

Endosperm formation in aposporous *Crataegus* (Rosaceae, Spiraeoideae, tribe Pyreae): parallels to Ranunculaceae and Poaceae

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Summary

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- Apomixis in *Crataegus* is primarily aposporous and requires pollination. The embryo sac is of the *Polygonum* type. A combination of meiotically unreduced embryo sacs with apparently reduced pollen would violate the usual requirement for a 2 : 1 ratio of maternal to paternal contributions to the endosperm. We therefore investigated the origin of endosperm in seeds of sexual diploids and apomictic polyploids of the sister genera *Crataegus* and *Mespilus*.
- Flow-cytometric DNA measurements from embryo and endosperm in mature seeds were converted to ploidy levels using leaf-tissue information.
- The diploids had triploid endosperm. In c. 60% of seed from polyploids, one sperm apparently contributes to the endosperm, while 25% or more may involve two sperm. Additional results suggest that trinucleate central cells also occur. Fertilization of meiotically unreduced eggs is indicated.
- The ratio of maternal to paternal contributions to the endosperm in these apomictic *Crataegus* is not constrained to 2 : 1. They thus resemble some *Sorbus* (Pyreae) and very distantly related *Ranunculus* (Ranunculaceae). It is suggested that *Paspalum* (Poaceae) may have similarly flexible endosperm ploidy levels.

Key words: apomixis, apospory, *Crataegus* (hawthorn), endosperm balance requirement, Maloideae, *Paspalum*, Pyrinae, *Ranunculus*.

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Introduction

Although angiosperm apomixis – asexual seed production – can reduce genetic variation in the short term, plants with pseudogamous gametophytic apomixis (including many *Crataegus* L.) require pollination and have long been of interest to plant breeders because of their tendency to produce seedlings with new ploidy levels (Clausen, 1961). Because apomixis is apparently always facultative (Nogler, 1984), the sexual pathway remains viable: embryos with a higher chromosome number can result from fertilization of a meiotically unreduced egg cell, and parthenogenesis can produce embryos with half the chromosome number of the mother plant. In the Rosaceae subfamily Spiraeoideae (Juss.) Arn., the potential for ploidy-

level increase is of particular interest in tribe Pyreae Baill., in the pome-bearing subtribe Pyrinae Dumort. (traditionally referred to as subfamily Maloideae C. Weber; Campbell *et al.*, 2006) where apomictic tetraploids are quite common, but pentaploids and hexaploids are rare, and higher polyploids have not been recorded (Kalkman, 2004; Talent & Dickinson, 2005). This is in contrast to some apomictic clades in subfamily Rosoideae (Juss.) Arn., where 28-, 16-, and 14-ploid plants have been recorded in *Alchemilla* L. *sensu lato*, *Potentilla* L. *sensu lato*, and *Rubus* L., respectively (Kalkman, 2004).

The endosperm tissue of the seed is commonly triploid in sexual diploid angiosperms, including the Rosaceae, because highly diversified clades in both monocots and eudicots have the *Polygonum* type of embryo sac, where the endosperm

forms from fertilization of the binucleate central cell (Maheshwari, 1950). Meiotically unreduced apomictic embryo sacs in Rosaceae generally also have the *Polygonum*-type morphology (Nogler, 1984; Czapik, 1996), but pollen meiosis is largely normal, so the usual requirement for a strict 2 : 1 ratio of maternal to paternal contributions to the endosperm tissue would be violated unless there were a genetic or developmental alteration (Quarin, 1999; Vinkenoog & Scott, 2001). One possible solution to this endosperm-balance problem of pseudogamous gametophytic apomixis is that both sperm contribute to the endosperm tissue (Nogler, 1984; Savidan, 2000), and in that case, ploidy-level increase through fertilization of the egg cell cannot occur. Other solutions to the endosperm balance problem that do not sequester both sperm have been observed or suggested (Nogler, 1984): meiotically unreduced pollen, noninvolvement of one of the central cell nuclei in endosperm formation, autonomous endosperm development without fertilization, or a genetic alteration to the endosperm-balance requirement. In those plants in which the endosperm persists in the mature seed, such as the Rosaceae (Péchoutre, 1902; Corner, 1976; Aldasoro *et al.*, 2005), the ploidy levels of the embryo and endosperm can indicate the fate of the two sperm (Matzk *et al.*, 2000; Nogler, 1984). For species with low seed set, such as many apomictic *Crataegus*, such information from mature seed is potentially of greater value than observations of early embryology, but no reports of the endosperm ploidy levels in mature seed of apomictic Pyrinae have, to our knowledge, been published.

In some apomictic Rosaceae (including *Amelanchier* Medik. and *Cotoneaster* Medik. in Pyrinae), the central cell nuclei do not fuse before fertilization, and this behaviour contrasts with the fusion of the nuclei in diploid sexual relatives (Hjelmqvist, 1962; Czapik, 1983, 1985a, 1985b; Campbell *et al.*, 1985, 1987). Thus it appeared that some apomictic Pyrinae achieve a 2 : 1 ratio of maternal to paternal contributions in the endosperm by matching one unreduced central-cell nucleus with one reduced sperm. Endosperm formation in *Crataegus* was unknown, however, because nuclear fusion before fertilization apparently did not occur in either apomictic polyploid or sexual diploid species (Muniyamma & Phipps, 1979a; Muniyamma & Phipps, 1985). In a triploid apomictic *Sorbus* (Pyrinae; Jankun & Kovanda, 1987), fertilization of partly fused central cell nuclei has been observed, and chromosome counts from the early endosperm of a diploid apomictic *Sorbus* were also consistent with two central cell nuclei and either one or two sperm (some ovules had 5x chromosome counts and others 6x; Jankun & Kovanda, 1988). Variation in the number of sperm that combine with the central cell has also been noted in apomictic *Ranunculus auricomus* L. (Ranunculaceae) where variation relates to the maternal genotype (Rutishauser, 1954; Nogler, 1984), but the relationship of the different endosperm ploidy levels to the viability of the seed has not been established as far as we are aware.

If apomictic *Crataegus* follows the *Sorbu/Ranunculus* pattern, then one sperm may sometimes be available to fertilize the egg cell, and sexual and apomictic seeds would differ in the ploidy level of the endosperm (i.e. in sexual seeds of tetraploids the endosperm should be 6x, contrasting with 10x or 12x in apomictic seeds). However, if the two central cell nuclei do not fuse either before or after fertilization, then cytometry of seeds could not be a useful tool for surveying the breeding system (because the endosperm of both sexual and apomictic tetraploids would be 6x). Two subcases of this latter scenario would have different implications for the potential for the egg cell to be fertilized: (i) if two separate fertilization events result in a composite endosperm tissue, then the embryo could not be fertilized; or (ii) if the second central-cell nucleus and the second sperm are not involved in formation of the endosperm, then the second sperm might be able to contribute to the embryo.

Although the diploids *Crataegus punctata* and *C. monogyna* have been repeatedly seen to be self-incompatible (Love & Feigen, 1978; Donovan, 1981; Celotti, 1995), a dissenting statement (Yeboah Gyan & Woodell, 1987) is that *C. monogyna* flowers form fruit when enclosed before anthesis in mesh bags (the rate of seed formation was not stated). A possible explanation for such experimental results is that some diploid individuals have autonomous apomixis, which does not require fertilization of the endosperm, and this phenomenon might also explain the surprising fertility of some pollen-sterile triploid North American *Crataegus* (Dickinson, 1983). Although in some Asteraceae the highly variable processes within embryo sacs have seemed to indicate that autonomous endosperm development from the antipodals or from the nucellus is possible, in *Hieracium* there is now good evidence that autonomous endosperm develops only from the central cell (Tucker *et al.*, 2003). Thus, if autonomous endosperm occurs in *Crataegus*, the ploidy level is most likely equivalent to either one or two unreduced nuclei, and less likely to be some other multiple of the somatic-cell ploidy level.

Crataegus and *Mespilus* form a clade within the Pyrinae, but there is little molecular resolution between these two genera (Campbell *et al.*, in press; Evans, 1999; Lo *et al.*, in press; Potter *et al.*, in press). *Mespilus* comprises only two species, a diploid and a triploid (Talent & Dickinson, 2005), but *Crataegus* probably consists of approx. 140 species worldwide (Phipps *et al.*, 2003). *Crataegus* is almost exclusively diploid, triploid, or tetraploid, apparently with rare pentaploids, and with one recorded hexaploid (Talent & Dickinson, 2005). Morphological taxonomy of *Crataegus* suggests that transitions between ploidy levels have occurred within many of the taxonomic series in the genus, and within some of the morphological species. We have speculated (Talent & Dickinson, 2005) that either the ploidy level is slowly increasing or, alternatively, the ploidy levels are almost exclusively locked into a diploid–triploid–tetraploid network.

Three questions for this study are as follows:

- Do open-pollinated diploids have triploid endosperm, or is some unusual origin of this tissue indicated that might predispose the genus to the evolution of apomixis or polyploidy?
- Can pollen-sterile triploids and isolated self-incompatible diploids produce seeds consistent with autonomous apomixis?
- Do the ploidy levels of the embryos and endosperm of mature seeds from apomictic triploids and tetraploids indicate that unreduced egg cells can be fertilized, or rather that they invariably develop by parthenogenesis?

Flow cytometry was our method of choice for ploidy-level determinations because of the relative ease with which large surveys of breeding systems could be conducted if the data proved to be informative.

Materials and Methods

Plant material

The ploidy levels of all the mother trees studied here were established previously (Talent & Dickinson, 2005). The diploid samples (Table S1) include four species that are frequently encountered in our fieldwork in Ontario (introduced *C. monogyna*, and native *C. punctata*), in the Rocky Mountains (*C. saligna*), and America's Pacific Northwest (*C. suksdorfii*). *C. brachyacantha* (Louisiana) and *Mespilus germanica* (Caucasus and northern Turkey) were included because of their apparent phylogenetic propinquity (Lo *et al.*, in press) and because seed samples were made available by C. S. Reid (Louisiana Department of Wildlife and Fisheries, LA, USA) and the Morton Arboretum, respectively. The triploid samples (Table S1) comprise individuals of *C. crus-galli sensu lato*, *C. succulenta*, and *C. pruinosa sensu lato*, taxa that have been shown to include triploid as well as tetraploid individuals, and *C. ?grandis*, which may be exclusively triploid (Muniyamma & Phipps, 1979b; Dickinson & Phipps, 1986; Talent & Dickinson, 2005). Tetraploid samples from Ontario (Table S1) belong to *C. crus-galli sensu lato*, *C. macracantha*, *C. submollis*, and *C. douglasii*, all species that are common and readily identifiable in the province. The Ontario *C. douglasii* were compared with the same species from the Rocky Mountains and Pacific Northwest as well as possible relatives and/or sympatric species that are tetraploid (Table S1): *C. okennonii*, *C. castlegarensis*, *C. rivularis*, and *C. chrysocarpa*.

The source trees are labeled with aluminum tags, and voucher specimens are filed in the Green Plant Herbarium of the Royal Ontario Museum (TRT) with the exception of trees ELA1 through ELA9, black-fruited *C. douglasii* (the only black-fruited hawthorn in Ontario), which were collected only as fruit material for flow cytometry. Where possible, we have collected both spring and autumn vouchers because these are needed for accurate identification of most *Crataegus* species.

Most of the seeds analyzed were open-pollinated. We also carried out tests for the autonomous production of seeds by male-sterile triploids, and for autogamy and self-fertility in

pollen-fertile diploids and tetraploids. These experiments, which involved preparing inflorescences as if for pollination, with or without manual transfer of self pollen, also contributed seeds to the study reported here.

Pollination experiments did not involve emasculation because it appears likely that a resulting hormonal disturbance could affect development of the flower and/or pollen-tube behaviour (Celotti, 1995; Greyson & Tepfer, 1967; Greyson & Raman, 1975; Williams & Knox, 1982; Dickinson & Phipps, 1986). Pollination was done at the 'popcorn stage', when petals are separating but still closed, which is *c.* 12 h before anthesis. Petals were removed from the 10 most mature buds on the inflorescence, pollen was applied to the stigmas using the eraser end of a new pencil, and then all unpollinated buds from the inflorescence were removed before enclosing it in a mesh bag. Pollen for hand-pollinations was obtained as follows: anthers from unopened flowers at or 1–2 d before the popcorn stage were removed and gently dried at *c.* 30°C for 2 h, then transferred to a small vial and frozen. The vial was warmed to ambient temperature, and then shaken to release the pollen from the anthers onto the walls of the vial, whence it was easily transferred to the pencil eraser.

All harvested fruit appeared to be full-sized, though not necessarily ripe, and the embryo was clearly visible in the seed. With the exception of an open-pollinated collection from tree D668 (Table S1), all of the harvested seeds were not old enough to appear dry. In section *Crus-galli* (*C. punctata* and *C. crus-galli*), the embryo develops very late, and harvest was delayed until the middle of July or later to ensure embryo development.

Flow cytometry

Flow cytometry of *Crataegus* seeds was based on our experience with leaf tissue (Talent & Dickinson, 2005). The flow cytometer, located in the University of Toronto Faculty of Medicine, is a Becton-Dickinson machine from BD Biosciences, model FacsCalibur, with a red diode laser for detecting propidium iodide DNA stain. The dimensions of the laser beam, at 15 × 61 µm (BD Biosciences, 2005), are large relative to the plant nuclei, at 7–10 µm. With these relatively tiny nuclei, the fluorescence intensity (height) measurement is appropriate to indicate the total DNA content in a single nucleus, although the width or area measurements could misrepresent aggregates as polyploid nuclei (Ormerod, 1994). The side-scatter measurement plotted against fluorescence intensity (height) has proved to be useful (Matzk *et al.*, 2000) in order to reject the signals from particles other than intact nuclei, and doublet signals (nuclei that are stuck together) were removed using a plot of height against width, as is normal practice (Ormerod, 1994). Histograms of the fluorescence height of the selected particles are then used to select subpopulations for statistical analysis.

Following Burton & Husband (1999), two buffers were used for flow cytometry, as for *Crataegus* leaf tissue (Talent & Dickinson, 2005): a chopping buffer, buffer A, from Bino

et al. (1992), and a staining buffer, buffer B, from Arumuganathan & Earle (1991). Buffer A contains 15 mM HEPES, 1 mM EDTA, 80 mM potassium chloride, 20 mM sodium chloride, 300 mM sucrose, 0.2% w/v Triton X-100, 0.1% β -mercaptoethanol and 0.5 mM spermine. Stain-free buffer B contains 10 mM magnesium sulfate heptahydrate, 1 mg ml⁻¹ dithiothreitol, 10% w/v Triton X-100 and 0.24% DNAase-free RNAase. To make buffer B with stain for one sample, 5 μ l of 2% propidium iodide stock solution (5 mg ml⁻¹) was added to 175 μ l of the stain-free buffer. However, because of solubility problems in the propidium iodide stock solution, we routinely add twice as much of the stain solution.

For preparation of the *Pisum* DNA standard (Burton & Husband, 1999; Talent & Dickinson, 2005), approx. 0.5 cm² of leaf tissue was chopped with a razor blade in 0.5 ml of buffer A over crushed ice. Then 1.0 ml of buffer A was added before filtering first through coarse fabric of *c.* 200 μ m mesh, then through 37 μ m mesh, and finally through 15 μ m mesh. The filtrate was centrifuged for 20 s at approx. 7600 *g* (13 000 rpm in a Hermle benchtop centrifuge), and the pellet resuspended in 170 μ l of staining buffer.

Preparation of seed tissues requires more care than does that of *Crataegus* or *Pisum* leaf, to remove a viscous precipitate. The volume of chopping buffer was increased to reduce the viscosity that can destroy the nuclei during filtering, and centrifuging times were increased (as for *Crataegus* leaf tissue). A whole or partial seed was chopped in 0.5 ml of chopping buffer, then 2.5 ml of chopping buffer was added before filtering through 37 μ m mesh. The filtrate was centrifuged at approx. 7600 *g* for 4 min, and the pellet was resuspended in 170 μ l of staining buffer. The resuspension was allowed to stand on ice for 20–30 min, after which the supernatant was used for flow cytometry and any precipitate was discarded.

Seed calculations

The seeds come from species that apparently vary slightly in their basic DNA amounts, but the ploidy levels of leaf tissue are readily distinguished in the following ranges: diploid, 1.37–1.67 pg DNA; triploid, 2.05–2.51 pg; tetraploid, 2.74–3.34 pg; pentaploid, 3.42–4.18 pg (Talent & Dickinson, 2005). Extending this estimate to the hexaploid range (4.10–5.02 pg) results in a slight overlap but still permits most embryo ploidy levels to be distinguished. The initial calculation of the DNA amounts in each seed was relative to a separate preparation of *Pisum sativum*, a well-established standard with a DNA amount close to the somatic tissue values established for *Crataegus* (Dickson *et al.*, 1992; Talent & Dickinson, 2005), but sufficiently different to permit clear differentiation from the unknown, for ploidy levels up to *c.* 10x. A *Pisum* standard can be leaf tissue from any cultivar of garden pea (Greilhuber & Ebert, 1994; Bennett & Leitch, 1995; Price & Johnston, 1996; Temsch & Greilhuber, 2001).

The variance of the fluorescence from particles observed by the cytometer is sensitive to how well the preparation is flowing through the machine and will increase if the machine is partly clogged. The mean of the measurements may also be affected. There is no independent measure of obstruction in the machine (Ormerod, 1994), but the variance is a useful indicator that can be used to exclude poor preparations. We used the standard deviation calculated according to the method contributed by Warren Lamboy (Dickson *et al.*, 1992), which takes into account the distributions of both the *Crataegus* signal and the *Pisum* standard. Where subscripts 1 and 2 refer to the sample and the standard, respectively,

$$\text{adjusted variance, } \sigma^2 = \sigma_1^2/\mu_2^2 + \sigma_2^2 \times \mu_1^2/\mu_2^4$$

$$\text{adjusted mean, } \mu = 8.84 \times \mu_1/\mu_2$$

If the ratio σ^2/μ was greater than 15% for any measurement (embryo or endosperm), then that measurement was excluded (a total of four seeds, one each from *C. ?grandis*, *C. crus-galli*, *C. macracantha*, and *C. submollis*).

The seeds were initially dissected into separate endosperm and embryo preparations (the color and texture of the different components are distinct: the embryo is opaque white, and the endosperm translucent). Because the ploidy levels of the endosperm might vary by a small proportion (by the contribution of a single sperm nucleus), it was essential to calibrate the signals as precisely as possible, preferably against a standard subjected to identical staining conditions. Therefore, after initially testing the method, we prepared the embryo, endosperm, and seed coat together for many of the seeds and used the embryo as an internal standard for the endosperm, in combination with a separate check that the ploidy level of the embryo, calculated using the external *Pisum* standard, was well within a euploid range.

The ploidy level of the embryo was used to adjust the endosperm measurement from the same seed as follows:

$$\text{Endosperm}_{\text{adjusted}} = \frac{\text{Endosperm}_{\text{measured}} \times \text{DNA}_{\text{midpoint}}}{\text{Embryo}_{\text{measured}}}$$

where the DNA midpoints are 1.52 pg for diploids, 2.28 pg for triploids, 3.04 pg for tetraploids, 3.80 pg for pentaploids, and 4.56 pg for hexaploids. The adjusted endosperm measurements were then used to assess the ploidy level of the tissue and whether fertilization had occurred.

Results

Crataegus and *Mespilus* seeds have a thick endosperm (15 cell layers), but largely crushed tegmen and testa, with two cell layers remaining (Corner, 1976). The seed coat is firmly attached to the endosperm tissues, and the endosperm is separated only with considerable damage, whereas the embryo

separates readily from the surrounding tissues (the endosperm and seed coat). Thus, it was of practical interest to discover whether maternal contamination from the seed coat is present in flow cytometric preparations of endosperm with the seed coat. Preparations of the integuments from young seed of (diploid) *C. punctata* produced diploid signals, and from (tetraploid) *C. submollis* produced tetraploid signals, but preparations from the mature seed coat were not successful. This lack of signal is probably related as much to damage to nuclei because of the texture of the tissue as to the small number of intact cells.

Seeds from diploids have triploid endosperm

By separately dissecting and analyzing the embryo and the remaining tissues (endosperm and seed coat) from diploid mother plants, it was established that the two preparations have different DNA amounts that appear to be, respectively, diploid and triploid (Fig. 1). No diploid maternal signal could be distinguished in the second preparation (endosperm and seed coat). Although there are 'echoes' at multiples of the main

fluorescence peak, these do not prevent distinguishing the two main signals (diploid and triploid) when an entire seed is prepared as a single sample (Fig. 1c). The echoes do not represent particles stuck together (Ormerod, 1994), which can be established by considering the size of the particles relative to the diameter of the laser beam (as discussed in the Materials and Methods section). The secondary peak at twice the base DNA amount would correspond either to cells arrested in the G2 phase of the cell cycle or alternatively to some endopolyploid cells, as Bino *et al.* (1993) found in seeds of other taxa. The peak at three times the base signal and the probable peak at four times (Fig. 1) definitely suggest endopolyploidy. Neither of these higher signals could be localized to any particular section of the embryo, but were equally apparent in the radicle and in the tips and central portion of the cotyledons when these components were prepared separately. Thus, we conclude that the embryo preparation and the endosperm preparation with the seed coat each represents cells of a single base ploidy level, plus some endopolyploid cells.

In order to estimate the ratio of DNA in embryo and endosperm better, a sample of 87 open-pollinated seeds from

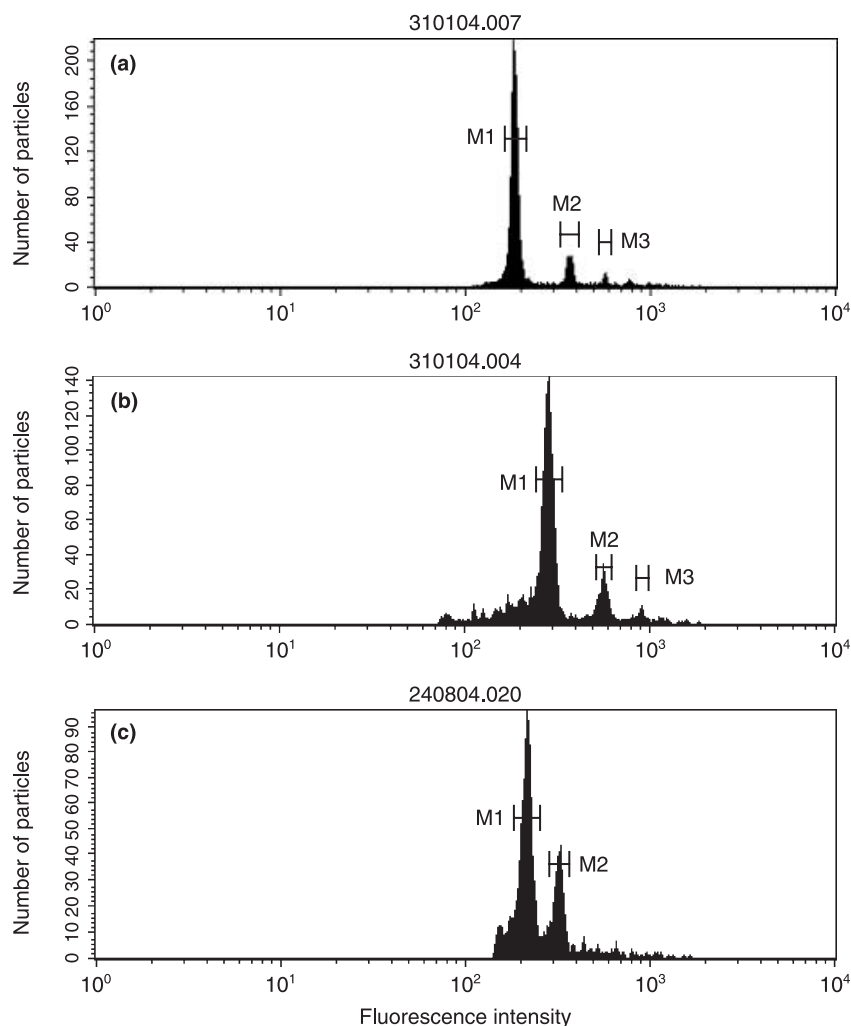


Fig. 1 Cytometry of seeds from diploids. A fresh seed can easily be dissected to separate the embryo from the remainder (the endosperm and seed coat), and three to four fluorescence peaks can appear in the signal, with the higher peaks occurring at multiples of the main peak (2 \times , 3 \times , etc.). (a) A separately prepared embryo from an open-pollinated seed from diploid *Crataegus punctata*, tree NT42G; the three major fluorescence peaks (labelled M1, M2, M3) correspond to ploidy levels 2 \times , 4 \times , and 6 \times . (b) In the remaining tissues from the same seed, the three major fluorescence peaks (M1, M2, M3) correspond to ploidy levels 3 \times , 6 \times , 9 \times ; no maternal signal from the seed coat (expected to be 2 \times or a multiple thereof) is evident. (c) In an entire open-pollinated seed from *C. brachyacantha*, tree OUAC 1-2, the two major fluorescence peaks (M1, M2) correspond to ploidy levels 2 \times and 3 \times and are interpreted as embryo and endosperm signals, respectively.

four diploid North American *Crataegus* species and one diploid European species from each of *Crataegus* and *Mespilus* were compared (Figs 2, 3). Of these seeds, 84 produced both of the apparently diploid and triploid signals; two produced only a diploid signal; and in one seed the second signal spanned the triploid and tetraploid ranges. These three seeds were interpreted as preparation failures and discarded. The embryo measurements vary slightly according to species (Fig. 3), and the variation is largely consistent with the leaf measurements from the mother plants. *C. brachyacantha* registers more DNA than the other species (Talent & Dickinson, 2005) and *M. germanica* and *C. punctata* tend towards high and low measurements, respectively. A discrepancy between the measurement from the mother and seeds from *C. monogyna* tree NT116 may be caused by a lack of replication of the measurement from the mother, or alternately to DNA variation within the species. The two seeds from *C. saligna* (tree D2004-08) are consistent with one another and with the mother tree, which suggests that this individual has a heritable high DNA

content, but three other individuals of this species had DNA measurements within the diploid estimate, at the high end of the range (Talent & Dickinson, 2005).

The endosperm measurements were standardized using the embryo of the same seed, as described in the Materials and Methods section (Fig. 2). Neither the mean nor the median of the endosperm measurements from the six species justifies rejecting the hypothesis that the standardized DNA amount in the endosperm of diploid seeds is 2.28 pg DNA. ($\bar{X} = 2.293$, $t_{0.05(2),83} = 1.837$, $P(2.280 \leq \mu \leq 2.306 \text{ pg}) = 0.95$, but because the distribution is skewed to the right (median $M_{\text{sample}} = 2.289$), a more appropriate test is comparison to a binomial distribution with $P = 0.5$ (Zar, 1999), which gives $P(2.271 \leq \text{median}_{\text{population}} \leq 2.300) \geq 0.95$). Thus, the DNA amount in the endosperm is 1.5 times the DNA amount in the embryo, and there was no effect, from partial DNA replication, or from other stain-absorbing chemicals in the seed, to prevent consistent measurement of triploid endosperm in seeds from these diploids.

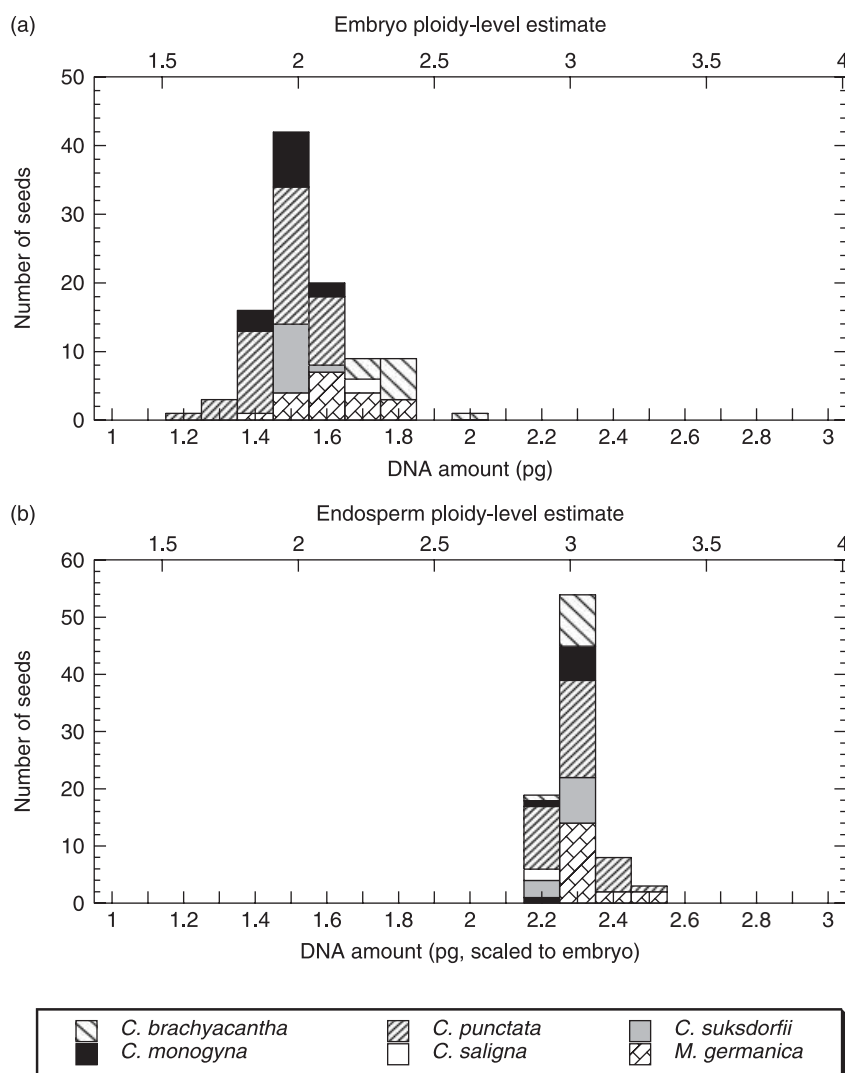
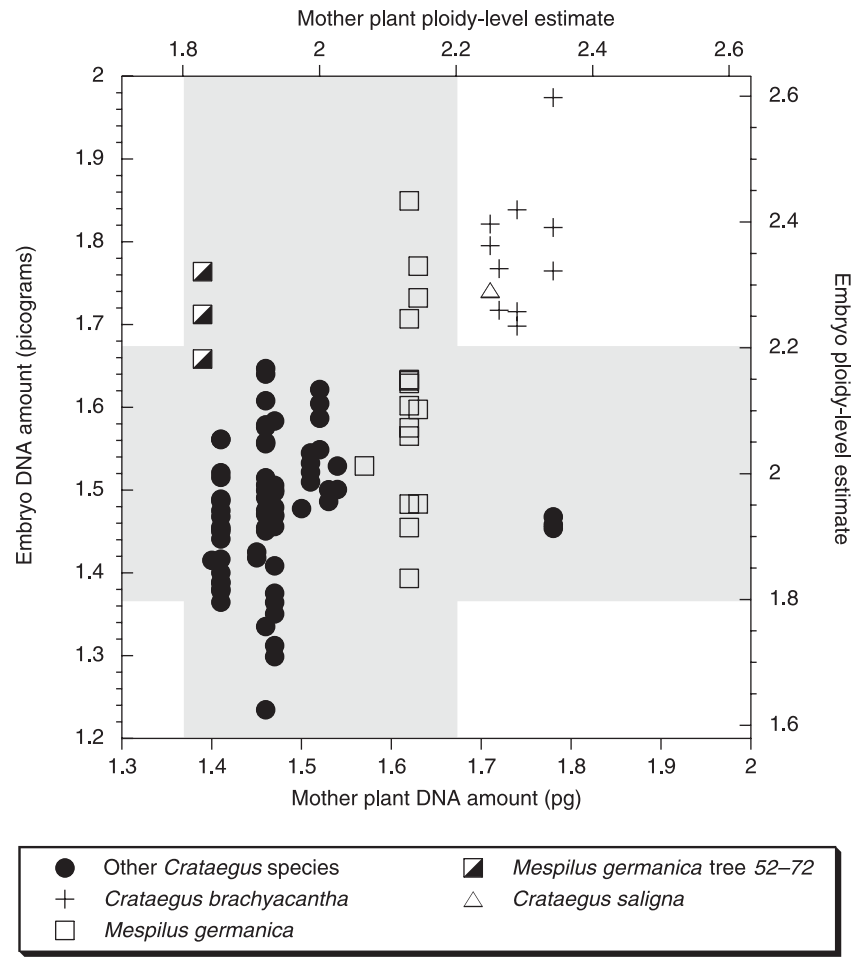


Fig. 2 Nuclear DNA measurements from seeds of various diploid and near-diploid species. Nuclear DNA measurements from the embryo and endosperm were compared from 84 open-pollinated seeds from six diploid species of *Crataegus* and *Mespilus*. (a) Embryo DNA measurements (unaltered). (b) The endosperm measurements from the same seeds were adjusted to standardize the embryo DNA amount to 1.52 pg, the midpoint approximation previously established for diploid *Crataegus* and *Mespilus*.

Fig. 3 DNA measurements from seed embryos are compared with their diploid and near-diploid mothers. The ploidy levels of the embryos from six diploid species of *Crataegus* and *Mespilus* (Fig. 2) were compared with their mother plants and with the previously established DNA estimate for diploids of most *Crataegus* and *Mespilus* species (range 1.368–1.672 pg, ranges shown by shading; Talent & Dickinson, 2005). *Crataegus brachyacantha* had a higher DNA amount than the other species, and measurements from *Mespilus germanica* were variable. Other high measurements represent one tree each of *C. monogyna* and *C. saligna*, each with two seeds.



Autogamy and autonomy

We tested for autogamy and/or autonomous apomixis under the conditions that would prevail in pollination experiments without emasculation (Yeboah Gyan & Woodell, 1987; Macklin, 2001), that is, each inflorescence was enclosed in a mesh bag that excludes insects (with the possible exception of

thrips; Kearns & Inouye, 1993). It appears likely that the conditions within the bags could contribute to pollen transfer on pollen-fertile species (M. Purich, pers. comm.), even without any of the special modifications of flower structure that effect self-pollination in other taxa. Isolated flowers from most of the diploid and tetraploid species tested set a few seeds (Table 1). Diploid *C. monogyna* and *C. punctata* have often

Table 1 Seeds set on isolated, unemasculated, flowers of diploids and tetraploids

Taxon	Number of trees	Number of flowers	Number of locules (estimated) ^a	Number of fruit	Number of seed
Diploids					
<i>Crataegus monogyna</i>	4	193	196	2	3 ^b
<i>C. punctata</i>	3	138	442	7	6
Tetraploids					
<i>C. crus-galli</i>	3	163	333	16	8
<i>C. macracantha</i>	8	363	865	81	43
<i>C. submollis</i>	1	80	373	0	–

^aThe number of pistils (locules) in the flowers was estimated using the average number of pyrenes in mature open-pollinated fruit from the same trees (between 50 and 100 fruit per tree). Although two ovules occur in each locule, almost invariably at most one seed develops; the obturator of the upper ovule is not associated with the micropyle (Celotti, 1995; Evans & Dickinson, 2005), which apparently prevents fertilization.

^bThis sample included a fruit that is an unusual for this species, with two pyrenes and two seeds.

been reported to be self-incompatible (Donovan, 1981; Dickinson, 1983; Dickinson & Phipps, 1986; Celotti, 1995; Purich, 2005), and in this experiment they converted only 2% of the flowers into fruit, with at most one seed in each fruit. The endosperm ploidy levels from nine of those seeds for which results were obtained (six from *C. punctata* and three from *C. monogyna*) were triploid, consistent with fertilization by self pollen.

Of the two male-sterile triploid species used in the experiment, a sample of 100 open-pollinated fruit from *C. ?grandis* contained nine seeds, but 162 fruit from *C. succulenta* contained no seeds (Table 2). Accordingly, we isolated flowers only on *C. ?grandis* (100 flowers), and no fruit or seeds resulted. We conclude that the quite high fruit set that is a regular feature of both these species is due in part to parthenocarpy, possibly subsequent to pollination that does not result in seeds. Whereas the *C. succulenta* trees appear to be isolated from sources of *Crataegus* pollen (but pollen from *Malus* and *Pyrus* may be available), the *C. ?grandis* tree is surrounded by tetraploid *C. crus-galli* and *C. conspecta*, and, of these, *C. conspecta* pollen can produce seed on *C. ?grandis* (Dickinson, 1983; Dickinson & Phipps, 1986). Thus, seeds did not form at a high rate via fully autonomous apomixis, but the possibility remained that autonomous endosperm development might depend on (appropriate) pollination, as has been noted, for example in *Hieracium* (Bicknell *et al.*, 2003). A test for autonomous apomixis therefore required endosperm-ploidy data from the seeds. Endosperm with the ploidy levels expected from autonomous development (3x or 6x in triploids), would not be found by flow cytometry of whole seeds because the signal would be mistaken for an endopolyploid echo from a triploid embryo. When the embryo was removed from the preparation of 13 samples (all from *C. ?grandis*), no 3x or 6x endosperm was discovered, and, as discussed below, open-pollinated seeds from various triploids commonly had endosperm ploidy levels consistent with fertilization. In the tetraploids, seeds with the possibility of 4x or 8x endosperm (where there was no 10x or higher signal) are likely to represent poor tissue preparation, and they constitute fewer than 11% of samples. Endosperm

measurements from tetraploid *C. crus-galli* also gave no indication that autonomous apomixis can occur: the embryo was removed from the preparation of 58 samples, and no 4x or 8x endosperm was discovered. Thus, the techniques used here did not reveal that autonomous endosperm from the central cell is a major component of apomixis in these species of *Crataegus*.

Seeds from tetraploids have variable endosperm

In seeds from tetraploids, the small echo signals from the embryo (at two times and three times the main peak) lie close to the endosperm signal, but their constant position permits the interpretation of almost all seed measurements (Fig. 4). The embryos from the tetraploids were almost invariably tetraploid (Figs 5–8), but hexaploid embryos were found in three of the four samples (Figs 5, 7, 8). This could be explained by an unreduced egg cell that has been fertilized by meiotically reduced pollen, or by a reduced egg cell that has been fertilized by meiotically unreduced pollen.

In one seed from *C. macracantha*, the endosperm signal is hexaploid (Fig. 5b), which suggests that a sexual embryo sac was involved. In other seeds from this species, the endosperm measurements fall into classes that appear to be at 10x, 12x, 14x, and 16x (Fig. 5).

The seeds from *C. crus-galli* var. *pyracanthifolia* are more variable in both embryo and endosperm measurements than those from *C. macracantha*, with suggestions of aneuploidy. Of the 86 seeds (Fig. 6), eight were dissected as just the embryo. Nine seeds (from tree SC06 pollinated from tree NT72) appear to have 5x embryos (six yielded an endosperm signal). These measurements could be explained by chromosome loss at various stages of development (before formation of the embryo sac (one seed), or from the embryo only (three seeds), but this is highly speculative. In the remaining seeds from *C. crus-galli* the endosperm measurements appear to fall into a subset of the same groups seen with *C. macracantha*: 10x, 12x, 16x, with again one seed at 6x, suggestive of a sexual embryo sac.

Table 2 Seeds set by male-sterile triploids without hand-pollination

Taxon	Tree number	Year	Number of flowers isolated	Number of fruit	Mode (pyrenes per fruit)	Range (pyrenes per fruit)	Total seed
<i>Crataegus ?grandis</i> ^a	NT29	2000	–	100	2	2–4	9
	NT29	2004	100	0	–		–
<i>C. succulenta</i> ^b	NT221	2003	–	38	3	2–4	0
	NT241	2003	–	62	3	2–4	0
	NT242	2003	–	62	3	2–4	0

^aThe *C. ?grandis* NT29 is surrounded by tetraploid *C. crus-galli* and *C. conspecta*, of which *C. conspecta* is a compatible pollen source (Dickinson, 1983; Dickinson & Phipps, 1986).

^bThe *C. succulenta* trees NT221, 241, and 242 appear to be isolated from sources of *Crataegus* pollen.

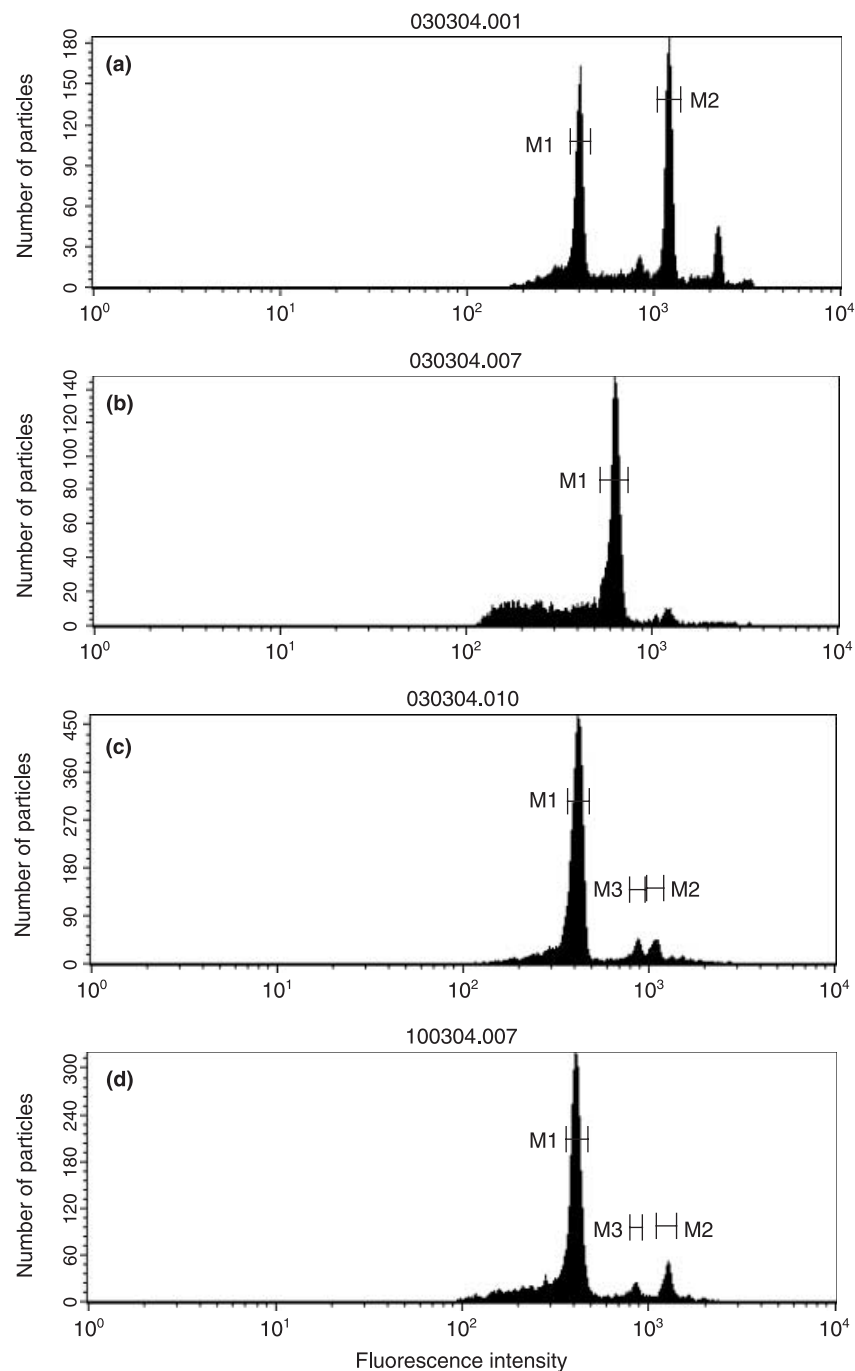


Fig. 4 Entire mature seeds from tetraploids show varying patterns. Seeds from tetraploids show different endosperm ploidy levels, even when all embryos were tetraploid. (a) The separately dissected embryo from a seed from tetraploid *Crataegus submollis* (tree NT26) showed a tetraploid signal (M1) with a strong internal DNA standard of *Pisum* nuclei (M2). (b) The endosperm and seed-coat preparation from the same seed as (a) has a strong 6x signal (M1) and a weak *Pisum* signal (unlabelled). Whole seed preparations (c, d) from *C. macracantha* var. *macracantha* (tree NT133) show a small peak (M3) at twice the fluorescence of the main peak (8x) that is interpreted as cells in the G2 phase of the cell cycle, and a third peak (M2), which occurs at different ploidy levels in different seeds, most commonly 10x, less often 12x or higher ploidy levels. When endosperm and seed coat are prepared separately from *C. macracantha*, the main fluorescence peak corresponds to peak M2 in the whole-seed preparations.

The embryo measurements from *C. submollis* (Fig. 7) are similar to those from *C. crus-galli* and *C. macracantha*, with the majority clustered around the tetraploid estimate, and a single 6x embryo. However, more of the endosperm measurements cluster close to 6x, with others in the 10x–12x range, and a single seed apparently with 14x endosperm. It is not possible to tell from these data whether the 6x endosperm means that this species is largely sexual. An alternative explanation is that the endosperm formation in some of the seeds differs from that in the other tetraploids, perhaps by

involving a single central cell nucleus rather than two (as in some apomictic Panicoid grasses; Warmke, 1954; Nogler, 1984).

The more diverse sample of *C. douglasii* with its tetraploid relatives and neighbors had overwhelmingly tetraploid embryos (Fig. 8). The endosperm measurements show a strong concentration of signals close to 10x and 12x, with rare higher values. There were three exceptional seeds. Two of the embryos from *C. castlegarensis* are close to hexaploid, which suggests that more investigation of this species might demonstrate a higher rate of fertilization. One embryo from *C. douglasii* measured

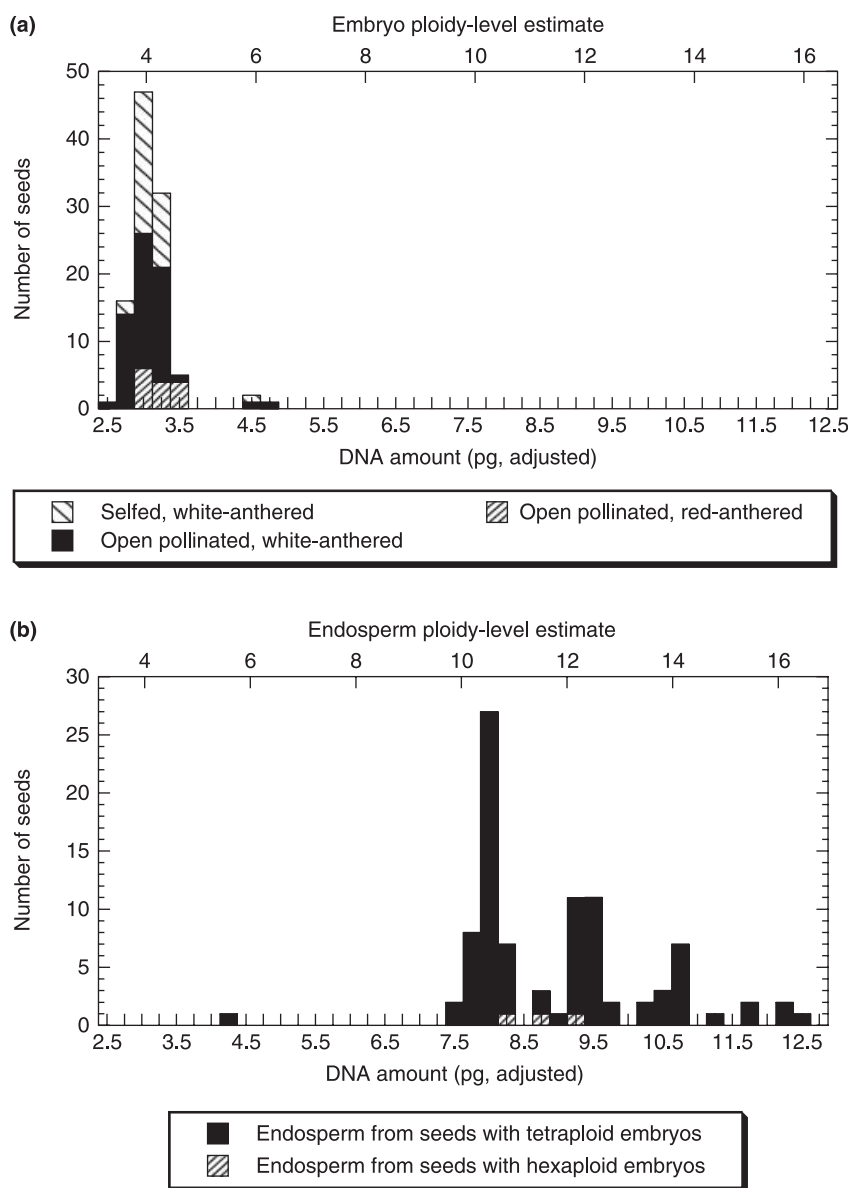


Fig. 5 Embryo and endosperm DNA measurements from tetraploid *Crataegus macracantha*. This combined sample of open-pollinated seed with the self-pollinated seeds derived from autogamy experiments comes from mother plants of *C. macracantha* var. *macracantha* (NT101, NT102, NT118, NT130, NT131, NT133, NT228, NT229, Durham county) and one mother plant of a red-anthered form of *C. macracantha* (NT30, Toronto). (a) The embryo DNA amounts were standardized using the leaf DNA measurements of the mother plant (tetraploid in all cases). (b) The DNA amounts of the endosperm were standardized by adjusting the DNA amount of the corresponding embryo to the midpoint of the estimated range for tetraploids (3.04 pg) or for hexaploids (4.56 pg).

octaploid, and the endosperm measured $12x$ – $13x$ (9.515 pg DNA), which suggests that this embryo was not fertilized by both sperm, but rather that DNA duplication occurred during early development.

Excluding *C. submollis*, because of the possibility that it is largely sexual, the most common endosperm DNA measurement occurring with $4x$ embryos was $10x$ (64%), which would be consistent with contributions from a meiotically unreduced binucleate central cell and one reduced sperm. Of the higher ploidy levels that occurred, the majority were $12x$ (22% $12x$ overall), consistent with fertilization by either two reduced sperm or one unreduced sperm. The readings higher than $12x$, given the error inherent in the cytometric measurement at higher ploidy levels (Talent & Dickinson, 2005), appear likely to represent $14x$, $16x$, and $18x$. Endomitotic duplication in endosperm is well known and could explain these values

(Nogler, 1984), but one or more extra central cell nuclei are sometimes observed in aposporous plants (Nogler, 1984; Jankun & Kovanda, 1986; Campbell *et al.*, 1987), including both sexual and apomictic *Crataegus* (Muniyamma & Phipps, 1984a, 1985). The $14x$ and $16x$ measurements can therefore be explained by a trinucleate central cell and one or two reduced sperm, and the $18x$ measurement could be explained by a tetranucleate central cell and one sperm.

Seeds from triploids have variable endosperm

The seeds from open-pollinated triploids (Table 2) were generally healthy in appearance but uneven in size, and further investigation of how the seed size relates to the ploidy levels of embryo and endosperm might be rewarding. The DNA measurements of the embryos (Fig. 9a) suggest two

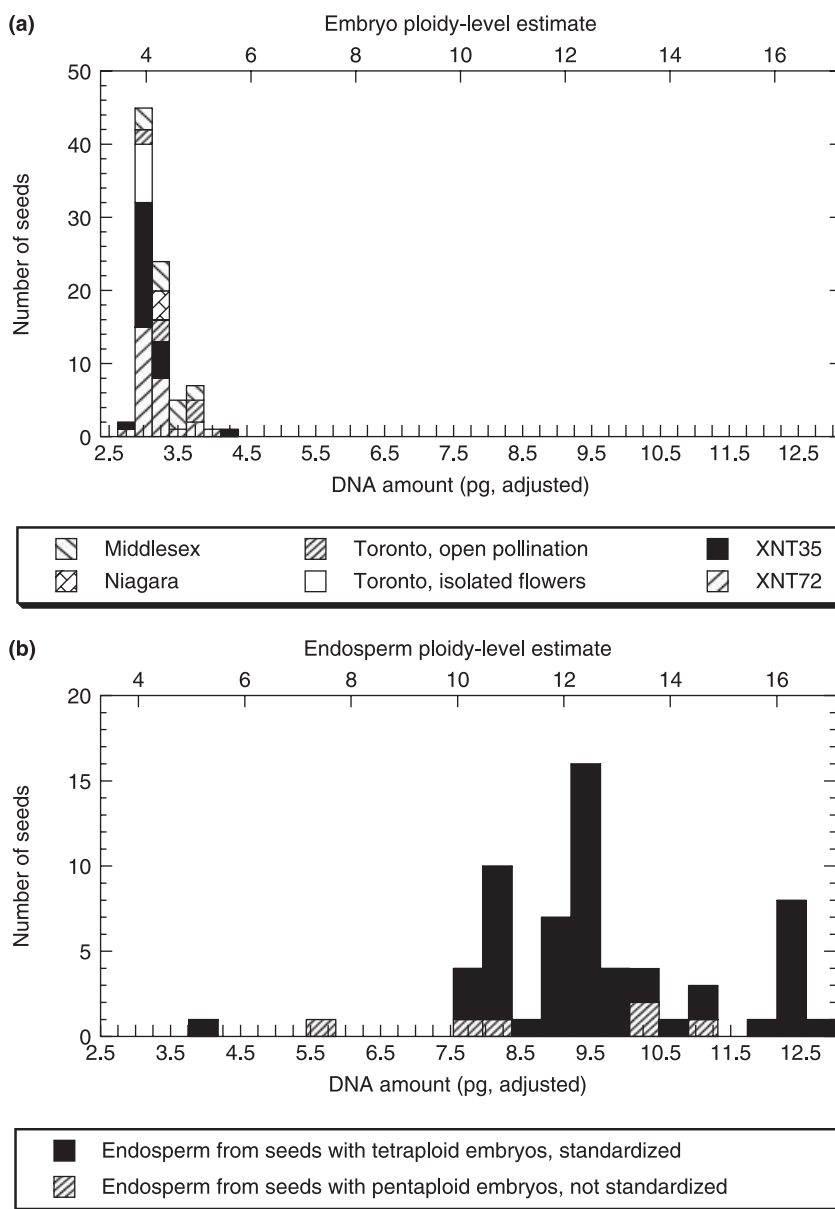


Fig. 6 Embryo and endosperm DNA measurements from *Crataegus crus-galli* var. *pyracanthifolia*. This combined sample includes the self-pollinated seeds derived from autogamy experiments (trees NT66 and SC06, Toronto), open-pollinated seeds from three localities (tree D668 from Middlesex county; NT35 from Niagara county; NT232 and NT234 from Toronto), and hand-pollinated seed (trees NT62 and NT66 from Toronto, pollinated from NT35 and NT72 from Niagara). The embryo measurements (a) were standardized using the DNA measurement from leaf tissue of each mother plant. A subset of the sample had tetraploid embryos and yielded both embryo and endosperm signals. The DNA amounts of the endosperm from these seeds (b) were standardized by adjusting the DNA amount of the embryo to the midpoint of the estimated range for tetraploids (3.04 pg). For six other seeds with apparently pentaploid embryos the measurements were not standardized.

concentrations close to (or a little below) $3x$ and $5x$. The seeds from *C. ?grandis* that had small embryos and appeared to be failing are not dramatically different. Two of the seeds from *C. pruinosa sensu lato* apparently lie at $4x$, outside the two concentrations from *C. ?grandis*. Thus, the embryo data from both species suggest that $3x$ embryos arise through parthenogenesis of meiotically unreduced egg cells, and that fertilization of these egg cells is also possible: the pollen sources for these *C. ?grandis* and *C. pruinosa* trees appear to be, respectively, a tetraploid and a diploid. Triploid *Crataegus* may therefore be capable of producing triploid, tetraploid, and pentaploid offspring.

When the endosperm measurements are matched to the embryo measurements, two particularly interesting concentrations of data points (Fig. 9b; groups α and γ) indicate, respectively, triploid embryos with decaploid endosperm, and pentaploid

embryos with octaploid endosperm. Thus, the majority of the seeds are consistent with a binucleate central cell and with either both sperm contributing to the endosperm (α) or one sperm contributing to each of the embryo and endosperm (γ). Parthenogenesis of the egg cell with single fertilization of the central cell is also suggested (Fig. 9b; group β), as is an aneuploid embryo sac presumably produced via meiosis (Fig. 9b; group δ). Two high endosperm DNA measurements suggest that entirely different origins of this tissue are possible.

Discussion

Crataegus seeds may be more amenable to analysis by flow cytometry than the seeds of many other plants, firstly because the endosperm tissue in the mature seed is sufficiently

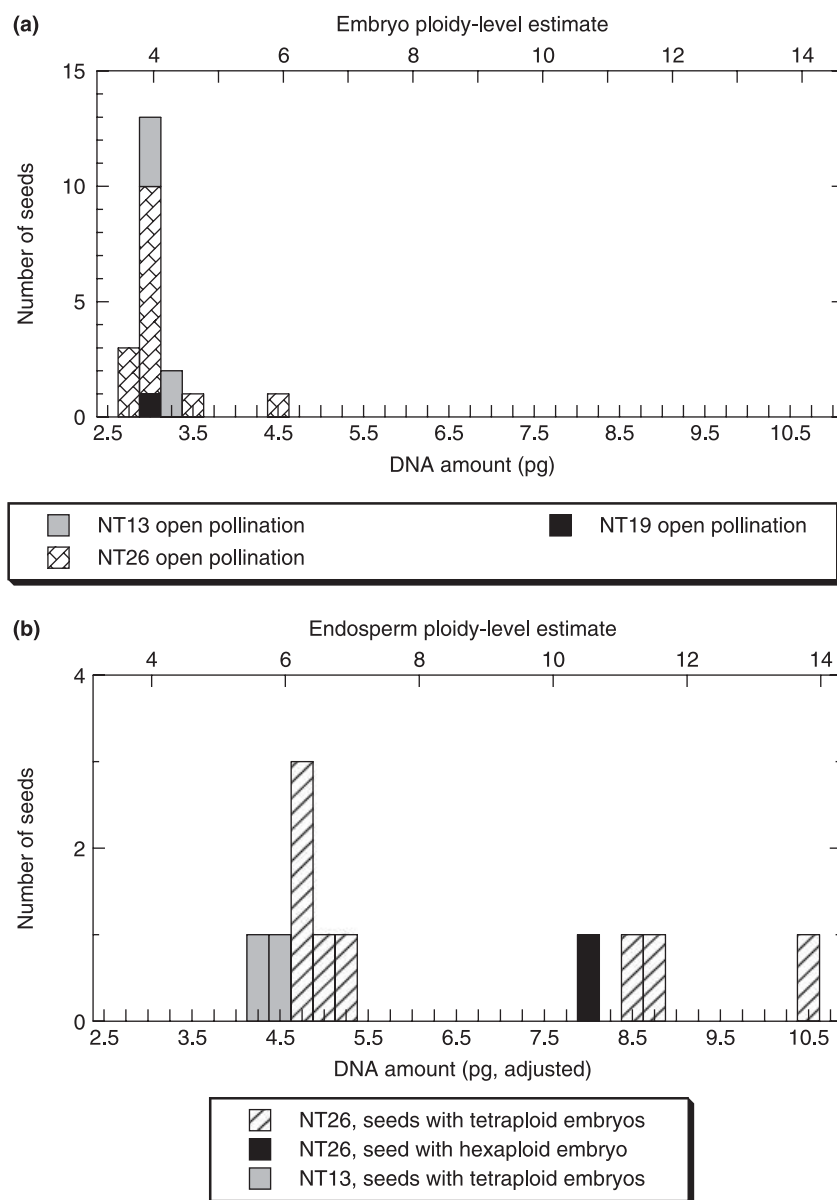


Fig. 7 Embryo and endosperm DNA measurements from tetraploid *Crataegus submollis*. This sample of 21 open-pollinated seeds comes from three mother plants (trees NT13, NT26, and NT19). The embryo measurements (a) were standardized using the DNA measurement from leaf tissue of each mother plant. A subset of 12 seeds (b) yielded both embryo and endosperm signals. The endosperm measurements were standardized using the embryo DNA measurement from the same seed, adjusted to the midpoint of the estimated range for tetraploids (3.04 pg) or for hexaploids (4.56 pg).

abundant for analysis, and secondly because complications from endopolyploidy are not very great. In a woody plant that is otherwise quite difficult to study (seed germination and propagation of cuttings are difficult, for example), flow cytometry will undoubtedly prove to be an important investigative tool. The same method may prove useful in other genera of Pyrinae, although many of these have smaller seeds and/or fewer endosperm cell layers (Péchoutre, 1902; Corner, 1976; Aldasoro *et al.*, 2005). Moreover, in all cases where there is substantial shedding of flowers after the pollination period (including 'June drop' in apples and other tree fruit) direct observations on seeds may more reliably indicate events during seed development than embryological observations on flowers, an unknown proportion of which are not directly related to seed formation.

Ploidy-level variation

All of the embryos from the diploid and near-diploid plants were similar to the maternal parent, with DNA variation according to the species involved. The tetraploid mother plants produced a majority of tetraploid embryos, and the triploids gave triploid, tetraploid, and pentaploid embryos.

The ploidy level of the endosperm tissue gave considerable additional insight into the origins of the various embryos. We confirmed that the endosperm tissue of the seeds from five diploid *Crataegus* and one diploid *Mespilus* was triploid, as expected with the contribution of a binucleate central cell and a single sperm. Although the methods we used to detect it were not ideal, we did not find any evidence that autonomous

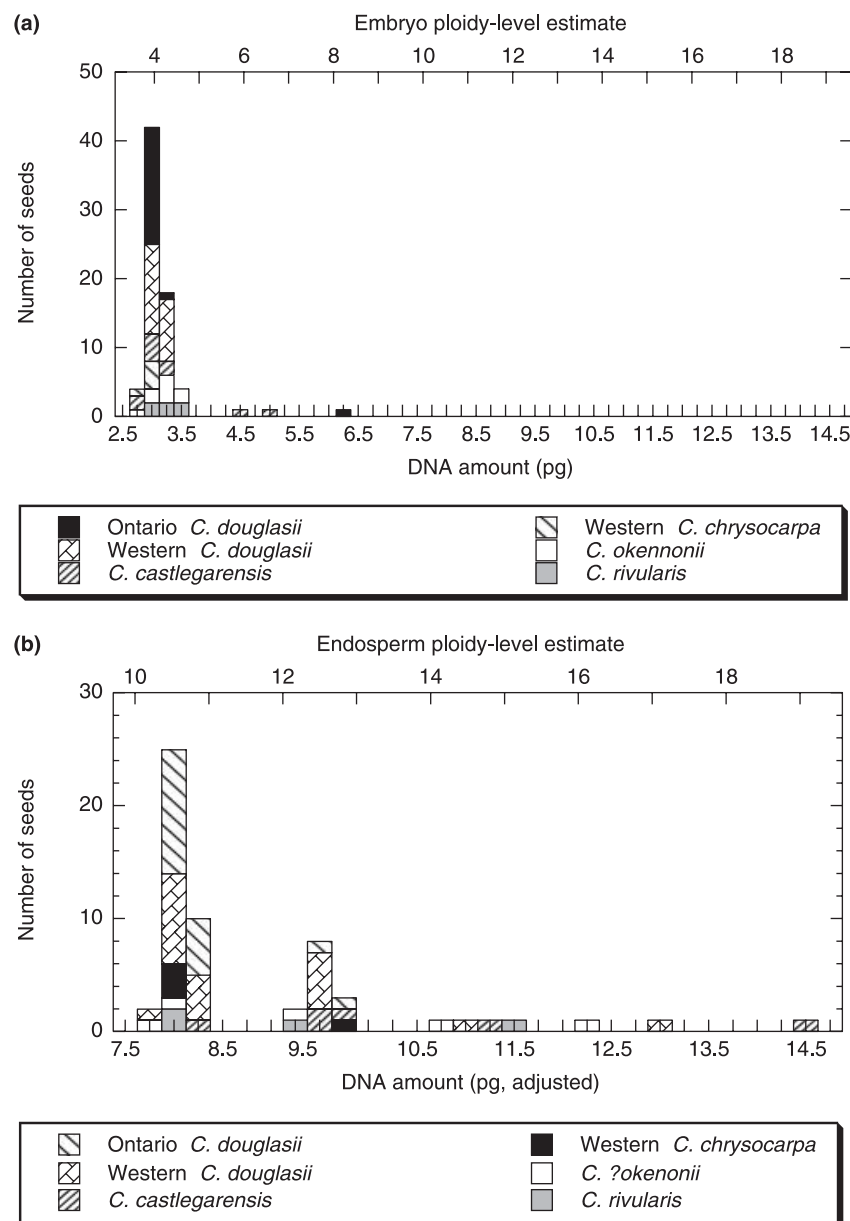


Fig. 8 Embryo and endosperm measurements from tetraploid *Crataegus douglasii*, its possible relatives in section *Douglasianae*, and sympatric *C. chrysocarpa* (section *Coccineae*). The plants in this sample are from the disjoint distribution of *C. douglasii*, and other tetraploids with which it is either sympatric in the Pacific Northwest (Talent & Dickinson, 2005) – *C. castlegarensis*, *C. okennonii*, as well as *C. chrysocarpa* with leaves similar to *C. douglasii*, but red fruit – or allopatric (*C. rivularis* from the Rocky Mountains). The embryo measurements (a) were standardized using the DNA measurement from leaf tissue of each mother plant. A subset of the seeds (b) yielded both embryo and endosperm signals. Six seeds that might have had an octaploid endosperm, which was not distinguished from a G2 signal from a tetraploid embryo, were omitted.

endosperm from any of the cells of the embryo sac can occur in the diploid (or polyploid) *Crataegus*. Some of the sexual diploids with triploid endosperm (*C. punctata* and *C. suksdorfii*) are considered closely related to apomictic polyploids (*C. crus-galli* and *C. douglasii*, respectively). It thus appears to be unlikely that a flexibility in the ploidy level of the endosperm in diploids, bypassing the 2 : 1 ratio of maternal to paternal contributions, is a necessary predisposing factor to the evolution of polyploid apomixis in this genus.

The apomictic tetraploid species produced tetraploid embryos along with a small number of hexaploids, one octaploid, and from *C. crus-galli* some pentaploids. The hexaploids would be consistent with fertilization of a meiotically unreduced egg cell, and the octaploid with genome duplication (not double

fertilization, since one sperm was apparently involved in fertilizing the endosperm). The pentaploid embryos cannot be fully explained, but chromosome loss (from a hexaploid embryo) might be occurring. One process that can involve chromosome loss is the formation of the restitution nucleus during diplosporous apomixis of the *Taraxacum* type (Leblanc *et al.*, 1995). Aposporous apomixis has been most frequently reported in *Crataegus* (Muniyamma & Phipps, 1979a,b, 1984a; Dickinson & Phipps, 1986; Smith & Phipps, 1988), but diplospory of the *Taraxacum* type has been reported in a single triploid (Muniyamma & Phipps, 1984b). An hypothesis of diplospory in the tetraploid, however, could explain only one of the six endosperm measurements from seeds with pentaploid embryos.

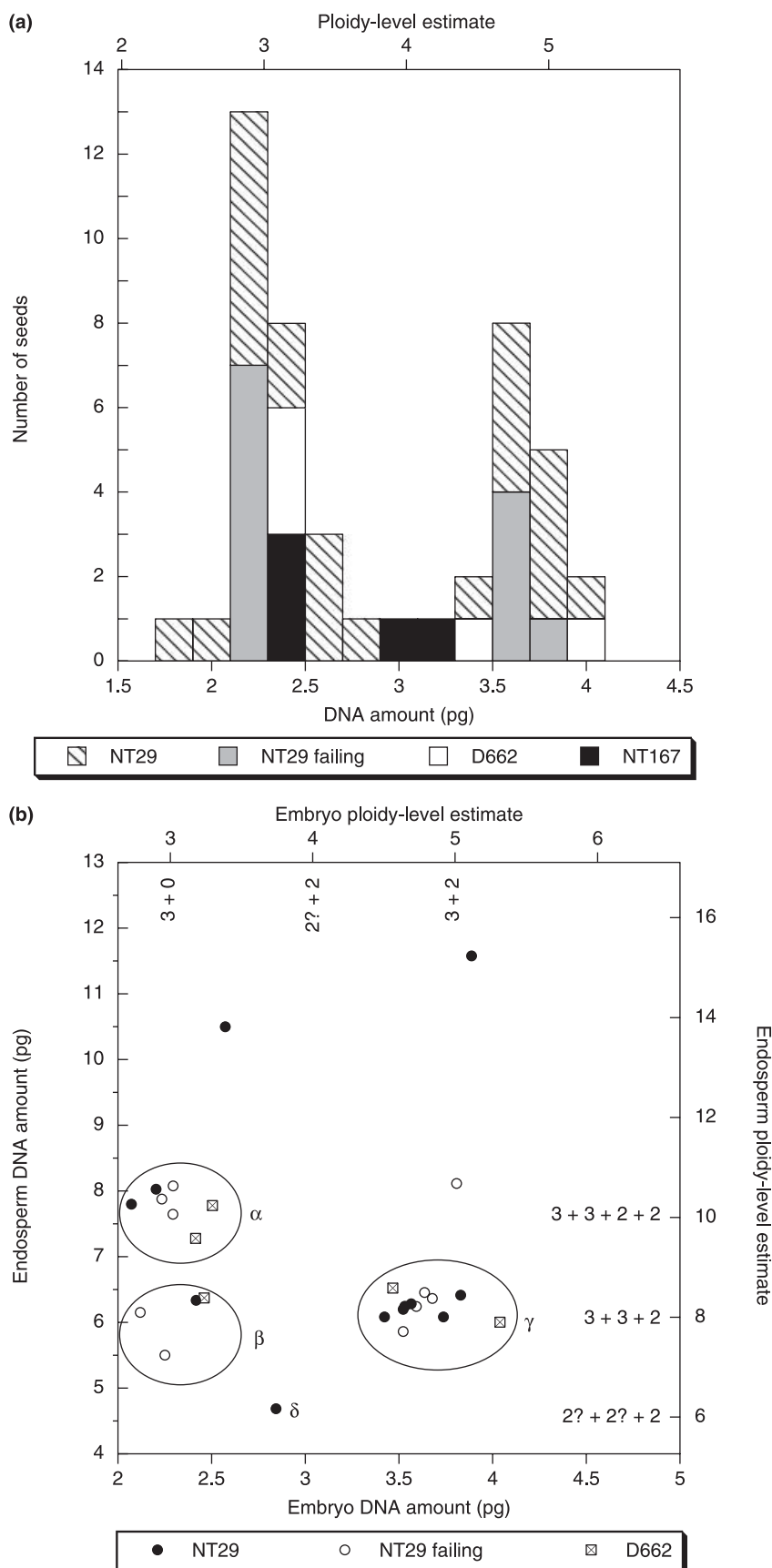


Fig. 9 Nuclear DNA measurements from seeds from triploids. We sampled 46 open-pollinated seeds from three triploids of *Crataegus* section *Crus-galli* (*C. ?grandis* tree NT29, and *C. crus-galli sensu lato* tree D662) and section *Pruinosae* (*C. pruinosa sensu lato* tree NT167). Of these, NT29 is male-sterile, D662 produced pollen with 34% stainability (Dickinson & Phipps, 1986), and NT167 was not observed in bloom. Seeds indicated as 'failing' had very small brownish embryos, and the endosperm was translucent and brownish; other seeds had white embryos of various sizes and translucent pale endosperm. The embryo measurements (a) were not standardized. Both embryo and endosperm signals were obtained from 27 of the seeds (b) from section *Crus-galli* only, the majority of which fall into three groups (groups α , β , and γ). '2?' indicates a possible aneuploid embryo sac with close to the diploid DNA amount. Along the axes, the ploidy levels of the inferred constituent gamete nuclei are indicated, with female gametes listed first (e.g. $3 + 3 + 2 + 2$ suggests that two triploid central-cell nuclei and two diploid sperm contributed to the observed ploidy level).

The few seeds available from open-pollinated triploid plants indicate considerable variation in the ploidy level of both embryo and endosperm, but there is evidence for fertilization of a meiotically unreduced egg cell by pollen from putative diploid or tetraploid neighbours. Thus, tetraploid or pentaploid embryos can arise in the seeds of triploids. The viability of the seeds may be quite low, however: they varied more in size than the seeds from the diploids and tetraploids. Future work will attempt to find more compatible pollen sources for these apparently fertile triploids to further map ploidy-level changes through seeds.

The pentaploid and hexaploid embryos observed in seeds from apomicts have their parallels in adult plants (Talent & Dickinson, 2005), but pentaploids have not often been noted, and there is only one record of a hexaploid adult. The hexaploid record is from an east Asian species, *C. pinmatifida* Bunge, that has also been recorded as diploid, triploid, and tetraploid (Gu & Spongberg, 2003), and whose breeding system is unknown. Thus, it appears likely that the pentaploid and hexaploid seeds can give rise to adult plants. It is not clear whether investigators may have overlooked pentaploid and hexaploid adults by concentrating their efforts on individuals that are most readily identifiable by morphological characteristics. Our sample of leaves and seeds from series *Douglasianae* provides a comparison of the ploidy levels from adults and embryos, but the few nontetraploid embryos were not statistically more prevalent than nontetraploid adults (33 adults were all tetraploid; 67 embryos included three nontetraploids; using Fisher's exact test (one-tailed), $P = 0.3095$). However, the small sample of seeds from tetraploid *C. castlegarensis* (*Douglasianae*) contained a higher proportion (20%) of hexaploid embryos, and therefore a larger survey of the ploidy levels of this species could be worthwhile. Such a survey might reveal hexaploid adult plants, or alternatively it might reveal that the survival and/or subsequent fertility of hexaploid seedlings is low.

Endosperm formation

Earlier experiments have shown that pollination is required for seed formation in apomictic tetraploid *Crataegus* (Dickinson & Phipps, 1986; Smith & Phipps, 1988; Dickinson *et al.*, 1996), including *C. crus-galli* var. *pyracanthifolia* as used here. The most likely origin for the 10x endosperm that commonly occurred in seeds from tetraploids is therefore from meiotically reduced pollen and a meiotically unreduced embryo sac, the same method of deriving the endosperm as in the sexual diploid species. Experiments with other pseudogamous apomicts have confirmed fertilization by demonstrating genetic differences between the endosperm in different ovules (Nogler, 1984). The largely 6x endosperm in *C. submollis* appears likely to have resulted from meiotically reduced (sexual) embryo sacs, also involving a binucleate central cell and a single sperm.

It has been suggested that the 12x endosperm seen with pseudogamous apomictic tetraploids involves both sperm (Nogler, 1984), and this may be the case with a minority of the seeds from the tetraploid *Crataegus*. The majority of the endosperm ploidy levels higher than 12x can be explained by an extra central cell nucleus; one or two extra central cell nuclei are sometimes observed in aposporous plants (Nogler, 1984; Jankun & Kovanda, 1986; Campbell *et al.*, 1987), including *Crataegus* (Muniyamma & Phipps, 1984a). It is possible, however, that the endosperm with high-ploidy levels (including 12x endosperm) derives from the poorly canalized development that is typical of apomictic plants, including *Crataegus* (Bicknell & Koltunow, 2004; Muniyamma & Phipps, 1979a; Koltunow *et al.*, 2000).

Our data indicate that one of the two sperm is frequently available to fertilize the egg of apomictic *Crataegus*. We do not therefore have a satisfying answer to the question of why *Pyrinae* do not appear to have undergone the ploidy-level increases evident in some apomictic *Rosoideae* (Kalkman, 2004). The answer to this question might involve a complex of factors, possibly including a more recent origin of the *Pyrinae* clade or infrequent fertilization of eggs that initiate parthenogenesis early. It is also possible that survival of individuals with high-ploidy levels is poor because the base chromosome number of *Pyrinae*, $x = 17$, is more than twice that of most *Rosoideae*, $x = 7$ (Kalkman, 2004). There are few data about the germinability of seeds from apomictic *Crataegus* (but in *C. crus-galli sensu lato*, even seeds from self pollinations can produce vigorous seedlings, although cross-pollinated seeds germinate more readily; T. A. Dickinson, unpublished). It is therefore possible that the seeds with 10x endosperm (some of which have fertilized embryos) are less viable than those with 12x endosperm. An endosperm-balance requirement does not prevent the formation of mature seed, but it is possible that there is an effect on germination.

Ploidy-level transitions

In the Panicoid grasses, a contrast has been noted (Naumova *et al.*, 1999; Quarin, 1999) between two subtly different types of species complexes: the so-called diploid–tetraploid–(di)haploid reproductive cycle exemplified by *Panicum* and the *Bothriochloa–Dichanthium* complex, and the so-called diploid–tetraploid–diploid cycle exemplified by *Paspalum*. Dihaploids (diploids) derived from tetraploids occur in both cases, but they are relatively common and fertile only with the second of these two cycles (*Paspalum*), where partly apomictic triploids and aneuploids also occur. It is thought that the triploids provide a bridge for genetic transfer between diploids and tetraploids that is lacking in *Panicum* and *Bothriochloa–Dichanthium*. The complex of ploidy levels in *Crataegus* is similar to that in *Paspalum*, and incidentally to that in *Ranunculus auricomus* (Nogler, 1984; Naumova *et al.*, 1999) and when what is known about the mode of endosperm

Table 3 Parallels between aposporous genera in three families

Taxon	Aposporous embryo sacs in diploids	Facultatively aposporous triploids, aneuploids	Endosperm ploidy of tetraploids ^b
Ranunculaceae, Ranunculoideae			
<i>Ranunculus auricomus</i>	Yes	Yes	10x, 12x, other
Rosaceae, Spiraeoideae, Pyreae, Pyrinae			
<i>Amelanchier</i> ^a	No data	–	6x
<i>Crataegus</i>	?	Yes	10x, 12x, other
<i>Sorbus eximia</i> ^a	Yes	?	5x, 6x ^b
Poaceae, Panicoideae, Andropogoneae, Sorghinae			
<i>Bothriochloa–Dichanthium</i>	Yes	No	6x
Poaceae, Panicoideae, Paniceae, Setariinae	Yes	No	6x
<i>Panicum</i>	Yes	No	6x
<i>Brachiaria decumbens</i>	Yes	No	6x
<i>Paspalum</i>	Yes	Yes	10x

^aEndosperm ploidy levels in these taxa were observed in early developmental stages or inferred from cell behaviour in the embryo sac.

^b*Sorbus eximia* includes diploid apomicts with endosperm ploidy levels comparable to 10x and 12x in tetraploids. Both apospory and diplospory occur in this species.

Sources: Reddy & D'Cruz (1968); Dickinson 1983; Nogler (1984); Campbell *et al.* (1987); Jankun & Kovanda (1988); Mabberley (1997); Naumova *et al.* (1999); Quarin (1999).

formation in these aposporous plants is added (Table 3), some other parallels are suggested. If endosperm formation in *Amelanchier* (Rosaceae) follows the pattern suggested by early embryology (Campbell *et al.*, 1987), then both the Panicoid grasses and the Pyrinae subtribe of Rosaceae include aposporous genera with two different solutions to the endosperm-balance problem. This duality would seem to indicate independent origins of apomixis in each genus, rather than a suite of apospory and endosperm genes ready to be activated by, for example, polyploidization. *Amelanchier* is a smaller genus than *Crataegus* (Kalkman, 2004), with relatively few diploids and very few triploids, and the structure of the genus therefore appears to resemble the structure of *Panicum* or *Bothriochloa–Dichanthium*.

Perhaps even more significant is that the predominantly 10x endosperm of tetraploid *Crataegus* may indicate a similarity between the mechanisms forming the predominantly 12x endosperm of *Ranunculus* (Rutishauser, 1954; Nogler, 1984), and the 10x endosperm seen in *Paspalum* (Table 3). The *Paspalum* data were obtained from chromosome counts in 20 nuclei, but the number of seeds was not specified (Quarin, 1999). Nogler (1972, 1984) found that in *Ranunculus auricomus* the proportion of 12x endosperm was genetically controlled independently from apomixis, which suggests that the lack of 12x chromosome counts from *Paspalum* might be a sampling artifact. Thus, it appears (Table 3) that there may be two basic approaches to the endosperm-balance problem with apospory. If the number of central-cell nuclei involved is reduced to one (which can be the result of a variety of earlier developmental alterations), then the diploids and tetraploids may be less likely to produce apomictic triploids. The second of the two alternative mechanisms is more flexible and possibly involves

a relaxation of the endosperm-balance requirement (as Quarin (1999) has suggested), which would allow either 10x or 12x endosperm in a tetraploid. This second putative pattern of endosperm origin would correlate in some way with the formation or persistence of triploids.

It would be a very curious result indeed to find that the taxonomic complexity of *Crataegus*, which was long ago attributed to the effect of polyploidy (Longley, 1924), is a consequence of the way in which the endosperm of polyploid apomicts is formed. The data suggest the formation of tetraploids from apomictic triploids, with consequent gene flow to tetraploids, and this would be related to the mode of endosperm formation in the triploids. It is not clear, however, how the formation of triploids could be related to the female function of apomictic tetraploids, as diploid mother plants pollinated from apomictic tetraploids would seem to be a much more likely route for triploid formation. The interploidy hybridization behaviour of *Crataegus* requires further study, and the Pyrinae offer a variety of other apomictic genera in which a possible connection between hybridization and endosperm formation could be tested (Kalkman, 2004).

An hypothesis concerning the second sperm

It has been speculated that the 12x endosperm of tetraploid *Ranunculus auricomus* and the 6x endosperm of diploid *Sorbus eximia* are explained by diversion of the second sperm to the central cell rather than to the egg (Nogler, 1972, 1984; Jankun & Kovanda, 1988). A hormonal model that could explain this is that an attractant is involved and it is normally produced by both the central cell and the egg cell and is essential to the fertilization process. In an apomict, the postulated attractant

might not be produced by an egg cell destined for parthenogenesis. Some experimental results consistent with this model involve embryo sacs of the *indeterminate gametophyte1* mutant of *Zea mays* L. (Poaceae), which can have multiple egg cells, and in this case the individual sperm behave as if they are separately attracted to the egg cells (Guo *et al.*, 2004). Alternatively, in aposporous *Cenchrus ciliaris* L. (Poaceae), Bashaw & Hanna (1990) report that they often observed 'the pollen tube with a sperm near the polars, but rarely saw a sperm anywhere near the egg', and they propose that failure to fertilize the egg cell might involve repulsion of the pollen tube. We have not seen any reports of the ploidy level of the endosperm in *Cenchrus ciliaris*, but this suggests a possible mechanism for double fertilization of the central cell if the pollen tube delivers both sperm to it. Therefore, although the mechanism is unclear, it appears likely that diversion of the second sperm to the central cell can occur in tetraploid *Crataegus* and *Paspalum* to give a 12x endosperm, as with *Ranunculus* and *Sorbus*.

If, as proposed, the sperm in the near-obligate apomictic *Crataegus* (*C. crus-galli*, *C. macracantha*, and *C. douglasii sensu lato*) are repelled by the parthenogenetic egg cell or both delivered to the central cell, then an explanation is needed for the greater incidence of 10x rather than 12x endosperm. Nogler (1984) noted a correlation with the maternal genotype in *Ranunculus*, and the possibility should therefore perhaps not be entirely discounted that the variation in endosperm ploidy level is the result of variation in the embryo sac; that is, for some embryo sacs, a single sperm can produce a viable endosperm, but for others both sperm are needed. We can only speculate as to what factors might produce a difference in the fertilization potential of the central cell. However, it has been suggested that a delay in the timing of nuclear fusion may correlate with apomixis in Rosaceae (Hjelmqvist, 1962; Czapik, 1983, 1985a; Campbell *et al.*, 1985, 1987; Czapik, 1985b). Fusion of the central cell nuclei before fertilization did not occur in an aposporous tetraploid *C. pruinosa* or in a sexual diploid *C. monogyna* (Muniyamma & Phipps, 1979a, 1985). It may be appropriate, however, to check for variation in the timing of nuclear fusion between embryo sacs in tetraploid apomictic *Crataegus*. Because *C. monogyna* is not known to have apomictic relatives, it may also be appropriate to check for early nuclear fusion among the North American diploid species that have apomictic relatives.

If two independent factors govern, respectively, embryo-sac formation and the number of sperm available to the central cell (and the second of these factors is perhaps the result of parthenogenesis), then a frequent mismatch between these two factors could contribute to the low seed set of apomictic *Crataegus*, which has been observed by some authors (Dickinson, 1983; Smith & Phipps, 1988; Macklin, 2001). Although it would be difficult to accomplish, further study of the multiple embryo sacs that develop at different rates in *Crataegus* (Muniyamma & Phipps, 1979a) and the failure rates of different

types of ovules would seem to be the best way to test whether there is a link between the endosperm ploidy level in the final seed and either the developmental origin of the embryo sac before fertilization or the parthenogenetic potential of the egg cell.

The possibility that the number of sperm contributing to the endosperm is dictated by a subtle difference in the origin of the female gametophyte is an interesting alternative to Quarin's model for *Paspalum* (Quarin, 1999), which holds that a new flexibility in the ratio of parental gametes contributing to the endosperm results from an increase in maternal ploidy level. The most fruitful sources of data to study these models further may come from the *Ranunculus auricomus* plants with predominantly 12x endosperm (Nogler, 1984). Larger surveys of endosperm ploidy levels in both *Crataegus* and *Paspalum* might also indicate whether they possess an independent genetic factor that affects the proportion of 12x vs 10x endosperm as in *Ranunculus*.

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Supplementary Material

The following supplementary material is available for the article online:

Table S1 Ploidy levels of individual trees

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-8137.2006.01918.x>
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