Theor Appl Genet (1997) 94:865–870

C. Pichot · M. El Maataoui

Flow cytometric evidence for multiple ploidy levels in the endosperm of some gymnosperm species

Received: 17 August 1996 / Accepted: 18 October 1996

Abstract Considered to be haploid tissue, the endosperm of coniferous trees has been extensively used by forest geneticists. Using laser flow cytometry, we show that endosperm ploidy level depends on the systematic position. The *Abies*, *Cedrus* and *Pinus* species tested exhibited uniform haploid endosperm compared to the diploid DNA content of the corresponding embryo. Endosperm of Cupressaceae contained multiple ploidy levels: *Cupressus arizonica*, *Juniperus oxycedrus* and *Thuja orientalis* endosperms exhibited a mixture of haploid–diploid nuclei, while *C. atlantica* and *C. sempervirens* endosperms contained six ploidy levels: 1C, 2C, 3C, 4C, 5C and 6C. Physiological and genetic implications of this original feature are discussed.

Key words Gymnosperms · Ploidy · Endosperm · Embryo · Flow cytometry

Introduction

In gymnosperm seeds, the diploid embryo is surrounded by a haploid tissue called endosperm (female gametophyte or megagametophyte) generated by the proliferation of one meiotic daughter cell (megaspore) which also produces the female gamete. Thus, the genotype of the endosperm corresponds exactly to the maternal contribution to the seed genotype.

Because of these specific features seeds of coniferous trees offer several interesting avenues of study, which have been largely used in basic genetic studies (O'Malley et al. 1979; Mitton 1983; Bergmann 1991): (1) the haploid endosperm makes it possible to study the inheritance and linkage of isozyme, protein or DNA markers by analysing segregations within progenies from heterozygous mother trees (Strauss and Conkle 1986; Tulsieram et al. 1992; Gerber et al. 1993; Pascual et al. 1993; Plomion et al. 1995; Gillet 1996); (2) mother-tree genotypes may be inferred from endosperm analysis (Morris and Spieth 1978); (3) allele frequencies and (4) heterozygosity levels can be estimated for two generations (maternal parents and seeds); (5) marker analyses of both the endosperm and embryo enable the paternal contribution to seed genotype to be determined in mating-system studies (Shaw and Allard 1982; Barret et al. 1987).

However, a survey of the literature on genetic studies using conifer endosperms has revealed that both the haploid nature and the genetic uniformity of this tissue are, in some cases, questionable. Studying the segregation of colour polymorphism in *Ginkgo biloba* endosperm O'Malley and Kelly (1988) reported mosaic endosperms. Similar unexpected features have also been reported with isozyme analyses. In *Pinus attenuata* endosperm, O'Malley et al. (1988) noted abnormal two-band phenotypes for three dimeric enzymes (alcohol deshydrogenase, 6-phosphogluconic deshydrogenase and phosphoglucose isomerase). The absence of the heterodimer bands has been interpreted to be the result of the probable mosaic nature of the endosperm, supposedly originating from genetically different megaspores. More recently, isozyme studies of *Cupressus sempervirens* always led to diploid phenotypes of the tissue surrounding the embryo (Raddi et al. 1990; Papageorgiou et al. 1993). The phenotypes were always similar to the mother-tree phenotypes, and this tissue was considered to be a diploid maternal tissue: the perisperm. These authors considered endosperm and

Communicated by P. M. A. Tigerstedt

C. Pichot (✉)
INRA, Unité de Recherches Forestières Méditerranéennes, Equipe de Génétique, Av. Vivaldi, 84000 Avignon, France

M. El Maataoui
Université d'Avignon, Laboratoire de Biologie Végétale Expérimentale, 33 rue L. Pasteur, 84000 Avignon, France

Table 1 Origin of the samples

Sample reference	Genetic origin	Geographic origin[a]
Cupressus sempervirens No. 1	1 tree var. *horizontalis*	Avignon (a), France
Cupressus sempervirens No. 2	1 tree var. *pyramidalis*	Afghanistan (n)
Cupressus sempervirens No. 3	1 tree var. *pyramidalis*	Afghanistan (n)
Cupressus atlantica	Rikt provenance	Haut Atlas Mountain (n), Morocco
Cupressus arizonica No. 1	1 tree	Oupia (a), France
Cupressus arizonica No. 2	1 tree	Bellegarde (a), France
Cupressus arizonica No. 3	1 tree	Bellegarde (a), France
Juniperus oxycedrus	1 tree	Avignon (n), France
Thuja orientalis	1 tree	Avignon (a), France
Cedrus libani	Provenance	Barouk (n), Lebanon
Abies cephalonica	Provenance	Vetina (n) Mainalon, Greece
Pinus halepensis	Provenance	St Etienne du Grès (n), France
Pinus pinaster	Provenance	Collo, Algeria
Pinus nigra	Provenance	Avignon (a), France
Pinus pinea	Provenance	Stes Marie de la Mer (n), France

[a] n, natural; a, artificial

perisperm to be closely connected and that the mass and enzyme activity of the perisperm were prevalent. This interpretation is not in agreement with the classical structure of mature conifer seeds (Ozenda 1982) where nucellus remnants (perisperm) are reduced to a thin layer of degenerating cells (El Maataoui, unpublished observations) probably without any enzymatic activity.

In spite of the frequent use of endosperm in genetic studies, investigations on its ploidy level have been very limited. In this context we used laser flow cytometry to quantify relative DNA contents of the embryo and endosperm of some coniferous trees, and especially of cypress.

Materials and methods

Plant material and tissue isolation

DNA content was separately assessed from embryo and endosperm nuclei of the conifer species referred in Table 1. Seeds were extracted from mature, open-pollinated cones and stored at 4°C until use. Embryos and endosperms were carefully excised from overnight-imbibed seeds under a dissecting microscope.

Extraction of nuclei

Embryo and endosperm tissues were finely chopped at 4°C with a sharp razor blade in PBS buffer, pH 7.4, containing 2.5 m*M* dithiothreitol and 0.05% triton X-100. The resulting slurry was filtered through a 30-μm nylon filter to eliminate cell debris. The suspension containing the nuclei was stained with 1% propidium iodide in PBS buffer (500 μl tissue extract + 50 μl 1% propidium iodide) and immediately measured using a Facscan (Becton Dickinson) laser flow cytometer equipped with an argon-ion laser tuned at a wavelength of 448 nm.

Measurements

Relative fluorescence histograms of DNA colouration measured by red fluorescence (FL2 Area) at 580 nm were registered. Mean and coefficient of variation of fluorescence peaks were estimated with the WinMDI software (version 2.0.4, copyright © 93–95 Joseph Trotter).

Results

All of the embryo samples produced a relative fluorescence histogram with only one peak, irrespective of the species tested (Fig. 1, Table 2) . For this reason we considered the embryos to be uniformly diploid structures and took their corresponding peaks as the control peaks for 2C DNA content. In *Pinus*, *Cedrus* and *Abies* species endosperm samples also produced one peak, and this peak was half the magnitude of that of the relative fluorescence peak of the embryo. On the contrary, all of the endosperm samples from the Cupressaceae family (genera: *Cupressus*, *Thuja* and *Juniperus*) exhibited multi-peak relative fluorescence histograms. Two peaks were observed for *Thuja orientalis*, *Juniperus oxycedrus* and *Cupressus arizonica* (3 genotypes) and six peaks for *C. sempervirens* (3 genotypes) and *C. atlantica* (1 genotype). The peaks were regularly spaced on the relative fluorescence scale and, when compared with histograms from the embryos (2C), corresponded to 1C, 2C, 3C, 4C, 5C and 6C. In endosperm samples producing a two-peak histogram, the first one was predominant (around 80%). Conversely, in samples producing a six-peak histogram, the second one was prevalent while the first peak contained only 15% of the scored events.

For those species of the genus *Cupressus*, estimations of 2C nuclear DNA sizes based on endosperms were more consistent than estimations based on embryos; the coefficients of variation were, respectively, 1.9% and 4.1% (Table 2).

Fig. 1 Histograms of flow cytometric analysis of embryos and endosperms of some coniferous species. Relative fluorescence expressed in arbitrary units

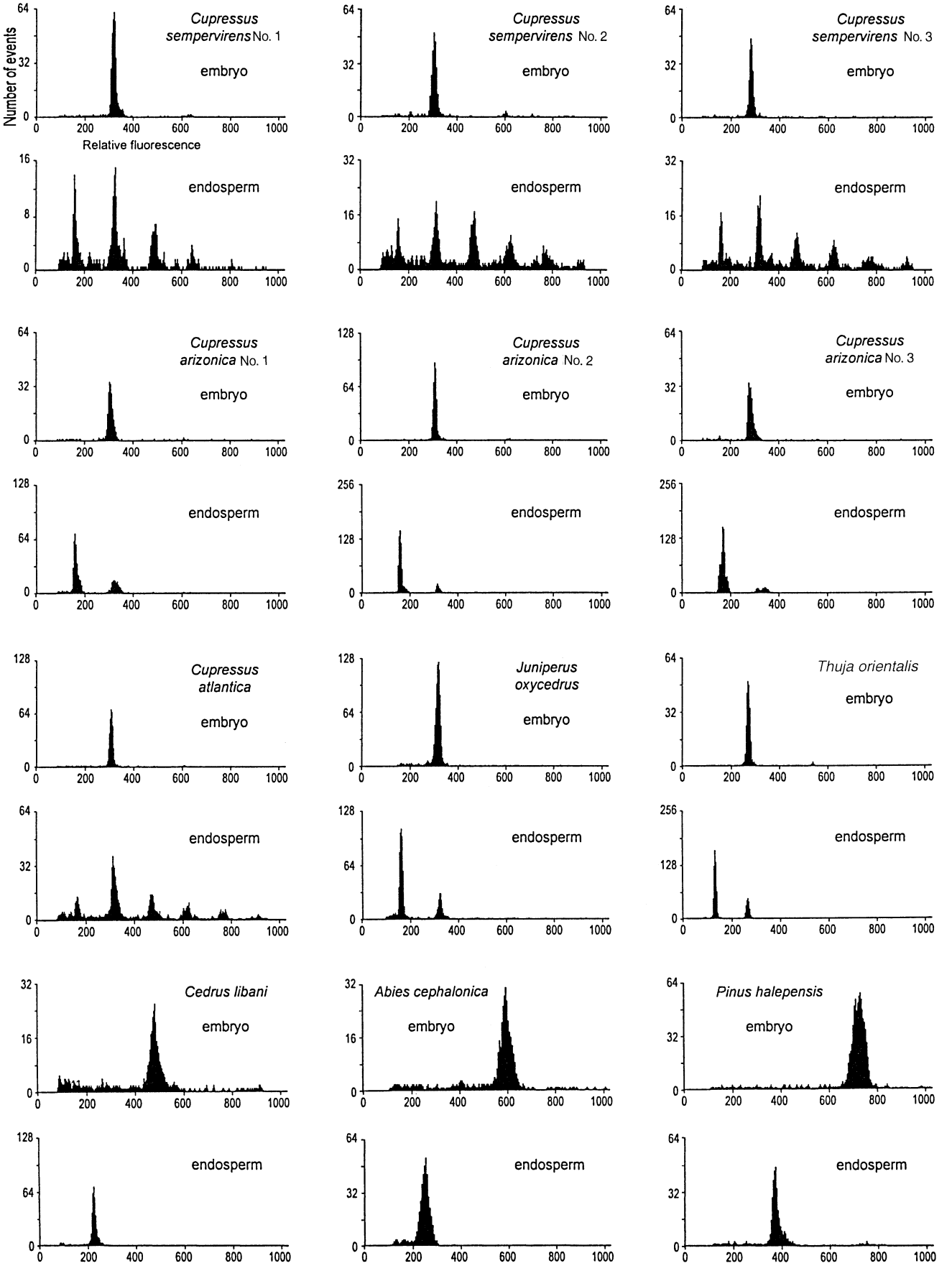
Number of events
Relative fluorescence
Cupressus sempervirens No. 1
embryo
endosperm
Cupressus sempervirens No. 2
embryo
endosperm
Cupressus sempervirens No. 3
embryo
endosperm
Cupressus arizonica No. 1
embryo
endosperm
Cupressus arizonica No. 2
embryo
endosperm
Cupressus arizonica No. 3
embryo
endosperm
Cupressus atlantica
embryo
endosperm
Juniperus oxycedrus
embryo
endosperm
Thuja orientalis
embryo
endosperm
Cedrus libani
embryo
endosperm
Abies cephalonica
embryo
endosperm
Pinus halepensis
embryo
endosperm

Table 2 Characteristics of the relative fluorescence peaks

Sample	Mean	C.V.[a]	Percentage of nuclei[b]
Cupressus sempervirens No. 1			
Embryo			
2C	320	2.0	100
Endosperm			
1C	157	3.6	29
2C	312	2.4	40
3C	465	1.6	20
4C	612	1.0	7
5C	762	1.3	2
6C	885	1.7	1
Cupressus sempervirens No. 2			
Embryo			
2C	300	2.2	100
Endosperm			
1C	157	3.2	11
2C	311	2.2	30
3C	465	1.6	27
4C	612	1.8	18
5C	759	1.3	10
6C	900	1.1	3
Cupressus sempervirens No. 3			
Embryo			
2C	282	2.2	100
Endosperm			
1C	157	2.7	16
2C	309	2.0	32
3C	459	1.8	24
4C	603	1.4	16
5C	747	1.2	8
6C	890	1.2	4
Cupressus atlantica			
Embryo			
2C	304	1.8	100
Endosperm			
1C	164	3.3	13
2C	310	2.9	51
3C	456	1.6	19
4C	600	1.3	10
5C	740	1.3	5
6C	885	0.8	2
Cupressus arizonica No. 1			
Embryo			
2C	300	2.5	100
Endosperm			
1C	161	4.0	71
2C	318	3.0	29
Cupressus arizonica No. 2			
Embryo			
2C	305	1.5	100
Endosperm			
1C	158	2.6	87
2C	311	1.4	13
Cupressus arizonica No. 3			
Embryo			
2C	282	2.4	100
Endosperm			
1C	168	5.2	86
2C	327	4.6	14
Juniperus oxycedrus			
Embryo			
2C	309	2.6	100
Endosperm			
1C	157	3.0	76
2C	311	2.0	24
Thuja orientalis			
Embryo			
2C	266	1.7	100
Endosperm			
1C	138	2.3	67
2C	273	1.8	33
Cedrus libani			
Embryo			
2C	479	2.8	100
Endosperm			
1C	229	2.3	100
Abies cephalonica			
Embryo			
2C	597	2.7	100
Endosperm			
1C	247	5.7	100
Pinus halepensis			
Embryo			
2C	723	3.0	100
Endosperm			
1C	374	3.0	100
Pinus pinaster			
Embryo			
2C	720	2.3	100
Endosperm			
1C	372	2.9	100
Pinus nigra			
Embryo			
2C	747	4.0	100
Endosperm			
1C	359	3.1	100
Pinus pinea			
Embryo			
2C	874	3.6	100
Endosperm			
1C	417	4.4	100

[a] Coefficient of variation
[b] Only within-peak events were scored

For multi-peak histograms, relative fluorescence increased slightly slower than ploidy level (Fig. 2).

Among the four *Pinus* species tested, *Pinus pinea* had a significantly higher nuclear DNA content (Table 2).

Discussion

The endosperm of gymnosperm seeds is considered to be a haploid tissue that originates from one megaspore.

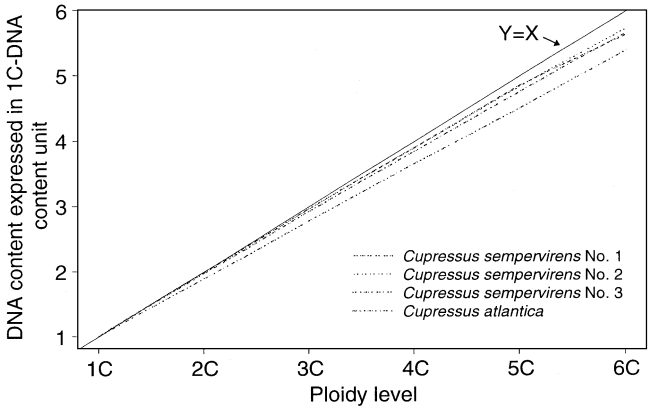

Fig. 2 Relationship between ploidy level and cytometric estimation of DNA content

All of the endosperm samples from the Pinaceae family (*Pinus*, *Cedrus* and *Abies*) that we studied had the expected haploid nuclear status. In *Pinus*, similar results were obtained by Wakamiya et al. (1993) for 18 North American *Pinus* species and *P. eldarica*, and by Bino et al. (1993) for *P. nigra*. The larger DNA amount of *P. pinea* can be related to the significant correlation between seed size and DNA content for the *Pinus* species studied by Walkamiya et al. (1993). To our knowledge, no results have been published on the seed ploidy levels of *Abies* and *Cedrus* species.

Our results reveal that the endosperm of all Cupressaceae species tested contains nuclei with various DNA contents corresponding to several ploidy levels. As expected, only one 2C ploidy level was observed for embryos. On the basis of their endosperm nuclear status, Cupressaceae species can be classified in two groups: species with two ploidy levels (1C and 2C for *Cupressus arizonica*, *Thuja orientalis* and *Juniperus oxycedrus*) and species with numerous ploidy levels (1C–6C for *C. sempervirens* and *C. atlantica*).

The origin of these multiple levels of ploidy in the Cupressaceae endosperm must be clarified considering that classically, in gymnosperms, the megagametophyte derives from one haploid cell (megaspore) and that, as compared to angiosperms, only one fertilisation occurs.

One interpretation could be that spontaneous endoreduplication or endopolyploidie as defined by Nagl (1978), frequently occurs in nutrient-storing tissues such as albumen or the cotyledons of angiosperms, which consequently increases their transcription and translation activities during storage-reserve elaboration (Nagl 1978; Queller 1983; Kowles et al. 1992). Moreover, natural polyploidisation is often observed in haploid tissues (Michaux-Ferrière and Soulié-Märsche 1987). In gymnosperms, endoreduplication has been described only for *Ginkgo biloba* (Avanzi and Cionini 1971) where cytophotometric observations (Feulgen method) revealed 1C to 64C ploidy levels.

Endoreduplication consists in doubling DNA content (Kowles et al. 1992) and thus should produce, from a haploid tissue, only 2C–4C–8C–16C . . . nuclei. Consequently, the ploidy levels that we oberved in the *C. sempervirens* and *C. atlantica* endosperm (1C–2C–3C–4C–5C–6C) cannot be due to simple endoreduplication. This particular process of 1C DNA increment at each step, could be due to endomitoses (Nagl 1995) followed by multiple nuclei fusions. This second possible explanation of endosperm polyploidy would fit with Schnarf's (1930 in Avanzi and Cionini 1971) and Ozenda's (1982) proposals and seems to be in agreement with our current cytological observations (unpublished results) which reveals that some developing *C. sempervirens* endosperm cells contained several free or fused nuclei, and sometimes fusing. In *Chamaecyparis nootkanensis*, Owens and Molder (1974) observed that during late female gametophyte differentiation the cells commonly become multinucleate.

Endoreduplication in the nutrient-storing tissues of the seed is classically considered to be an angiosperm feature (Friedman 1992). We therefore suggest that *C. sempervirens* and *C. atlantica* are evoluted gymnosperm species. Nevertheless, the processes leading to DNA increase seems to be quite different: merging of nuclei in *Cupressus vs* endoreduplication in angiosperms.

Our results confirm the conclusion derived from the isozyme analysis of *C. sempervirens* endosperm (Raddi et al. 1990; Papageorgiou et al. 1993), which suggested a non-haploid tissue but this tissue is not the diploid perisperm. The diploid heterozygote zymograms observed by these authors cannot be explained without there being a contribution of two different alleles. Considering that pollen nuclear DNA does not contribute to endosperm DNA (only one fertilization occurs in Gymnosperms), these alleles could derive from two (or more) megaspores (1C) from one or multiple meiosis, or from a mixture of one (or more) unreduced megasporocyste (2C) and one (or more) megaspores (1C). The present study does not allow us to test these two hypotheses.

The nuclear status of the endosperm in Cupressaceae is not always in accordance with what is expected for Gymnosperm species. Consequently, in this botanical family, knowledge of the genotypic origin of phenotype constitutes a prerequisite for genetic marker studies based on the endosperm. If the endosperm originates from several cells, the respective contributions of the different alleles must be considered with particular attention. Further investigations are needed to understand the cytological basis of endosperm development, particularly in Cypress.

Acknowledgements This work has been supported by the European Community in the framework of the ≪AIR-Cypress Program≫ coordinated by E. Teissier du Cros. We thank B. Fady and N. Michaux-Ferriere for critically reading the manuscript.

References

Avanzi S, Cionini PJ (1971) A DNA cytometric investigation on the development of the female gametophyte of *Ginkgo biloba*. Caryologia 24 : 105–116

Barrett JW, Knowles P, Cheliak WM (1987) The mating system in a black spruce clonal seed orchard. Can J For Res 17 : 379–382

Bergmann F (1991) Isozyme gene markers. In: Muller-Starck G, Ziehe M (eds) Genetic variation in European populations of forest trees. Sauerlander's Verlag, Frankfurt am Main, pp 67–78

Bino RJ, Lanteri S, Verhoeven HA, Kraak HL (1993) Flow cytometric determination of nuclear replication stages in seed tissues. Ann Bot 72 : 181–187

Friedman WE (1992) Evidence of a pre-angiosperm origin of endosperm: implication for the evolution of flowering plants. Science 255 : 336–338

Gerber S, Rodolphe F, Bahrman N, Baradat Ph (1993) Seed-protein variation in maritime pine (*Pinus pinaster* Ait) revealed by two-dimensionnal electrophoresis: genetic determinism and construction of a linkage map. Theor Appl Genet 85 : 521–528

Gillet EM (1996) Qualitative inheritance analysis of isoenzymes in haploid gametophytes: principles and computerized method. Silvae Genet 45 : 8–16

Kowles RV, Yerk GL, Srienc F, Phillips RL (1992) Maize endosperm tissue as an endoreduplication system. In: Setlow JK (ed) Genetic engineering, vol 14: Plenum Press, New York, pp 65–88

Michaux-Ferrière N, Soulié-Märsche I (1987) The quantities of DNA in the vegetative nuclei of *Chara vulgaris* and *Tolypella glomerata* (Charophyta). Phycologia 26 : 435–442

Mitton JB (1983) Conifers. In: Tanksley SD, Orton TJ (eds) Isozymes in plant breeding and genetic. Elsevier, Amsterdam, pp 443–472

Morris RW, Spieth PT (1978) Sampling strategies for using female gametophytes to estimate heterozygosity in conifers. Theor Appl Genet 51 : 217–222

Nagl W (1978) Endopolyploidy and polyteny in differentiation and evolution. Towards an understanding of quantitative and qualitative variation of nuclear DNA in ontogeny and phylogeny. Elsevier, North-Holland Publ, Amsterdam

Nagl W (1995) Cdc2-kinases, cyclins and the switch from proliferation to polyploidization. Protoplasma 188 : 143–150

O'Malley DM, Kelly JM (1988) Segregation of a megagametophyte color polymorphism in *Gingko biloba* L. J Hered 79 : 51–53

O'Malley DM, Allendorf FW, Blake GM (1979) Inheritance of isozyme variation and heterozygosity in *Pinus ponderosa*. Biochem Genet 17 : 233–250

O'Malley DM, Guries RP, Wheeler NC (1988) Electrophoretic evidence for mosaic 'diploids' in megagametophytes of knobcone pine (*Pinus attenuata* Lemm.). Silvae Genet 37 : 85–88

Owens JN, Molder M (1974) Pollination, female gametophyte and seed development of yellow cedar (*Chamaecyparis nootkanensis*) Can J Bot 53 : 186–189

Ozenda P (1982) Les gymnospermes. In: Gaussen H, Leroy JF, Ozenda P (eds) Précis de botanique, vol 2: végétaux supérieurs. Masson, Paris, pp 1–120

Papageorgiou AC, Bergmann F, Gillet E, Hattemer HH (1993) Genetic analysis of isoenzyme variation in mediterranean cypress (*Cupressus sempervirens* L.). Silvae Genet 42 : 109–111

Pascual L, Garcia FJ, Perfectti F (1993) Inheritance of isozyme variations in seed tissues of *Abies pinsapo* Boiss. Silvae Genet 42 : 335–340

Plomion C, Bahrman N, Durel CE, O'Malley DM (1995) Genomic mapping in *Pinus pinaster* (maritime pine) using RAPD and protein markers. Heredity 74 : 661–668

Queller DC (1983) Kin selection and conflict in seed maturation. J Theor Biol 100 : 153–172

Raddi S, Di Lonardo V, Sufra M (1990) The use of biochemical markers to distinguish cypress clones. In: Ponchet J (ed) Progress in EEC research on cypress diseases. Commission of the European Communities Report EUR 12493, pp 43–49

Shaw D, Allard RW (1982) Estimation of outcrossing rates in Douglas-fir using isozyme markers. Theor Appl Genet 62 : 113–120

Strauss SH, Conkle MT (1986) Segregation, linkage, and diversity of allozymes in knobcone pine. Theor Appl Genet 72 : 483–493

Tulsieram LK, Glaubitz JC, Kiss G, Carlson JE (1992) Single tree genetic linkage mapping in conifers using haploid DNA from megagametophytes. Bio/Technology 10 : 686–690

Wakamiya I, Newton RJ, Johnston JS, Price HJ (1993) Genome size and environmental factors in the Genus Pinus. Am J Bot 80 : 1235–1241