Evolutionary stasis in Euphorbiaceae pollen: selection and constraints

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Introduction

Given the Darwinian process of ‘descent with modification’, evolutionary change is to be expected as a common occurrence. Although such evolutionary change occurs in many cases, several examples of lineages exhibiting little morphological change for an individual character, for character complexes or for the whole organism are known (Futuyma, 2010). It may be inferred that a character exhibits little morphological change in a lineage when the rate of change of the character is slower than it theoretically could be (see Bradshaw, 1991; Williams, 1992; Futuyma, 2010 for examples). The amount of morphological variation of a character can be visualized by determining the theoretical morphospace of all the possible states that can exist for the character and then looking at how the morphospace has been filled during the course of evolution (Raup, 1966; McGhee, 2007). Morphospaces are delineated by the fact that all the members of a lineage have inherited a limited number of developmental modules (Breuker et al., 2006; Müller, 2007; Wagner et al., 2007) and that the morphological variation allowed by these modules is finite (Lauder, 1981; Wake & Larson, 1987; Raff, 1996; Salazar-Ciudad, 2006; Schlosser, 2007). Therefore, the evolution of a character may only occur within the limits allowed by these modules unless new modules are acquired and added to the previous ones. The utilization of morphospace allows testing for the occurrence of stasis or phylogenetic conservatism between related lineages that share common developmental modules. If it is found that, for a given character, a lineage explored a smaller subset of the morphospace than another lineage, it may be stated that this character exhibits stasis in the former relative to the latter.

Abstract

Although much attention has been paid to the role of stabilizing selection, empirical analyses testing the role of developmental constraints in evolutionary stasis remain rare, particularly for plants. This topic is studied here with a focus on the evolution of a pollen ontogenetic feature, the last points of callose deposition (LPCD) pattern, involved in the determination of an adaptive morphological pollen character (aperture pattern). The LPCD pattern exhibits a low level of evolution in eudicots, as compared to the evolution observed in monocots. Stasis in this pattern might be explained by developmental constraints expressed during male meiosis (microsporogenesis) or by selective pressures expressed through the adaptive role of the aperture pattern. Here, we demonstrate that the LPCD pattern is conserved in Euphorbiaceae s.s. and that this conservatism is primarily due to selective pressures. A phylogenetic association was found between the putative removal of selective pressures on pollen morphology after the origin of inaperturate pollen, and the appearance of variation in microsporogenesis and in the resulting LPCD pattern, suggesting that stasis was due to these selective pressures. However, even in a neutral context, variation in microsporogenesis was biased. This should therefore favour the appearance of some developmental and morphological phenotypes rather than others.

Keywords:
comparative analysis;
evo-devo;
microsporogenesis;
morphospace;
pollen aperture pattern.
Neo-Darwinians consider two classes of mechanisms supposedly involved in the lack of change: failure in the generation of variation – caused by developmental constraints – or failure in the fixation of new variation – caused by stabilizing selection (Charlesworth et al., 1982; Maynard Smith, 1983; Williams, 1992; Gould, 2002; Hansen & Houle, 2004; Eldredge et al., 2005). Evolutionary stasis may thus be explained by the fact that phenotypes corresponding to the unfilled parts of the morphospace are always counter-selected. An alternative is that developmental constraints (i.e., biases in the production of variant phenotypes or limitations on phenotypic variability caused by the structure, character, composition or dynamics of the developmental system – Maynard Smith et al., 1985) appeared during the history of the lineage, preventing switches from the filled parts of the morphospace to the unfilled ones. Such constraints may, for example, be expressed as the nonequiprobability of mutations, resulting in nonequiprobability of developmental changes (Stoltzfus & Yampolsky, 2009); the impossibility of some reversals to an ancestral state (Teotonio & Rose, 2001) or the easier derivation of some character states from a particular character state than others, for example, because of the structure of the genetic network or the nonlinearity of the genotype–phenotype map (Oster & Alberch, 1982; Bradshaw, 1991; Weill et al., 2004; Wagner, 2011). Alberch (1982) suggested a thought experiment to identify the roles of natural selection and developmental constraints to account for the empty spaces of the morphospace. It consists in removing the selective pressures acting on the character and studying variation of the character in this context. If the character remains unchanged, then constraints are likely to be acting. If it changes, then it is probable that evolutionary stasis was due to stabilizing selection (see Amundson, 1994; Raff, 1996: 298 for comments on this experiment). We used this approach to test for the action of developmental constraints and natural selection on the evolution of pollen grains.

Pollen grains are formed through microsporogenesis and microgametogenesis. Microsporogenesis results in a tetrad composed of four haploid microspores (future pollen grains), obtained through the division of a diploid mother cell by meiosis and the synthesis of intersporal walls composed of callose. Callose begins to be deposited on the borders and/or at the middle of the plane between two adjacent microspores and progressively separates their cytoplasmic. As a result, at the late microsporogenesis stage, some areas of the plane remain unfilled by callose. We suggest naming these areas last points of callose deposition (LPCD). The LPCD are variable in number and position, as three features of microsporogenesis can change the LPCD pattern (Ressayre et al., 2002a). These features are the cytokinesis type (two states – successive and simultaneous), the mode of callose deposition (four states – centripetal according to the tetrad, centripetal according to the cleavage plane, centrifugal according to the tetrat and centrifugal according to the cleavage plane) and the form of the tetrat (three states – tetrahedral, rhomboidal and tetragonal). By determining all possible combinations of variants of these features, a theoretical morphospace for the LPCD pattern can be elaborated. This morphospace contains sixteen possible states (tetrahedral and rhomboidal tetrads cannot be produced through successive cytokinesis). A subset of this morphospace is illustrated in Fig. 1 (only LPCD patterns observed in this study are shown).

All theoretically possible LPCD patterns do not appear to be observed equally in nature: some have never been observed, and others dominate large clades. For example, in early-divergent angiosperms and monocots, microsporogenesis features determining LPCD pattern are quite variable (Furness & Rudall, 1999a,b; Furness et al., 2002; Penet et al., 2005; Nadot et al., 2006, 2008; Sannier et al., 2006). In the eudicot clade, the huge majority of species have the same LPCD pattern (Wodehouse, 1935; Blackmore & Crane, 1998; Furness & Rudall, 2004; Nadot et al., 2008). This LPCD pattern is shown in Fig. 1c.1. Evolution of the LPCD pattern thus seems to represent a case of evolutionary stasis in eudicots relative to its evolution in early-divergent angiosperms and in the monocot clade. The stasis observed in eudicots could be caused by developmental constraints arising from the loss of the capacity to generate variation in the cytokinesis type, the mode of callose deposition and the tetrat form. Alternatively, selective pressure could also be involved as the LPCD pattern may determine the number and position of the apertures in the pollen wall (Wodehouse, 1935; Heslop-Harrison, 1968; Dover, 1972; Sheldon & Dickinson, 1983, 1986; Barnes & Blackmore, 1986; Blackmore & Barnes, 1990; Blackmore & Crane, 1998; Ressayre et al., 1998, 2002a, 2005; Ressayre, 2001; Blackmore et al., 2007; Albert et al., 2010). Apertures are thin areas of the outer pollen wall (the exine), through which the pollen tube will grow at germination. They are variable in number and position (e.g. Erdtman, 1952; Kesseler & Harley, 2004; Hesse et al., 2009). The aperture pattern (i.e. number and position of the apertures) has been found to influence the fitness of pollen grains (Dajoz et al., 1991, 1993; Till-Bottraud et al., 1999). Separate mechanisms are involved in the induction and the positioning of apertures. Induction is achieved through specific cellular mechanisms (reviewed in Ressayre, 2001) that prevent deposition of the exine matrix in the areas destined to become apertures (Scott et al., 2004). In eudicots with equatorial aperture patterns, positioning of the apertures in the pollen wall depends on the LPCD pattern, as apertures are located in the areas of the pollen wall adjoining LPCD (Blackmore et al., 2007). Figure 1 shows the aperture patterns expected for six different LPCD patterns, according to Ressayre et al. (2002a). The ‘typical’ LPCD pattern of eudicots may result in pollen grains with three equatorial apertures (Fig. 1c-1), and it may also lead to pollen
morphs with higher aperture numbers obtained through rearrangements of microtubule arrays (Ressayre et al., 2002b). This flexibility in the pollen aperture number associated with this LPCD pattern has been proposed as a possible key innovation underlying eudicot success (Furness & Rudall, 2004). Variation in aperture number allows pollen to adapt to different environments: increased aperture number increases the rapidity of pollen germination but decreases pollen longevity (Dajoz et al., 1991, 1993; Till-Bottraud et al., 1999). This putative adaptive flexibility could thus possibly explain the evolutionary stasis in the LPCD pattern in eudicots.

As pointed out by Mayr (1961), evolutionary changes are determined by two levels of causation: a proximate causation (expressed as a set of physiological changes) and an ultimate one (expressed as changes orchestrated by natural selection). These two types of causation may be identified for the LPCD pattern, in which changes are determined by bottom-up ontogenetic processes (microsporogenesis – the ‘proximal’ cause) and by downstream selective pressures (aperture pattern – the ‘ultimate’ cause). As suggested in Alberch’s hypothetical experiment (Alberch, 1982), studying variation of the LPCD pattern in cases where the selective pressures acting on it
have been removed should allow testing for the action of developmental constraints. In species producing pollen grains lacking apertures (inaperturate pollen), none of the cellular mechanisms known to induce apertures are present (Furness, 2007). Thus, we assume that at least some of the selective pressures acting on the mechanism positioning the apertures (i.e. the LPCD) are removed. If the observed stability of the LPCD pattern among eudicots is due to developmental constraints, it may be expected that the LPCD pattern will remain unchanged despite the loss of selective pressures. Conversely, if the LPCD pattern exhibits unusual variation in inaperturate species, this will strongly indicate that its stability was not due to developmental constraints but to stabilizing selection.

Inaperturate pollen evolved independently numerous times throughout the eudicots, although generally in groups containing a relatively small number of species (Furness, 2007). In Euphorbiaceae s.s. [sensu stricto is delimited here as Euphorbiaceae minus the recently segregated Peraceae (Wurdack & Davis, 2009)], inaperturate taxa fall into two separate groups of differing size: (i) c. 11 species of tribe Plukenetieae in subfamily Acalyphoideae (Gillespie, 1994) and (ii) c. 1500 species in subfamily Crotonoideae [i.e. the clade of ‘inaperturate crotonoids’ sensu Wurdack et al. (2005)]. The latter group is the focus here given its exceptional species richness. These two groups are widely separated in phylogenies of Euphorbiaceae (i.e. Wurdack et al., 2005; Tokuoka, 2007) and represent at least two losses of apertures. We have made new observations on the LPCD pattern in Euphorbiaceae s.s. and compared in a phylogenetic context the variation of the LPCD pattern among inaperturate and aperturate species. This comparison allows testing for the presence of relative developmental constraints. Under the hypothesis that developmental constraints are not acting on the LPCD pattern, we would expect to find a phylogenetic correlation between the loss of the apertures and the appearance of variation in the LPCD pattern. Such a correlation has been tested here using phylogenetic comparative methods (Pagel, 1994). This is, to our knowledge, the first time that the thought experiment of Alberch (1982) has been conducted empirically.

Material and methods

Assessment of aperture pattern and microsporogenesis features

Data on aperture pattern were scored from an extensive survey of the palynological literature for the Euphorbiaceae s.s. (Erdtman, 1952; Punt, 1962; Díaz Zavaleta & Palacios Chávez, 1980; Thanikaimoni et al., 1984; Nowicke, 1994; Lobreau-Callen & Suarez-Cervera, 1997; Nowicke et al., 1998; Takahashi et al., 2000; Suarez-Cervera et al., 2001; Nowicke & Takahashi, 2002; Sagun et al., 2006). For the study of microsporogenesis, a data set of microsporogenesis features was obtained using information from the literature for 16 species and our own observations for 17 additional species (Table 1). Data on microsporogenesis were obtained for aperturate species representing all the major clades of the family. For inaperturate species, data were obtained only for species of the ‘inaperturate crotonoids’. Data were not obtained for species of tribe Plukenetieae (clade A8) because these were not available from the living collections.

For the description of microsporogenesis, fresh material was obtained from the living collections of botanical gardens or from plants cultivated in a greenhouse at the Laboratoire Ecologie, Systématique, Evolution, Orsay, France (Table 1). Anthers were dissected from unopened buds of various sizes to find the ontogenetic stages corresponding to callose deposition and pollen assembled in mature tetrads. Anthers were squashed on a slide and mounted with aniline blue solution (aniline blue, 1 g L\(^{-1}\); K\(_4\)PO\(_4\), 21.2 g L\(^{-1}\); glycerol 187.4 g L\(^{-1}\)), according to a protocol modified from the study of Arens (1949). Aniline blue staining causes fluorescence of the intersporal callose walls when illuminated with UV light. Slides were observed under a Zeiss Axioshot fluorescence microscope (DAPI filter, excitation at 345 nm, emission at 425-nm-long pass). For each species, pollen morphology, tetrad form, cytokinesis type and the mode of callose deposition during intersporal wall formation were recorded. When more than one tetrad configuration was observed for a given species, a survey of the proportion of each tetrad form was carried out by counting a number of tetrads ranging from c. 200 to c. 300 per plant. Chi-square tests were performed using R Development Core Team, 2008) to test (i) whether each tetrad form was produced in similar proportions within plants and (ii) whether the distribution of the proportions of tetrad forms was equivalent among the heteromorphic species (i.e. species for which individuals produce more than one form of tetrad). Then, a phylogenetic framework was used to study the evolution of microsporogenesis in relation to aperture pattern.

Phylogenetic analysis

Phylogenetic relationships of the Euphorbiaceae s.s. species for which we acquired data on pollen morphology and microsporogenesis were obtained using DNA sequences of rbcL and trnL-F genes (Wurdack et al., 2005; Tokuoka, 2007; Sagun, 2008) (Table S1). Although molecular data were not available for some of our investigated species, the resulting species sampling is sufficient to yield statistically significant results with the test of Pagel (1994). We used the alignment matrix of the combined data for rbcL and trnL-F in Wurdack et al. (2005). Taxon sampling was modified by removing taxa that lacked morphological data and by adding new taxa...
Table 1 Studied specimens, with origin of species for which new data are provided, and references for data obtained from the literature.

<table>
<thead>
<tr>
<th>Species for which new data are provided</th>
<th>Species</th>
<th>Origin (accession number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acalypha inophylla (G. Forst.) P. Green</td>
<td>Museum national d’Histoire naturelle, Paris (# 52146)</td>
<td></td>
</tr>
<tr>
<td>Bocquollonia spicata Baill.</td>
<td>Jardin Botanique de la Ville de Paris (no AN)</td>
<td></td>
</tr>
<tr>
<td>Caperonia serrata (Turcz.) C. Presl</td>
<td>Cultivated, seeds from Royal Botanic Gardens Seed Bank, Kew (# 351517)</td>
<td></td>
</tr>
<tr>
<td>Croton californicus Müll. Arg.</td>
<td>Cultivated, seeds from Rancho Santa Ana Botanic Garden (# 20565)</td>
<td></td>
</tr>
<tr>
<td>Dalechampia roezliana Müll. Arg. (synonym of Dalechampia spatulata (Scheidw.) Baill.)</td>
<td>Jardin Botanique de la Ville de Paris</td>
<td></td>
</tr>
<tr>
<td>Eremocarpus setigerus (Hook.) Benth.</td>
<td>Cultivated, seeds from Hedgerow Farms (# C3YOLF1007)</td>
<td></td>
</tr>
<tr>
<td>Jatropha gossypifolia L.</td>
<td>Parc Botanique de Launay (no AN)</td>
<td></td>
</tr>
<tr>
<td>Jatropha integerrima Jacq.</td>
<td>Parc Botanique de Launay (no AN)</td>
<td></td>
</tr>
<tr>
<td>Jatropha multifida L.</td>
<td>Parc Botanique de Launay (no AN)</td>
<td></td>
</tr>
<tr>
<td>Jatropha podagrica L.</td>
<td>Museum national d'Histoire naturelle, Paris (# 18839)</td>
<td></td>
</tr>
<tr>
<td>Pediandrus thyrmaloides (L.) Pot. subsp. thyrmaloides</td>
<td>Royal Botanic Gardens, Kew (# 1984-2163)</td>
<td></td>
</tr>
<tr>
<td>Ricinus communis L.</td>
<td>Parc Botanique de Launay (no AN)</td>
<td></td>
</tr>
</tbody>
</table>

Species for which data were obtained from GenBank (i.e. from Tokuoka, 2007; Sagun, 2008) for which we acquired data on pollen and microsporogenesis. For the additional sequences, alignments were easily adjusted by eye using BioEdit version 7.0.5.3 (Hall, 1999), except for some ambiguous indels in the trnL-F sequences. These were removed unless informative substitutions were present. Outgroups were Humiriaceae (Vantarea guaianensis Aubl.) and Peraceae (Pera bicolor [Klotzsch] Müll. and Clitulia pulchella L.). They were chosen according to previous studies on the systematics of Euphorbiaceae s.s. and Malpighiales (Wurdack et al., 2005; Tokuoka, 2007; Wurdack & Davis, 2009). Rafflesiaeaceae have inaperturate pollen and have a phylogenetic placement near the root of Euphorbiaceae (Wurdack & Davis, 2009), but they lack plastid data and were not included here (see Discussion).

The phylogenetic relationships of species were inferred under the assumption of maximum likelihood (ML) using PhyML 3.0 (Guindon & Gascuel, 2003; Guindon et al., 2010). The most appropriate model of nucleotide substitution was determined with Modeltest version 3.7 (Posada & Crandall, 1998) to be the general time-reversible model (GTR + I + T). Parameter values were estimated during running, and node support was estimated with a ML bootstrap analysis with 100 replicates. The substitution rate matrix was 1.37049, 2.51808, 0.43019, 0.82579 and 3.43486. Nucleotide frequencies were (A) = 0.30530; (C) = 0.18853; (G) = 0.22164; and (T) = 0.28453. The estimated proportion of invariable sites was 0.469, and a gamma distribution was assumed for rates at invariable sites. The presented tree was obtained from a single run. A BioNJ starting tree was used, and tree topologies were estimated using the nearest neighbour interchange (NNI) method.

Analysis of character evolution

Pollens and microsporogenesis characters obtained from species sampled by us and from published information (summarized in Table 2) were optimized onto the most likely tree with relative branch lengths obtained from our ML analyses, using Mesquite (Maddison & Maddison, 2008). Characters were optimized using maximum parsimony (MP), and maximum likelihood using the Mk1 model which assumes that any particular change (from state 0 to 1 or state 3 to 2, for example) is equally probable. Maximum likelihood complements the qualitative result assessed with MP optimization, as it takes into account branch lengths and provides an estimate of the probability of the character state at each node. In addition, ML reconstruction is more conservative than MP in reconstructing ancestral states at nodes for which few data are available.

Three character states were described for the aperture pattern: apertures equatorial, global or absent. Microsporogenesis features were coded as binary characters as follows: (i) cytokinesis: simultaneous or heteromorphic...
Table 2. Records of tetrad form, mode of callose deposition and cytokinesis type in species of Euphorbiaceae s.s. investigated, according to their aperture pattern.

<table>
<thead>
<tr>
<th>Species</th>
<th>Aperture pattern</th>
<th>Tetrad form (percentage of each)</th>
<th>Callose deposition</th>
<th>Cytokinesis</th>
<th>Reference/Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acalypha ahitifolia</td>
<td>Equatorial</td>
<td>Td – Tg – Dc*</td>
<td>?</td>
<td>Simultaneous</td>
<td>Kapil (1960)</td>
</tr>
<tr>
<td>Acalypha brachystachya</td>
<td>Equatorial</td>
<td>Td – Tg – Dc*</td>
<td>?</td>
<td>Simultaneous</td>
<td>Kapil (1960)</td>
</tr>
<tr>
<td>Acalypha ciliata</td>
<td>Equatorial</td>
<td>Td</td>
<td>CpP</td>
<td>Simultaneous</td>
<td>Mukherjee (1964)</td>
</tr>
<tr>
<td>Acalypha indica</td>
<td>Equatorial</td>
<td>Td – Tg – Dc*</td>
<td>Centripetal</td>
<td>Simultaneous</td>
<td>Johri &amp; Kapil (1963)</td>
</tr>
<tr>
<td>Acalypha lanceolata</td>
<td>Equatorial</td>
<td>Td</td>
<td>Centripetal</td>
<td>Simultaneous</td>
<td>Thathachar (1952)</td>
</tr>
<tr>
<td>Acalypha malabarica</td>
<td>Equatorial</td>
<td>Td</td>
<td>Centripetal</td>
<td>Simultaneous</td>
<td>Mukherjee (1964)</td>
</tr>
<tr>
<td>Bocquielloua spicata</td>
<td>Equatorial</td>
<td>Td</td>
<td>CpP</td>
<td>Simultaneous</td>
<td>Fig. 4</td>
</tr>
<tr>
<td>Caperonia serrata</td>
<td>Equatorial</td>
<td>Td</td>
<td>CpP</td>
<td>Simultaneous</td>
<td>Fig. 4</td>
</tr>
<tr>
<td>Dalechampia roezliana</td>
<td>Equatorial</td>
<td>Td</td>
<td>CpP</td>
<td>Simultaneous</td>
<td>Fig. 4</td>
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<tr>
<td>Euphorbia dracunculoides</td>
<td>Equatorial</td>
<td>Td – Tg*</td>
<td>Centripetal</td>
<td>Simultaneous</td>
<td>Mukherjee (1960)</td>
</tr>
<tr>
<td>Euphorbia dulcis</td>
<td>Equatorial</td>
<td>Td – Tg – Dc*</td>
<td>?</td>
<td>?</td>
<td>Kapil (1961); Murgia et al. (1986)</td>
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<td>Euphorbia microphylla</td>
<td>Equatorial</td>
<td>Td – Tg*</td>
<td>Centripetal</td>
<td>Simultaneous</td>
<td>Mukherjee (1960)</td>
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<tr>
<td>Euphorbia petita</td>
<td>Equatorial</td>
<td>Td</td>
<td>?</td>
<td>?</td>
<td>Mukherjee (1965)</td>
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<td>Hevea brasiliensis</td>
<td>Equatorial</td>
<td>Td</td>
<td>Centripetal</td>
<td>Simultaneous</td>
<td>Rao (1964)</td>
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<td>Hura crepitans</td>
<td>Equatorial</td>
<td>Td</td>
<td>CpP</td>
<td>Simultaneous</td>
<td>Fig. 4</td>
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<td>Macaranga tanarius</td>
<td>Equatorial</td>
<td>Td</td>
<td>CpP</td>
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<td>Fig. 4</td>
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<td>Mercurialis annua</td>
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<td>Td</td>
<td>CpP</td>
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<td>Mercurialis perennis</td>
<td>Equatorial</td>
<td>Td</td>
<td>CpP</td>
<td>Simultaneous</td>
<td>Fig. 4</td>
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<td>Micrococa mercurlia</td>
<td>Equatorial</td>
<td>Td</td>
<td>?</td>
<td>Simultaneous</td>
<td>Rao (1962)</td>
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<td>Pedilanthus thyraloides</td>
<td>Equatorial</td>
<td>Td</td>
<td>CpP</td>
<td>Simultaneous</td>
<td>Fig. 4</td>
</tr>
<tr>
<td>Ricinus communis</td>
<td>Equatorial</td>
<td>Td</td>
<td>?</td>
<td>Simultaneous</td>
<td>Fig. 4</td>
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<tr>
<td>Bologna spicata</td>
<td>Inaperturate</td>
<td>Td (70 %) – Rh (25 %) – Tg (5 %)</td>
<td>Cpp/CpT/Cpt</td>
<td>Simultaneous</td>
<td>Fig. 6</td>
</tr>
<tr>
<td>Codiaeum variegatum</td>
<td>Inaperturate</td>
<td>Td – Rh – Tg</td>
<td>Cpp/CpT/Cpt</td>
<td>Heteromorphic</td>
<td>Albert et al. (2009)</td>
</tr>
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<td>Croton californicus</td>
<td>Inaperturate</td>
<td>Td (77 %) – Rh (19 %) – Tg (4 %)</td>
<td>Cpp</td>
<td>Simultaneous</td>
<td>Fig. 6</td>
</tr>
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<td>Emrocopinus setigerus</td>
<td>Inaperturate</td>
<td>Td (51 %) – Rh (29 %) – Tg (19 %)</td>
<td>Cpp/CpT</td>
<td>Simultaneous</td>
<td>Fig. 6</td>
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<td>Jatropha curcas</td>
<td>Inaperturate</td>
<td>Td – Rh</td>
<td>Centripetal</td>
<td>Simultaneous</td>
<td>Liu et al. (2007)</td>
</tr>
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<td>Jatropha gossypilolia</td>
<td>Inaperturate</td>
<td>Td (89 %) – Rh (10 %) – Tg (1 %)</td>
<td>Cpp/CpT</td>
<td>Simultaneous</td>
<td>Fig. 5</td>
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<td>Jatropha integerrima</td>
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<td>Td (61 %) – Rh (32 %) – Tg (7 %)</td>
<td>Cpp/CpT</td>
<td>Simultaneous</td>
<td>Fig. 5</td>
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<td>Jatropha multifida</td>
<td>Inaperturate</td>
<td>Td (72 %) – Rh (24 %) – Tg (4 %)</td>
<td>Cpp/CpT</td>
<td>Simultaneous</td>
<td>Fig. 5</td>
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<td>Jatropha podagrica</td>
<td>Inaperturate</td>
<td>Td (83 %) – Rh (15 %) – Tg (2 %)</td>
<td>Cpp/CpT</td>
<td>Simultaneous</td>
<td>Fig. 5</td>
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<td>Manihot esculenta</td>
<td>Global</td>
<td>Td (80 %) – Rh (20 %)</td>
<td>Cpp/CpT</td>
<td>Simultaneous</td>
<td>Fig. 6</td>
</tr>
</tbody>
</table>

Td, tetrahedral; Tg, tetragonal; Rh, rhomboidal; Dc, decussate; CpP, centripetal according to the cleavage plane; CpT, centripetal according to the tetrad; Cpp, centrifugal according to the tetrad. * denotes the literature reports on tetrads that have been reinterpreted (see Results). ? denotes missing data.

Correlation between aperture pattern and microsporogenesis

To test for correlated evolution between aperture pattern and microsporogenesis, the aperture pattern was binary-coded (apertures equatorial vs. global/absent), as correlation tests can only be performed on binary traits. The coding of microsporogenesis was the same as that used for character optimization. This coding is suitable for our aim of testing whether there is variation in microsporogenesis once aperture pattern is not equatorial.

The test for correlated evolution was carried out using the BAYESDISCRETE method of the BAYESTRAITS computer package (Pagel, 1994; Pagel & Meade, 2006). BAYESDISCRETE analyses correlated evolution among pairs of binary traits. The result of the test does not rely upon any reconstruction of the ancestral character states (Pagel, 1994). The method consists in comparing two models of evolution: one in which the traits evolve independently and one that assumes correlated evolution. Evidence of correlated evolution is found when the model assuming correlated evolution fits better than the ‘independent’ model. The two methods of analysis

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implemented in BayesDiscrete [ML and Markov chain Monte Carlo (MCMC)] were used. In the ML method, the program estimates the likelihood of the two models. The difference between them is tested using a likelihood ratio statistic \( \text{LRS} = 2(\log\text{-likelihood(dependent model)} - \log\text{-likelihood(independent model)}) \), which is nominally distributed as a \( \chi^2 \) (4 d.f.). The MCMC method estimates the Bayesian posterior distributions of the likelihoods of the data given the model (dependent or independent). The log-harmonic mean of the likelihoods is compared between two runs (one assuming correlated evolution and the other independent evolution). Relative fit of the two models is tested using the Bayes factor \( \text{BF} = 2(\log\text{harmonic mean(better model)} - \log\text{harmonic mean(worse model)}) \). Any positive value of the BF favours the other independent evolution. Conventionally a ratio > 2 is taken as ‘positive’ evidence, > 5 is ‘strong’ and > 10 is ‘very strong’ evidence. Analyses were run with the default values of the software.

To account for phylogenetic uncertainty and branch length heterogeneity in the analyses, LRS and BF were estimated from a sample of 100 tree topologies obtained from a Bayesian analysis. This sample was obtained by analysing our alignment matrix with Bayesian inference using MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The evolutionary model was set to the GTR + I + \( \Gamma \) model with gamma-distributed rate variation across sites and an estimated proportion of invariable sites. Analyses were performed as two independent runs of two million generations, with sampling every 100 generations. Likelihood values reached a plateau within 500 000 generations. These were deleted as burn-in. The tree files of the two runs were combined in a single file from which 100 trees were randomly sampled.

**Results**

**Phylogenetic analysis**

Maximum-likelihood analyses resulted in a single tree with a score of log-likelihood \(-19082.20\) (Fig. 2). The phylogeny obtained with our analysis recognized all the subclades (Adenoclinaceae s.l.; Articulated crotonoids; C1–2; Pseudanthial; H1–2; Alchorneoids; A1–8) found in the two previous studies on Euphorbiaceae s.s. (Wurdack et al., 2005; Tokuoka, 2007). The three major clades in the previous studies (Crotonoideae s.l.; Euphorbioideae; and Acalyphoideae s.s.) also coincided with those of our analysis except that our analysis suggests the monophyly of the Crotonoideae s.l., whereas the other two exclude the Adenoclinaceae from this group. Crotonogynopsis groups with the Alchorneoids instead of expected clade A3 (i.e. Wurdack et al., 2005; Tokuoka, 2007), but this difference does not affect our conclusions. A substantial difference is in the relationships between the major clades. The Wurdack et al. (2005) analysis grouped Euphorbioideae with Crotonoideae s.l., whereas both Tokuoka’s study and the current study group Euphorbioideae with Acalyphoideae s.s. This difference may be due to the species sampling, which is higher in the Wurdack et al.’s (2005) analysis relative to our own and to that of Tokuoka (2007). Regardless, relationships between these clades are poorly supported in all the analyses. Changes in the relationships between these clades do not affect the results of our comparative analysis.

**Optimization of the pollen aperture pattern**

Our survey of the palynological literature provided data on aperture pattern for 779 Euphorbiaceae species representing 256 genera. Of these, only species for which phylogenetic data were available were utilized [101 species, representing 91 of the 152 genera included in the phylogenetic analysis of Euphorbiaceae s.s. by Wurdack et al. (2005)]. This sorting considerably reduced the sampling, but it allowed us to work in a phylogenetic framework. Although fewer species were included in this study, the sampling is an adequate representation of the full phylogenetic tree of Wurdack et al.’s (2005) study, as it contains taxa representing all the terminal clades in their analysis, except for the small Erismantheae clade.

Of the 101 species in the analysis, the majority (73) produce pollen with equatorial apertures (usually three apertures, but sometimes more). To determine whether the predominance of this character state is due to phylogenetic conservatism or to homoplasy, the aperture pattern was optimized onto our phylogeny (Fig. 3a). The two methods used for the optimization of the aperture pattern (MP and ML) were congruent: all the character states inferred at each node in MP were statistically supported in ML using the likelihood decision threshold of two [i.e. a state was preferred when its log-likelihood differed by two units or more from the one of the other states, as suggested by Pagel (1999)], except for two nodes at the base of Crotonoideae s.l. where there was disagreement between the two methods. Figure 3a shows that aperture pattern is plesiomorphically equatorial and underwent two (maybe three) independent transitions across the tree. In the clade formed by inaperturate crotonoids + articulated crotonoids + Gelonieae, parsimony optimization indicates that the aperture pattern changed to a global pattern and subsequently to an inaperturate one in the inaperturate crotonoids. In clade A8, it was transformed directly to an inaperturate pattern. One reversal to the equatorial pattern was inferred, in the articulated crotonoids.

**Description of microsporogenesis in Euphorbiaceae**

Our observations (summarized in Table 2) reveal that the species of Euphorbiaceae studied that produce pollen with an equatorial aperture pattern differ in aspects of microsporogenesis from those species that have an
Fig. 2 Relationships of the species of Euphorbiaceae investigated in this study. The tree was obtained from a combined analysis of the markers rbcL and trnL-F using a maximum-likelihood reconstruction method (log-likelihood = -19 082.20). ML-estimated branch lengths are shown. Numbers next to branches are the maximum-likelihood bootstrap values (only values > 50 are shown). The names of major clades follow the study of Wurdack et al. (2005).
### Aperture pattern

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### Tetrad form

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**Fig. 3** Mirror tree of Euphorbiaceae s.s. comparing optimizations of the aperture pattern (a) and tetrad form (b). Colour of the boxes near species names indicates the state of the corresponding character (box absence indicates data absence). Branch colours indicate the ancestral states obtained using MP optimization. Pie charts on the nodes indicate the ancestral states obtained using ML. Pie chart colours represent the probability that this node is at a particular state. Analyses were conducted on the phylogram shown in Fig. 2 but are shown here on a cladogram for clarity. The names of major clades follow the study of Wurdack *et al.* (2005).
inaperturate or a global pattern: all the species with equatorial apertures share the same microsporogenesis character states (tetrahedral tetrads, CnP mode of callose deposition and simultaneous cytokinesis). All the species with inaperturate pollen or with global apertures show intraindividual variation (i.e. heteromorphism) in one or two microsporogenesis features: tetrad form and mode of callose deposition. Detailed microsporogenesis sequences for each species are given below.

**Species with equatorial apertures**

All the species investigated with an equatorial aperture pattern produce pollen grains with three equatorial apertures (Fig. 4a, d, g, j, m, p, s, v), except *Caperonia*...
serrata, which produces pollen with six apertures (Fig. 4y). All these species produce pollen grains that result from tetrahedral tetrads in which apertures are arranged in Fischer’s configuration (i.e. apertures of neighbour grains of the tetrad are grouped in pairs (Fischer, 1889); Fig. 4b, e, h, k, n, q, t and z). Arrangement of the apertures in the tetrad could not be determined in Macaranga tanarius because in this species the microspores are released from the tetrad very early. Tetrads are formed by the simultaneous synthesis of six intersporal cleavage planes (Fig. 4c, f, i, l, o, r, u, x and z). Cytokinesis is thus of the simultaneous type. During formation of the intersporal cleavage planes, callose deposition begins at the borders of each of the six cleavage planes, and deposition progresses centripetally towards the centre of each plane (Fig. 4c, f, i, l, o, r, u, x and z). Callose is thus always deposited according to the CpP mode (Fig. 1c-1).

Species with inaperturate or global aperture patterns

The four species of Jatropha examined (Jatropha podagrica (Fig. 5a–d), Jatropha integerrima (Fig. 5e–h), Jatropha gossypiifolia (Fig. 5i–l) and Jatropha multifida – Fig. 5m–p) produce inaperturate pollen (Fig. 5a, e, i, m). Several types of tetrad are involved in pollen production in this genus: these four species produce, within the same stamen, tetragonal, rhomboidal and tetrahedral tetrads, as shown in Fig. 5 (b, f, j, n: tetragonal tetrads; c, g, k, o: rhomboidal tetrads; d, h, l, p: tetrahedral tetrads). Assessment of the proportion of each tetrad type (Table 2) revealed that the four species produce a majority of tetrahedral tetrads (61–89%), a relatively high number of rhomboidal tetrads (10–32%) and a few tetragonal ones (1–7%). Variation in the mode of callose deposition was observed for J. podagrica, J. integerrima and J. gossypiifolia. In these species, both CpP (Fig. 5c, f, j) and CpT (Fig. 5d, h, l) modes of callose
deposition were found. This variation was observed within the same stamen for *J. podagrica* and *J. integerrima*, whereas it was observed at the scale of the plant for *J. gossypiifolia*. Callose deposition in *J. multifida* was found to follow the CpP mode (Fig. 5p). As in the case of the triaperturate species, cytokinesis type is simultaneous in all the *Jatropha* species investigated (Fig. 5c, d, f, h, j, l, o, p).
Fig. 6 Pollen morphology and microsporogenesis for three inaperturate and one panto-aperturate species of Euphorbiaceae. (a)–(d) Croton californicus. (a) Inaperturate pollen. (b) Mature tetragonal tetrad. (c) Rhomboidal tetrad during callose wall formation. Callose deposition follows the CpP mode (Fig. 1b-1, section). Callose begins to be deposited at the border of each plane (arrows) and progresses towards the centre of each plane. (d) Planes 4–6 of a tetrahedral tetrad during callose wall formation. Callose deposition follows the CpP mode (Fig. 1c-1, section).

(e)–(h) Baloghia inophylla. (e) Inaperturate pollen. (f) Mature tetragonal tetrad. (g) Rhomboidal tetrad during callose wall formation. Callose deposition follows the CpT mode (Fig. 1b-2, whole tetrad). Callose begins to be deposited at the borders of the tetrad (arrows) and progresses towards the centre of the tetrad (asterisk). At the stage represented here, the centre of the tetrad remains empty of callose. (h) Planes 1–3 of a tetrahedral tetrad during callose wall formation. Callose deposition follows the CpP mode (Fig. 1c-1, whole tetrad). Callose (c) begins to be deposited at the border of each plane and progresses towards the centre of the plane (asterisks). At the stage represented here, the centre of each plane remains empty of callose. (i)–(l) Eremocarpus setigerus. (i) Inaperturate pollen. (j) Tetragonal tetrad during callose wall formation. Only two of the four cleavage plane formations are visible on this view (the two other planes are orthogonal to the picture plane and thus callose deposition cannot be observed). Callose (c) begins to be deposited at border of the cleavage plane and progresses towards the centre of each plane (asterisk). (k) Rhomboidal tetrad during callose wall formation stage. Callose deposition follows the CpT mode (Fig. 1b-2, whole tetrad). (l) Planes 4–6 of a tetrahedral tetrad during callose wall formation. Callose deposition follows the CpP mode (Fig. 1c-1, section). (m)–(p) Manihot esculenta. (m) Panto-aperturate pollen. (n) Rhomboidal tetrad during callose wall formation (same configuration as in c). (o) Planes 1 and 2 of a tetrahedral tetrad during callose wall formation (same configuration as in h). (p) Planes 4–6 of a tetrahedral tetrad during callose wall formation. Callose deposition follows the CpT mode (Fig. 1c-2, section). Callose begins to be deposited at the border of the tetrad and progresses towards the centre of the tetrad (arrow). All photographs are taken using fluorescence microscopy except (a), which is with light microscopy. Scale bars 10 \( \mu \)m.
Microsporogenesis in *Croton californicus* (Fig. 6a–d), *Baleghia inophylla* (Fig. 6e–h) and *Eremocarpus setigerus* (Fig. 6i–l) is similar to that in *Jatropha*. These species also produce inaperturate pollen (Fig. 6a, e, i). Again, these species produce, within the same stamen, three tetrad configurations, as shown in Fig. 6 (b, f, j: tetragonal tetrads; c, g, k: rhomboidal tetrads; d, h, l: tetrahedral tetrads). As in *Jatropha*, the different forms of tetrad were produced in unequal proportions [tetrahedral tetrads (51–77%), rhomboidal tetrads (19–25%) and tetragonal ones (4–19%)]. Callose deposition in *C. californicus* follows the CpP mode exclusively (Fig. 6c–d). In *B. inophylla* and *E. setigerus*, both CpT (Fig. 6g, k) and CpP (Fig. 6h, l) modes of callose deposition were observed. This variation was found within the tetrads produced by a single flower. Cytokinesis is simultaneous (Fig. 6c, d, g, h, k, l).

*Manihot esculenta* (Fig. 6m–p) produces pollen with panto-aperturate pollen (Fig. 6m). Variation was found in tetrad form, as this species produces tetrahedral (Fig. 6o–p) and rhomboidal (Fig. 6n) tetrads, in unequal proportions (80% and 20%, respectively). Tetragonal tetrads were not observed. Callose cleavage planes were synthetized through CpP (Fig. 6n–o) and CpT (Fig. 6p) modes. Cytokinesis is simultaneous (Fig. 6n–p).

A regular pattern was found for variation in tetrad form: all species with an inaperturate or global aperture pattern produced a majority of tetrahedral tetrads. Within each species, the distribution of the proportion of each type of tetrad presents a significant departure from 1/3 (36 < χ^2_3 < 296; \( P < 10^{-7} \)) (Table S2). This variation was not homogeneous at the interspecific level as the species studied differ significantly from each other in the distribution of their respective tetrad-form proportions (\( \chi^2_{14} = 198.8; P < 2.10^{-16} \)).

### Optimization of microsporogenesis

Our observations coupled with data recorded in the literature were used to optimize microsporogenesis features on our tree. Prior to optimization, some descriptions recorded from literature were reinterpreted in the light of current knowledge about relations between aperture pattern and microsporogenesis. In particular, some studies report the presence in species with an equatorial aperture pattern of tetragonal and decussate tetrads in addition to tetrahedral ones (Table 2). We suspect that the description of tetragonal and decussate tetrads probably results from observational artefacts and/or terminological confusion. A decussate tetrad is a tetragonal tetrad for which one cleavage plane underwent a 90° rotation (Fig. S1). Once they are mature, decussate tetrads may be indistinguishable from tetrahedral ones. Indeed, in these two types of tetrad, microspores are arranged in a very similar way (Fig. S1). The only way to distinguish between them is to look at cytokinesis and callose deposition. Tetrahedral tetrads are formed by simultaneous cytokinesis through the synthesis of six cleavage planes separating the microspores (Figs 1 and S1). Decussate tetrads can only be obtained from tetragonal tetrads produced by successive cytokinesis through the synthesis of three cleavage planes (Fig. S1: Blackmore *et al.*, 2007; Nadot *et al.*, 2008). Tetragonal tetrads can also be produced by simultaneously synthesizing four cleavage planes, but this configuration cannot result in a decussate tetrad. In the literature, cytokinesis and/or the callose deposition stage was described for all the species supposed to produce decussate and/or tetragonal tetrads (except for *Euphorbia dulcis*). Cytokinesis was simultaneous and occurred through the formation of six cleavage planes in all the species for which data were available (Table 2). Consequently, the occurrence of decussate tetrads in these species is not plausible because the authors who described decussate tetrads never reported the dyad stage that characterizes successive cytokinesis (Table 2). The occurrence of tetragonal tetrads is also questionable because callose deposition stages with three or four cleavage planes were never described. Based on the fact that cytokinesis was described as simultaneous in all species, we conclude that the reports of decussate and tetragonal tetrads in these species, in addition to tetrahedral, are incorrect. We consequently coded the character as tetrahedral in these species. Callose deposition was always described as centripetal, without a precise description of the mode (except for *Acalypha ciliata*, which is CpP). The resulting data matrix is shown in Table S1.

Figure 3b shows the optimization of tetrad form on our tree using ML and MP. The optimization conducted by ML recognizes that variation in tetrad configuration may have appeared at the base of the clade formed by inaperturate and articulated crotonoids. MP reconstruction fails to identify the point at which variation may have appeared, due to the lack of data for the more basal clades. However, optimization of the tetrad form on the Wurdack *et al.*’s (2005) topology (in which the Acalyphoideae s.s. clade is external relative to the Crotonoideae s.l. and Euphorbioideae) using ML and MP methods indicates unambiguously that the ancestral state is tetrahedral and that variation may have appeared at the base of the clade formed by inaperturate and articulated crotonoids (Fig. S4). In the articulated crotonoid clade, the panto-aperturate *M. esculenta* exhibits variation in tetrad form but, as the related triaperturate *Hevea brasiliensis* is tetrahedral, the ancestral state for the clade could not be resolved unambiguously. Both MP and ML analyses recognize the ‘equatorial’ clade (Acalyphoideae s.s. + Euphorbioideae) as being plesiomorphically tetrahedral, but this result needs to be treated with caution due to the large amount of missing data.

Results are similar for the mode of callose deposition (Figs S2 and S5). Both reconstruction methods suggest that the CpP mode of callose deposition is fixed for the
‘equatorial’ clade and that variation may have appeared at the base of the clade formed by inaperturate and articulated crotonoids. Again, inferences regarding the most basal nodes of the tree must be treated with caution due to the large amount of missing data. Finally, cytokinesis is exclusively simultaneous for all taxa, except for the inaperturate Codiaeum variegatum, which has simultaneous and successive cytokinesis (Fig. S3).

**Test for correlated evolution**

The tests for correlated evolution between traits indicate correlated changes in two of the three pairs of traits tested. LRS were relatively high for the pairs of characters aperture pattern/tetrad form (LRS₄ = 17.16; \( P = 0.0018 \)) and aperture pattern/callosose deposition (LRS₄ = 11.5; \( P = 0.021 \)) relative to that for the pair aperture pattern/cytokinesis (LRS₄ = 2.01; \( P = 0.73 \)). LRS were significant for the first two pairs, indicating that in these, the model for which the two characters are allowed to evolve independently is a worse fit for the data than the model in which the characters evolve in a correlated fashion. Similar results were obtained with the MCMC method. Bayes factor values were 15.3, 1.14 and 5.2, respectively, for the pairs of characters aperture pattern/tetrad form; aperture pattern/callosose deposition; and aperture pattern/cytokinesis. According to Pagel & Meade (2006), such values suggest ‘very strong’ evidence of correlated evolution for the pair of character aperture pattern/tetrad form, weak evidence for the pair aperture pattern/callosose deposition and no evidence for the pair aperture pattern/cytokinesis.

**Discussion**

Increasingly, in the literature, an important role is given to mutational and developmental systems in limiting the direction that can be taken by a lineage during its evolution (e.g. Bradshaw, 1991; Lynch, 2007; Stoltzfus & Yampolsky, 2009; Braendle et al., 2010; Futuyma, 2010). Other studies are inclined to argue that limitations on morphological diversity are caused by the action of natural selection rather than mutational biases or developmental constraints (e.g. Beldade et al., 2002; Eldredge et al., 2005; Wiens et al., 2006). In this study, we show that limitations in the evolution of pollen morphology and in the evolution of its developmental system are likely to be caused by selective pressures acting on pollen morphology. However, even in the absence of these selective pressures, a bias in the variability of the developmental system remains.

**Stasis in pollen morphology and development**

The aperture pattern is relatively conserved in the Euphorbiaceae s.s.: most species in this group (for which palynological data are available) produce pollen grains with (generally three) equatorial apertures. Our optimization of pollen aperture pattern indicates that this is the plesiomorphic condition and thus that the predominance of this pattern is not due to homoplasy. Within Malpighiales, inaperturate pollen also occurs in Linaceae, Malpighiaceae, Phyllanthaceae, Rafflesiaceae and Salicaceae (Furness, 2007). These occurrences, with the exception of Rafflesiaceae, map to well-nested clades within the most robust phylogenies for each family (i.e. Chase et al., 2002; Kathiriarachchi et al., 2005; Davis & Anderson, 2010; McDill & Simpson, 2011) and therefore represent losses that do not effect optimizations presented here. Rafflesiaceae are highly modified parasites that have been placed either sister to Euphorbiaceae s.s. or sister to Peraceae + Euphorbiaceae s.s. (Davis et al., 2007; Wurdack & Davis, 2009). Rafflesiaceae pollen is reported to be inaperturate, and its microsporogenesis needs further investigation (see Furness, 2007, 2008). Although not included in this study, it appears to represent an independent loss of apertures and would not impact our conclusions. Only two (possibly three) departures from the equatorial aperture pattern were observed. Euphorbiaceae s.s. comprise c. 6300 species (Wurdack et al., 2005). This group is quite diverse with respect to other morphological, phytochemical and palynological features (Punt, 1962; Webster, 1994; Wurdack et al., 2005) and is ancient with an estimated divergence of 110–69 Ma (Davis et al., 2005; Van Ee et al., 2008; Forest & Chase, 2009). Despite such ‘potentialities’ for change (high number of species, observed change in other features and ancient divergence time), aperture pattern remains relatively conserved as shown by our optimization. This result is in agreement with general palynological data, suggesting that aperture pattern is quite conserved at the eudicot scale. Although important diversity is known for aperture pattern, most genera of eudicots have pollen grains with (generally three) equatorial apertures (Wodehouse, 1935; Erdtman, 1952). Such conservatism may be considered as an example of evolutionary stasis (Muller, 1984), as this pollen morphology is a synapomorphy for the eudicot clade (Donoghue & Doyle, 1989; Doyle & Hotton, 1991), and its first occurrence in the fossil record corresponds approximately to 124 Myr (Hughes & McDougall, 1990). Here, the pattern suggested at the eudicot scale is confirmed for Euphorbiaceae s.s. using representative taxon sampling and a phylogenetic framework.

Evolutionary stasis in pollen aperture pattern was found to be associated with stasis in its developmental system (i.e. microsporogenesis and LPCD). All the species producing pollen with equatorial apertures investigated here were found to undergo microsporogenesis via the same LPCD pattern, which is the LPCD accounting for pollen with three or more equatorial apertures (illustrated in Fig. 1c-1). Our optimizations suggest that the species of the Euphorbioideae and Acalyphoideae s.s.
clades producing pollen with equatorial apertures are associated with this LPCD pattern. These results need to be treated with caution due to the limited data obtained for microsporogenesis, but are nevertheless consistent with the results of other workers indicating that equatorial apertures develop from this LPCD pattern (Ressayre et al., 2002a; Blackmore et al., 2007). The ancestral state for microsporogenesis is not resolved for the basal nodes of our topology. However, an optimization of microsporogenesis characters on the topology of Wurdack et al. (2005) (which is based on a larger taxon sample and in which the Acalyphoideae s.s. clade diverged before Crotonoideae s.l. and Euphorbioideae) indicates unambiguously that the ancestral state for microsporogenesis and LPCD is the one accounting for pollen with three or more equatorial apertures. Whereas this single LPCD predominates in eudicots, several LPCD have been described in the monocots (Wodehouse, 1935; Blackmore & Crane, 1998; Furness & Rudall, 2004; Nadot et al., 2008). The results shown here support this general view.

Bias on the fixation of variant phenotypes caused by selective pressures

It might be hypothesized that developmental constraints are acting on the LPCD pattern and selective pressures are acting on the aperture pattern. So both processes might account for the evolutionary stasis of the entire system, as the LPCD pattern is part of the ontogenetic determination of the aperture pattern (Furness & Rudall, 2004). Discrimination between these two hypotheses has been achieved here by realizing the thought experiment of Alberch (1982). For this, studying microsporogenesis in species with an inaperturate or a global aperture pattern is appropriate because in these, a partial or total disconnection of aperture position from microsporogenesis occurs (Wodehouse, 1935; Blackmore & Crane, 1998; Furness, 2007). Putative selective pressures due to aperture positioning may not have any effect on microsporogenesis and on the resulting LPCD pattern in these species.

Species of Euphorbiaceae s.s. investigated with inaperturate or global aperture patterns were found to be variable, at the level of the plant, for the three microsporogenesis features determining the LPCD pattern. For tetrad form, intraindividual variation was found in all nine inaperturate species examined and also in a species with a global pattern. This suggests that variation in tetrad form may occur easily and that there are no constraints on this feature, as suggested previously for some monocots (Nadot et al., 2006; Pereira Nunes et al., 2009). Variation in the mode of callose deposition was found in six of the nine species for which data for this character were available. This indicates that variation in this character may appear relatively easily in the context of a loss of the equatorial aperture pattern. The cytokinesis type seems to be less labile, as variation in this feature was found in only one inaperturate species. The association between the occurrence of the inaperturate or global pattern and the occurrence of variation in the LPCD pattern is not due to chance, as shown by the comparative analysis. We found a significant phylogenetic association between loss of the equatorial aperture pattern and appearance of variation in two of the three microsporogenesis features studied here (tetrad form and mode of callose deposition). This test is informative despite the fact that it is based on a single evolutionary transition from triaperturate to inaperturate pollen. The robustness of such a test depends on the number of phylogenetically independent occurrences of the two binary traits for which a correlation is being tested and on the number of nodes of the phylogenetic tree used: the higher the number of nodes of the tree, the lower the probability that changes in the two characters occur at the same node by chance if the two traits evolve independently. The appearance of variation in tetrad form and mode of callose deposition is thus not independent of the release of selective pressures on the LPCD due to loss of the equatorial pattern.

In the context where the LPCD pattern is disconnected from aperture patterning, six different LPCD patterns are observed here (Fig. 1) and many others were described in Codiaeum variegatum with inaperturate pollen (Albert et al., 2009). Where the LPCD pattern determines the aperture pattern (i.e. in species possessing equatorial apertures), a single LPCD pattern is observed (Fig. 1c-1). A possible explanation for this is that in the context where the aperture pattern and LPCD pattern are connected, selective pressure acting on the system prevents variation. A putative selective pressure is that acting on aperture number. Thus, according to Ressayre et al. (2002a), the five alternative LPCD patterns result in pollen grains with one, two or (rarely) three apertures (Fig. 1), whereas the predominant LPCD pattern results in pollen with three or more apertures (Ressayre et al., 2002b). Variation in aperture number has been shown to be linked to pollen grain fitness. It may be that the predominant LPCD pattern has been positively selected because of the flexibility in aperture number it provides (Furness & Rudall, 2004).

Bias in the production of variant phenotypes caused by developmental constraints

Variation in LPCD pattern occurs where it may be assumed that some selective pressure has been released. However, this variation appears to be partially biased because only six LPCD patterns (of the sixteen that are possible) are observed. Two biases remain. Firstly, LPCD patterns resulting from a switch in cytokinesis were rarer than those resulting from a switch in tetrad form or callose deposition (bias 1). Secondly, LPCD patterns resulting from a change in two or three microsporogen-
esis features were rare or absent, respectively, whereas changes resulting from a change in a single microsporogenesis feature were observed in all the inaperturate species (bias 1). Additional selective pressures and/or developmental constraints are thus likely to be acting on the system. It may be assumed that where microsporogenesis is connected to aperture pattern, developmental phenotypes (LPCD patterns) and corresponding pollen phenotypes (aperture patterns) resulting from a switch in cytokinesis should be rarer than those resulting from a switch in tetrad form or mode of callose deposition (bias 1). In addition, phenotypes resulting from a switch in two or three microsporogenesis features should be rarer than those resulting from a switch in only one feature (bias 2). These biases may constitute an example of a developmental constraint sensu Maynard Smith et al. (1985), that is, a bias in the generation of phenotypic variation due to the properties of the developmental system.

It would be interesting to assess microsporogenesis and LPCD in other inaperturate lineages (e.g. in Apocynaceae). This would provide additional phylogenetically independent samples to conduct the thought experiment of Alberch and would allow to test whether the biases observed here are produced repeatedly. Assessing the molecular basis of variation in microsporogenesis would allow to investigate whether this variation is constrained by the topology of the gene network (Wagner, 2011), as well as to test for the presence of molecular signatures of selection on genes functionally linked to microsporogenesis. To date, knowledge about the molecular pathways involved in the control of microsporogenesis does not allow to carry out such studies (but see Magnard et al., 2001).

Conclusion

The pattern observed here indicates that evolutionary stasis in pollen aperture morphology and development may be due, in part, to stabilizing selection because removing the presumed target of selection permits the appearance of variation. However, not all possible variants occur, indicating that constraints also contribute for stability. In the debate concerning selection and constraints, it appears that neither of these two factors can totally account for evolutionary stasis. It is the interaction between them that may determine the direction of evolution.

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References


### Supporting information

Additional Supporting Information may be found in the online version of this article:

- **Table S1** Genbank accession numbers (or source) of the sequences used for the phylogenetic analysis; and matrix used for the optimization and for the test of correlated evolution
- **Table S2** Number of tetrads for each type of tetrad observed in the heteromorphic species, and results of the chi-square tests, testing if each type of tetrad is produced in similar proportions within a plant
- **Figure S1** Number and position of callose cleavage planes, relative to the arrangement of the microspores in the tetrad and to the cytokinesis type
- **Figure S2** "Mirror tree of Euphorbiaceae s.s. comparing optimizations of the aperture pattern (a) and mode of callose deposition (b)
- **Figure S3** Mirror tree of Euphorbiaceae s.s. comparing optimizations of the aperture pattern (a) and cytokinesis type (b)
- **Figure S4** Mirror tree of Euphorbiaceae s.s. comparing optimizations of the aperture pattern (a) and tetrad form (b) using the topology of Wurdack *et al.* (2005)
- **Figure S5** Mirror tree of Euphorbiaceae s.s. comparing optimizations of the aperture pattern (a) and mode of callose deposition (b) using the topology of Wurdack *et al.* (2005)

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