Chapter XVI

Apomixis and hybridization in Rosaceae subtribe Pyrinae Dumort.: a new tool promises new insights

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The knowledge base about certain genera of the pome-bearing clade of Rosaceae (subfamily Spiraeoideae (Juss.) Arn., tribe Pyreae Baill., subtribe Pyrinae Dumort., traditionally referred to as subfamily Maloideae C. Weber), e.g., Malus Mill. and Pyrus L., is considerable because of their great economic importance. However, what is known relates primarily to the domesticated species and much less is known about the much more numerous wild species. Several small genera appear to be sexual diploids, and the larger genera that have been studied (Amelanchier Medik., Cotoneaster Medik., Crataegus L., Malus Mill., and Sorbus L. s.l.) are taxonomically complex because they include apomictic polyploids as well as sexual diploids. Multiple taxonomic sections of the largest genera also include both apomictic polyploids and sexual diploids. In Crataegus, we hope to shed light on how often apomixis has arisen, and on the nexus between polyploidy, hybridization, and apomixis. Flow cytometric DNA measurement boosts the efficiency of ploidy-level determination, allowing us to survey cytological variation in wild populations. Flow cytometry also reveals the ploidy levels of embryos and endosperm in mature seeds, and these indicate whether the embryo sac was meiotically unreduced. Whereas embryological observations of apomixis can be difficult to interpret, the cytometric data give a simpler picture and are more indicative of the final reproductive outcome. Our results indicate that, in Crataegus, one of the two meiotically reduced sperm from diploids or tetraploids is frequently available to fertilize the unreduced egg cells of apomictic triploids and tetraploids, yielding higher-ploid offspring. This improved ability to survey ploidy level and breeding system will help us to interpret the results of molecular and genetic studies.

KEYWORDS: Crataegus, endosperm balance, flow cytometry, Maloideae, Rosaceae, polyploidy, pseudogamous apospory, Pyreae, Spiraeoideae.
APOMIXIS AND TAXONOMY IN ROSACEAE SUBTRIBE PYRINAЕ (= MALOIDEAE S.S.)

The Rosaceae are perhaps the third most popular Angiosperm family for the study of gametophytic apomixis, after Asteraceae and Poaceae (Nogler, 1984; Czapik, 1996); pseudogamous apospory is most common in the family, and diplospory also occurs (Muniyamma & Phipps, 1984; Jankun & Kovanda, 1988; Kalkman, 2004). As in other Angiosperm families, apomictic species are almost always polyploid and often of hybrid origin.

Apomixis is notable in several tribes of Rosaceae including tribe Pyreaе (until recently generally known as the apple subfamily Maloideae s.l.). The Pyreaе are a monophyletic group of about 25 genera which arose from a Gillenia-like ancestor by polyploidization (Evans & Campbell, 2002). The majority of these are the fleshy-fruited genera in subtribe Pyrinaе (Potter & al., in press) that appear to have radiated rapidly and have limited barriers to inter- and intrageneric hybridization. The genera of Pyrinaе vary in their taxonomic complexity, although it is difficult to assess how many species exist because of differences in taxonomic opinion (Robertson & al., 1991). A number of small genera appear to contain sexual diploids only (Rhaphiolepis Lindl., Pyracantha M. Roem, Chaenomeles Lindl., Cydonia Mill.) or sexual diploids with occasional polyploids and aneuploids (Pyrus L. s.s.), and these contrast with the larger genera Sorbus L., Malus Mill., Amelanchier Medik., Cotoneaster Medik., and Crataegus L., in which apomictic tetraploids are common. Apomixis in Pyrinaе is, with rare exceptions (e.g., Jankun & Kovanda, 1988), confined to polyploids, that must have arisen repeatedly from the diploid species of the various genera (Nogler, 1984; Czapik, 1996; Savidan, 2000). It appears likely that the sizes of the apomictic genera also vary. Crataegus and Cotoneaster are notoriously complex, with many hybrids or clonal forms; recent conservative estimates suggest that taxonomic revisions could reduce the number of species in each genus to perhaps 150 (Phipps & al., 2003; Kalkman, 2004), a number similar to that in Sorbus s.l. In other apomictic genera the number of species may be much smaller (e.g., Amelanchier = 20, Malus = 40; Kalkman, 2004).

Asexually reproducing genotypes of Pyrinaе may be mistaken for species because of their frequency in landscapes, as well as their local abundance. In some genera the morphological species, as currently defined, may contain a mixture of ploidy levels, in some cases with geographically distinct sexual and apomictic components, or partly apomictic individuals (e.g., Muniyamma & Phipps, 1985). Reproductive isolation and species relationships need to be understood in the context of polyploidy, apomixis, and hybridization. Such studies rely heavily on chromosome counts from the parent trees, but in some genera this approach is barely practical because of the difficulty of obtaining suitable tissue samples\(^1\) with meta-

\(^1\)Root tips for chromosome counts can be difficult to obtain from adult plants of Pyrinaе, and it is most common to use pollen meiosis or mitosis in ephemeral tissues such as petals or filaments; in Crataegus, none of these tissues are available for more than one week per year (Longley, 1924; Byatt & Murray, 1977; Muniyamma & Phipps, 1979b; Ptak, 1986; Dickinson & al., 1996).
phase figures. Flow cytometric measurement of nuclear DNA content, if it could be reliably translated to ploidy levels, therefore promised to improve the efficiency of studies of apomixis, with consequent benefits for studies of phylogenetic relationships and species richness in the genera of Pyrinae.

HYBRIDIZATION, TAXONOMY, AND ENDOSPERM OF APOMICTIC PYRINAE

The paradox of genera with gametophytic apomixis is that on the one hand they largely avoid sexual reproduction by producing asexual seed, but on the other hand the apomictic “species” in some genera appear to hybridize quite freely. The sexual pathways apparently always coexist with apomixis (Nogler, 1984), and hybridization can result either through fertilization of meiotically reduced egg cells, or through fertilization of unreduced apomictic egg cells to give new higher ploidy levels (Clausen, 1961). However, diploid-diploid hybridization has frequently been implicated in the origin of apomixis, or its influence has been assumed (e.g., Camp, 1942; Stebbins, 1980; de Wet, 1987; Carman, 1997). In the genera of Pyrinae, there is little evidence that we are aware of that apomixis has arisen subsequent to hybridization of diploids, as Camp surmised (1942). An alternative model, championed by Fagerlind (1944), emphasized the potential for gametophytic apomixis to facilitate hybridization and polyploidy and inferred the existence of diploid (possibly non-hybrid) apomicts. The suggestion of diploid apomixis, whether as a rare phenomenon in otherwise sexual plants, or in rare apomictic diploids, is attractive because apomixis occurs in several related genera of the subtribe, which suggests that some genetic predisposition to apomixis pre-dates the differentiation of these genera. However, the strikingly greater prevalence of apomixis in polyploids, which has been observed not just in the Pyrinae but in all apomictic Angiosperms that have been studied, requires a separate explanation.

We conjecture that the taxonomic complexity of some of the apomictic genera in Pyrinae might be an interesting feature, possibly reflecting (at least in part) speciation through hybridization, rather than reflecting greater evolutionary age or differences in taxonomic perspective. All genera of Pyrinae wherein gametophytic apomixis occurs include diploids and polyploids (Kalkman, 2004). Ploidy levels higher than 4x appear to be very rare (rare pentaploids and/or hexaploids have been noted in *Malus*, *Cotoneaster*, and *Crataegus*; Gu & Spongberg, 2003; Kalkman, 2004; Talent & Dickinson, 2005), which suggests that fertilization of meiotically unreduced egg cells might be rare (or alternatively that chromosome loss regularly occurs or that high ploidy levels confer low viability). In *Sorbus* s.l., the genus whose species origins are best understood, hybridization involving either one or two copies of the *S. aria* genome appears to be related to the spread of apomixis (Liljefors, 1953; Nelson-Jones & al., 2002). Some of these hybridizations clearly involve fertilization of unreduced female gametes in apomictic triploids to produce tetraploids (Robertson & al., 2004). We speculate that differences between apomic-
Talent & Dickinson

Tic genera in their propensity to hybridize might relate to differences in endosperm formation, and hence in the availability of the second sperm to fertilize the egg. Savidan (2000) has pointed out that the Ranunculus-type of endosperm formation\(^1\) is an example of a mechanism that, by sequestering both sperm, prevents fertilization of the egg cell. We have sought to obtain data on endosperm formation in Crataegus in order to address this question.

**TAXONOMIC PROBLEMS ASSOCIATED WITH APOMIXIS IN CRATAEGUS**

Taxonomic complexity in Crataegus is well-documented (e.g., Phipps & Muniyamma, 1980) and derives in part from the very large number of names published, mainly as a result of the activities of North American taxonomists in the period 1896–1910, and in part from the relatively limited palette of ecological specialization and morphological variation that the genus exhibits. Current taxonomic opinion attempts to grant species status to groups of related apomictic or partly apomictic variants rather than to individual morphotypes, and with this conservative approach there are probably only 140–200 species worldwide, distributed between about 40 series, or a smaller number of more inclusive sections (Phipps & al., 2003). Taxonomic complexity has been linked to hybridization (Phipps & al., 2003), and to the occurrence of gametophytic apomixis and polyploidy (reviewed in Dickinson, 1999). In the latter argument apomixis and self-compatibility (the gametophytic self-incompatibility of diploids breaks down in polyploids; Dickinson & Phipps, 1986; Smith & Phipps, 1988; Dickinson & al., 1996; Macklin, 2001) mean that distinctive genotypes can reproduce with little or no genetic change from one generation to the next. Combined with the ecology of the genus—vertebrate endozoochory based on fleshy, few-seeded fruits often produced in large numbers, and seedling establishment largely restricted to disturbed sites—the result is that such genotypes may be encountered in large numbers, often many of them together at a single site. Such local aggregations (topodemes) can thus give the appearance of a panmictic population if the prevailing breeding system is not taken into account (Dickinson & Campbell, 1991; Dickinson, 1999). North American black-fruited hawthorns (Crataegus section Douglasianae Loudon) provide an example of this situation that we and our co-workers are currently studying.

North American black-fruited hawthorns include three taxa that appear to consist exclusively (C. brachycantha Sarg. & Engelm. and C. saligna Greene, both currently placed in series Brevispinae Beadle by Phipps & al., 2003) or partially [C. suksdorfii (Sarg.) Kruschke, as currently understood, in series Douglasianae

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\(^1\)The Ranunculus-type endosperm forms from the central cell with two unreduced polar nuclei, and both sperm, so that a tetraploid apomict forms 12x endosperm. We suggest later, however, that it may be inappropriate to ignore the proportion of 10x endosperm formed with a single sperm. The proportion of the different endosperm types was seen to be under independent genetic control from other aspects of apomixis in Ranunculus (Nogler, 1972).
(Loudon) Rehd.) of diploid individuals (Talent & Dickinson, 2005). All three share the apparently plesiomorphic stamen-number state of approximately 20 stamens per flower. Molecular phylogenies (Lo & Dickinson, 2005; E. Lo, unpubl.) indicate that *C. brachycantha* is distantly related to the other two species, however, and so it will not be considered further here. *Crataegus saligna* and *C. suksdorfii* are allopatric, whereas *Crataegus saligna* is sympatric with *C. rivularis* Nutt. *Crataegus suksdorfii* comprises diploids, triploids and tetraploids and is sympatric with the much more widely distributed *C. douglasii* Lindl. *Crataegus douglasii* and *C. rivularis* are both black-fruited and belong in series *Douglasianae* as well, but both have been shown to be tetraploids (Talent & Dickinson, 2005); both exhibit the apomorphic state of having approximately 10 stamens per flower. While *C. rivularis* appears to be morphologically uniform across its range (Phipps, 1999), recent fieldwork in the Pacific northwest has suggested that variants of *C. douglasii* warrant recognition as species (Phipps & O’Kennon, 1998, 2002). This is reminiscent of the early twentieth century phase of *Crataegus* taxonomy in that the emphasis is placed on drawing attention to morphological distinctness while information about breeding system and ploidy level is lacking. Both gametophytic apomixis and self-compatibility have been demonstrated in *C. douglasii* (Dickinson & al., 1996). Variation in molecular markers in *C. douglasii* and some of its variants is currently being studied (E. Lo, unpubl.), but given that there is evidence along the lines described below that these variants reproduce apomictically (Fig. 1; Talent, 2006), their recognition as species warrants re-examination.

**POLYPLOIDY IN CRATAEGUS**

In numerous North American taxonomic series, morphologically distinct sexual diploid and apomictic polyploid “species” co-occur; some “species” consist of multiple ploidy levels (e.g., *C. suksdorfii* in series *Douglasianae*), but others that are geographically widespread seem to consist of only one ploidy level (Muniyamma & Phipps, 1985; Talent & Dickinson, 2005). Diploid-diploid hybridization is well known in Europe (Raunkiaer, 1925; Christensen, 1992a, b, 1996), but it is unclear whether it commonly occurs in North America (Talent & Dickinson, 2005). It is also currently unclear whether apomixis is as widespread among Eurasian *Crataegus* as in North America, though aposporous embryo sacs have been noted in European triploids (Ptak, 1989). Therefore, we chose to reinvestigate and to challenge the established wisdom (Camp, 1942) that diploid-diploid hybridization increased the incidence of meiotically unreduced gametes, from which largely sterile triploids formed a foundation for the evolution of apomixis as an escape from sterility. Camp’s model has considerable plausibility, but was built on an assumption of widespread diploid hybridization, with triploid and subsequent tetraploid derivatives of these hybrids. Our initial questions were to investigate polyploidy within related (taxonomic) groups, and to check whether triploids could be found among diploid-diploid hybrids in our sample.
Fig. 1. Comparison of DNA measurements from endosperm of seeds with tetraploid embryos from tetraploid *Crataegus* in six taxonomic series¹.

¹From seeds from tetraploid mother plants we selected those with tetraploid embryos (embryo DNA measurements by flow cytometry in the range 2.74 to 3.34 pg; Talent & Dickinson, 2005). Only those seeds that yielded both embryo and endosperm signals were retained, while six seeds with hexaploid embryos and six seeds that might have had an octaploid endosperm (which was not distinguishable from a G2 signal from a tetraploid embryo) were omitted. The sample of 213 seeds includes the taxonomic series *Douglasianae* (*C. douglasii*, *C. rivularis*, *C. castlegarenis* J.B. Phipps), *Purpureofructi* (*C. okennonii* J.B. Phipps), *Rotundifoliae* (*C. chrysocarpa* Ashe), *Molles* (*C. submollis*), *Crus-galli* (*C. crus-galli* var. *pyracanthifolia*), and *Macracanthae* (*C. macracantha* Lodd. ex Loudon). Most seeds were from open pollination, but since these were scarce, 35 self-pollinated seeds of *C. macracantha* and the eight seeds that resulted from intra-species, intra-ploidy handpollinations of *C. crus-galli* were included. The DNA measurements from the endosperm were standardized by adjusting the DNA amount of the embryo to the midpoint of the estimated range for tetraploid *Crataegus* (3.04 pg; Talent & Dickinson, 2005). The flow-cytometric DNA measurements from mature seeds were converted to ploidy levels using information from leaf tissue (Talent & Dickinson, 2005):

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\text{Endosperm}_{\text{adjusted}} = \frac{\text{Endosperm}_{\text{measured}} \times \text{DNA mid/point}}{\text{Embryo}_{\text{measured}}}
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DNA measurements by flow cytometry from most of the 40 taxonomic series in *Crataegus* proved to be comparable, although there is some small variation in C-values (Talent & Dickinson, 2005). Many of the published chromosome counts in *Crataegus* are approximate (Meyer, 1915; Longley, 1924), and chromosome counts are sometimes considered unreliable because of the small number of cells examined, because seedlings have been used as surrogates for the parent plants, and because of uncertain identifications or inadequate taxonomic coverage (Merxmüller, 1970; Nelson-Jones & al., 2002; Funk & al., 2005). Nevertheless, we found that a reexamination of the older data was extremely valuable. The old data combined with our flow cytometric survey give a very consistent picture of multiple taxonomic series where diploids, triploids, and tetraploids coexist. To the single published higher (hexaploid) chromosome count (Gu & Spongberg, 2003), we added evidence of pentaploids. Comparison of ploidy levels with morphology suggests that both autopolyploidy and allopolyploidy might be contributing to the taxonomic complexity.

We did not identify any further instances of diploid-diploid hybridization in North America, and consequently surveyed only two Euro-American hybrid complexes (*C. monogyna* Jacq. × *C. punctata* Jacq, 10 trees; *C. monogyna* × *C. suksdorfii*, 11 trees). The cytometric measurements confirmed the limited data from chromosome counts (Wells, 1985) that these hybrids were diploid like their parents. Thus we have not yet uncovered any evidence to support Camp’s (1942) view that later generations of hybrids derived from diploids may contain triploid individuals. Rather, the survey data tended to support the view that many triploids are morphologically similar but not identical to tetraploids, and might represent diploid-tetraploid hybrids.

**SEED FORMATION IN CRATAEGUS**

The requirement of many (pseudogamous) apomicts for fertilization of the endosperm is a puzzle that has received insufficient attention, particularly in those taxa like the Rosaceae whose embryo sacs have the same morphology whether they are sexually or apomictically derived (Nogler, 1984; Czapik, 1996; Savidan, 2000). Pollen meiosis is usually normal (Nogler, 1984), and this is also true of *Crataegus* (Longley, 1924; Muniyamma & Phipps, 1979b; Dickinson & al., 1996). The mismatch between the unreduced female gametes and the reduced male gametes means that the requirement with a *Polygonum*-type embryo sac for a 2:1 ratio of maternal to paternal genome copies in the endosperm is bypassed in some way, but little is known about how this occurs. Some Rosaceous apomicts (including *Amelanchier* and *Cotoneaster* in Pyrinae) differ from their diploid sexual relatives in that the polar nuclei do not fuse before fertilization (Liljefors, 1953; Hjelmqvist, 1962; Czapik, 1983, 1985a, b; Campbell & al., 1985, 1987; Jankun & Kovanda, 1987; Campbell & Wright, 1996). Although in some cases the fusion of the second polar nucleus has been seen to occur after fertilization (Jankun & Kovanda, 1987),
it has been assumed that non-fusion prevails in some species and that the resulting ratio of maternal to paternal genome copies in the endosperm is 2:1 as in diploids. The situation in _Crataegus_, however, was not understood because fusion did not occur before fertilization in both apomictic polyploid and sexual diploid species (Muniyamma & Phipps, 1979a, 1985).

_Crataegus_ is difficult to work with in many ways: as well as the other difficulties of the woody Rosaceae, it is difficult to propagate by seed (Flemion, 1938), and its low seed set means that embryological observations may be a poor indicator of its actual mode of reproduction (Dickinson, 1983). However, its seeds are moderately large and have abundant and persistent endosperm (Péchoutre, 1902; Corner, 1976, Aldasoro & al., 2005). We found that the ploidy levels of both embryo and endosperm can be established by flow cytometry of the entire tissue (Talent, 2006), and that endoreduplication in the tissue represents a small number of cells (similar to results from other genera; Bino & al., 1993; Matzk & al., 2000). We therefore used flow cytometry to imitate the experiments on endosperm ploidy levels that have been so informative about _Ranunculus auricomus_ (Rutishauser, 1954; Nogler, 1972), and to test whether inter-ploidy pollinations produce inter-ploidy crosses.

All of the diploid _Crataegus_ studied (four North American species and one European species) had the triploid endosperm that indicates the contribution of one sperm and two central-cell nuclei (Talent, 2006). Some of these diploids (_C. punctata_ Jacq. and _C. suksdorfii_) are considered closely related to apomictic polyploids (_C. crus-galli_ L. and _C. douglasii_, respectively), so it appears unlikely, as in the other plants where gametophytic apomixis has been studied (Nogler, 1984), that a change in the derivation of the endosperm in diploids is a necessary pre-disposing factor to the evolution of polyploid apomixis.

Earlier experiments have shown that pollination is required for seed formation in apomictic tetraploid _Crataegus_ (Dickinson & Phipps, 1986; Smith & Phipps, 1988) including _C. crus-galli_ L. var. _pyracanthifolia_ (G. Don) Wood, one of the taxa included in our endosperm measurements. In the triploid and tetraploid apomicts, we did not find evidence of autonomous endosperm with a ploidy level to match the meiotically unreduced central cell, and conclude that such occurrences are at least rare1. Instead we found mostly 10x endosperm from tetraploids (Talent, 2006) and 8x endosperm in triploids pollinated from tetraploids (Talent, 2006). The most likely origin for the endosperm is therefore from meiotically reduced pollen and a meiotically unreduced embryo sac with a binucleate central cell (Nogler, 1984), the same derivation as expected and inferred from ploidy-level data from the diploid species. Comparison of endosperm ploidy levels in seeds produced using pollen from diploids and from tetraploids on the same mother plant support-

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1We found it necessary for good calibration of the endosperm signal to measure it in the same sample preparation as the embryo from the same seed, and this technique means that a signal from the endosperm could be overlooked if it happened to occur at a multiple of the basic embryo signal. The seeds where this possibility arose are likely to represent poor tissue preparation in which the endosperm was destroyed; they constitute close to 10% of samples. However, when the embryo and endosperm were prepared separately, no endosperm was discovered that measured the same as or double the embryo signal.
ed the inference that fertilization by a single unreduced sperm is involved (Talent, 2006). We do not know whether the seeds with the apparently unbalanced 10x endosperm (with a 4:1 ratio of maternal to paternal contributions) are capable of germination.

A minority of the seeds of tetraploid Crataegus had 12x endosperm (25% 12x; 60% 10x; Fig. 1), which might mean that endosperm-formation sequestered both sperm, or that a single meiotically unreduced sperm sometimes effects fertilization, or that endosperm formed from a trinucleate central cell without fertilization. For these and the exceptional high-ploid endosperm, it would be helpful to confirm that fertilization has occurred by demonstrating genetic differences between the endosperm in different ovules (similar to the experiments that have already been done with other pseudogamous apomicts; Nogler, 1984), but experiments showing that pollen from diploids produced a greater proportion of 10x endosperm tend to support the fertilization inference (Talent, 2006).

From tetraploid Ranunculus auricomus, Rutishauser and Nogler observed more 12x than 10x endosperm (69% 12x; 22% 10x; Nogler, 1984), and Nogler (1972) demonstrated that the proportion differed depending on the maternal genotype. The Ranunculaceae and the Rosaceae are not closely related in the Eudicots (Stevens, 2001–onwards), but a larger survey of aposporous Crataegus would be a useful step towards assessing whether both sperm contribute to the endosperm in a proportion of seeds that varies between different individuals or different taxa. If both sperm are required to produce a “balanced” endosperm in certain species, then hybridization through fertilization of the egg cell would not be possible (Savidan, 2000).

Two individuals of a typically tetraploid species (C. submollis Sarg.) were found to produce about 40% of seeds with 6x rather than 10x or 12x endosperm. Pollination experiments using pollen from an unrelated diploid (C. punctata) produced seeds with triploid embryos, confirming that the mother trees are partly sexual. This same pollination produced a small proportion (10%) of diploid embryos, and the endosperm of these seeds was pentaploid, indicating that parthenogenesis occurs within sexual embryo sacs and that these embryos are dihaploids. In this tetraploid, fertilization of a meiotically unreduced egg is moderately common, and pentaploid and hexaploid embryos can result depending on the ploidy level of the pollen source. Thus, the ploidy levels of embryos show a mixture (diploids, triploids, tetraploids, with rare pentaploids and hexaploids) similar to that found in the surveys of adult trees in the genus as a whole. The reproductive behaviour of the pentaploids and hexaploids is as yet unknown.

Interploidy pollinations varied in their success rates. Some diploid-tetraploid pollinations produced triploids, and some triploid-diploid pollinations produced tetraploids (Talent, 2006). We deduce that a major change in endosperm-balance number with polyploidy (triploidy or tetraploidy) permits these inter-ploidy hybridizations, possibly due to the general relaxation of endosperm-balance requirements due to the high ploidy level of the central cell that was inferred by Quarin in polyploid Paspalum notatum Fluegge (Quarin, 1999). It appears likely
that gene flow from sexual diploids via apomictic triploids to apomictic tetraploids is possible in *Crataegus*. More work is needed to confirm that diploid-tetraploid and triploid-diploid crosses occur in the wild, to check for pollen compatibility between a wide range of taxa, and to study how flowering times overlap. In series *Douglassianae* such studies will shed light on the relationship between the 10- and 20-stamen *Crataegus* found in mixed populations (tetraploid and triploid, respectively, or both tetraploid; Talent & Dickinson, 2005; E. Lo, unpubl.). These studies will also help clarify the origin of isolated, high elevation topodemes of exclusively triploid individuals currently identified as *C. suksdorfii* (Dickinson & al., unpubl.).

It is still unclear how many times apomixis has arisen in *Crataegus*. Most of the tetraploids studied were near-obligate apomicts, but a small proportion of sexual embryo sacs occurred (perhaps 1%). We would expect that these sexual embryo sacs, as well as those in the partly sexual *C. submollis*, could hybridize with other tetraploids. There is currently no direct evidence that tetraploid-tetraploid hybridization occurs, although taxonomists have long suggested that some tetraploid forms are hybrids between other tetraploids (e.g., Eggleston, 1923). We also have no data as yet about whether tetraploids can result directly from diploids either from two unreduced gametes or from somatic polyploidization. We also do not know whether the dihaploid seeds from *C. submollis* can grow into fertile adults, perhaps entering into hybridization with other diploids or triploids. Harlan & Cielarier (1961) concluded that in the Panicoid grasses “the net effect of apomixis is as a long-range binding force, permitting gene exchange among widely divergent materials and knitting together large groups which would drift apart if sexual reproduction alone were operating”. The genus *Crataegus* would be a very suitable subject for testing whether apomixis can have such a binding effect in a genus of Pyrinae.

**MUCH-NEEDED TOOLS**

We now have a better understanding of the interbreeding of diploids, triploids, and tetraploids in *Crataegus*, and a strong suggestion that the many triploids in the genus are a conduit for gene flow from diploids to tetraploids. Flow cytometry for ploidy-level determination has become one of the much-needed tools that, in parallel with molecular data, will enable us to elucidate relationships within and between populations. Flow cytometry also permits us to assess whether the effective breeding system of individual plants is sexual or apomictic (to the stage of mature seed) from the ploidy level of the endosperm tissue. With those seed data in hand, microscopic examination of embryo-sac development and microdensitometry can now be undertaken in a new light. The confusion of multiple embryo sacs in young ovules (Muniyamma & Phipps, 1979a) can now be usefully compared to mature seeds, opening the way for experiments that compare early to late developmental stages and evaluate, for example, the effects of different pollen sources.
Crataegus is certainly an excellent subject for apomixis research, but its taxonomy is currently in a nearly unmanageable state. There are good revisions of most of the species from Europe and Asia, but there is considerable confusion surrounding both the biology and the nomenclature of the North American taxa, that is only now in the process of being resolved (Phipps & al., 2003). In the quest for a phylogeny, traditional taxonomy and nomenclature are an essential tool. Eugenia Lo is now constructing phylogenies (Lo & Dickinson, 2005) using coding and non-coding DNA sequences. There is considerable concordance between chloroplast and nuclear trees that supports an overall hierarchical structure in the genus; a detailed investigation of reticulate relationships, however, is just beginning. These recent successes with data gathering suggest that Crataegus can soon emerge from the ignominious status of a taxonomic nightmare to become the provider of numerous separate groups where the evolution of asexuality can be compared.

APOMIXIS IN PYRINAE

We have a tentative explanation for the taxonomic complexity of Crataegus in the successful crosses that produce triploids from diploids (pollinated from unrelated tetraploids) and tetraploids from triploids (pollinated from unrelated diploids) (Talent, 2006). We may also be close to an explanation for what appears to be the relative taxonomic simplicity of some smaller apomictic genera of Pyrinae (Amelanchier, Malus, possibly Photinia; Kalkman, 2004), but much more comparative work with the different genera is needed.

In some apomictic genera of Rosoideae (Czapik, 1985b) and in some apomictic Amelanchier (Pyrinae; Campbell & al., 1987; Campbell & Wright, 1996), the polar nuclei do not fuse to form a secondary nucleus before fertilization, and this contrasts with the nuclear fusion of some related sexual species. It was thought that a 2:1 ratio of maternal to paternal genome copies in the endosperm was thereby preserved with apomixis, and that the endosperm from a tetraploid apomict would be hexaploid. In Crataegus, where non-fusion was observed in both apomictic polyploid and sexual diploid species (Muniyamma & Phipps, 1979a, 1985), the data from mature seed indicate that nuclear fusion did occur. Similar measurements from mature seeds of other Pyrinae are needed to test whether one of the polar nuclei can be omitted from the endosperm of any of the other genera.

If, as we suspect, the polar nuclei of tetraploid aposporous Amelanchier never fuse, unlike those of Crataegus that apparently fuse at some point close to fertilization, then the fate of the second sperm in Amelanchier needs to be investigated. If the second sperm fertilizes the second polar nucleus to produce a compound hexaploid or 12x endosperm, then this would imply that no sperm is available to fertilize the egg cell. If instead the second polar nucleus degenerates (as in some eight-nucleate embryo sacs of Dichanthium annulatum (Forssk.) Stapf; Reddy & D’Cruz, 1968), then the second sperm might be available. The predominance of 10x endosperm in Crataegus and our data to show that the egg cell can be fertilized,
might therefore offer a clue that “the Crataegus problem” (Eggleston, 1910; Palmer, 1932; Camp, 1942) is the result of a particularly flexible solution to the endosperm-balance problem with pseudogamous apospory. Thus, hybridization in this genus may be largely a result, rather than the trigger, of apomixis. On the other hand, if the fertilization of unreduced egg cells in triploids does not occur in some other genera, then gene flow from sexual diploids via apomictic triploids to apomictic tetraploids would be blocked. The implications for tokogenic and cladogenic processes of such a difference in potential gene flow are undoubtedly complex. However, a hint that there is a difference in the conduits for gene flow within genera such as *Amelanchier* and *Crataegus* may be what we see when individual taxonomists attempt to compare the taxonomic complexity of genera (e.g., Robertson & al., 1991; Kalkman, 2004).

If there are indeed two different solutions to the endosperm-balance problem with apospory in the Pyrinae, this would seem to indicate independent origins of at least this component of apomixis in each genus, rather than a suite of apospory and endosperm genes ready to be activated by, for example, polyploidization. On the other hand, there is a possibility that apomixis has arisen only once or a few times per genus (as may be the case in *Sorbus*; Liljefors, 1953; Nelson-Jones & al., 2002). Early embryology may be showing us a poorly canalized process that is less comparable between genera than the final product. There is a need for ploidy-level data from the endosperm and embryo of mature seeds of other Pyrinae comparable to the data that we are obtaining from *Crataegus*. By comparing which of the mechanisms of apomixis can produce a mature seed, we may gain a better understanding of the evolution of apomixis in the genera of Pyrinae, and of the processes of cladogenesis in the subtribe. Ultimately, the results have the potential to also shed light on the origin of the Pyraeae through polyploidization.

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