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# Unreduced diploid nuclei in *Cupressus dupreziana* A. Camus pollen

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**Abstract** Size and DNA content of pollen of *Cupressus dupreziana* A. Camus, a highly endangered Mediterranean conifer, were assessed by cytomorphological observations and flow cytometric analyses and then compared to *C. sempervirens* L. pollen. Mature *C. dupreziana* pollen was composed of two uninucleated types of pollen grains differing in size. Around 35% of the grains exhibited a size similar to *C. sempervirens* pollen, while 65% exhibited a larger diameter. However only one peak of fluorescence was detected by flow cytometry. DNA content of *C. dupreziana* pollen was twice the DNA content of *C. sempervirens* pollen. Comparison of DNA contents of mature and germinating pollen revealed that mature pollen of both species were arrested in the G2 phase. Comparison with the DNA content of somatic tissue (2C) provided evidence for the production of unreduced pollen in *C. dupreziana.* This unexpected feature in gymnosperms is discussed in terms of reproductive strategy of this species.

**Key words** 2n pollen · *Cupressus dupreziana* · Gymnosperms · Flow cytometry · Reproduction

## Introduction

The Cupressaceae family is the largest coniferous taxon with 19 genera distributed in both the Northern and Southern Hemispheres (Biswas and Johri 1997). Most of these genera are monotypic or only consist of a few species, exhibiting a narrow geographic distribution (Miller 1977). Accordingly, it has been proposed that such genera could be regarded as relicts (Biswas and Johri 1997). The genus *Cupressus* includes at least 25 species (Ducrey et al. 1999), but some of them are also endangered, such as *Cupressus dupreziana* A. Camus. This Mediterranean species is the most notable of the trees surviving in the mountainous region of the Tassili N'Ajjer desert, in the center of the Sahara, Algeria (Balachowsky 1955; Simonneau and Debazac 1961). Its natural stands are likely to disappear due to the absence of *in situ* regeneration. Additionally a previous study showed that only 10% of the seeds collected from *in situ* growing trees contained an embryo, suggesting anomalies in the sexual reproductive process (Pichot et al. 1998). In order to explain this feature and define a biological basis for an adequate conservation strategy, we decided to study the reproductive biology of this species.

We have recently shown that, as for the two other Mediterranean cypresses (*C. sempervirens* L. and *C. atlantica* Gaussen), *C. dupreziana* endosperm exhibits multiple ploidy levels (Pichot and El Maâtaoui 1997; Pichot et al. 1998; El Maâtaoui and Pichot 1999). However, in contrast to the odd and even ploidy levels (1C, 2C, 3C, 4C, 5C ...) observed in *C. sempervirens* and *C. atlantica*, only even levels (2C, 4C, 6C ...) were found in *C. dupreziana.* In gymnosperms, the endosperm is expected to be haploid since it results from the development of the megagametophyte, which derives from one meiotic spore (Singh 1978; Pennel 1988; Biswas and Johri 1997). Given that the megagametophyte produces the gametes through archegonia, the absence of haploid nuclei in *C. dupreziana* endosperm raises the problem of the origin of the female gametes and, consequently, of their involvement in embryogenesis. Analysis of isozyme variability among open-pollinated progenies led us to reject the hypothesis of a maternal apomictic origin of the embryos (Pichot et al. 2000). The absence of maternal alleles in most of the embryo zymograms suggested a strictly paternal origin of the embryo nuclear DNA and

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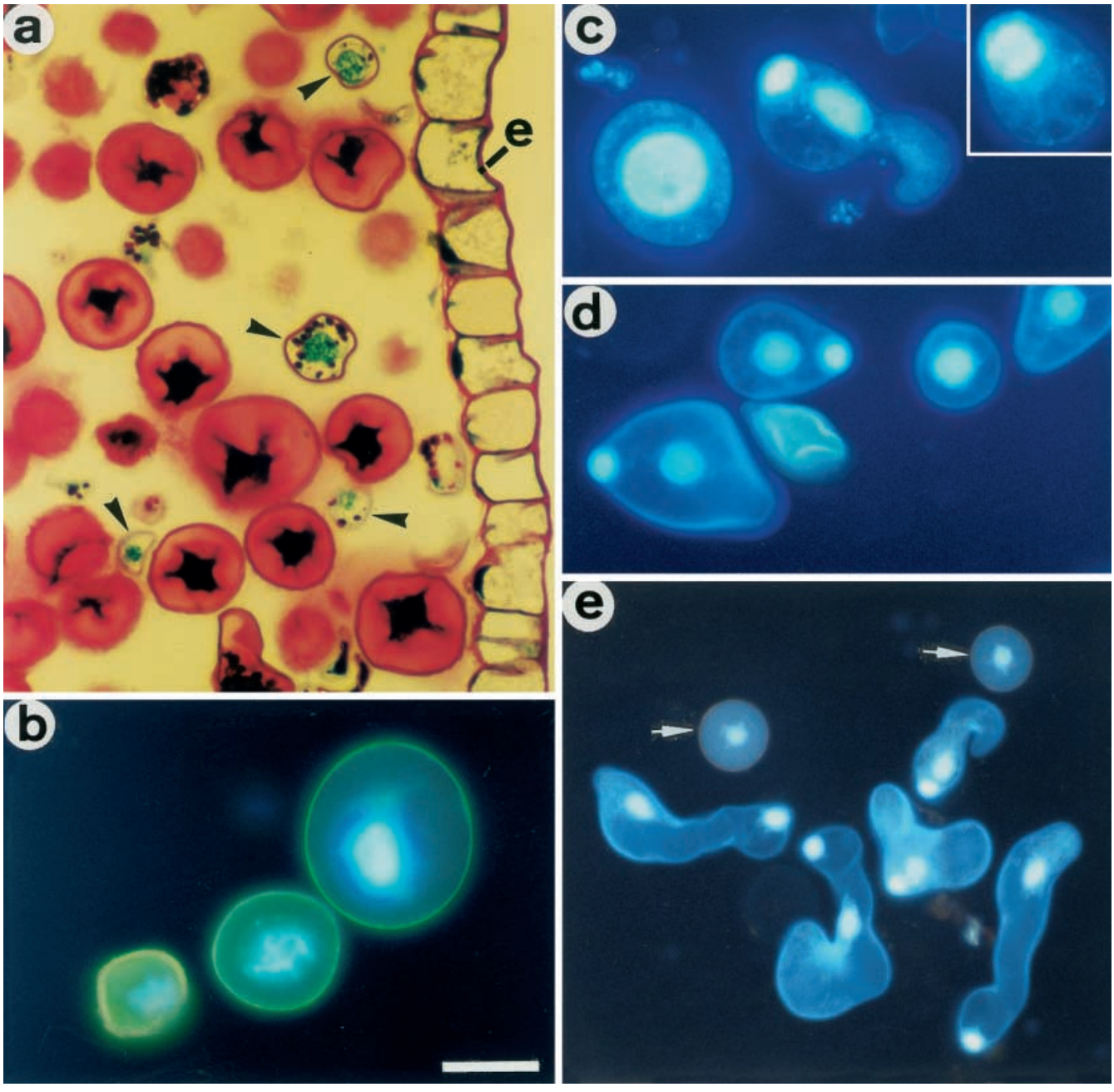


**Fig. 1a–e** Comparative cytomorphology of pollen in *Cupressus dupreziana* and *C. sempervirens*. **a** Section of a microsporangium of *C. dupreziana* before pollen shedding, showing two types of pollen grains. The small grains (*arrowheads*) contain numerous amyloplasts around the nuclei. *e*=Epidermis. **b** Mature pollen of *C. dupreziana* illustrating size heterogeneity. **c** Pollen of *C. dupreziana* after 3 days of culture on a germination medium. The imbibition results in exine-free cells. The nucleus becomes voluminous (*left*) and divided (*right*), producing a two-celled male gametophyte. *Inset* shows the telophase step of nuclear division. **d** Pollen of *C. sempervirens* after 3 days of culture as control. A two-celled male gametophyte is also generated. Note the reduced nuclear size compared to *C. dupreziana* germinating pollen presented in **c**. **e** Pollen of *C. dupreziana* after 10 days of culture showing well-developed pollen tubes and intact grains corresponding to the small cytotype (*arrows*). **a** Periodic acid-Schiff's reagent – Naphtol blue-black staining, **b**–**e** DAPI staining, *Bar* (**b**) 20 µm (**a**–**d**) 40 µm (**e**)

led us to hypothesize a "paternal apomixis." In this context, the size and DNA content of *C. dupreziana* pollen were studied by cytological approaches and flow cytometric analyses and then compared to those of *C. sempervirens* pollen.

## Materials and methods

### Plant material

Somatic tissues and mature pollen were collected from two *Cupressus dupreziana* trees, referred as "dup-est" and "dup-ouest", planted in southern France and from one *C. sempervirens* tree. This latter species was used as control, since it exhibits a conform sexual reproduction.

### Pollen structure, size, and germination

For each pollen tree, we estimated the diameter of 150 pollen grains imbibed in distilled water for 30 min using a light microscope. Histological studies were carried out on microsporangia excised from opening male cones of *C. dupreziana* "dup-est." These were fixed, dehydrated, and prepared for light microscopy as previously described (El Maâtaoui and Pichot 1999).

For fluorescence microscopy, mature and germinating pollen were mounted in a drop of the staining solution ("High resolution DNA kit, type T", PARTEC, Münster, Germany) containing the DNA-specific fluorochrome DAPI (4′,6-diamidino-2-phenylindole). Preparations were viewed under epifluorescent illumination (480 nm).

Pollen germination was obtained by placing mature microsporangia in petri dishes containing a germinating medium. This consisted of 0.7% agarose and 20% sucrose in distilled water that had been autoclaved for 20 min at 110°C, 1.5 bar. For microscope observations, samples were harvested at 3 and 10 days after culture initiation.

### DNA content

Relative DNA content of pollen nuclei was estimated by flow cytometry for the different pollen samples and compared to the DNA content of somatic nuclei (2C standard) as detailed in Table 1. Nuclei were extracted and stained using the High resolution DNA kit, type T (PARTEC, Münster, Germany). Somatic tissue and germinating pollen (10 days) were chopped with a razor blade, while mature pollen was gently squashed, in 0.5 ml of solution A of the kit. Samples were then filtered through a 30-µm CellTrics™ filter and stained in 1.5 ml of solution B (4′,6-diamidino-2-phenylindole, DAPI staining) of the DNA kit. The DNA content of the nuclei was evaluated using a PARTEC Ploidy Analyser PA equipped with a HBO lamp for UV excitation. Measurements were assigned according to relative fluorescence intensity. Results were stored in histogram-type data files and studied with the WINMDI software (version 2.7, copyright© 93–98 Joseph Trotter).

## Results

### Pollen structure and size

Main representative results on the structure and the morphology of *C. dupreziana* pollen are presented in Fig. 1a–e. Sections, stained to reveal polysaccharides and nucleoproteins, showed two cytotypes of pollen grains (Fig. 1a). The predominant cytotype consisted of voluminous uninucleated grains exhibiting a thick intine encompassing a wrinkled, highly condensed cytoplasm. The second cytotype was represented by smaller and apparently conform cells showing well-structured walls and normal cytoplasm. In these cells, numerous amyloplasts were observed surrounding the nucleus. Whole mounts using the specific fluorochrome DAPI revealed the presence of nuclei in both pollen cytotypes (Fig. 1b). Observations on 3-day cultured pollen showed nuclear division, resulting in a two-celled male gametophyte, followed by pollen-tube initiation (Fig. 1c). Similar behaviors were also observed in *C. sempervirens*, but the fluorescence intensity suggested that the nuclei were of a smaller size (Fig. 1d). Observations of 10-day cultured *C. dupreziana* pollen showed more developed male gametophytes with elongated pollen tubes containing one of the two nuclei. Some intact, non-germinated pollen grains were also observed (Fig. 1e).

As shown in Fig. 2a-c, the distribution of the diameter of *C. sempervirens* pollen was unimodal and averaged 25.8 µm. In contrast, *C. dupreziana* pollen exhibited a bimodal distribution. For the two *C. dupreziana* trees tested (dup-

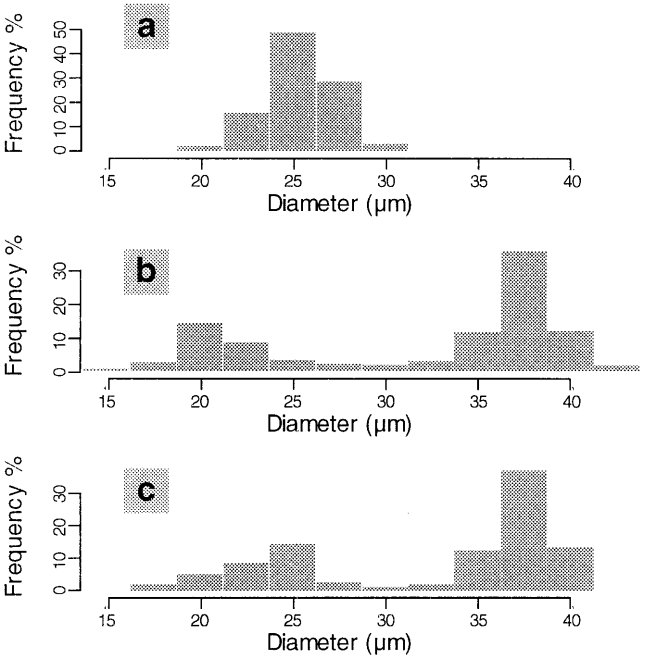


**Fig. 2a–c** Histograms of pollen diameter frequency. **a** *Cupressus sempervirens* pollen as control. **b** *C. dupreziana* dup-est pollen. **c** *C. dupreziana* dup-ouest pollen. Note the two populations of pollen diameters observed for both *C. dupreziana* samples as compared to the unimodal distribution of the control

**Table 1** Origin of the *Cupressus* samples analyzed by flow cytometry for the estimation of nuclear DNA content

| Tree | Mature pollen (M) | Germinating pollen (G) | Somatic tissue (S) | Mixture |
|---|---|---|---|---|
| *C. dupreziana* | | | | |
| dup-est | +[a] | + | + | M+G and M+S |
| dup-ouest | + | – | – | –- |
| *C. sempervirens* | | | | |
| Control | + | + | + | M+S |
| Mixture dup-est + control | + | + | – | – |

[a] +, Samples analyzed; –, samples not analyzed

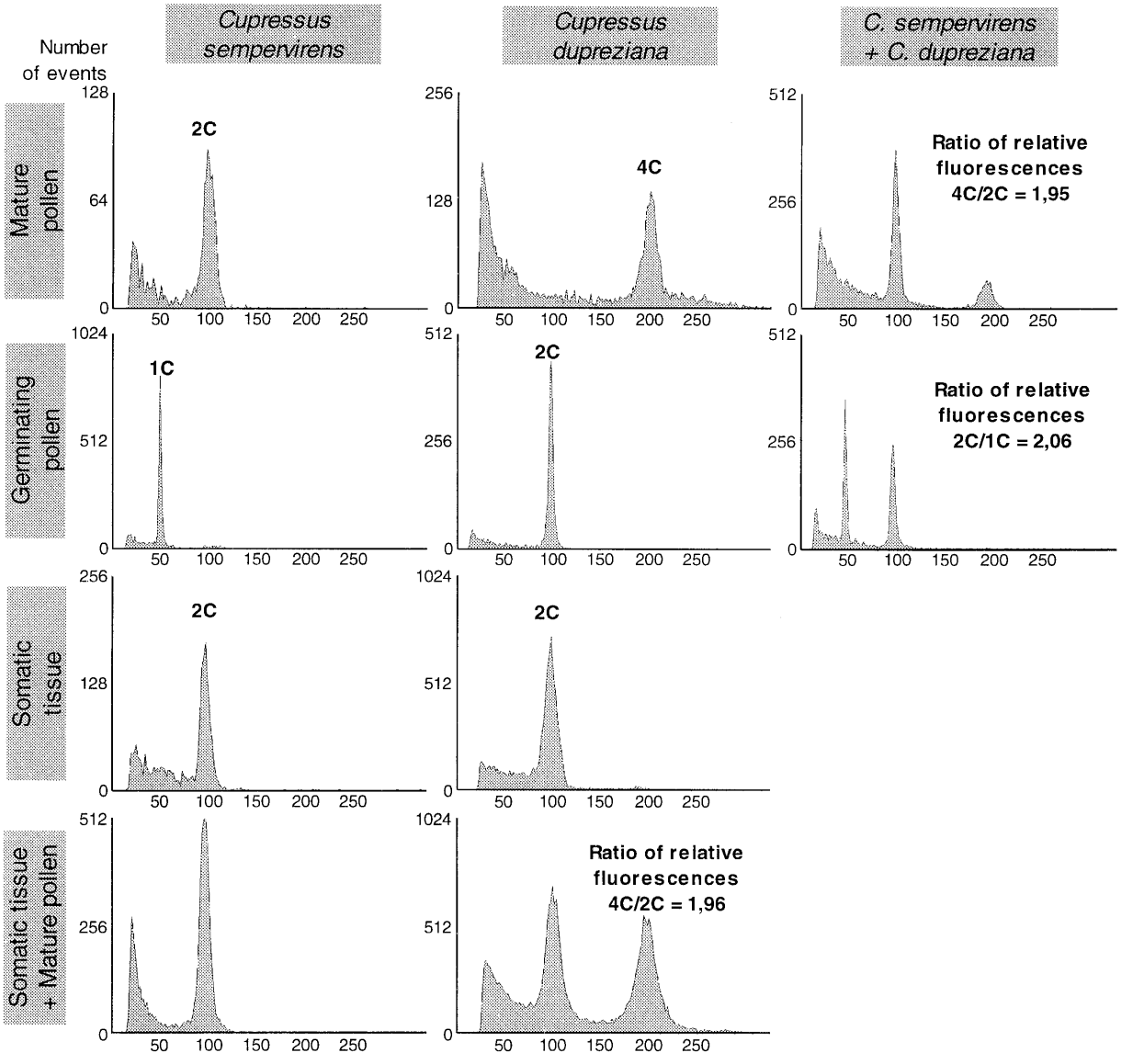


**Fig. 3** DNA content of nuclei from pollen and somatic tissue. *First column Cupressus sempervirens, second column C. dupreziana, third column* mixture of the two species. *First row* Mature pollen, *second row* germinating pollen, *third row* somatic tissue, *fourth row* mixture of mature pollen and somatic tissue. Histograms represent the number of events or nuclei (*ordinate*) per relative fluorescence intensity expressed in arbitrary units (*abscissa*). For each peak, the mean fluorescence value is expressed in C units, whereby 1C is the amount of the haploid genome. *C. dupreziana* mature pollen produced one peak corresponding to 4C DNA content. Comparison with the 2C DNA peak exhibited by *C. dupreziana* germinating pollen and somatic tissue indicates that mature pollen grains are arrested in the G2 phase and originate from unreduced microspores. Histograms produced by *C. sempervirens* samples show that the pollen originate from reduced microspores. Histograms produced by the mixture of the two species show that DNA content of *C. dupreziana* pollen is twice that of *C. sempervirens* pollen

ouest and dup-est), the diameter of most of the pollen grains (around 65%) was more than 30 µm, with a mean value of 37.3 µm, while the diameter of the smaller pollen was close to (for dup-ouest tree) or slightly less than (for dup-est tree) the diameter of *C. sempervirens* pollen.

## Ploidy analysis

The histograms of relative fluorescence observed for the different samples are presented in Fig. 3. All somatic and pollen samples (except mixtures) produced one peak of fluorescence. For somatic samples of the two species as well as for *C. sempervirens* mature pollen, peaks were located at similar positions, corresponding to a 2C DNA content. *C. dupreziana* mature pollen produced a peak corresponding to 4C DNA, as confirmed by the analysis of the mixture of the two species: the ratio of the two relative fluorescence peaks (4C/2C) was 1.95. In both species, the DNA content of nuclei extracted from germinat-

ing pollen was half that of mature pollen nuclei. Thus, in *C. dupreziana*, nuclei of germinating pollen and nuclei of somatic tissue had a similar DNA content (2C). The mixture of germinating pollen from the two species produced a two-peak histogram with a peak ratio estimated at 2.06.

## Discussion

Our observations show that *C. dupreziana* mature pollen contains two types of pollen grains that differ in their size. The smallest *C. dupreziana* pollen proved to be similar to *C. sempervirens* pollen. The diameter of the largest *C. dupreziana* pollen reported here is in agreement with previous observations of Allemand (1979) who considered this pollen as aberrant: 38 µm *vs* 28 µm for *C. sempervirens.* It is commonly assumed that, for a given species, pollen size is correlated to the nuclear DNA content (Jones 1990; Negri et al. 1995; Ramsey and Schemske 1998). This property allows reduced and unreduced pollen to be differentiated. It is therefore likely that the largest *C. dupreziana* pollen grains are unreduced.

According to our flow cytometry results, the DNA content of *C. dupreziana* pollen is twice that of *C. sempervirens*. This feature was observed both in mature and germinating pollen samples. Before germination, mature pollen grains of both species are uninucleated cells (Fig. 1a-c), with a 2C DNA content for *C. sempervirens* and a 4C DNA content for *C. dupreziana.* Our analysis of germinating pollen revealed 1C and 2C DNA contents, respectively. It is thus clear that in both species, mature pollen is arrested in the G2 phase. When germinating, the pollen nucleus resumed mitosis, producing two sister nuclei (Fig. 1c). A comparison of the DNA content between pollen and somatic tissue confirmed the existence of reduced 1n pollen in *C. sempervirens* and unreduced 2n pollen in *C. dupreziana*. Despite the presence of two morphological types of pollen grains suggesting two ploidy levels in *C. dupreziana*, no peak corresponding to haploid nuclei was detected by flow cytometry, neither from mature pollen nor from germinating pollen. At the present stage of our investigation, the reason for this feature remains unknown. However, our results clearly demonstrate the production of unreduced pollen, a phenomenon already observed in several flowering plants (reviewed in Bretagnolle and Thompson 1995) but, to our knowledge, never reported in gymnosperms.

In angiosperms, unreduced pollen production is considered to be the main evolutionary process in species polyploidization (Veilleux 1985; Bohac and Jones 1994; Ramsey and Schemske 1998). Unreduced pollen is intensively used in breeding programs of polyploid species such as *Solanum tuberosum* (Peloquin et al. 1989) or *Vaccinium* spp (Shoemaker-Megalos and Ballington 1988) and in interspecific crosses (Ishizaka 1998). According to Shoemaker-Megalos and Ballington (1988) and Tavoletti et al. (1991), the production of 2n gametes in *Vaccinium* and *Alfalfa* is an inherited trait, but there is no correlation between 2n male gametes and 2n female gamete production. The possible production of 2n female gametes has not been yet assessed in *C. dupreziana*.

Bretagnolle and Thompson (1995) recently reviewed the different meiotic anomalies leading to 2n gametes in plants. Depending on the meiotic stage at which these anomalies occur, unreduced gametes are either called first-division restitution (FDR) or second-division restitution (SDR) 2n gametes. In SDR, the two sister chromatids are conserved in the same gamete, while the FDR gamete possesses two non-sister chomatids. If a random localization of loci on the chromosomes is hypothesized, FDR gametes conserve most of the parent's non-additive value: around 80% with crossing-over and 100% without (Hutten et al. 1994; Bretagnolle and Thompson 1995; Buso et al. 1999). In *C. dupreziana*, the retention of heterozygosity of the pollen tree had to be checked by the analysis of genetic marker variability among single 2n pollen grains. A high conservation of pollen tree heterozygosity would be interpreted as a result of FDR. Cytological studies of microsporogenesis are in progress and will provide information on the ontogenesis of the two pollen types and on the meiotic anomalies involved in the production of unreduced pollen.

The production of 2n pollen is influenced by both genetic and environmental factors. A systematic survey of the seven 2× *Vaccinium* species revealed a widespread occurrence of 2n pollen as well as a large variability in this trait (Ortiz et al. 1992). At the individual plant level, the frequency of 2n pollen is variable but generally low (Bretagnolle and Thompson 1995). Without totally rejecting the possibility of the production of reduced pollen, we demonstrated that in the two *C. dupreziana* studied trees, the viable pollen contained diploid unreduced nuclei. Pollen collected from other trees has to be studied in order to know if some individuals produce viable reduced pollen. The production of only unreduced viable pollen would seriously compromise the possibility of conform sexual reproduction of *C. dupreziana*, as well as interspecific hybridization with other *Cupressus* species, and would lead to the extinction of this endangered species (Vrijenhoek 1998). The biological significance of this particular feature in gymnosperms seems to be cryptic, but would be consistent with our hypothesis of paternal apomixis mentioned above.

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