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Breeding Systems in Diploid and Polyploid Hawthorns (*Crataegus*): Evidence from Experimental Pollinations of *C. monogyna*, *C. subsphaerica*, and Natural Hybrids

Dominika Vašková * and Vladislav Kolarčik

Institute of Biology and Ecology, Faculty of Science, P. J. Šafárik University, Mánesova 23, SK-041 54 Košice, Slovakia; vladislav.kolarcik@upjs.sk

* Correspondence: vaskova.nika610@gmail.com

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Abstract: Background and Objectives: Polyploidisation and frequent hybridisation play an important role in speciation processes and evolutionary history and have a large impact on reproductive systems in the genus *Crataegus*. Reproductive modes in selected diploid and polyploid taxa in eastern Slovakia were investigated and analysed for the first time. Materials and Methods: Diploid, triploid, and tetraploid hawthorns were tested for self-pollination, self-compatibility, and self-fertilisation. Pollination experiments were performed within and between diploid and triploid species to determine the possibilities and directions of pollen transfer under natural conditions. Seeds from crossing experiments and open pollinations were analysed using the flow cytometric seed screen method. Results: These experiments demonstrated that sexual reproduction, cross-pollination, and self-incompatibility are typical of the diploid species *Crataegus monogyna* and *C. kyrtostyla*. Seeds produced by self-fertile tetraploid *C. subsphaerica* were derived from both meiotically reduced and unreduced megagametophytes. Conclusions: Experimental results concerning triploid *C. subsphaerica* and *C. laevigata* × *C. subsphaerica* are ambiguous but suggest that seeds are almost exclusively created through apomixis, although a few sexually generated seeds were observed. In the genus *Crataegus*, pseudogamy is a common feature of polyploid taxa, as in all cases pollination is essential for regular seed development. Research Highlights: We suggest that all studied *Crataegus* taxa produce reduced pollen irrespective of ploidy level. Moreover, we emphasise that triploids produce apparently aneuploid pollen grains as a result of irregular meiosis. They are also capable of utilising pollen from 2*x*, 3*x*, or 4*x* donors for pseudogamous formation of endosperm.

Keywords: breeding systems; *Crataegus*; flow cytometry; gene flow; pollination; polyploidy; pseudogamy; self-fertilisation

1. Introduction

Polyploidisation and hybridisation have played an important role in the evolution and speciation of angiosperms [1–8]. Both processes can often affect not only genomic, morphological, and physiological properties in plants [9] but are also associated with changes in the reproductive system [10–14]. In certain species, the shift from diploid to polyploid biotypes can be correlated with changes from sexuality to asexuality [12,15,16] or from self-incompatibility to self-compatibility [17–22]. Most asexually reproducing plants are polyploids and possibly hybrids [23,24]. Sexual reproduction has several benefits in the formation of genetic variability, which allows adaptations to ecological and climatic changes and improves survivability [25,26]. Despite these advantages, many plants, mainly in the families *Asteraceae* Bercht. et J. Presl, *Poaceae* Barnhart, and *Rosaceae* Juss., reproduce apomictically [12].

The success of apomictic plants is mainly because of their ability to occupy more geographical areas than their sexual progenitors [15,27–29], to fix successful genotypes [30], and to prevent reduced fitness because of loss of recombination and infertility in odd polyploids [14]. Apomixis (agamospermy) is an asexual reproduction via seeds, resulting in a progeny genetically identical to the maternal plant [31]. It is well known as a way to maintain hybrid genotypes and facilitate reproductive isolation from parental taxa [22,32]. Therefore, apomixis is essential for the formation of new species arising by polyploidy and hybridisation [12,15,33].

Extensive hybridisation, polyploidy, and apomixis have been observed in *Crataegus* L. (hawthorn), a monophyletic genus [34], which belongs to the family *Rosaceae*, subfamily *Amygdaloideae* Arn., tribe *Maleae* Small. [35] and subtribe *Malinae* Reveal [36–38]. The genus *Crataegus* is taxonomically complex and the number of species varies between 150 and 1200, depending on the interpretation of species boundaries by different authors [39–43]. The morphological differences between many species are relatively minor [44,45]. Hawthorns are deciduous, small trees and ruderal shrubs primarily distributed in the temperate regions of Europe, Asia, Africa, and North America [41,42]. Hawthorns grow abundantly in regions with high light intensity, alongside rivers or lakes or in anthropogenic areas. Flowers are insect-pollinated, and small, fleshy fruits containing 1–5 seeds [42,46] are an important source of food, not only for different kinds of animals but also for humans [47,48]. Seeds are produced either by allogamy, which is common in diploid hawthorns, when self-fertilisation is prevented by gametophytic self-incompatibility [18,49], or by autogamy and apomixis common in polyploids [50]. Gametophytic self-incompatibility found in the family *Rosaceae* [51–53] is a genetically conditioned biochemical reaction used by plants to prevent self-fertilisation and consequent inbreeding depression [54].

For the genus *Crataegus*, the occurrence of diploids, triploids, and tetraploids prevails, although pentaploids and one hexaploid have been recorded [55]. Seeds of diploid hawthorns are known to form sexually with triploid endosperm tissue; having the Polygonum type of embryo sac, the endosperm develops from fertilisation of the binucleate central cell [56]. In contrast, triploids and tetraploids primarily reproduce by gametophytic apomixis [10]. In unreduced megagametophytes with the Polygonum type morphology [57], an embryo develops from an unreduced egg parthenogenetically, either without (autonomously) or with pollination where fertilisation of polar nuclei by one or two sperm cell nuclei is essential (pseudogamy) for successful development of endosperm [18,31,58,59]. Many angiosperms require balanced dosages of maternal-to-paternal genome contributions (2m:1p) to the endosperm for successful seed development [14,60]. Overrepresentation of either parental genome could affect embryo and endosperm sizes and lead to endosperm failure and consequently, seed abortion [61,62]. This problem is bypassed in many apomictic *Asteraceae*, where the development of endosperm does not require fertilisation [31], whereas in some apomictic *Poaceae*, megagametophytes contain one rather than two central cell nuclei [63]. Fertilisation of the central cell by one or both sperm cells seems to be necessary for apomictic polyploid *Crataegus* [10]; thus, the endosperm balance requirement appears to be relaxed or absent in apomicts [11].

Extensive research on the genus *Crataegus* (e.g., sect. *Coccineae* Loudon and sect. *Douglasia* Loudon) has been conducted in North America [10,11,45,50,55,64–68], including the first pollination experiments on hawthorns described by Brown [69] and Love and Feigen [70]. Mating systems in European hawthorns have been studied with the emphasis mainly on hybridisation [71–74] and on embryology [75,76]. We present an analysis of reproductive systems in *Crataegus* sect. *Crataegus* occurring in Slovakia (central Europe), where we performed controlled and open pollinations between taxa of diploid and triploid ploidy levels and tested for self-pollinations of tetraploid species to determine the mating system of seed progeny, self-compatibility, and pollen transfer between different ploidy levels. Consequently, the ploidy levels of the embryo and endosperm were evaluated by the flow cytometric seed screen (FCSS), which can reveal whether the embryo sac originated meiotically or apomeiotically and whether fertilisation of the egg cell and central cell had occurred. To further understand the fertilisation process in megagametophytes, the ploidy level of the pollen was determined.

2. Materials and Methods

2.1. Plant Material

The mother trees used for pollination experiments in this paper, *Crataegus monogyna* Jacq., *C. subsphaerica* Gand. s.l., *C. kyrtostyla* Fingerh. (*C. monogyna* × *C. subsphaerica*), and hybrid *C. laevigata* × *C. subsphaerica*, originated from three localities in eastern Slovakia (Table 1): Košice, Botanical garden of Pavol Jozef Šafárik University (population code BOTZ), Prešov, south-eastern margin of village Hermanovce (GOCAL), and north-eastern margin of village Hermanovce (PS-VYH, VYH). Trees were selected for their accessibility and had inflorescences easily reachable from the ground. Voucher specimens of 13 trees (out of 14) used in our experiments are deposited in the herbarium of Botanical Garden of P. J. Šafárik University in Košice (KO). The ploidy levels of all trees and their pollen were investigated (see below). Fruits produced after pollinations were collected from all 14 individual trees. In total, 232 seeds were analysed from 248 mature fruits collected.

Table 1. Number of seeds produced by controlled (Contr. Poll.) and open pollinations (Open Poll.) which were analysed using the flow cytometric seed screen (FCSS) and localities of the *Crataegus* individuals analysed in the study. Cytotype diversity at sites based on Kolarčik et al. (unpublished).

Locality, Date of Experiments, Population Codes, Cytotype Diversity [%]	Taxon	No. Trees	FCSS No. Seeds	
			Contr. Poll.	Open Poll.
Slovakia, Prešov, south-eastern margin of village Hermanovce, 49°02′31.7″, 21°01′15.3″; 30.5.2017–31.5.2017; GOCAL; 2*x*–0.00, 3*x*–34.04, 4*x*–65.96 (*n* = 47)	*C. kyrtostyla* (2*x*)	2	4	11
	C. subsphaerica (3*x*)	1	7	5
Slovakia, Prešov, north-eastern margin of village Hermanovce, 49°03′06.8″, 21°00′22.5″; 30.5.2017-31.5.2017; VYH, PS-VYH; 2*x*–30.00, 3*x*–20.00, 4*x*–50.00 (*n* = 10)	*C. laevigata* × *C. subsphaerica* (3*x*)	2	0	8
	C. subsphaerica (4*x*)	5	21	30
Slovakia, Košice, Botanical garden of Pavol Jozef Šafárik University, 48°44′09.3″, 21°14′08.6″; 27.4.2017–2.5.2017; BOTZ; 2*x*–88.89, 3*x*–11.11, 4*x*–0.00 (*n* = 9)	*C. monogyna* (2*x*)	2	49	41
	C. kyrtostyla (2*x*)	1	2	20
	C. subsphaerica (3*x*)	1	12	22
TOTAL		14	95	137

2.2. Determination of Ploidy Level of Crataegus Mother Trees and Their Pollen

Flow cytometry (FCM) was used to establish the ploidy levels of 14 hawthorn trees and their pollen. Sample preparation for flow cytometry was done as per the modified protocol of Loureiro et al. [77]. Briefly, cell nuclei were simultaneously isolated from the leaves or petioles of target individual and the reference standard *Solanum lycopersicum* L. 'Stupnické polní tyčkové rané' (2C = 1.96 pg DNA, [78]) in 1 mL of general purpose buffer (GPB, [77]) in Petri dish by using the chopping technique [79]. The suspension was then filtered through a 42 µm nylon filter and supplemented with 2 µL β-mercaptoethanol, 30 µL RNAse and 30 µL propidium iodide. After approximately 30 min incubation at 4 °C, the fluorescence signal was measured by using a Partec CyFlow ML (Partec Gmbh, Münster, Germany) flow cytometer (Institute of Biological and Ecological Sciences, P. J. Šafárik University in Košice, Slovakia) equipped with a 532 nm (150 mW) green laser. Measurements were performed with FloMax ver. 2.70 (Partec Gmbh, Münster, Germany). The quality of histograms was ascertained according to the following criteria: 1300 particles recorded, CV below 5% and symmetric peaks of both sample and standard. DNA content (2C value) was determined according to the following formula: DNA amount in the sample = DNA amount in the internal standard × ((G_0/G_1 peak mean of sample)/(G_0/G_1 peak mean of internal standard)) (G_0/G_1 refers to the population of nuclei in G_0 or G_1 phases of the cell cycle). The ploidy level was then inferred on the basis of the obtained DNA amount interpreted with the help of the DNA amount ranges for the cytotypes provided by Talent and Dickinson [55]: 1.37–1.67 pg for diploids, 2.05–2.51 pg for triploids, 2.74–3.34 pg for tetraploids, and 3.42–4.18 pg for pentaploids.

Pollen was obtained from all studied mother trees, and nuclei were extracted using the general filter bursting method [80]. Whole flowers (3–5) with dehiscing anthers were placed in 0.5 ml GPB

and then vortexed for a few seconds. Flowers were removed, and the pollen suspension was passed through a first filter into a clean tube to prevent debris from entering the sample. This filter (100 µm) was large enough to allow pollen to pass through. The suspension was then passed through a second filter (30 µm), which had pore size small enough that the pollen grains were collected on it but nuclei could pass through. The pollen grains were then gently rubbed against the filter for 20 s, using a glass rod with a rounded end. GPB (0.5 ml) was added for the second time to help nuclei pass through the filter. Next, the nuclei were stained and incubated as described above and the samples were analysed using flow cytometry. To determine the ploidy level of pollen grains, the signal of somatic nuclei from the same individual was then used as a reference for pollen grain nuclei signals.

2.3. Pollination Experiments

Pollination experiments were performed between diploid and triploid *Crataegus* individuals (Figure 1) to observe pollen transfer between different ploidy levels. Flowers of two tetraploid *C. subsphaerica* trees were bagged in pollinator exclusion bags to prevent cross-pollination and to determine whether tetraploids are self-compatible and able to self-fertilise.

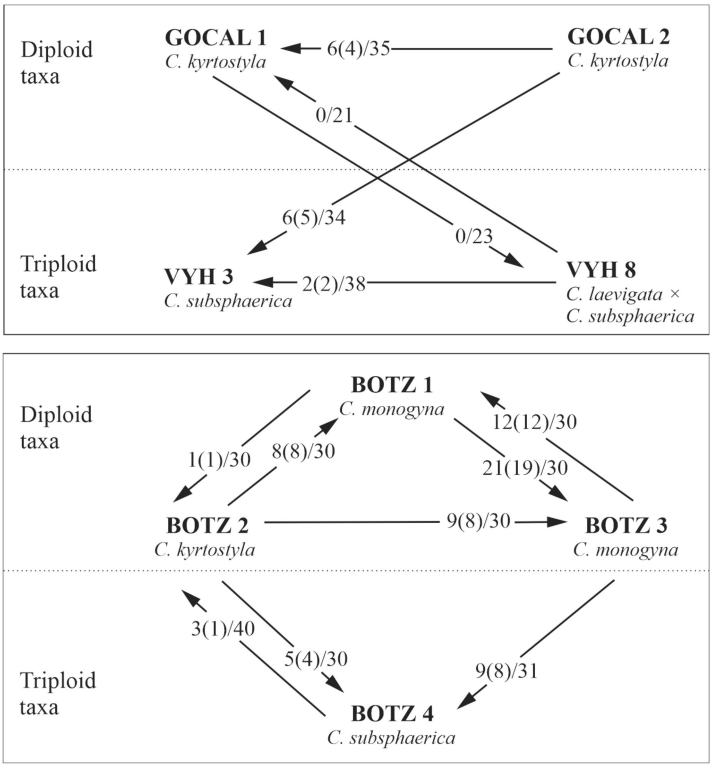

Figure 1. Schemes of pollination experiments on selected diploid and triploid *Crataegus* individuals. Arrows represent the direction of pollen transfer to the mother tree, and fractions indicate the number of seeds produced in all the treated flowers (number of seeds successfully measured using FCSS is given in brackets).

The pollinations included (a) self-pollination to test for self-incompatibility (intact flowers bagged and left for self-pollination); (b) tree pollinated by pollen of different cytotype (cross-pollination); (c) tree pollinated by pollen of the same cytotype but different species (cross-pollination); (d) tree pollinated by its own pollen (controlled self-pollination), and (e) flowers left for pollination without the use of pollinator exclusion bags (open pollination). Firstly, flowers at the 'balloon' stage (Figure 2) were collected from all studied trees, knowing cross-pollination had not happened yet. Flowers were left to dry overnight to release pollen, which was used for pollination the following day. Consequently, manual pollination of flowers (at the 'balloon' stage) was performed by brushing

dehiscing anthers against stigmas of mother trees until pollen could be seen on the surface of the stigma. Immediately after pollination, inflorescences were covered with pollinator exclusion bags to prevent cross-pollination. In these pollination experiments, emasculation was not done because it could cause parthenocarpy [79,81,82] and possibly affect reproduction [11]. Fruit formation was monitored, and fruits were collected when they turned red (ripe). Seeds were used to determine reproductive modes using the flow cytometric seed screen method [83]. In total, the number of pollinated flowers was 1354, whereas the number of flowers pollinated on one tree was approximately 180, and certain flowers were left for open pollination. Seeds from experimentally manipulated and open-pollinated flowers were counted and monitored for viability; only large, firm seeds filled with white embryo and endosperm were considered viable.

Figure 2. Flowers of *C. laevigata* × *C. subsphaerica* at different stages of opening; flowers at the 'balloon' stage before anthesis used for pollination experiments are indicated by white arrows.

2.4. The Flow Cytometric Seed Screen

The flow cytometric seed screen has become an important method for identification of reproductive modes in flowering plants [83]. It provides information about embryo sac origin by estimating embryo and endosperm DNA content. This method thus facilitates distinction between meiotically and apomeiotically developed megagametophytic, parthenogenetic, and zygotic origins of an embryo and autonomous or pseudogamous development of endosperm and detection of meiotic irregularities [82–84].

Flow cytometry was used to analyse 232 seeds from collected fruits. The seeds were either analysed whole (sample containing both embryo and endosperm tissue) or the endosperm and embryo were prepared separately. The same FCM protocol as described above was applied. The nuclei of endosperm may sometimes be hidden among nuclei of the reference standard on FCM records in cases when the whole seed is used for sample preparation. Therefore, one of the reference standards (*Solanum pseudocapsicum* L., 2C = 2.59 pg DNA [85]; *Solanum lycopersicum*) was used for the determination of embryo ploidy level. In few cases, the ploidy level of plants (sample + reference standard) was analysed via preparation of 'bulked' samples consisting of two, three, or even five individual seeds to accelerate the process. Ploidy levels determined in this way then served as a reference for the determination of endosperm ploidy level in separate endosperm + embryo measurements (without the reference standard). In cases where the whole embryo was used for the first sample, the second sample contained endosperm and reference standard tissue. Estimated DNA ploidies of endosperm and embryo were acquired and compared to determine the mode of reproduction for each analysed seed. The potential presence of endopolyploidy in seed tissues may generally compromise the results

of FCSS measurements [84,86]; however, this was shown to be minimal in hawthorn seeds, allowing accurate identification of embryo and endosperm nuclei on FCSS records [10].

3. Results and Discussion

3.1. Cytogenetic Screening of Mother Trees and Their Progeny

Analysis of the DNA content and ploidy level of both leaves and seeds yielded good-quality histograms with high-resolution peaks (Figure 3). Results of ploidy level determinations for mother trees are given in Table 1. Apart from 15 seeds where the embryo or endosperm signals were not detected (and therefore omitted from further analyses), endosperm and embryo ploidy levels could be very well distinguished (Supplementary Materials Table S1 and Table S2).

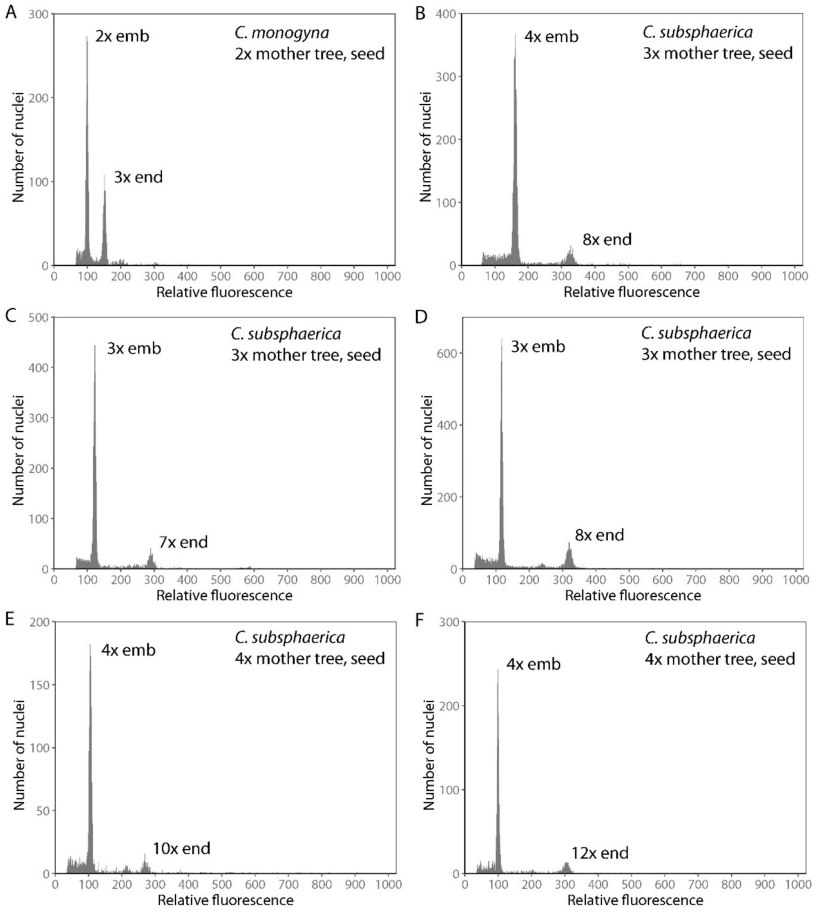

Figure 3. Selected flow cytometric histograms of *Crataegus* seeds that originated sexually (**A**,**B**) and apomictically (**C**–**F**). The first peak represents the embryo nuclei and their ploidy level and the second peak represents the endosperm nuclei and their ploidy level.

3.2. The Flow Cytometric Screen of Crataegus Pollen

FCM screening of pollen always allows recognition of two peaks, which correspond to the nuclei of vegetative and generative cells. The quality of pollen FCM records was slightly lower than that of FCM records of somatic cells. Clearly, diploid mother trees produced reduced pollen, as evidenced by the presence of 1*x* vegetative and 2*x* generative nuclei on FCM histograms (Figure 4; mitosis of the generative cell has already occurred in mature binucleate pollen grain typical for *Rosaceae* [80,87,88]). Similarly, polyploids also produced reduced pollen; we recorded approximately 1.5*x* vegetative and 3*x* generative nuclei in triploids and further, 2*x* vegetative and 4*x* generative nuclei in tetraploids. We did not record any '4*x*', '6*x*', or '8*x*' peaks on FCM histograms for diploid, triploid, and tetraploid

hawthorns, respectively, which could potentially correspond to generative nuclei of unreduced pollen. This indicates that unreduced pollen is extremely rarely produced in investigated hawthorns. To our knowledge, this is the first ploidy level investigation of pollen grains in *Crataegus* except the successful test on *C. macrosperma* Ashe performed by Kron and Husband [80]. Comparable results were obtained in *Sorbus* L., a genus with similar breeding systems to *Crataegus* [89]. Their data suggest production of reduced pollen and absence of unreduced pollen in studied taxa.

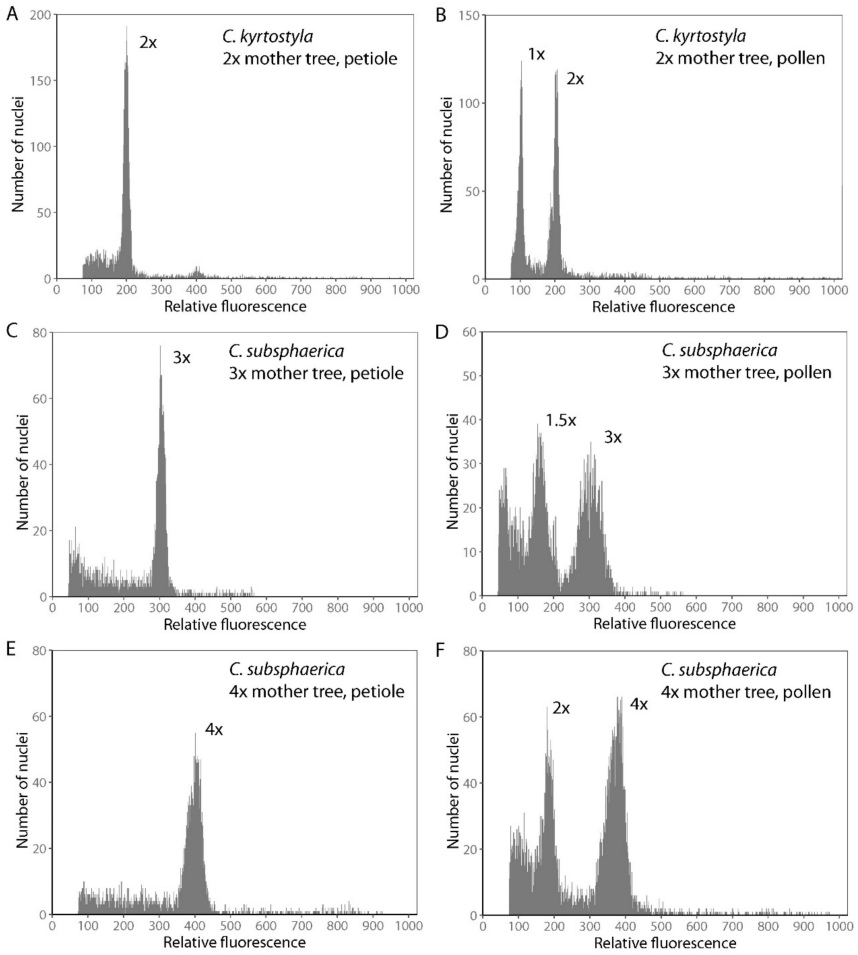

Figure 4. Selected flow cytometric histograms of pollen grains of diploid (**A**,**B**), triploid (**C**,**D**), and tetraploid (**E**,**F**) mother trees. Histograms on the left represent the FCM of petiole nuclei and histograms on the right represent the FCM of pollen nuclei where the first peak corresponds to vegetative cell nuclei and the second peak to generative cell nuclei.

3.3. Pollination Experiments

In total, 13 pollinations were performed between diploid and triploid *Crataegus* trees, and flowers from two tetraploid trees were covered with pollinator exclusion bags and tested for self-pollination and self-compatibility. Two out of 13 cross-pollinations resulted in no seeds (Figure 1), including reciprocal crosses of diploid *C. kyrtostyla* (GOCAL 1) with the triploid hybrid *C. laevigata* × *C. subsphaerica* (VYH 8). The experiment on the tree GOCAL 2 was destroyed, and no seeds from this individual were obtained. The reproductive systems in diploid, triploid, and tetraploid trees were also observed with open pollinations.

3.4. Seed-Set Analysis and Reproductive Modes in Diploid Crataegus Mother Trees

Pollination experiments in diploid *C. monogyna* and *C. kyrtostyla* included self-pollinations, open pollinations, and pollinations with pollen from diploid and triploid species. Self-pollinations when

inflorescences were enclosed in pollinator exclusion bags resulted in four seeds out of 606 pollinated flowers/648.4 carpels (pyrenes in fruits, approximately 0.7%/0.6%; number of pyrenes per fruit was comparable in diploids, 1.07 pyrenes per fruit (n = 383 analysed fruits)). Note, that only two seeds were measured successfully using FCSS. The result suggests self-incompatibility in diploid *Crataegus* taxa. Diploid *C. monogyna* was considered to be self-incompatible in other studies as well [81,90–92]. Self-incompatibility in the family *Rosaceae* is of the gametophytic type [52,53], defined by germination of the pollen grain on the stigma and consequent cessation of pollen tube growth in the style [93]. Both seeds were analysed by the FCSS method and yielded the diploid signal for the embryo (1.46, 1.48 pg DNA) and the triploid signal for the endosperm (2.22, 2.24 pg DNA), as expected in normal sexual reproduction. Similar results were reported in a North American experiment [10], where only 2% of self-pollinated flowers (from diploid *C. punctata* Jacq. and *C. monogyna*) produced fruits, and seeds originated sexually.

Pollination of a diploid mother tree with pollen from another diploid individual of the same species (*C. monogyna* × *C. monogyna* and *C. kyrtostyla* × *C. kyrtostyla*) resulted in 39 seeds out of 95 pollinated flowers/102 carpels (approximately 36.8%/34.3%, note: 35 seeds measured successfully using FCSS). Pollination of diploid *C. kyrtostyla* by diploid *C. monogyna* resulted in only one seed out of 30 pollinated flowers/32 carpels (approximately 3.3%/3.1%) while pollination of diploid *C. monogyna* by diploid *C. kyrtostyla* was more successful and resulted in 17 seeds out of 60 pollinated flowers/64 carpels (approximately 28.3%/26.6%; note: Sixteen seeds measured successfully using FCSS). After analysis, all produced seeds yielded the diploid embryo signal and the triploid endosperm signal, which indicates sexual reproduction. In these seeds, the DNA content ranged from 1.40 to 1.57 pg for diploid embryos and from 2.11 to 2.38 pg for triploid endosperms. Pollination of diploid *C. kyrtostyla* by triploid *C. subsphaerica* resulted in three seeds from 40 pollinated flowers/42.8 carpels (approximately 7.5%/7.0%). Only a single seed yielded a good-quality histogram and it originated sexually, with 2x embryo (1.56 pg) and 3x endosperm (2.33 pg). We speculate that this seed originated via self-fertilisation; note that we did not remove anthers from diploid mother trees. We analysed the ploidy level of the embryo and endosperm in 72 seeds to determine modes of reproduction in diploid *Crataegus* taxa without our interference (open pollination). After separate analysis of each seed, we observed diploid peaks for the embryo and triploid peaks for the endosperm on every histogram, which proves that seeds of diploid trees originate exclusively sexually, and double fertilisation by reduced 1x pollen occurred (Table 2). The ploidy-level screening of pollen from diploid trees performed in the present study supports this explanation. Sexual reproduction in diploid *Crataegus* taxa was also reported in other studies [10,11,75]. Sexual reproduction in diploids is rather a rule among angiosperms, and only rarely exceptions have been evidenced, e.g., in *Paspalum* L. [94] or *Boechera* Á.Löve et D.Löve [95–97]. In the *Rosaceae* family, where *Crataegus* belongs, diploids are mostly sexual, as observed by FCM in *Amelanchier* Medik. [98], *Potentilla* L. s.l. [82], *Rubus* L. [25,99], and *Sorbus* [100], but few exceptions were reported [e.g., 82,98].

3.5. Seed-Set Analysis and Reproductive Modes in Triploid Crataegus Mother Trees

Similarly, as in diploid *Crataegus* taxa, self-pollination in triploid *C. subsphaerica* and *C. laevigata* × *C. subsphaerica* resulted in no viable seeds out of 282 pollinated flowers. We suggest that this result indicates poor pollen quality resulting from irregular meiosis in triploid species (see Section 3.2, Supplementary Materials Figure S1), although gametophytic self-incompatibility or general lower fruit production in triploids (as a result of shift in flowering phenology and consequent period of unfavourable climatic conditions) are also possible explanations. Although cross-pollinations of the 3x × 3x type resulted in only two seeds out of 38 pollinated flowers, pollen viability tests would still be necessary to exclude male infertility. Male sterility (no pollen produced) and male infertility (i.e., poorly stainable pollen produced) were recorded in triploid *Crataegus* [11]. Two seeds (38 pollinated flowers/40.7 carpels, approximately 5.3%/4.9%; number of pyrenes per fruit was 1.07 (n = 297 analysed fruits)) were produced from pollination of a *C. subsphaerica* mother tree by *C. laevigata* × *C. subsphaerica*

(3*x* × 3*x*). Flow cytometry revealed a 3*x* embryo signal (2.40, 2.46 pg) and a 9*x* endosperm signal (7.20, 7.29 pg). Seeds with 3*x* embryos and 9*x* endosperms were also recorded in other studies in the genera *Crataegus* [66] and *Sorbus* [89,100], where triploids use not only 1*x* or 2*x* but also 1.5*x* or 3*x* sperm cells for fertilisation. This indicates their apomictic origin when embryo developed parthenogenetically and endosperm possibly resulted from fertilisation of binucleate central cell (6*x*) either by one unreduced sperm cell (3*x*) or by two sperm cells with the aneuploid cytotype (approximately 1.5*x*). Our pollen ploidy-level screening indicates that the 3*x* trees produced regular pollen with approximately 1.5*x*. High CV values for both peaks (of vegetative nuclei and generative nuclei), compared with that for the peak of somatic nuclei (see Figure 4), may be a result of the presence of various aneuploid pollen grains ranging from 1*x* to 2*x*, with the highest probability of 1.5*x*. These data suggest that some irregular male meiosis occurs in triploids. Triploids are generally meiotically unstable and can often produce aneuploid gametes with incomplete or unbalanced chromosome sets [101–103]. In polyploid plants, aneuploidy is an extremely frequent phenomenon [104,105] and especially odd-numbered polyploids or hybrids may have pollen with high rates of aneuploidy [80]. Aneuploid pollen grains were recorded, e.g., in triploid *Arabidopsis thaliana* L. (Heynh.), and proven to be fertile after crossing experiments with diploids and tetraploids [106] and in allotriploid poplar (*Populus alba* × *P. berolinensis* 'Yinzhong'), where pollen fertility is also suggested [107].

Pollinations of triploid individuals with pollen from diploids resulted in 20 well-developed seeds. All originated only on *C. subsphaerica*, out of all 95 pollinated flowers/100.7 carpels (approximately 21.1%/19.7%, note: 17 seeds measured successfully using FCSS; *C. subsphaerica* (1.06 pyrenes per fruit, *n* = 297 analysed fruits), *C. laevigata* × *C. subsphaerica* (2.11 pyrenes per fruit, *n* = 299 analysed fruits)). Seeds were found with different ploidy levels of embryos and endosperms. In this case, we can observe that triploids of the genus *Crataegus* are almost exclusively apomictic because only three seeds originated sexually, whereas 14 seeds were produced via apomixis. Two seeds with aneuploid approximately 3.7*x* embryos and approximately 5*x* endosperms possibly originated sexually when the egg cell and central cell were fertilised by unreduced sperm cells. A single seed, which yielded a 4*x* embryo signal and a second 8*x* signal, probably originated sexually (B_{III} hybrid) when the egg cell was fertilised with a 1*x* sperm cell, and the second signal might be either the G_2 phase of the embryo nuclei or, less probably, the endoreduplicated nuclei (Figure 4B). Fertilisation of an unreduced triploid egg cell, although rare because of the probable low viability of the seeds, was documented in the genera *Crataegus* [10,11] and *Sorbus* [89,100]. In pseudogamous apomictic plants, if the unreduced egg cell is fertilised by a sperm cell, ploidy levels can increase [10,31,108,109]. The other 14 seeds were produced via apomixis with 3*x* embryos and 6*x*, 7*x*, and 8*x* endosperms. One seed with a 3*x* embryo and 6*x* endosperm possibly originated autonomously without fertilisation by sperm cells in unreduced megagametophyte, or the 6*x* peak could represent the G_2 phase of embryonic nuclei or endoreduplicated nuclei. The origin of another single seed was probably pseudogamous apomixis when a 3*x* embryo developed parthenogenetically and 7*x* endosperm was produced from fertilisation of the binucleate central cell by a single 1*x* sperm cell. The majority of seeds were derived from meiotically unreduced megagametophytes: A 3*x* embryo developed parthenogenetically and 8*x* endosperm originated from the fertilisation of the binucleate central cell (6*x*) by two reduced sperm cells (1*x*), originating from diploid pollen donors (or by one unreduced sperm cell (2*x*), but note extremely low production of unreduced pollen grains in diploids).

Similarly, as in diploids, we analysed ploidy levels of embryos and endosperms in triploid individuals produced from open pollinations. The analysis of open-pollinated seeds yielded 3*x* embryos and endosperms with five different ploidy levels (5*x*, 7*x*, 8*x*, 9*x*, and 10*x*), and the 8*x* endosperm was the most common. All seeds are probably of apomictic origin. A single seed with a 3*x* embryo and 5*x* endosperm probably originated via apomixis where the embryo developed parthenogenetically, while the endosperm resulted from fertilisation of a single, 3*x* central cell with two reduced sperm cells (1*x*) from a diploid. This seed could have also originated sexually, where the egg cell and the central cell were fertilised by reduced sperm cells (Table 2). The inferred origin of seeds with

3*x* embryos and 7*x*, 8*x*, and 9*x* endosperms corresponds to the same cases in 3*x* × 3*x* crosses mentioned above. The origin of a single seed with a triploid embryo and decaploid endosperm is consistent with a binucleate central cell and two reduced 2*x* sperm cells contributing to the endosperm (originating most probably from 4*x* plants). Seeds with 3*x* embryos and 10*x* endosperms are rare but have been recorded in the genera *Crataegus* [10,11,66] and *Sorbus* [89,100]. The majority of seeds in pollination experiments in triploid *Crataegus* are suggested to develop unreduced megagametophytes, and the observed variation in ploidy levels of embryo and endosperm stems from the natural cross-compatibility of triploids; they can utilise pollen from 2*x*, 3*x*, and possibly even from 4*x* donors (Table 2) for pseudogamous development of functional endosperm.

3.6. Seed-Set Analysis and Reproductive Modes in Tetraploid Crataegus Mother Trees

Self-pollination experiments were performed in tetraploid *Crataegus* individuals where intact flowers of tetraploid *C. subsphaerica* that were enclosed in pollinator exclusion bags produced 24 seeds out of 101 flowers/102 carpels (approximately 23.8%/23.5%; number of pyrenes per fruit was comparable in tetraploids, 1.01 pyrenes per fruit (n = 164 analysed fruits)). Note, that histograms of three seeds did not yield embryo or endosperm signals. After FCSS analysis, histograms of 21 seeds yielded tetraploid embryo signals (3.22–3.32 pg DNA) and 10*x* or 12*x* endosperm signals (8.02–10.30 pg DNA), which indicates the formation of unreduced megagametophytes. The majority of seeds developed endosperms with the 12*x* ploidy level. Embryos developed parthenogenetically wherein the binucleate central cell was fertilised (pseudogamy) by one meiotically reduced sperm cell (10*x*) or by one unreduced or two reduced sperm cells (12*x*). These experiments indicate that tetraploid *Crataegus* taxa are pseudogamous apomicts able to self-pollinate and that pollination is essential for seed formation, as reported in other studies [10,11,110].

Open pollination resulted in 30 seeds with different ploidy levels of embryos: Most were tetraploid, one was diploid, and another one was hexaploid. The endosperm ploidy levels greatly varied; 6*x*, 8*x*, 10*x*, 11*x*, 12*x*, 14*x*, and 16*x* were recorded. Open-pollinated apomictic tetraploids as well as triploids seem to always require fertilisation of the central cell involving either one or two sperm cells [10,11]. The majority of seeds produced by open pollination are suggested to be apomictic: 4*x* embryos (and a single 6*x* embryo with 10*x* endosperm) with 10*x*, 11*x*, 12*x*, 14*x*, and 16*x* endosperms were recorded. The origin of seeds with 10*x* and 12*x* endosperms is proposed above. A single seed had 11*x* endosperm, suggesting fertilisation with pollen from triploids (either one unreduced 3*x* sperm cell or two reduced, probably approximately 1.5*x* sperm cells). Endosperm ploidy levels higher than 12*x* can be explained by the presence of a trinucleate central cell, which was observed in *Crataegus* [111]. Endosperms of these four seeds are then suggested to develop through fertilisation of a trinucleate central cell by either one reduced 2*x* sperm cell (14*x*) or by one unreduced 4*x* or two reduced 2*x* sperm cells (16*x*). A single seed with a 6*x* embryo and 10*x* endosperm was probably derived from fertilisation of both an unreduced egg cell and a binucleate central cell by a reduced 2*x* sperm cell. Seeds with 6*x* embryos representing B_{III} hybrids seem to be rare [10], and the prevailing ploidy level of the embryo in tetraploids is 4*x*. In addition to seeds of apomictic origin, reduced megagametophytes were observed in tetraploid seeds. A single seed with a 4*x* embryo and 6*x* endosperm was likely to have resulted from a reduced megagametophyte where both the egg cell and the central cell were fertilised by reduced 2*x* sperm cells. Another seed with a reduced embryo sac was recorded where the embryo ploidy level was 2*x* and could therefore have developed parthenogenetically, whereas the endosperm with ploidy level 8*x* was derived from fertilisation of the central cell by either one unreduced 4*x* sperm cell or two reduced 2*x* sperm cells. These results indicate that tetraploid *Crataegus subsphaerica* frequently produce unreduced megagametophytes, but sometimes sexual megagametophytes occur (Table 2).

Table 2. Summary of results of pollination experiments in the genus *Crataegus*, including type of crosses, number of seeds and pollinations, hypothesized seed, embryo and endosperm origin, and embryo, endosperm, egg, central cell, and pollen ploidies. In case of alternative explanations, the most probable is given in bold.

Type of Pollination/ Ploidy Level ♀× ♂	Number of Seeds (Number of Pollinations)	Seed Origin	Embryo Ploidy	Endosperm Ploidy	Egg Ploidy	Central Cell Ploidy	Pollen Ploidy (Sperm Cell)	Embryo Origin (egg Cell + Sperm Cell)	Endosperm Origin (Central Cell + Sperm Cell)
2*x* (intact flowers bagged)	1 (475)	Sexual	2*x*	3*x*	1*x*	2*x*	1*x*	(1*x*) + 1*x*	(2*x*) + 1*x*
2*x* × 2*x* (self-pollination)	1 (131)	Sexual	2*x*	3*x*	1*x*	2*x*	1*x*	(1*x*) + 1*x*	(2*x*) + 1*x*
2*x* × 2*x* (cross-pollination)	52 (185)	Sexual	2*x*	3*x*	1*x*	2*x*	1*x*	(1*x*) + 1*x*	(2*x*) + 1*x*
2*x* (open pollination)	72	Sexual	2*x*	3*x*	1*x*	2*x*	1*x*	(1*x*) + 1*x*	(2*x*) + 1*x*
2*x* × 3*x* (cross-pollination)	1 (40)	Sexual	2*x*	3*x*	1*x*	2*x*	1*x*	(1*x*) + 1*x*	(2*x*) + 1*x*
3*x* (intact flowers bagged)	0 (221)	/	/	/	/	/	/	/	/
3*x* × 3*x* (self-pollination)	0 (61)	/	/	/	/	/	/	/	/
3*x* × 3*x* (cross-pollination)	2 (38)	Apomictic	3*x*	9*x*	3*x*	6*x*	1.5*x*	(3*x*) + 0	(6*x*) + 3*x* or **(6*x*) + 2×1.5*x***
3*x* (open pollination)	1	Apomictic [a]	3*x*	5*x*	3*x*	3*x*	1*x*	(3*x*) + 0	(3*x*) + 2×1*x*
	2	Apomictic	3*x*	7*x*	3*x*	6*x*	1*x*	(3*x*) + 0	(6*x*) + 1*x*
	22	Apomictic	3*x*	8*x*	3*x*	6*x*	2*x*	(3*x*) + 0	(6*x*) + 2*x*
	8	Apomictic	3*x*	9*x*	3*x*	6*x*	1.5*x*	(3*x*) + 0	(6*x*) + 1×3*x* or **(6*x*) + 2×1.5*x***
	2	Apomictic	3*x*	10*x*	3*x*	6*x*	2*x*	(3*x*) + 0	(6*x*) + 2×2*x*
3*x* × 2*x* (cross-pollination)	1	Apomictic	3*x*	6*x*	3*x*	6*x*	/	(3*x*) + 0	(6*x*) + 0
	1	Apomictic	3*x*	7*x*	3*x*	6*x*	1*x*	(3*x*) + 0	(6*x*) + 1*x*
	12 (118)	Apomictic	3*x*	8*x*	3*x*	6*x*	1*x*	(3*x*) + 0	(6*x*) + 1×2*x* or **(6*x*) + 2×1*x***
	2	Sexual [b]	~3.7*x*	~5*x*	~1.7*x*	~3.4*x*	~2*x*	~(1.7*x*) + ~2*x*	~(3.4*x*) + ~2*x*
	1	Sexual, B_{III}	4*x*	/	3*x*	/	/	(3*x*) + 1*x*	/
4*x* (intact flowers bagged)	2 (101)	Apomictic	4*x*	10*x*	4*x*	8*x*	2*x*	(4*x*) + 0	(8*x*) + 2*x*
	19	Apomictic	4*x*	12*x*	4*x*	8*x*	2*x*	(4*x*) + 0	(8*x*) + 1×4*x* or **(8*x*) + 2×2*x***

Table 2. *Cont.*

Type of Pollination/ Ploidy Level ♀× ♂	Number of Seeds (Number of Pollinations)	Seed Origin	Embryo Ploidy	Endosperm Ploidy	Egg Ploidy	Central Cell Ploidy	Pollen Ploidy (Sperm Cell)	Embryo Origin (egg Cell + Sperm Cell)	Endosperm Origin (Central Cell + Sperm Cell)
4*x* (open pollination)	1	Apomictic, haploid parthenogenesis	2*x*	8*x*	2*x*	4*x*	2x	(2*x*) + 0	(4*x*) + 1×4*x* or **(4*x*) + 2×2*x***
	1	Sexual	4*x*	6*x*	2*x*	4*x*	2*x*	(2*x*) + 2*x*	(4*x*) + 2*x*
	1	Sexual, B_{III}	6*x*	10*x*	4*x*	8*x*	2*x*	(4*x*) + 2*x*	(8*x*) + 2*x*
	4	Apomictic	4*x*	10*x*	4*x*	8*x*	2*x*	(4*x*) + 0	(8*x*) + 2*x*
	1	Apomictic	4*x*	11*x*	4*x*	8*x*	3*x*	(4*x*) + 0	(8*x*) + 3*x*
	18	Apomictic	4*x*	12*x*	4*x*	8*x*	2*x*	(4*x*) + 0	(8*x*) + 1×4*x* or **(8*x*) + 2×2*x***
	3	Apomictic	4*x*	14*x*	4*x*	12*x*	2*x*	(4*x*) + 0	(12*x*) + 2*x*
	1	Apomictic	4x	16*x*	4*x*	12*x*	2*x*	(4*x*) + 0	(12*x*) + 1×4*x* or **(12*x*) + 2×2*x***

[a] Alternative explanation—the origin of a seed with a 3*x* embryo and 5*x* endosperm is sexual, where 2*x* egg cell and 4*x* central cell were fertilised each with a reduced 1*x* sperm cell.
[b] This case is unclear, the origin of seeds with a ~3.7*x* embryo and ~5*x* endosperm is possibly sexual, where ~1.7*x* egg cell and ~3.7*x* central cell were fertilised each with abnormal aneuploid ~2*x* sperm cell of triploid (self-fertilisation) or euploid 2*x* sperm cell of diploid.

4. Conclusions

In this first analysis of reproductive systems of *Crataegus* taxa in Europe and *Crataegus* sect. *Crataegus* overall (except *C. monogyna* introduced to North America), we performed controlled and open pollinations to establish that the diploid species *C. monogyna* and *C. kyrtostyla* are self-incompatible; however, after being pollinated by pollen from other diploid or triploid trees, they produced viable seed progeny. The flow cytometric seed screen method was used to identify ploidy levels of trees and their pollen grains and modes of reproduction, providing fast, accurate analysis with relatively low expanses. Flow cytometry analyses demonstrated that seeds produced by both controlled and open pollinations originated sexually in these diploids. Our data suggest that polyploid taxa are almost exclusively pseudogamous apomicts. The results of self-pollination in triploid *C. subsphaerica* and *C. laevigata* × *C. subsphaerica* reflect either self-incompatibility or more likely, poor pollen quality. Conversely, tetraploid *C. subsphaerica* can use their own pollen for fertilisation, i.e., they are self-compatible. To determine reproductive systems, particularly in the polyploid *Crataegus*, measurements of the ploidy levels of pollen used for fertilisation are essential. We observed that mother trees more frequently produce reduced pollen, as evidenced on FCM histograms. The study indicates that reproductive systems of central European diploid and polyploid *Crataegus* species of sect. *Crataegus* are similar to those of hawthorns in North America of sect. *Douglasia*, sect. *Coccineae*, sect. *Crus-galli* Loudon or sect. *Pruinosae* (Sarg.) Eggl. Apomixis and polyploidisation documented in the study, as well as the hybridisation inferred from morphological comparisons performed in other studies, are probably responsible for the taxonomic complexity of European hawthorns. This genus can provide valuable insights into reproductive interactions between cytotypes. The next step should be to determine the interactions among several apomictic tetraploid species with partial sexuality and to experimentally test for interspecific cross-compatibility and hybridisation.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/12/1059/s1. Table S1: FCM results of cross-pollination experiments in studied *Crataegus* taxa. Table S2: FCM results of open pollinations in studied *Crataegus* taxa. Figure S1: Light microscopy photographs of *Crataegus* pollen grains of diploid (*C. kyrtostyla*), triploid (*C. laevigata* × *C. subsphaerica*) and tetraploid (*C. subsphaerica*) accessions.

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