspond to single or combined threads protruding above the superficial sheet of the perispore. The surface of both faces mainly differs in the presence of the larger cones, 0.9–2.0 μm high (Plate IV, 17, 18) resulting from protrusions of the whole outer part of the perispore, from the equator to the distal face (Plate V, 20). The sporoderm comprises this perispore forming the ornamentation, an exospore tangentially divided into two parts in distal and lateral areas, and a fibrillar endospore.

The perispore (P) (Plate V, 19–21) is strongly contrasted when observed with TEM. It is 1.0–2.2 μm thick distally and 0.5–1.0 μm proximally. It is single-layer. Its outer surface consists of a thin sheet, made up of threads more or less regularly juxtaposed and closely fused, showing small discontinuities (Plate V, 21). The bulk of this layer has a lacunose structure. It consists of thin threads, circular in section, 33 nm in diameter which have a less contrasted core zone (Plate V, 21). The threads are branched several times.

The exospore is less contrasted than the perispore and is made up of flattened elements, 95 nm thick in section, tangentially arranged. These units have a less electron dense central zone and a dark outer zone (Plate V, 21).

The outer part (Eo) is 0.3–1.0 μm thick with a laminar structure. The units anastomose between them. At the apertural zone the Eo units decrease in number and accompany the perispore in the formation of the supra-laesural expansion. The inner part (Ei) is 0.2–0.5 μm thick, apparently mostly compact, except for the evident laminae only located at both sides of the apertural region ('pluristratified zones') (Plate V, 19). An equatorial–distal separation of the exospore (Plate V, 21) is evident.

The fibrillar endospore is 0.3–1.0 μm thick, surrounds the entire spore lumen (Plate V, 19, 21). It forms an onciform thickness (On) below the apertural differentiation (Plate V, 19).

4. Discussion and conclusions

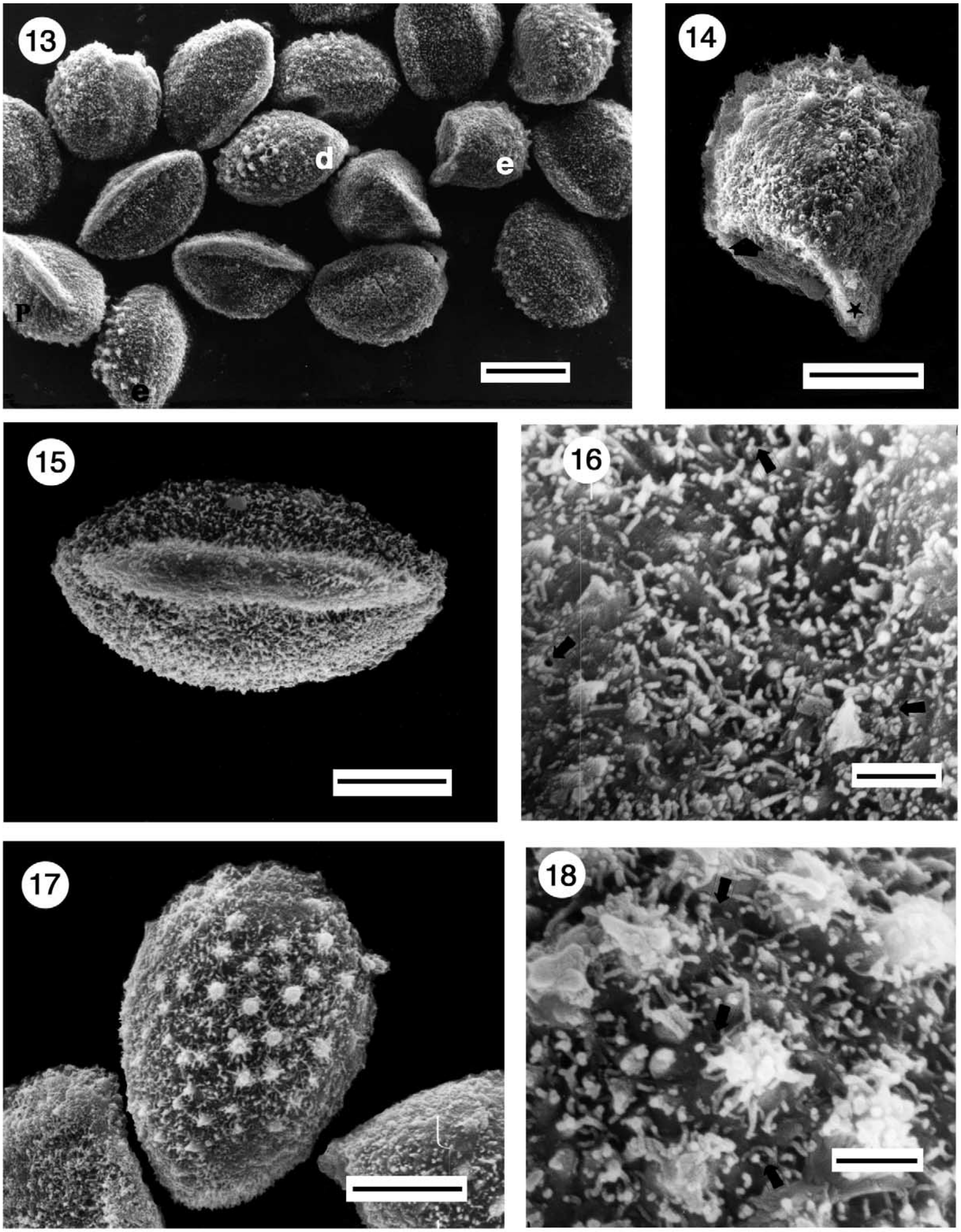
Like megaspores of the Selaginellaceae those of the Isoetaceae have a unique type of exospore (Robert et al., 1973; Pettitt, 1966) which is different from all the other exospore types present in both extant and fossil Pteridophyta. This exospore type is shared (Lugardon et al., 2000) by

Plate III. TEM sections of the megaspore wall in non-apertural and apertural regions

11a. The sporoderm consists of a silica cover (Si), the exospore with an outer part (Eo) with lacunae that are partly filled with silica and an inner part (Ei) and the endospore (En). There is a space (G) between laminae of the inner part of the exospore. Scale bar: 1 μm.

11b. Thick section of a megaspore in equatorial view taken with LM. A separation between parts of the exospore is evident along the equatorial and distal regions (arrows). The separation (G) is wider at the distal part. Portions of the silica cover (Si) can be distinguished on the exospore in proximal and distal surfaces. A nucleus surrounded by a small portion of cytoplasm is evident under the aperture. A narrow stripe of cytoplasm is surrounding a central large vacuole (v). At this stage an endospore is not evident yet. Scale bar: 50 μm.

12. Detail of the apertural area. Both the silica cover (Si) and the outer part of the exospore (Eo) are modified at this level while the structural elements (usually unbroken but accidentally ruptured in the sectioned area) of the inner part of the exospore (Ei) have the same tangential orientation as in the non-apertural region. Scale bar: 1 μm.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the megaspores of the other groups of fossil lycopsids.

Isoetes megaspores, like some of those of *Selaginella* and spores of some homosporous ferns, have a zone or cingulum, which is higher than the rest of the elements of the surface (Tryon, 1986).

Some *Isoetes savatieri* specimens show a transition in ornamentation in both mega and microspores which could probably be related to geographic distribution. This topic is now under research in this taxon.

The megaspores of *Isoetes savatieri*, which possess a white cover when seen with a stereoscopic microscope, have a sculpture made up of ridges that are more strongly impressed on the distal face. In connection with the previous paragraph, Morbelli (1980) defined the megaspore ornamentation in this species as scabrate proximally and with projections of wide bases and blunt apex distally. This author also interpreted the sporoderm as consisting of a perine and an exine which are distinguishable by their color and nature.

A similarity has been found between *Isoetes savatieri* megaspores and those of *Isoetes setacea* Delille (Robert et al., 1973) regarding the surface background that is constituted in both by discontinuities and single or fused rods.

The interpretation of the sporoderm structure in megaspores of *Isoetes savatieri* in this case coincides with that of Taylor (1992, 1993) for other species of *Isoetes*. Taylor divides the wall into a siliceous cover and an exospore with two strata: outer and inner, and the latter is subdivided into two layers: the inner separable layer (isl) and the outer layer. The isl was also recognized in *Selaginella* megaspores (Taylor, 1989) as it was also mentioned by other authors such as Morbelli and Rowley (1999); Rowley and Morbelli (1995) and Tryon and Lugardon (1991).

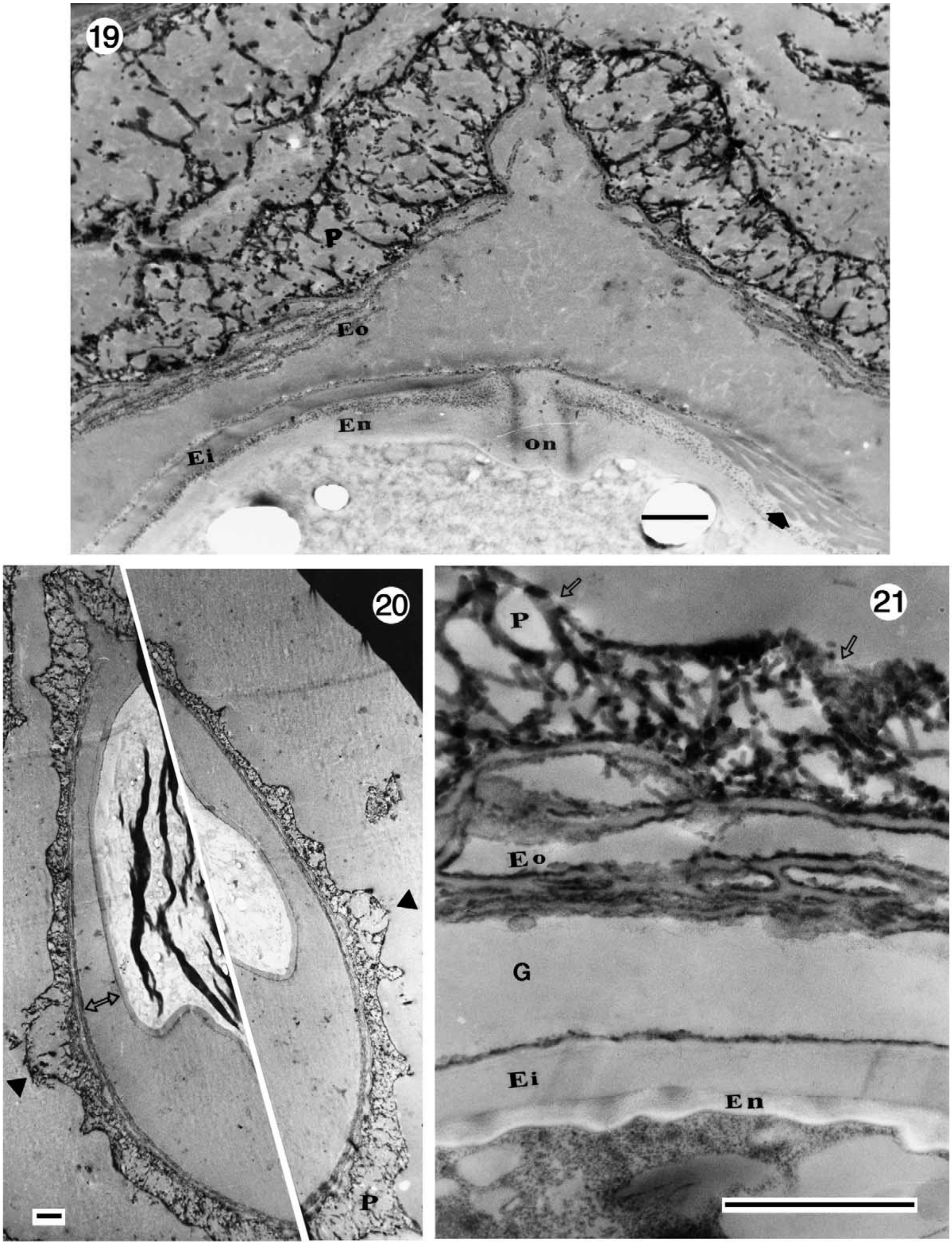
The differentiation of zones within the megaspore exospores in section as defined here for *Isoetes savatieri* was also described for other species including *Isoetes setacea* (Robert et al., 1973), *Isoetes melanopoda* (Taylor, 1992), *Isoetes velata*, *Isoetes durieui*, *Isoetes flaccida*, *Isoetes riparia*, *Isoetes lacustris* and *Isoetes echinospora* (Taylor, 1993).

According to our interpretation the same kind of structural material that constitutes the siliceous cover in *Isoetes savatieri* fills the outer strata of the lacunar exospore. The presence of siliceous material in the same stratum was also reported by Taylor (1992, 1993) for *Isoetes echinospora* and *Isoetes durieui* and Tryon and Lugardon (1991) for *Isoetes andicola* and *Isoetes setaceum*.

Rod structural units of the silica cover in megaspores of *Isoetes savatieri*, as defined here, were found to be surprisingly similar to the siliceous 'rodlets' present between the structural elements of the exospore in megaspores of *Selaginella convoluta* (Arn.) Spring as described by Morbelli and Rowley (1993). Both structures are tubular, circular in section, 30–40 μm in diameter, with a core of lower contrast and a superficial striated pattern. Tryon and Lugardon (1978) suggested a dis-

Plate IV. Microspores as seen with SEM

13. Several microspores in the proximal (p), equatorial (e) and distal (d) views. The polar shape is elliptic and the equatorial shape is convex at the proximal face and hemispheric at the distal face. The difference in ornamentation of both surfaces is evident. Scale bar: 20 μm.
14. Equatorial view showing the proximal expansion of the laesura (star). The distal ornamentation consists of cones. An equatorial ridge (arrow) is evident. The proximal ornamentation is low. Scale bar: 10 μm.
15. Proximal view showing echinulate sculpture. The supra-laesural expansion is evident. Scale bar: 10 μm.
16. Detail of the proximal sculpture showing a variety of elements such as small cones and microechinae on a perforated background (arrows). Scale bar: 2 μm.
17. Distal view. The ornamentation consists of large cones and microechinae on a perforated background (arrows). Scale bar: 10 μm.
18. Detail of the distal sculpture showing large cones, shorter elements of the same shape and microechinae. Scale bar: 2 μm.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tinction regarding the silica deposits of *Selaginella* and *Isoetes* megaspores because the small, often discontinuous deposits in *Selaginella* strongly contrast with the usually very important silica accumulations in *Isoetes*, but in this work the authors have not approached the question of the resemblance or differences of the 'silica structural elements' in both genera. Lugardon (1973) studied the microspores of four species of *Isoetes* and determined that the inner part of the exospore, like in a number of *Selaginella* species, has numerous laminae (plates) irregularly anastomosed in the interradial area. The author named them 'pluristratified or laminated zones'. The term was adopted by us in the description.

Tryon and Lugardon (1991) also recognized an exospore with two laminated zones (Zp) adjacent to the aperture in *Isoetes echinospora*, *Isoetes setaceum*, *Isoetes durieui* and *Isoetes andicola* microspores.

The term and concept of 'para-exospore' (sensu Lugardon, 1972) has not been adopted in the description of the microspore sporoderm in *Isoetes savatieri*. According to Lugardon (1973) in *Isoetes brochoni*, *Isoetes durieui*, *Isoetes echinospora* and *Isoetes setaceum*, the para-exospore is the wall consisting of anastomosed laminar elements that loosely covers the exospore and shows a narrow discontinuity in the apical area of the laesurae. As confirmed in posterior reports of ontogenetical studies (Lugardon, 1990; Tryon and Lugardon, 1991; Uehara et al., 1991), this wall is essentially made up of the same material as the outer layer of the subjacent exospore. Moreover, Lugardon (1990) stressed that 'The ontogenic features suggest very close relationships between the exospore and para-exospore, the latter appearing, in many respects, as a detached part of the exospore'. In the present study, we have regarded and described the laminar layer as the outer part of the microspore exospore. The difficulty in distinguishing a para-exospore in microspores could be related to the stage of development which the spores were going through when they were studied, since, according to Lugardon (1972), this wall when mature has the same texture and contrast as the outer portion of the exospore. Our interpretation of the exospore agrees with that of Uehara et al. (1991) who considered the para-exospore as being part of the exospore and named it 'outer exospore' in *Isoetes japonica*.

A noticeable similarity was observed between the exospore structure and organization of microspores and megaspores of *Isoetes savatieri*. Both exospores consist of superimposed laminae, which are anastomosed to other laminae of different planes. The same coincidence was observed by Pettitt (1966) in both kinds of spores in *Isoetes echinospora*. Likewise, the inner part of the microspore exospore and the inner part of the megaspore exospore do not take part in the formation of the apertural fold. In both types of spores, an equatorial–distal separation of laminae, which is located within the exospore, is present.

Plate V. Microspore wall ultrastructure and apertural organization with TEM

19. A section through the sporoderm in the apertural area. The perispore (P) consists of ramified threads. The outer zone of the exospore (Eo) is made up of interconnected thin elements. The inner zone of the exospore (Ei) has 'pluristratified zones' on either side of the aperture, which are evident in this micrograph only on the right side (arrow). The outer part of the exospore (Eo) and the perispore take part in the formation of the supra-laesural expansion. The endospore (En) is visible under the inner part of the exospore and forms an onciform thickening (On) below the very thin, non-folded apertural differentiation. Scale bar: 1 μm.

20. Microspore in section. The proximal pole is at the top left. The perispore (P) has a lacunose structure and is thicker distally. Its equatorial expansion is marked by arrowheads. Two exospore parts structurally different are evident (two-headed arrow). The outer part of the exospore is laminar. Scale bar: 1 μm.

21. Detail of the sporoderm in section. The perispore (P) is composed of thin threads. The outer part of the exospore (Eo) has a laminar structure with laminae fused with others of different levels. The inner part of the exospore (Ei) has a compact homogeneous structure. A separation (G) between the two parts of the exospore is evident. The endospore (En) is marked. Scale bar: 1 μm.

On the surface, both types of spores also share the presence of discontinuities. These are through the silica cover on megaspores and through the sporopolleninous perispore of microspores. Both types of spores are more prominently ornamented on the distal face, which results in an increase of spore contact along surface.

On the other hand, the TEM micrographs of megaspores and microspores showed differences in the exospore with respect to the contrast of the laminae edges. In megaspores the contrast is uniform throughout the section, but in microspores the laminae edges are more contrasted than the central zone. It was noticed that the material of these edges has characteristics similar to those of the perisporal material, as was also reported in the microspores of several other *Isoetes* species (Lugardon, 1972; Tryon and Lugardon, 1991; Uehara et al., 1991).

The TEM studies carried out in *Isoetes savatieri* have not provided conclusive evidence for the existence of an organic template within the megaspore's siliceous cover. According to Robert et al. (1973) such a thick mineral layer should have a morphogenesis problem, since modern methods of detection had not been enough to detect an organic network. Taylor (1993) observed a lightly staining residue on the megaspore surface after treatment with HF on three species of *Isoetes*. This author referred that materials as probable remnants of an organic template.

The similarity between *Isoetes* and *Selaginella* is less evident with respect to microspore sporoderm (Lugardon, 1990). According to Lugardon (1990), the sporoderm of *Isoetes* microspores is similar to that of some *Selaginella* species like *S. selaginoides* (L.) Link based on the presence of an exospore with lamellar zones, a para-exospore and a space between both layers.

Futures studies of sporoderm ontogeny of both mega and microspores of *Isoetes savatieri* will provide more information about the origin, pattern, presence/absence of an organic template as well as the origin and function of spaces (gaps) within the exospores.

The lack of studies like this on *Isoetes savatieri* in which mega and microspores of a single species are compared is quite significant.

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