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Multiple Developmental Pathways Leading to a Single Morph: Monosulcate Pollen (Examples From the Asparagales)

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• **Background and Aims** Early developmental events in microsporogenesis are known to play a role in pollen morphology: variation in cytokinesis type, cell wall formation, tetrad shape and aperture polarity are responsible for pollen aperture patterning. Despite the existence of other morphologies, monosulcate pollen is one of the most common aperture types in monocots, and is also considered as the ancestral condition in this group. It is known to occur from either a successive or a simultaneous cytokinesis. In the present study, the developmental sequence of microsporogenesis is investigated in several species of Asparagales that produce such monosulcate pollen, representing most families of this important monocot clade.

• **Methods** The developmental pathway of microsporogenesis was investigated using light transmission and epifluorescence microscopy for all species studied. Confocal microscopy was used to confirm centripetal cell plate formation.

• **Key Results** Microsporogenesis is diverse in Asparagales, and most variation is generally found between families. It is confirmed that the whole higher Asparagales clade has a very conserved microsporogenesis, with a successive cytokinesis and centrifugal cell plate formation. Centripetal cell wall formation is described in Tecophilaeaceae and Iridaceae, a feature that had so far only been reported for eudicots.

• **Conclusions** Monosulcate pollen can be obtained from several developmental pathways, leading thus to homoplasy in the monosulcate character state. Monosulcate pollen should not therefore be considered as the ancestral state unless it is produced through the ancestral developmental pathway. The question about the ancestral developmental pathway leading to monosulcy remains open.

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Key words: Aperture pattern, Asparagales, cell wall formation, development, microsporogenesis, monosulcate pollen.

INTRODUCTION

The pollen grain is the male gametophytic phase of flowering plants. Its morphology is highly diversified, from the texture and structure of its wall to its ornamentation and general shape. Apertures, the areas where the external layer (exine) is thinner than over the rest of the grain, represent one of the most variable features of pollen grains (Erdtman, 1947). Apertures play a crucial role during pollen germination, since they are the areas through which the pollen tube emerges. Other aperture functions have been described in the literature. These include harmomegathy, which accommodates pollen volume variation during pollen dehydration prior to anther dehiscence and rehydration on the floral stigma (Wodehouse, 1935; Heslop-Harrison, 1976). Pollen water content status at dispersal is a phenomenon known to influence survival, longevity and germination ability, and to vary among angiosperms (Franchi *et al.*, 2002).

Apertures vary in shape, number and location. Shapes are usually furrows or pores, but other morphologies are also found, such as a zonalsulcus (Walker and Doyle, 1975), a ring-like aperture extended around the pollen grain, or a trichotomosulcus (Erdtman, 1952) with three branches that are joined to form a Y-like structure. The number of apertures usually ranges from one to several. When there is only one polar furrow, the pollen is called monosulcate

(Erdtman, 1952). When three apertures are present, the pollen is named trisulcate (or tricolpate, a characteristic of the eudicots clade), trisulcate or triporate (Punt *et al.*, 1994). Occasionally, pollen grains with more apertures are found. Sometimes no aperture is present, as in inaperturate pollen (Iversen and Troels-Smith, 1950), a condition that is not infrequent in the monocots (Furness and Rudall, 2000b).

The variation in aperture pattern is not randomly distributed in angiosperms, but reflects the phylogeny of this group to some extent. Thus, eudicots are known as the tricolpate clade, because this pollen morphology is synapomorphic for this group (Chaloner, 1970; Crane *et al.*, 1995); monocots generally have monosulcate or monoporate pollen grains, although there are numerous exceptions (Furness and Rudall, 1999, 2000a); basal angiosperms also frequently have monosulcate pollen (Kuprianova, 1966; Walker, 1974; Zavada, 1983; Sampson, 2000; also see Furness and Rudall, 2004 for a review of aperture type distribution in angiosperms). Monosulcate pollen is considered to be plesiomorphic in monocots and basal angiosperms (Furness and Rudall, 1999; Sampson, 2000). This is in agreement with the fact that the oldest known unambiguous fossilized angiosperm pollen is monosulcate (Doyle, 1969; Walker and Doyle, 1975).

Although observation of mature pollen is very well documented, the ontogeny of aperture pattern is still poorly understood at present. Aperture pattern is determined during

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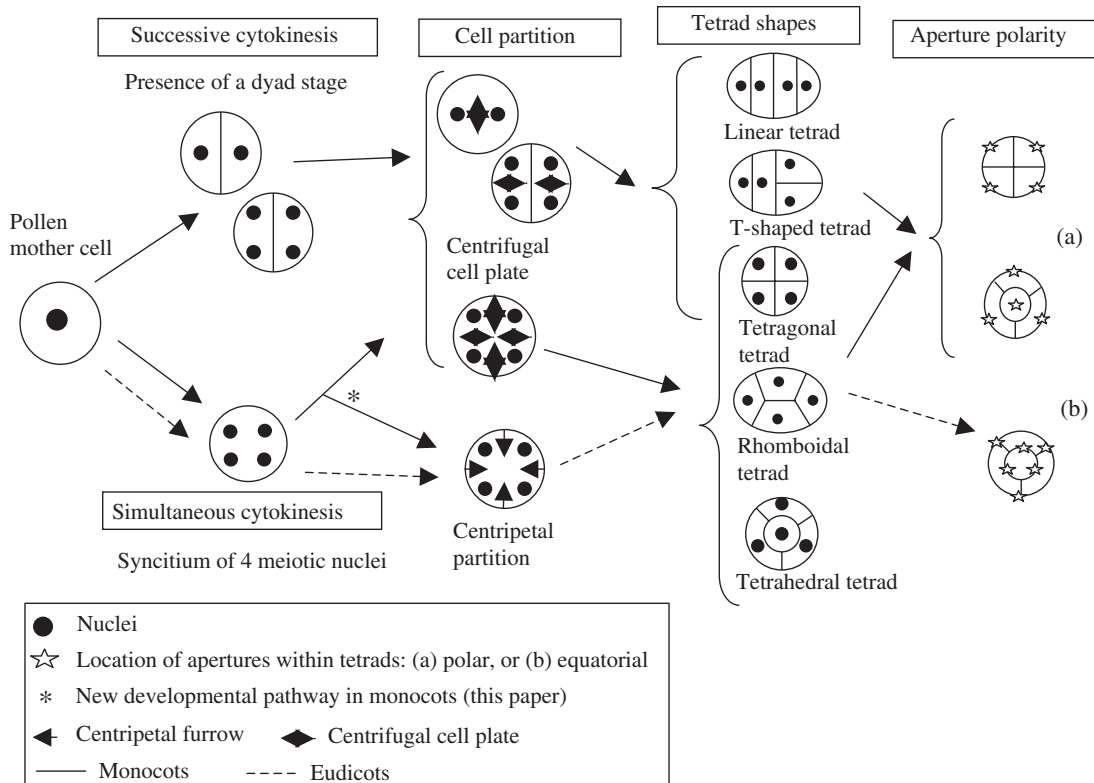


FIG. 1. A schematic presentation of the main developmental pathways of microsporogenesis in angiosperms.

microsporogenesis and can already be observed in mature tetrads. Some key features of aperture pattern have been identified, including the role played by meiotic spindles (Sheldon and Dickinson, 1986; Brown and Lemmon, 1992; Ressayre *et al.*, 2002b) and the shape of the tetrad that results from the combination of meiosis and cytokinesis, as discussed throughout the pollen literature (Rudall *et al.*, 1997; Blackmore and Crane, 1998; Ressayre *et al.*, 2003). An ontogenic model of aperture pattern that explicitly takes into account consequences of tetrad shapes and early microsporogenesis events to predict aperture type (Ressayre *et al.*, 2002a) was used as the basis of the present work. Early microsporogenesis events are important to establish the shape of the tetrad, the polarity within the tetrad and consequently to determine the aperture pattern of the pollen grain.

Among these developmental events, the cytokinesis type is the most described characteristic of microsporogenesis in monocots, and it is known to be diverse in this group.

While some pollen morphologies such as trichotomosulcate pollen seem to be correlated with a simultaneous cytokinesis (Rudall *et al.*, 1997; Harley, 2004), it is known that monosulcate pollen can be produced via both successive or simultaneous cytokinesis, providing some evidence that monosulcate pollen arises from different developmental arrangements. This has been described for species belonging to the Asparagales (Huynh, 1976; Rudall *et al.*, 1997), Dioscoreales and Poales (Furness and Rudall, 1999; 2000a). Successive cytokinesis is characterized by a transitory dyad

stage (Longly and Waterkeyn, 1979b), and the tetrad is constrained to tetragonal, decussate, T-shaped or linear shapes (Fig. 1). This type of cytokinesis is particularly common in monocots (Rudall *et al.*, 1997; Furness and Rudall, 1999). Simultaneous cytokinesis, on the other hand, is mostly associated with tetrahedral tetrads. This type is found in monocots, but is the rule in eudicots (Rudall *et al.*, 1997; Furness and Rudall, 1999; Furness *et al.*, 2002). This view on microsporogenesis, which associates centrifugal cell plates and sometimes successive cytokinesis with monocots, and centripetal cytoplasm partitioning and sometimes simultaneous cytokinesis with eudicots (Fig. 1) is well established in the literature (for examples, see Dover, 1972; Harley and Baker, 1998; Harley, 2004). Results from this study, however, suggest that this view is rather a conventionally held generalization.

Despite the fact that cytokinesis and pollen morphology are well described, only limited data are currently available concerning the entire sequence of microsporogenesis in the Asparagales. We have thus chosen to investigate microsporogenesis in this group for several reasons: a robust molecular phylogeny is now available for this group (Chase *et al.*, 1995; Rudall *et al.*, 1997; Fay *et al.*, 2000; see also Fig. 2), monosulcate pollen is the most common pollen morphology, and cytokinesis is already known to vary within this order (Rudall *et al.*, 1997). Within Asparagales, simultaneous cytokinesis is considered to be the ancestral condition, with a reversal to successive cytokinesis in the 'higher' Asparagales clade (Chase *et al.*, 1995; Rudall *et al.*, 1997).

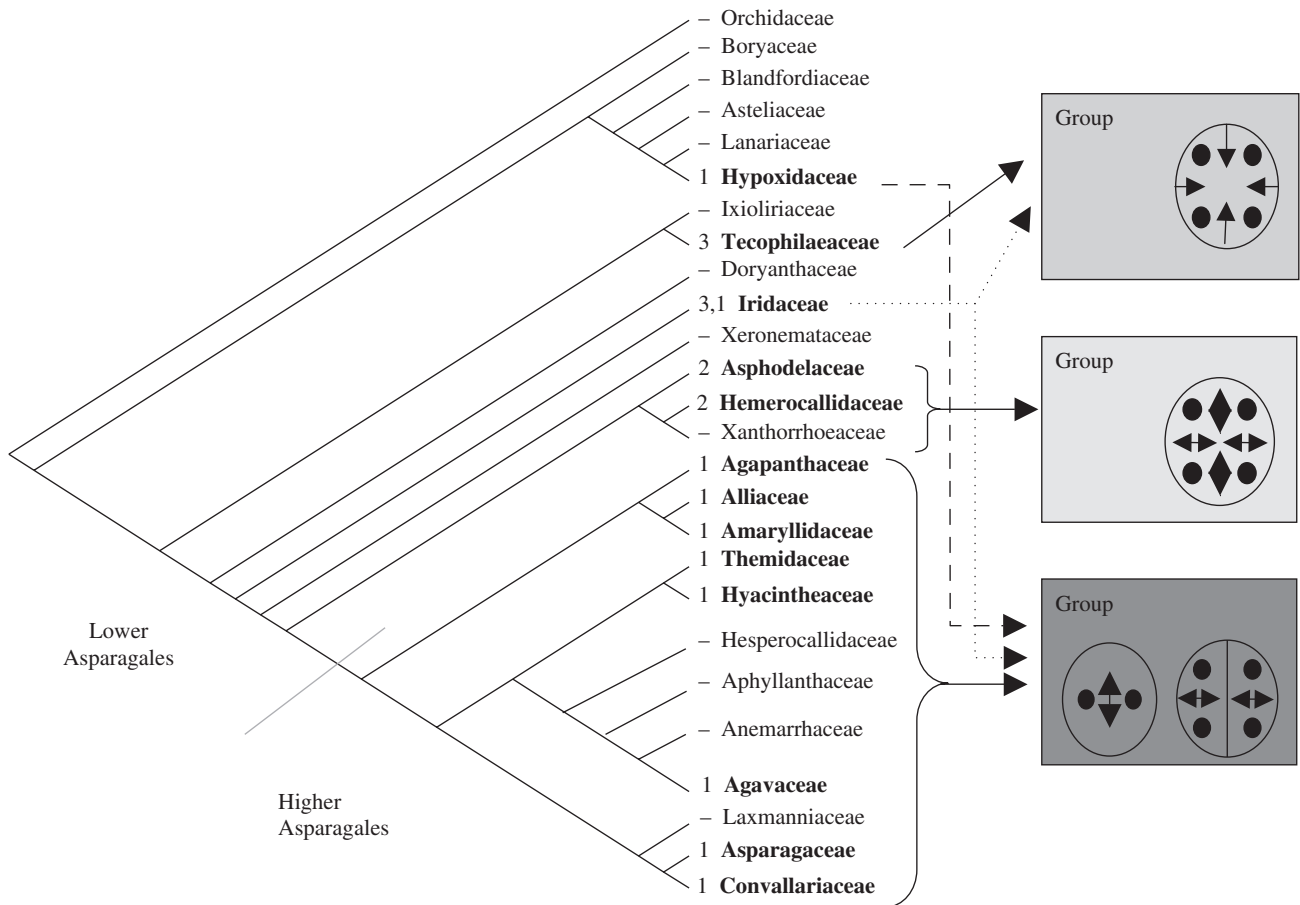


FIG. 2. A phylogenetic reconstruction of the affinities between families in the Asparagales (APG, 2003). Families of which representative species were included in the present study are indicated in bold. They were assigned to the different groups according to the way cytoplasm is partitioned: group 1, successive division/centrifugal cell plates; group 2, simultaneous division/centripetal infurrowings; and group 3, simultaneous division/centrifugal cell plates. The groups encountered in a family (1, 2 or 3) are indicated at the tips of the phylogeny. A minus (–) indicates that the family was not investigated in this study.

The successive/simultaneous cytokinesis dichotomy has been useful to distinguish Anthericaceae (now placed within Agavaceae, APG II, 2003), displaying successive cytokinesis, and Asphodelaceae, which have simultaneous cytokinesis (Dahlgren *et al.*, 1985; Stedje and Nordal, 1994; Kativu, 1996). Monosulcate pollen is found as the usual condition in most families in the lower asparagoids, from Hypoxidaceae (Rudall *et al.*, 1997) to the better documented Asphodelaceae (Diaz Lifante, 1996; Xiong *et al.*, 1998; Kosenko and Sventorzetskaya, 1999), but also in Iridaceae (Goldblatt *et al.*, 1991; Goldblatt and Le Thomas, 1992, 1993; Pinar and Dönmez, 2000). The higher Asparagales also mainly have monosulcate pollen (Rudall *et al.*, 1997).

We made a deliberate choice to focus in this paper on species producing monosulcate pollen, in which we investigated variation during microsporogenesis in order to address the following key questions: what are the possible ontogenic trajectories leading to monosulcate pollen (in other words, how do Asparagales acquire the monosulcate pollen type)? Does microsporogenesis display more variation than just cytokinesis? What are the consequences of the developmental diversity on the presumed monosulcate ancestry of monocot pollen?

MATERIAL AND METHODS

Microsporogenesis was investigated using material collected from plants grown in botanical gardens, plants collected on the campus of the University Paris-Sud (Orsay, France) or plants collected on the campus of the University Joseph Fourier (Grenoble, France) (Table 1).

Microsporocytes were extracted from one anther and immediately squashed in aceto-carmin to determine the stage of microsporogenesis. Depending on the stage observed, different subsequent staining techniques were used. Aceto-carmin (67.5 mL acetic acid, 0.75 g carmin, 0.025 g ferrous acetate, made up to 150 mL with distilled water) was used to investigate cytokinesis. A syncytium stage with four meiotic nuclei resulted in the species being assigned to the simultaneous type. If the second meiotic nuclear division occurred after the first cytokinesis event had taken place, and if a dyad stage was identified, the species was assigned to the successive type. Anilin blue (modified from Arens, 1949, with the addition of glycerol to 15 % of the final volume) was used to visualize callose wall formation and to determine the shape of the tetrad. In some cases, it was even possible to discern apertures within

TABLE 1. A summary of the families, species, voucher specimens and origin of the material studied

Family	Species	Voucher	Origin
Lower Asparagales			
Hypoxidaceae	<i>Hypoxidia maheensis</i> Friedmann	810110	CBNB, Brest (France)
Tecophileaceae	<i>Cyanella alba</i> Herb.	510/67	Kirstenbosch (RSA)
	<i>Cyanella orchidiformis</i> Jacq.	9809wf2	BulbArgence (France)
Iridaceae	<i>Babiana angustifolia</i> Sweet	Cultivated (no voucher)	Kirstenbosch (RSA)
	<i>Babiana disticha</i> Ker Gawl.	655/74	Kirstenbosch (RSA)
	<i>Chasmanthe floribunda</i> (Salisb.)N.E.Br.	856/83	Kirstenbosch (RSA)
	<i>Ferraria crispa</i> Burm.	9810AC12	BulbArgence (France)
	<i>Freesia alba</i> (Baker)Gumbl.	Cultivated (no voucher)	Stellenbosch (RSA)
	<i>Iris pseudoacorus</i> L.	Wild	UPS (France)
	<i>Ixia lutea lutea</i> Baker	177/78	Kirstenbosch (RSA)
	<i>Libertia chilensis</i> Klotzsch	980085	CBNB, Brest (France)
	<i>Libertia formosa</i> Graham	AUT-2001-JAR-032	Serres d'Auteuil (France)
	<i>Moraea bipartita</i> L.	1002/71	Kirstenbosch (RSA)
	<i>Moraea aristata</i> (Houtt.)Asch. & Graebn.	1252/82	Kirstenbosch (RSA)
	<i>Tritonia securigera</i> Ker Gawl.	9810AC66	BulbArgence (France)
	<i>Watsonia aletroides</i> Ker Gawl.	(No voucher)	BulbArgence (France)
Hemerocallidaceae	<i>Hemerocallis fulva</i> L.	Cultivated (no voucher)	Grenoble University (Fr.)
Asphodelaceae	<i>Aloe globulifera</i> Graessn.	Chèvreloop	Chèvreloop (France)
	<i>Asphodeline liburnica</i> Reichb.	AUT-1999-JAR-043	Serres d'Auteuil (France)
	<i>Trachyandra muricata</i> Kunth	Wild (no voucher)	Langebaan (RSA)
	<i>Trachyandra</i> sp.	Wild (no voucher)	Langebaan (RSA)
	<i>Bulbine alooides</i> Willd.	431/35	Kirstenbosch (RSA)
	<i>Bulbinella nutans nutans</i> Th.Dur.et Schinz	1309/84	Kirstenbosch (RSA)
	<i>Kniphofia praecox</i> Baker	172/76	Kirstenbosch (RSA)
Higher Asparagales			
Alliaceae	<i>Allium altaicum</i> Pall.	Al 233	ESE (France)
	<i>Allium ursinum</i> L.	Wild (no voucher)	UPS (France)
Amaryllidaceae	<i>Narcissus poeticus</i> L.	PPF-2000-NAT-014	Serres d'Auteuil (France)
	<i>Agapanthus umbellatus</i> L'Hér.	Cultivated (no voucher)	Stellenbosch (RSA)
Themidaceae	<i>Triteleia ixioides ssp scabra</i> Greene	9510HD1	BulbArgence (France)
Hyacinthaceae	<i>Albuca nelsonii</i> N.E.Br.	9809 kb	BulbArgence (France)
	<i>Eucomis autumnalis</i> (Mill.) Chittenden	671/83	Kirstenbosch (RSA)
	<i>Hyacinthus non-scriptus</i> L.	Wild (no voucher)	UPS (France)
	<i>Ornithogalum longibracteatum</i> Jacq.	528/93	Kirstenbosch (RSA)
	<i>Veltheimia bracteata</i> Harv. Ex Baker	Cultivated (no voucher)	Kirstenbosch (RSA)
Agavaceae	<i>Yucca agavoides</i> Horl. Ex Carr.	Cultivated (no voucher)	UPS (France)
	<i>Arthropodium cirrhatum</i> G.Benn.	Kew	RBG Kew (England)
	<i>Chlorophytum pauciflorum</i> Dammer	Wild (no voucher)	West Coast (RSA)
	<i>Hosta</i> sp.	Cultivated (no voucher)	UPS (France)
Convallariaceae	<i>Polygonatum multiflorum</i> Kunth	Wild (no voucher)	UPS (France)
	<i>Convallaria majalis</i> L.	EDB-2000-VIV-0135	UPS (France)

the tetrads. Congo red (Stainier *et al.*, 1967) was used to stain pollen grains or microspores just before their release from the tetrad, in order to determine the aperture type, shape and position. Aceto-carmin and congo red preparations were observed under transmission light with a Zeiss Axiophot microscope. Anilin blue preparations were observed using epifluorescence microscopy (epifluorescence Zeiss Axiophot microscope used with filter set 01; excitation 345, emission 425 nm long pass) or confocal microscopy.

Whenever possible, the developmental sequence during microsporogenesis, as presented in the results, was established on the basis of data obtained from a single individual, although several individuals were usually investigated. For species in which each plant produces only a few flowers per year, such as in some Iridaceae, it was not possible to infer the complete developmental sequence from only one individual.

RESULTS

The observations reveal that monosulcate pollen (see, for example, the monosulcate pollen of *Veltheimia bracteata* Harv., Fig. 3L) can be obtained through seven different developmental pathways. We divided these different pathways in three main groups, according to their similarity in microsporogenesis. We have chosen to illustrate the developmental pathway typical of each group by presenting the developmental sequence of one or a few species. Group 1, named 'higher Asparagales' is illustrated by *Allium altaicum*. Group 2, 'Asphodelaceae' (and sister groups), is illustrated by *Bulbinella nutans*, *Trachyandra muricata* and *Hemerocallis fulva*. Group 3, 'centripetal group' is illustrated by *Cyanella orchidiformis*, *Iris pseudoacorus* and *Tritonia securigera*. Table 2 lists the data obtained for the species of Asparagales investigated in this study, according to their developmental pathways.

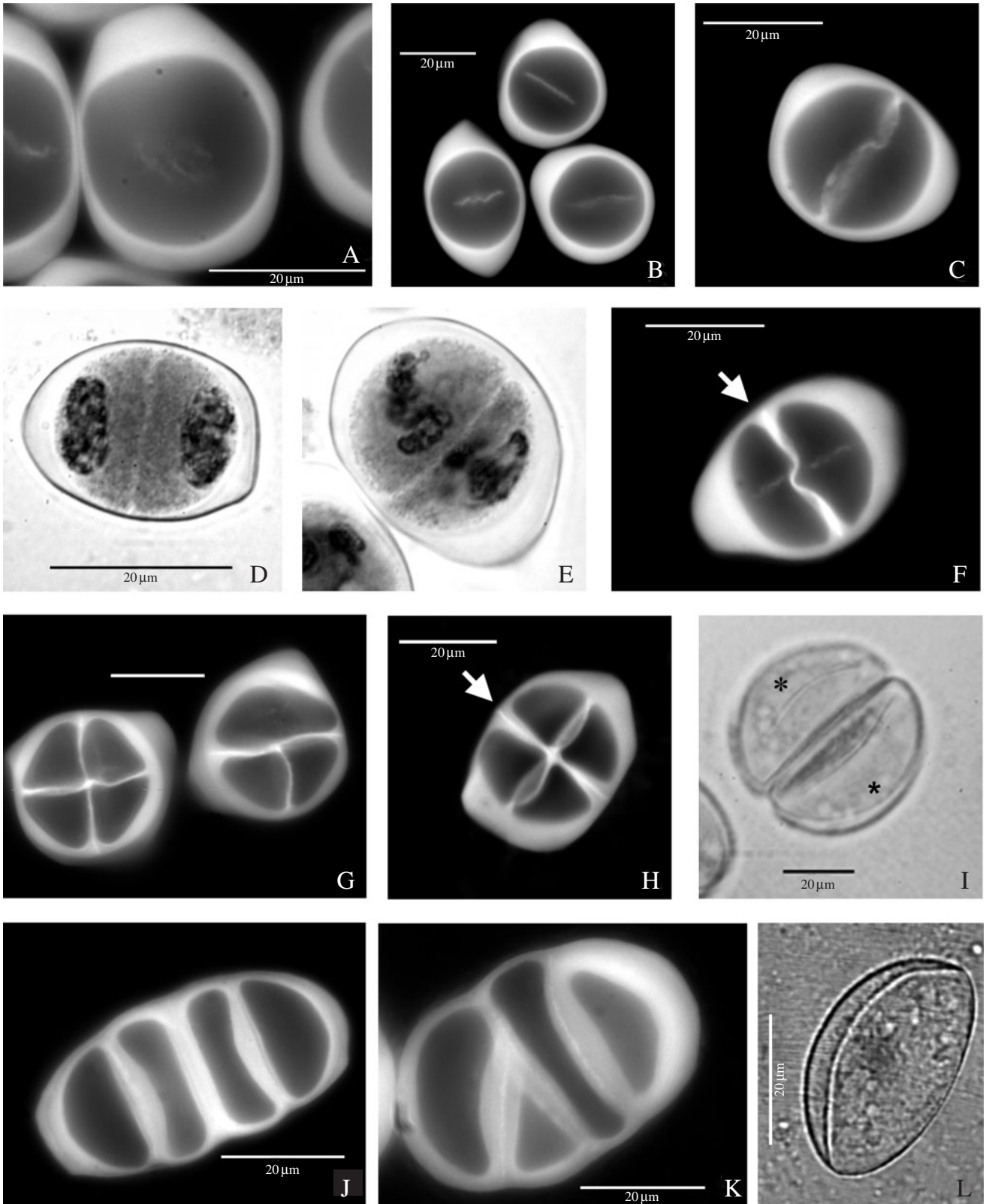


FIG. 3. Group 1, 'Higher Asparagales'. Figs 3A to 3L: Microsporogenesis in *Allium altaicum*. (A, B) Centrifugal cell plate of the first cytoplasmic division. (C, D) Dyad stage. (E) Metaphase of second meiotic division. (F) Centrifugal cell plates of the second cytoplasmic division. (G) Tetragonal and decussate tetrads. (H) Later tetragonal tetrad with extra callose deposits (indicated by an arrow). (I) Mature tetrad with (polar) apertures visible (indicated by asterisk). (J, K) Alternative tetrad shapes in *Allium nelsonii*. (J) Linear tetrad and (K) irregular T-shaped tetrad. (L) Monosulcate pollen (*Veltheimia bracteata*). (A–C, F–H, J, K) Anilin blue staining; (D, E) aceto-carmin staining; (I, L) congo red staining. Scale bars = 20 μm.

TABLE 2. Summary of the different types of pollen development recorded in this study. Each developmental type is represented by one species (left column), while other species with similar developmental pathways are listed in the last column to the right

Species	Cytokinesis	Cell wall formation	Tetrad shape	Particular features	Species with similar development type
<i>Allium altaicum</i>	Successive	Centrifugal	Tetragonal/decussate	Occasionally with additional tetrad morphologies: linear, T-shaped or other successive-derived tetrads	<i>A. ursinum</i> <i>Agapanthus umbellatus</i> <i>Albica nelsonii</i> <i>Arthropodium cirrhatum</i> <i>Chlorophytum pauciflorum</i> <i>Eucomis autumnalis</i> <i>Hyacinthus non-scriptus</i> <i>Hypoxidia maheensis</i> <i>Hosta</i> sp. <i>Moraea aristata</i> <i>Moraea bipartita</i> <i>Narcissus poeticus</i> <i>Ornithogalum longibracteatum</i> <i>Polygonatum multiflorum</i> <i>Convallaria majalis</i> <i>Triteleia ixioides</i> ssp <i>scabra</i> <i>Veltheimia bracteata</i> <i>Yucca agavoides</i> <i>Aloe globulifera</i> <i>Asphodeline liburnica</i> <i>Bulbine alooids</i> <i>Kniphofia praecox</i> <i>Trachyandra</i> sp.
<i>Bulbinella nutans</i>	Simultaneous	Centrifugal	Tetrahedral (irregular)	–	–
<i>Trachyandra muricata</i>	Simultaneous	Centrifugal	Mostly tetragonal or decussate, rarely tetrahedral	–	–
<i>Hemerocallis fulva</i>	Intermediate between successive and simultaneous	Centrifugal	Tetragonal, decussate or Tetrahedral (irregular)	Curved cell plates	–
<i>Cyanella orchidiformis</i>	Simultaneous	Centripetal	Mostly tetrahedral (irregular)	–	<i>Cyanella alba</i> <i>Ferraria crispa</i> <i>Freesia alba</i> <i>Libertia chilensis</i> <i>Libertia formosa</i> <i>Babiana angustifolia</i>
<i>Iris pseudacorus</i>	Simultaneous	Centripetal	Tetragonal, decussate or tetrahedral (irregular)	Callose ring	<i>Babiana disticha</i> <i>Chasmanthe floribunda</i> <i>Ixia lutea lutea</i> <i>Watsonia aleitroides</i>
<i>Tritonia securigera</i>	Intermediate between successive and simultaneous	Centripetal	Tetragonal, decussate or tetrahedral (irregular)	Callose ring	–

Group 1: Allium altaicum, a microsporogenesis pathway typical of higher Asparagales

In *Allium altaicum*, cytokinesis is of the successive type (Fig. 3A–F), as is shown by the characteristic dyad stage (Fig. 3C–E). Cell plates grow centrifugally during both the first cytoplasmic partition event (Fig. 3A, 3B) and the second partition (Fig. 3F). Secondary callose deposits are observed in this species (Fig. 3F, 3H), and are abundant on the first cell plate. We use the term ‘extra callose deposits’ for the deposits that are formed after cytokinesis is completed. Although their biochemical nature may differ from the cell plates (Longly and Waterkeyn, 1979a, b), this has not been investigated. Tetrads are tetragonal or decussate in equal proportions (Fig. 3G). Apertures are located at the distal pole of microspores within the tetrads (Fig. 3I, indicated by an asterisk).

All investigated species from the higher Asparagales clade display the same developmental sequence: successive cytokinesis with centrifugal cell plate formation and polar apertures. Some species from the Lower Asparagales, such as *Hypoxidia maheensis* (Hypoxidaceae), *Babiana angustifolia* and *B. disticha* (Iridaceae), present the same developmental pathway. In several species, linear or T-shaped (or derived) tetrads (Fig. 3J and 3K, respectively, in *Albuca nelsonii*) are observed in addition to the tetragonal and decussate tetrads. It was not possible to see apertures within these tetrads so we do not know if they result in monosulcate pollen, but only monosulcate pollen grains are observed at maturity. Variable amounts of extra callose are laid onto cell plates in mature tetrads in higher Asparagales, either on the first cleavage wall (Fig. 3F, 3H), or on all the cleavage walls (Fig. 3J, 3K).

Group 2: Bulbinella nutans, a microsporogenesis typical of Asphodelaceae

This species displays a simultaneous cytokinesis (Fig. 4E). Cytoplasmic partitioning is achieved by the formation of centrifugal cell plates (Fig. 4A–D). The resulting tetrads are irregular tetrahedral (Fig. 4F). Secondary callose deposits are recorded (Fig. 4G, H). Apertures are located distally within tetrads (Fig. 4H).

Most species studied from the Asphodelaceae have the same type of microsporogenesis: simultaneous cytokinesis, centrifugally growing cell plates, irregular tetrahedral tetrads, and polar apertures. This type was also observed in *Hemerocallis*, the only genus of Hemerocallidaceae that produces monosulcate pollen. Minor variation in the microsporogenesis developmental sequence were detected in the following species.

Trachyandra muricata (Asphodelaceae). This species has the same developmental sequence as the other Asphodelaceae, but the tetrads formed are mostly tetragonal instead of irregular tetrahedral. Meiotic nuclei are squarely arranged at the end of meiosis (Fig. 4I), and our observations reveal that only four cleavage planes are formed during cytokinesis (Fig. 4J–L) instead of six as in the other species investigated (Fig. 4C).

Hemerocallis fulva (Hemerocallidaceae): *a special case of cytokinesis*. This species displays centrifugal cell plates

that grow simultaneously, although one cell plate starts growing before the others and is generally larger than the others (Fig. 4M–O). This makes cytokinesis appear like a quasi-successive partitioning, but a true dyad stage is never observed. Cytokinesis in *Hemerocallis fulva* is therefore intermediate between successive and simultaneous partitioning. Tetrads are either tetragonal (Fig. 4Q) or irregular tetrahedral. Cell plates curve and fold back in some cells (Fig. 4P), so that the resulting tetrad can assume different shapes (Fig. 4R), and appear to be slightly irregular tetrahedral. Aperture location is clearly visible in the late tetrad stage with aniline blue staining (Fig. 4R, indicated by an asterisk) as it is marked as a footprint in the callose wall (Fig. 4Q, indicated by an asterisk). Apertures are also polar. Extra callose deposits are present but less conspicuous than in Asphodelaceae. The microsporogenesis in this species differs from the situation found in all other Hemerocallidaceae, which have regular tetrahedral tetrads and produce trichotomosulcate pollen (Rudall *et al.*, 1997).

Group 3: ‘Centripetal group’, Cyanella orchidiformis (Tecophilaeaceae)

Cyanella orchidiformis displays a simultaneous cytokinesis that proceeds centripetally (Fig. 5A–C), apertures are located at the distal pole (Fig. 5G, H). This species exhibits a large variety of tetrad shapes, ranging from quasi-tetragonal (Fig. 5D) to irregular tetrahedral (Fig. 5E) or rhomboidal (Fig. 5F), and the pollen grains are always monosulcate (Fig. 5G, H, apertures indicated by an asterisk). Extra callose is deposited on the cell plates (Fig. 5D–F). A similar developmental pathway was observed in several species of the Iridaceae (*Freesia alba*, *Libertia chilensis*, *Libertia formosa* and *Ferraria crispa*). Minor variation from this pathway was recorded in several species of this family.

The callose ring in Iris pseudoacorus (Iridaceae). *Iris pseudoacorus* is peculiar in possessing a callose ring extending around the pollen mother cell, which is visible during meiosis (Fig. 5I, J, but see also Fig. 5M in *Tritonia*). This callose ring is a continuous callose deposit running around the pollen mother cell, which forms during meiosis between the two nuclear divisions. Such a structure is already known to occur, for example in *Magnolia* (Huynh, 1976). Although this callose ring could be misleading and make cytokinesis appear to be successive (Fig. 5I), the cytoplasmic partitioning does not take place before meiosis is completed (Fig. 5J). Cytokinesis is therefore simultaneous, and cytoplasmic partitioning is achieved through centripetally growing infurrows (Fig. 5K). Most tetrads are tetragonal or decussate, occasionally irregular tetrahedral. Apertures are polar (Fig. 5L, indicated by an asterisk).

A similar developmental sequence was observed in several species of Iridaceae, including *Ixia lutea* Baker var. *lutea*, *Babiana angustifolia*, *Babiana disticha*, *Chasmanthe floribunda* and *Watsonia aletroides*. The striking feature of this development type is the presence of this callose ring in all of these species.

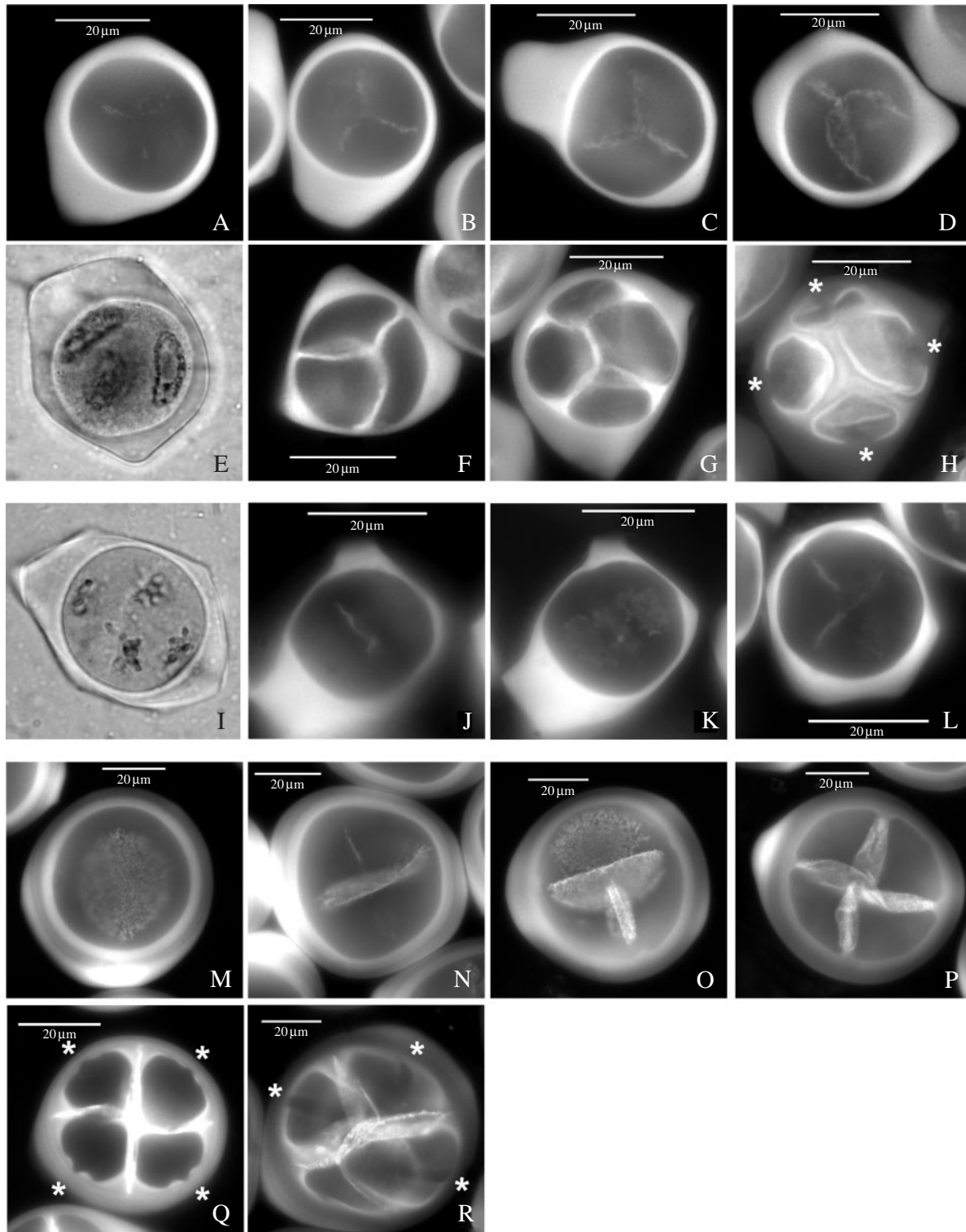


FIG. 4. Group 2, 'Asphodelaceae' (and sister groups). (A–H) Microsporogenesis in *Bulbinella nutans*. (A–D) Cell plates expanding centrifugally and simultaneously. (E) Syncytium stage with four meiotic nuclei. (F) Irregular tetrahedral tetrad, with two large and one small walls. (G, H) The same tetrad illuminated with only a DAPI filter and with a IRTC filter respectively; note extra callose deposits and polar aperture location (indicated by asterisk). (I–L) Microsporogenesis in *Trachyandra muricata*. (I) Telophase of second meiotic division, with nuclei on the same plane. (J, K) Simultaneous centrifugally expanding cell plates (different height within one tetrad). (L) Another example of simultaneous centrifugally expanding cell plates. (M–R) Microsporogenesis in *Hemerocallis fulva*. (M) First centrifugal cell plate. (N, O) Centrifugal growth of secondary cell plates, showing that the first cell plate still does not close the pollen mother cell (no dyad formed) at this stage. (P) Tetragonal tetrad, with slightly curved cell plates. (Q) Mature tetragonal tetrad, with extra callose deposits and polar apertures visible (indicated by asterisk). (R) Mature irregular tetrahedral tetrad with polar apertures visible (indicated by asterisk). (A–D, F–H, J–R) Anilin blue staining; (E, I) aceto-carmin staining. Scale bars = 20 μm .

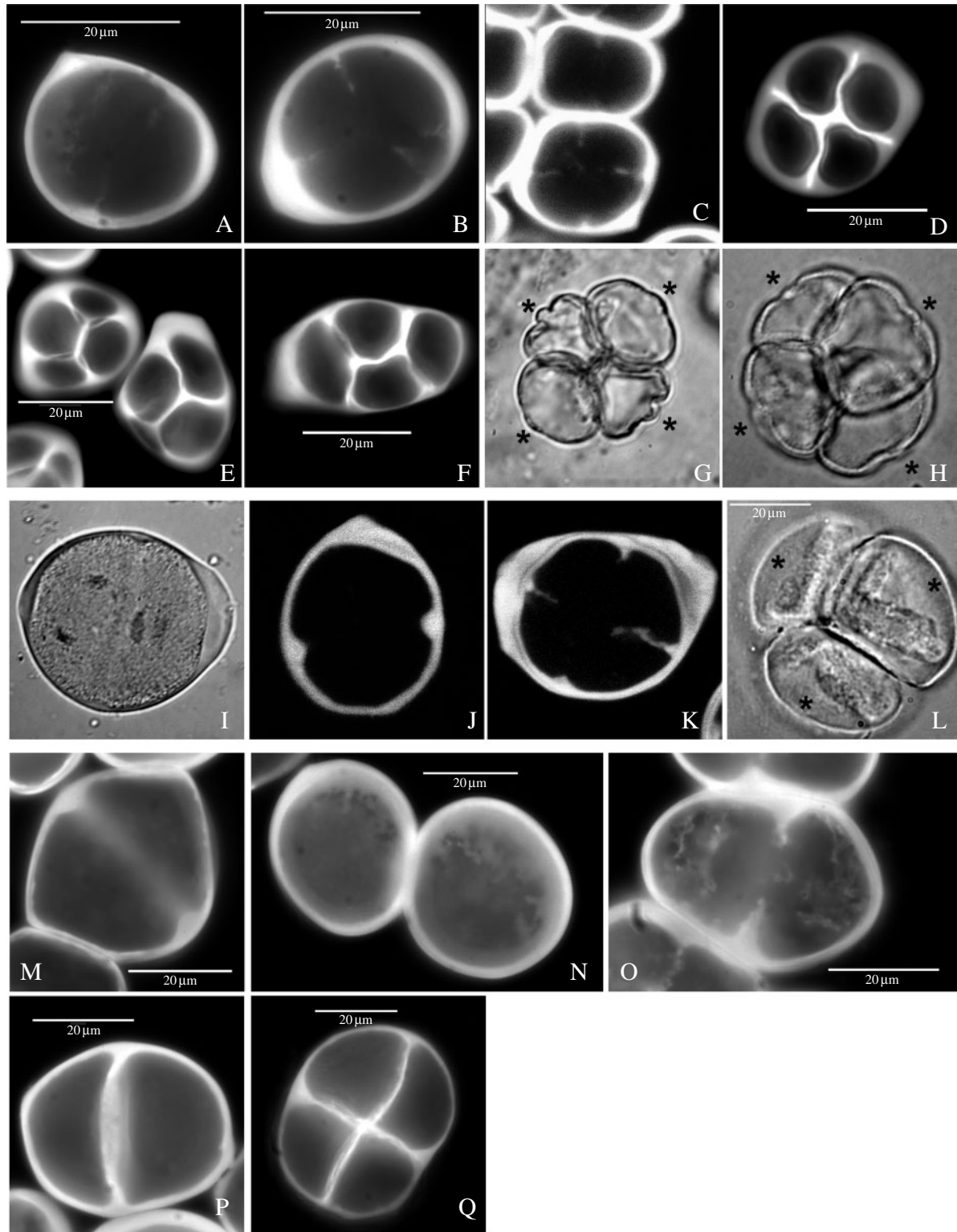


FIG. 5. Group 3, 'Centripetal group'. (A–H) Microsporogenesis in *Cyanelia orchidiformis*. (A, B) Beginning of cytoplasmic division with centripetal infurrowings. (C) Confocal microscopy; centripetal partition observed at the median section of the cell. (D–F) Tetrad shapes: quasi-tetragonal (D), tetrahedral (E) and rhomboidal (F). (G, H) Polar apertures, in rhomboidal (G) or irregular tetrahedral tetrads (H). (I–L) Microsporogenesis in *Iris pseudoacorus*. (I) Telophase of second meiotic division, with a callose ring. (J) Confocal microscopy; callose ring observed at the median section of the cell. (K) Confocal microscopy; centripetal cell partitioning, observed at the median section of the cell. (L) Polar apertures in an irregular tetrahedral tetrad (indicated by asterisk). (M–Q) Microsporogenesis in *Tritonia securigera*. (M) Callose ring stage. (N) Centripetal infurrowing growth from the callose ring. (O) Simultaneous centripetal infurrowing. (P) A cell in a true dyad stage. (Q) Tetragonal tetrad. (A–F, J, K, M–Q) Anilin blue staining; (I) acetato-carmin staining; (G, H, L) congo red staining. Scale bars = 20 μm .

Simultaneous and successive cytokinesis both occurring in Tritonia securigera (Iridaceae). A developmental sequence similar to *Iris pseudoacorus* was observed in *Tritonia securigera*: formation of a callose ring during meiosis (Fig. 5M), cytokinesis by centripetal division (Fig. 5N, O) with mostly tetragonal tetrads (Fig. 5Q) and polar apertures. However, the cytoplasmic partitioning starts very soon after the callose ring is elaborated (Fig. 5N), leading to a dyad stage in some cells (Fig. 5P), while other cells are partitioned simultaneously (Fig. 5O). Cytokinesis is therefore intermediate between successive and simultaneous, depending on the mother cell.

DISCUSSION

Our study shows that microsporogenesis in the Asparagales is actually more diverse than has been previously thought. Variation in Asparagales does not only concern the type of cytokinesis, but also the way in which the cytoplasm is partitioned. While certain of these developmental pathways develop into different aperture types (data not shown), we focused on microsporogenesis pathways leading to the production of monosulcate pollen. Seven different developmental pathways were identified, all of which eventually result in the formation of this aperture type. Some pathways are very similar (in *Iris* and *Tritonia*, microsporogenesis is related to the group 3 ‘centripetal group’, and in *Trachyandra* and *Hemerocallis*, it is related to the group 2 ‘Asphodelaceae group’). The combination of cytokinesis (successive or simultaneous) and cytoplasmic partitioning (centripetally or centrifugally) determines four different pathways. These four combinations also vary with regard to the tetrad shape. The tetrad shape is linked to the type of cytokinesis: successive cytokinesis usually leads to tetragonal or decussate tetrads, while simultaneous cytokinesis usually leads to tetrahedral tetrads. The three most frequent combinations are (group 1) successive division/centrifugal cell plates, (group 2) simultaneous division/centripetal infurrowings and (group 3) simultaneous division/centrifugal cell plates, respectively found in 18, 13 and eight of the 40 species investigated. These three combinations correspond to the most widespread view about microsporogenesis in angiosperms (Fig. 1), as presented in the Introduction, but development actually varies far beyond these limits, with additional developmental features (such as intermediate types of cytokinesis or the presence of a callose ring during meiosis). Moreover, the Iridaceae and Tecophilaeaceae are characterized by the combination of simultaneous cytokinesis and centripetal cell plates, a combination of features which, to our knowledge, has never been described in monocots before. This combination is, however, known to characterize eudicot microsporogenesis.

Variation in microsporogenesis will be discussed with respect to each different step: cytokinesis type, cell wall formation, and tetrad shape, before summarizing our results about the observed patterns in microsporogenesis in Asparagales.

Cytokinesis type

The evolution of cytokinesis in Asparagales has been studied by Rudall *et al.* (1997). Our results, which are

based on the study of different species, confirm that the higher Asparagales clade, represented here by eight families (Alliaceae, Amaryllidaceae, Agapanthaceae, Themidaceae, Hyacinthaceae, Agavaceae, Asparagaceae and Convallariaceae, see also Fig. 2) is characterized by successive cytokinesis. Both types of cytokinesis occur in lower Asparagoids, although the simultaneous condition is more frequent. As successive cytokinesis in lower Asparagoids is found in several species from different families, for example in Asteliaceae (Rudall *et al.*, 1997), in Iridaceae and in Hypoxidaceae (Rudall *et al.*, 1997; our results), it has been argued that switches in cytokinesis are frequent in this group, and that this character is labile. Simultaneous cytokinesis has evolved independently several times in monocots, and may increase the efficiency of pollen production (Furness and Rudall, 2000a). The lack of simultaneous cytokinesis in the large ‘higher’ Asparagales clade may then indicate that the ability to switch back from successive to simultaneous has been lost or that this developmental switch is deleterious in this group.

Theoretically, transitions in cytokinesis should not imply dramatic mutations as no functional change is needed, but simply a delay in cytoplasmic partitioning during meiosis. Thus such a transition can potentially arise rapidly. Two cases from our data (namely *Hemerocallis* and *Tritonia*) nevertheless suggest that transitions from simultaneous to successive cytokinesis can happen progressively. The developmental pathway observed in *Hemerocallis fulva* could represent a transition between simultaneous and successive cytokinesis. Generally, during cytokinesis, cleavage planes are located equidistantly between the nuclei; the cell walls are formed at the intersection between microtubules radiating from each meiotic nucleus. In simultaneous cytokinesis, the number of cleavage planes ranges from four to six (Ressayre *et al.*, 2002a). Four cleavage planes are formed in a tetragonal (or decussate) tetrad as observed in *Trachyandra muricata* (Fig. 4I–L), five cleavage planes are formed in a rhomboidal tetrad (Fig. 5F), and six in a tetrahedral tetrad (Fig. 4C for the cleavage planes, 4F and G for the tetrahedral tetrads). Normal successive cytokinesis involves three cleavage planes: the first one is between the nuclei resulting from the first meiotic division, leading to the dyad stage, and the other two are between the nuclei resulting from second meiotic division. The resulting tetrad is generally tetragonal (other possible tetrad shapes are illustrated in Fig. 1). Only three cell plates are observed in *Hemerocallis fulva* (Fig. 4O) as in the case of a successive cytokinesis, despite the fact that it is a simultaneous species. The formation of one of the cell plates is initiated before the others, but a true dyad stage is never observed. On the other hand, *Tritonia securigera* displays a transition from simultaneous toward successive cytokinesis with centripetal cytoplasmic partitioning. As illustrated, some pollen mother cells go through a true dyad stage (Fig. 5P), which is characteristic of successive cytokinesis, while others are partitioned simultaneously (Fig. 5O). The timing of cell partitioning in this species is variable, and therefore seems to be transitional if we consider the population of cells. Evolution from simultaneous towards successive cytokinesis could then possibly involve transitory

stages in which cytoplasmic partitioning is initiated before the second meiotic division, until a true dyad stage is retained. This may also be a way to canalize tetrad shape with simultaneous cytokinesis and therefore stabilize pollen into monosulcate morphology, as tetragonal tetrads always lead to monoaperture when apertures are polar.

Cell wall formation

The occurrence of centripetal cell partitioning in monocots is recorded here for the first time. This type of partitioning was observed in two families from the lower asparagoids, namely Tecophilaeaceae (Fig. 5B, C) and Iridaceae (Fig. 5K, N, O). In both families it constitutes the main way in which pollen mother cells divide. Centripetal cytoplasmic partitioning is known to occur in basal angiosperms, for example among the magnoliids (Brown and Lemmon, 1992), and is known to be general in eudicots (Furness and Rudall, 2004). To date, only centrifugal cell plates had been recorded in monocots.

Another striking result associated with centripetal infurrowings during cell partitioning is the presence of a callose ring in several Iridaceae species that display simultaneous cytokinesis. Although the exact role of this callose ring in meiosis is still unclear, we noted that the shape of the tetrads seemed to be constrained to tetragonal when this ring is present. In most cases, the partitioning process starts on the callose ring, making the resulting tetrad look like a tetrad obtained through successive cytokinesis, i.e. tetragonal or decussate.

The mode of cytoplasmic partitioning also allows us to distinguish between lower asparagoids and higher Asparagales. In this study, only centrifugal cell plates were recorded in higher Asparagales, while in lower asparagoids both centrifugal and centripetal cell wall formation were observed. In this latter group, centrifugal cell plates are usually observed when cytokinesis is successive, such as in *Hypoxidia maheensis* (Hypoxidaceae) or *Moraea aristata* and *M. bipartita* (Iridaceae). Thus successive cytokinesis appears to be strongly correlated with centrifugal cell plates. Dyads are occasionally observed in *Tritonia securigera* (Iridaceae, Fig. 5P), which has cell walls that grow centripetally, but most cells in this species are partitioned simultaneously after the completion of nuclear divisions. Successive cytokinesis could then constrain species to adopt centrifugal cytoplasmic partitioning, since the pattern displayed by *Tritonia* seems to be rare, at least within Asparagales. In contrast, simultaneous cytokinesis is associated with either centripetal or centrifugal cell wall formation within this order. Centrifugal cell plate formation coupled with simultaneous cytokinesis is reported here for the Asphodelaceae and Hemerocallidaceae. This is particularly interesting since these families, together with Xanthorrhoeaceae, form a clade (Xanthorrhoeaceae *sensu lato*; APG II, 2003) which is sister to the higher Asparagales (Fig. 2). In the higher Asparagales, cell plate growth is also always centrifugal but it only occurs through successive cytokinesis. Asphodelaceae, Hemerocallidaceae and Xanthorrhoeaceae could thus represent an intermediate

step in the fixation of the developmental pathway characteristic of higher Asparagales.

Tetrad shape

A variety of tetrad shapes were observed in this study. The tetrad shape is sometimes used to infer the cytokinesis type of a species. In most cases, the shape of a tetrad is indeed correlated with the type of cytokinesis. For example, tetrahedral tetrads can never be obtained through successive cytokinesis, since the occurrence of a dyad stage constrains the shape of the resulting tetrads. In this study, species with successive cytokinesis mostly produced tetragonal or decussate tetrads, although linear or T-shaped tetrads were also recorded (Fig. 3J, K). The transitory syncytium stage in simultaneous cytokinesis theoretically allows nuclei to adopt any spatial arrangement, but a tetrahedral configuration is the most common and characteristic. However, the four meiotic nuclei can adopt any other configuration, thereby giving rise to different tetrad shapes. Linear tetrads resulting from simultaneous cytokinesis were observed in a moss (Shimamura *et al.*, 1998), and we observed tetragonal tetrads resulting from simultaneous cytokinesis in *Trachyandra muricata*. Linear and tetragonal tetrads are generally obtained from a successive cytokinesis. Thus tetrad shape should not always be considered as a reliable criterion for inferring the type of cytokinesis, and one should avoid drawing conclusions solely on the basis of tetrad shape. The observation of callose walls in tetrads stained with anilin blue nevertheless allows the distinction between successive and simultaneous cytokinesis in some cases. In the successive type, extra callose is usually deposited onto the cell plate that is formed first, but not on the other two, a feature which has already been described by Huynh (1967). When cytokinesis is simultaneous, such as in *Trachyandra*, all four cell plates (or none) were covered equally with such extra callose deposits. We believe that the only way to determine the type of cytokinesis followed is through direct observation of cell wall growth, and the possible presence of a dyad stage. The observation of tetragonal or decussate tetrads alone is not a sufficient criterion.

Variation in tetrad shape may result from locular space constraints. The study of tetrad packaging in the anther could help to understand why some tetrad types are more frequent in some species, since such constraints in space could modify tetrad shape. Other criteria may nevertheless account for tetrad shape, such as the initial shape of microsporocytes (varying from spherical to ellipsoidal), which can modify the relative position of the nuclei and consequently the shape of the future tetrad.

In this study, species with simultaneous cytokinesis always had irregular tetrahedral tetrads. Interestingly, since pollen aperture pattern seems to be conditioned by tetrad shape, regular tetrahedral tetrads are associated with the presence of trichotomosulcate pollen (Rudall *et al.*, 1997; S. Nadot, unpublished data), while irregular tetrahedral tetrads are always associated with monosulcate pollen. This change of pollen morphology is ascribed to a modification in the relative positions of the nuclei at the end of meiosis. It may be the consequence of

selection for a better-adapted pollen morphology. Other such correlations between the production of monosulcate and trichotomosulcate pollen through simultaneous cytokinesis have been reported (Rudall *et al.*, 1997; Harley, 2004) and more data are needed to address this question.

Interestingly, Iridaceae species with a callose ring may represent another example of the fixation of a developmental event canalizing pollen morphology. In these species, the majority of tetrads are tetragonal or almost tetragonal. This particular shape could result from a constraint due to the callose ring and might have been selected as being more efficient for constraining tetrads into tetragonal shapes and therefore for producing monosulcate pollen. Monosulcate pollen can also result from irregular tetrahedral tetrads, but in this case it is often associated with the production of trichotomosulcate pollen resulting from regular tetrahedral tetrads. If there is a cost for producing two different pollen (for example, if one morph is locally less efficient in fertilization), we should expect developmental options that reduce the occurrence of a morphology to be selected. This may be the case for species with a callose ring. The case of *Hemerocallis fulva* and its semi-simultaneous cytokinesis may be another example of such a mechanism that constrains tetrad shape and therefore apertural type. The co-occurrence of monosulcate and trichotomosulcate pollen should then be considered as a direct consequence of the developmental pathway.

Pattern in microsporogenesis

Microsporogenesis in the Asparagales is relatively conservative at the family level, except in the Iridaceae. The higher Asparagales clade is characterized by a highly conserved developmental pathway: successive cytokinesis with centrifugal cell plate growth. In this group, the only variation in this study concerns the shape of tetrads, which are mostly tetragonal or decussate, but also linear or T-shaped in some species. The latter shapes of tetrads can be rare (*Hyacinthus non-scriptus*, *Hosta sp.*, *Polygonatum multiflorum* and *Convallaria majalis*) or relatively frequent (*Albuca nelsonii*). Asphodelaceae are characterized by simultaneous cytokinesis associated with centrifugal cell plate development, resulting in monosulcate pollen grains. We did not investigate enough species of Hypoxidaceae and Tecophilaeaceae to generalize our observations in these groups. However, since microsporogenesis appears conservative within most families, we expect other species from these families to present a pattern similar to the species described here. Iridaceae was the only family in which monosulcate pollen was obtained via different developmental pathways. Most of the pathways present in this family are also present in other Asparagales families. However, the presence of a callose ring formed during meiosis is unique to the Iridaceae and therefore could represent a diagnostic character of this family within the Asparagales.

Incidentally, the described similar developmental groups correlate more or less with phylogeny, indicating that dramatic differences in microsporogenesis are generally

discerned only at macroevolutionary levels, while developmental differences between related species (generally within the family level) are less marked.

Microsporogenesis leading to monosulcate pollen is thus highly diverse, with some developmental pathways limited to families, while others are encountered throughout the Asparagales. This journey through development raises questions about pollen morphology: monosulcate pollen is considered ancestral among the monocots. If several developmental pathways correlate with this morphology, the character is homoplastic, and only the morphology associated with the ancestral developmental pathway can be considered as ancestral. If developmental events that determine monosulcate apertures are so diverse, which developmental type really constitutes the ancestral condition? This needs further in-depth investigations.

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