The relation of meiotic behaviour to hybridity, polyploidy and apomixis in the Ranunculus auricomus complex (Ranunculaceae)

Birthe Hilkka Barke (birthe-hilkka.barke@biologie.uni-goettingen.de)
Georg-August-Universitat Gottingen Geowissenschaftliches Zentrum https://orcid.org/0000-0001-5379-6055

Kevin Karbstein
Georg-August-Universitat Gottingen

Mareike Daubert
Georg-August-Universitat Gottingen

Elvira Hörandl
Georg-August-Universitat Gottingen

Research article

Keywords: developmental biology, gametophytic apomixis, hybrid, meiosis, PMC, polyploidy, Ranun-culus

Posted Date: November 19th, 2019

DOI: https://doi.org/10.21203/rs.2.17395/v1

Version of Record: A version of this preprint was published at BMC Plant Biology on November 17th, 2020. See the published version at https://doi.org/10.1186/s12870-020-02654-3.

Abstract

Background Hybridization and polyploidization are powerful evolutionary factors that are associated with manifold developmental changes in plants such as irregular progression of meiosis and sporogenesis. The emergence of apomixis, which is asexual reproduction via seeds, is supposed to be connected to these factors and was often regarded as an escape from hybrid sterility. However, the functional trigger of apomixis is still unclear. Recently formed di- and polyploid Ranunculus hybrids as well as their parental species were analysed for their modes of mega- and microsporogenesis by microscopy. Chromosomal configurations during male meiosis were screened for abnormalities. Developmental abnormalities were documented qualitatively and collected quantitatively for statistical evaluations.

Results Allopolyploids showed significantly higher frequencies of erroneous microsporogenesis than homoploid hybrid plants. Among diploids, F 2 hybrids had significantly more disturbed meiosis than F 1 hybrids and parental plants. Chromosomal aberrations included laggard chromosomes, chromatin bridges and disoriented spindle activities. Meiotic failure appeared to be much more frequent in female compared to male development.

Conclusions Results suggest diverging selective pressures on female and male meiosis, with only minor effects of hybridity on male development, but fatal effects on the course of megasporogenesis. Hence, pollen development continues without major alterations, while selection will favour alternatives to the female meiotic pathway. Relation of investigated meiotic errors with the observed occurrence of apospory in Ranunculus hybrids identifies disturbed female meiosis as potential elicitor of apomixis in order to rescue these plants from hybrid sterility. Meiotic disturbance appears to be stronger in neopolyploids than in homoploid hybrids, which may contribute to the prevalence of apomixis in polyploid plants.

Background

In all eukaryotic organisms, meiosis is the core of sexual reproduction, which ensures recombination and thus evolution and speciation [*e.g.* 1]. This type of cell division manages to half the chromosome number of a diploid organism in order to produce four haploid gametes. Meiosis requires one step of DNA replication followed by two chromosome segregation processes [meiosis I and II, 2]. The most important and therefore tightly controlled part of the whole mechanism is the formation of crossing overs among homologous chromosomes facilitating genetic recombination during meiosis I [3]. Exact chromosome segregation is strictly required since unbalanced gamete formation can lead to cell death, sterility or aneuploidy [4].

Interspecific hybridization is a frequent phenomenon in plants [5], which results either in offspring with a doubled chromosome number (allopolyploids) or in diploid hybrids [homoploids, 6, 7]. Hybridisation creates a versatile range of hybrids with each different genotypes and divergent fitness [5]. Hybrids generally have a negative connotation and are even termed as “hopeful monsters” because of their reduced fitness [8]. This means that these plants are often inviable or sterile, while suffering from a lack

of mating partners due to isolation *e.g.* through divergent ploidy levels [5, 8]. The strongest effects of hybridization on plant fertility are usually found in F_1 hybrids [8, 9]. The combination of divergent chromosomes can oblige mispairing and –segregation at meiosis, depending on the differences between parental species. Strong discrepancies are assumed to result in deleterious consequences for sporogenesis, gametophyte development and gamete formation [7, 10, 11].

However, fertility of plant hybrids is highly variable, and eventually subsequent generations can establish novel evolutionary lineages [5]. Historically, homoploid hybrid speciation was assumed as rarely arising phenomenon [*e.g.* 5, 12], because of missing concrete identification evidences [7]. The importance of this topic among evolutionary biologists grew, while only a few cases of homoploid hybrid plants are known [13]. Best documented and described natural homoploid hybrids belong to the taxa *Helianthus, Senecio, Doronicum* and *Iris* [*e.g.* 14–17]. Homoploid hybrids possess half of the chromosome set of each parent, which strongly limits reproductive isolation of these hybrids. Speciation of homoploid hybrids is unlikely because gene flow is not efficiently suppressed, as it is in allopolyploids [8, 12] but reproduction isolation can be achieved by spatial isolation, karyotype and/ or ecological divergence [7].

Polyploid plants have to organize and maintain functionality with more than two complete chromosome sets. Neopolyploids are therefore considered to be genetically and phenotypically unstable and prone to meiotic errors [10]. Such errors get less over generations because the polyploid chromosome set becomes stabilized by cytological diploidization that acts on gamete formation [18]. During this meiotically-driven mechanism genetic and chromosomal configuration is drastically restructured *e.g.* redundant chromosomes are eliminated and gene duplicates can get disposed or new functions can be assigned [Neofunctionalization, 4, 10]. Autopolyploids are the result of restitutional meiosis, gaining unreduced gametes that develop into plants with increased ploidy level, often via a triploid bridge [19]. In contrast, allopolyploids are not only caused by unreduced gamete formation, but additionally by a hybridization event of two species. Meiosis in autopolyploids is disturbed due to fact that such plants are equipped with more than two copies of each chromosome, which favours the emergence of homologous multi- and univalents, while allopolyploids are able to develop regular bivalents during prophase I [11]. Indeed, the frequency and likelihood of allopolyploids recognizing one or more homeologous pairing partners fundamentally depends on sequence divergences of the parental genomes. Difficulties in chromosome alignment and synapsis still occur on regular basis in young diploid hybrids due to the forced pairing of even homeologous partners. Overall, polyploid plants with hybrid origin tend to behave during meiosis as diploids, because the homologs derived from the same parent can form bivalents [4, 10]. This way, the problems of homeolog pairing can be avoided.

Apomixis, which is asexual seed formation, circumvents meiosis in various different developmental pathways [20]. One common form of apomixis involves mitotic embryo sac (ES) development out of a somatic nucellar cell (apospory), resulting in clonal, maternal egg cells [21]. This specialized mode of reproduction is able to avoid negative effects of allopolyploidy on meiosis and is in natural populations often regarded as an escape from hybrid-caused sterility [10, 20, 22]. Indeed, most apomicts are

polyploids and/ or hybrids but how apomixis is triggered in natural plant populations is still under debate [23].

However, in the context of meiotic errors, apomictic reproduction seems to represent a powerful tool in saving plants from deleterious consequences like chromosome mispairing and –segregation upon hybridization and (allo-) polyploidization. In plants, apomixis only affects female development, where meiosis is difficult to observe directly. In male development, however, no specific developmental pathways evolved in apomictic plants, and pollen is mostly meiotically reduced. Meiosis research, especially those studies including cytological investigations, is in plants traditionally done on pollen mother cells (PMCs) only because of easier observation [*e.g.* 2, 24, 25]. Due to these technical reasons, only a few empirical studies are available on a possible correlation of meiosis behaviour and expression of apomixis [26, 27]. It is further unclear whether male meiosis can be regarded as predictor for female meiosis and development, when they occur in the same hermaphroditic plant.

The *Ranunculus auricomus* complex includes about 800 described species [28]. The vast majority of these species are apomictically reproducing polyploids, while a small number of species are diploid ($2n$ = 16) and tetraploid ($2n$ = 32) sexuals [29–33]. The sexual species, *R. notabilis, R. carpaticola,* and *R. cassubicifolius,* are obligate outcrossers [32, 34] and can be regarded as progenitors of the whole polyploid complex [28, 35, 36]. *R. notabilis* and the more closely related species pair *R. cassubicifolius/ carpaticola* represent two genetically and morphologically distinct lineages that separated c. 900,000 years ago [28, 35, 37, 38]; all three taxa occur in geographical isolation [map in 28]. Functional apomixis in *Ranunculus* demands effective coupling of apomeiosis and parthenogenetic egg cell generation [21]. Unsuccessful linkage of these two crucial steps towards apomictic reproduction can result in increased offspring ploidy [21, 39].

In fact, cytological analysis in the *R. auricomus* complex has been performed on either female or male development focusing on gametogenesis and following processes such as pollen quality determination [21, 29, 40, 41]. Reduced female fertility of F_1 hybrids between *R. notabilis* and *R. cassubicifolius/ carpaticola* has been observed in experimental crosses [34], and apospory has been observed in F_1 and F_2 hybrids [32, 39]. The present study provides an analysis of chromosomal behaviour in *Ranunculus* pollen mother cells (PMCs) during sporogenesis and beyond. This allows a comparative evaluation of development in di- and polyploid natural sexual and apomictic species as well as of two synthetic, diploid and polyploid hybrid generations that represent an intermediate phase between sexuality and apomictic reproduction. Additionally, these results are qualitatively and quantitatively compared to disturbances of megagametogenesis in di- and polyploid F_2 hybrid plants that have shown different frequencies of apospory and asexual seed formation [39]. Hence, we expected an increase in abnormal microsporogenesis, not only within synthetic, diploid and polyploid *Ranunculus* hybrids but also in young natural polyploids with hybrid background. Results, however, suggest different meiotic behaviour in diploid versus polyploid plants, and also different selective constraints for female and male development.

Results

Male development

In order to determine whether the hybrid character or the ploidy level of *Ranunculus* plants has an influence on the male gametes during meiotic division, more than 10,000 PMCs were analysed for abnormalities (Table 3). The overall frequency of abnormal meiosis in tested male gametes was 5.42 %, while the remaining 94.58 % resulted in four normal microspores of the same size (Table 3, Fig. 1d). Although, the comparison of abnormal meiotic cell division between the three different plant generations (parents, F_1 and F_2 hybrids) did not show significant differences, a significantly higher frequency of faulty microsporogenesis was found in polyploid samples (mean 8.59 % ± 9.84 STD, median 3.73 %, p = 0.012) compared to diploid ones (mean 2.09 % ± 3.05 STD, median 1.43 %; Table 3, Fig. 3a). In addition, erroneous male gamete formation in all hybrid plants was analysed, including the young, natural hybrid, revealing significantly more failures during sporogenesis in allopolyploid samples (mean 16.11 % ± 17.58 STD, median 13.33 %, p = 0.006) in contrast to homoploid *Ranunculus* individuals (mean 2.11 % ± 3.19, median 1.44 %; Table 3; Fig. 3b).

Various abnormalities at different meiotic stages were identified in male gametes of all *Ranunculus* hybrid generations independently of ploidy levels (Table 3). Irregularities included lagging chromosomes and chromatin bridges at metaphase I (Fig. 1f). At anaphase I laggards and sticky chromosomes and disoriented spindle activities were detected (Fig. 1g, h, i, Fig. 2a - e). Disoriented spindle activity as well as scattered chromosomes occurred during anaphase II (Fig. 1j). In addition, micronuclei were formed during telophase II (Fig. 1 k, Fig. 2f - h). The consequence of the described failures during male sporogenesis led to the formation of dyads, triads and polyads, instead of a microspore tetrad (Fig. 1l—q). In turn, incompletely separated and heterogeneous-sized microspores resulted in *Ranunculus* pollen grains of different sizes, of which the micronuclei-derived pollen grains are much smaller than normal pollen (Fig. 1r - t).

Female development

Female meiosis of three polyploid *Ranunculus* F_2 hybrid individuals, derived from two different crosses (G1 * G9, G16A * I2A), was analysed for signs of abnormal, aposporic development. Overall, development of 186 ovules was evaluable because of the small number of formed flower buds by polyploid synthetic F_2 hybrids and the difficulties to find the developmental stadium of interest. Normal megasporogenesis was detected in 48.92 % of the ovules (Table 4). Regular meiotic division was indicated by the presence of a functional megaspore (FM) at the end of the germ line, closest to the chalazal pole, while the other three meiotic products were already aborted. Additional to this, apospory was identified in 12.37 % of the analysed F_2 ovules (Table 4). Characteristic for this type of meiosis bypass is the occurrence of an aposporous initial cell close to the FM, which is known to dominate development from that point on and results in the abortion of the FM. The remaining 38.71 % of the analysed ovules were found to be dead (Table 4). Furthermore, a comparison of di- and polyploid F_2 hybrid samples for failure during meiotic cell

division was done, which resulted in non-significant differences between these two groups ($p = 0.241$, Mann-Whitney-U test).

Comparison of male and female meiosis in synthetic *Ranunculus* F_2 hybrids

Developmental irregularities were observed in F_2 hybrids of both, female and male meiosis, at different percentages (Fig. 3c, Table 3, 4). Therefore, the frequencies of abnormal male and female sporogenesis were analysed for differences, revealing a significantly stronger defective meiosis on the female than on the male side of development (Fig. 3c).

Generalized linear mixed effect model analysis of sporogenesis in *Ranunculus*

In order to uncover and recess potential connections between the occurrence of deleterious errors in sporogenesis and certain characteristics of the studied plants, GLMM and Chi-squared analyses were performed (Table 5; Supplementary Data Table S1). Polyploid *Ranunculus* plants showed a significantly higher frequency of erroneous microsporogenesis than diploid samples ($p < 0.001$), and a similar negative relation was observed for hybridization. According to this, hybrid plants of the F_2 generation developed significantly more abnormal male gametes than plants of the non-hybrid parent ($p < 0.05$) and the F_1 generation ($p < 0.01$). In addition, accumulative effects of *ploidy level* and *generation* were explored by GLMM, indicating weakly but non-significant increased failures of microsporogenesis in diploid *Ranunculus* F_2 hybrids compared to both, polyploid parent plants ($p = 0.08$) and polyploid F_1 hybrids ($p = 0.09$). The impact of polyploidy on developmental behaviour was additionally investigated in female sporogenesis of F_2 hybrids, inferring no significant differences ($p = 0.46$). Furthermore, the total F_2 dataset, comprising mega- and microsporogenesis measurements, was consecutively tested for an influence of *ploidy level* and *sex* on gamete formation. A highly significant relation between errors during female sporogenesis and plant polyploidy was observed ($p < 0.001$) as well as between faulty microsporogenesis in diploid F_2 hybrids and megasporogenesis in polyploid F_2 plants ($p < 0.001$).

Chi-squared tests were done to support GLMM analyses, obtaining corroborative results (Supplements). Highly significant differences in microsporogenesis performance were detected between di- and polyploid *Ranunculus* plants of the parental ($X^2 = 119.78$, $df = 1$, $p < 0.001$), the F_1 hybrid ($X^2 = 8.42$, $df = 1$; $p = 0.01$), and the F_2 hybrid ($X^2 = 43.32$, $df = 1$, $p < 0.001$) generation (Supplementary Data Fig. S2b). In addition, similar significant differences in error frequency were observed between male and female sporogenesis of F_2 hybrids ($X^2 = 470.82$, $df = 1$, $p < 0.001$; Supplementary Data Fig. S2c).

Discussion

Hybridization and polyploidization are known to have substantial effects on male and female reproductive programs in angiosperm plants [11]. Although hybridization was recently shown to play an important role in the onset of apospory in diploid *Ranunculus* plants, its interaction with meiotic behaviour remained unclear [32, 39]. The investigation of chromosomal behaviour at meiosis plus male

and female sporogenesis in *Ranunculus* allows first insights into the role of meiosis and sporogenesis for occurrence of apomictic reproduction in hybrid and polyploid plants.

In this study, microsporogenesis progression in di- and polyploid *Ranunculus* plants of natural and hybrid origin were analysed to identify deviations during reproduction that mediate abnormal development products. Through a combined analysis of acetic-orcein and DAPI staining, irregularities in polyploid flower buds were identified as significantly higher as in diploid plant tissue. This is striking as the great majority of diploid plants studied here were F_1 and F_2 hybrids, which did not differ significantly from their parental diploid species, in regard to frequency of erroneous male sporogenesis (Table 3). Limited viability and fertility of young hybrids are extensively described and therefore, poor hybrid fitness is often taken for granted in case of natural hybrid progeny [12, 15], whereas F_2 hybrid performance is often worse than the situation in F_1 progeny but hybrids are not invariably less fit than their parents [9, 42]. Investigations on the influence of polyploidy, in *Ranunculus* hybrid plants only (including the natural allopolyploids), revealed a significantly increased frequency of disturbed microsporogenesis versus diploid hybrids (Table 5, Fig. 3c).

Overall, 5.42 % of all analysed samples showed an altered course of male sporogenesis (Table 3) with manifold error types, of which problems in bivalent and spindle formation and orientation are thought to be the most dramatic ones, because they led to abnormally shaped microspores and beyond (Fig. 1l - t). A significantly greater proportion of irregularly developed sporads were observed in polyploid *Ranunculus* plants (mean 13.28 %, P = 0.012), which led to the conclusion that polyploidization in combination with hybridization favours malfunctions in male reproductive development rather than hybridization alone (Fig. 3a). The production of dyads, triads and polyads seems to be due to various problems during microsporogenesis. Since meiosis is described to be very sensitive to unbalanced chromosome segregation, it is likely that either chromosome mispairing led to the formation of uni- and multivalents or erroneous spindle activities resulted in unusual gamete generation and pollen [10, 43]. This assumption is supported by the observation of anaphases with an odd number of spindle poles (Fig. 3i). Nevertheless, chromosome mispairing cannot be ruled out, because unbalanced chromosome segregation was regularly detected as well (Fig. 1j). In rare cases, plants showed incomplete cell plate assembly, forming unseparated aggregations of poly-nucleated microspores and in consequence, dyad pollen grains (Fig. 3r - s). Sporads, equipped with more than the normal quantity of four meiotic products, were believed to originate form unsuccessful chromosome division that again could be associated with defective spindle function. The detection of dwarf-microspores could be correlated to their genomic content, since in *Arabidopsis* and other model plants pollen size is positively connected to their DNA content [44]. However, this link to genome size was not yet demonstrated in *R. auricomus,* but microscopic pollen studies revealed dwarf and malformed pollen in apomictic taxa [29]. Quantitative pollen analyses in apomictic *Ranunculus kuepferi* found a great variation in pollen size, and dwarf pollen in tetraploids to be inviable [45]. The observed abnormalities during male gamete development seem to be relatively common phenomena in polyploid Ranunculaceae. Kumar *et al. [*46] characterized meiotic progression in tetraploid *Ranunculus* species, collected at the Himalayas. Consistent with the present data, they found several

severe meiotic problems including chromosome stickiness, laggards as well as disoriented bivalents. Additionally, they identified cases of cytomixis [46]. In cytomixis, the transfer of chromatin or even whole chromosomes via cytoplasmic channels or intercellular bridges can be achieved, presuming adjacent microspores [47, 48]. For example the disoriented chromosome in Figure 1g may be the result of either mispairing plus subsequent missegregation or can be caused by cytomictic transfer. Cytomixis is known to be able to lead to differentially sized pollen grains, which was identified in this setup as well [Fig. 1t, 46]. To estimate whether the obtained results are the consequences of synthetically generated polyploid *Ranunculus* hybrids, additionally, an adapted tetraploid (*R. cassubicifolius,* parent species) and a potential young, natural allopolyploid (Table 1) were included. It is assumed that the latter plants represent natural crosses between *R. notabilis* and *R. variabilis* due to phenotypical reasons as well as due to the fact that a *R. variabilis* population occurs nearby [49]. The frequency of abnormal microsporogenesis was found to be consistent with data of the *Ranunculus* hybrids made by hand-pollination (F_1; F_2; Table 1; Supplementary Data Table S1). This finding shows that irregularities can be triggered by hybridization events but can get significantly stronger, when it is combined with polyploidization as well (Table 1, Fig. 3a, b).

Furthermore, the age and degree of diploidization seems to play a crucial role for meiosis function, because the tetraploid *R. cassubicifolius,* which is at least 80,000 years old [35], displayed very low frequencies of abnormal male gamete development that are similar to that of diploid *Ranunculus* material (Table 1). Polyploidy is common in angiosperms and these plants are regarded as evolutionary fit, which might be due to a long diploidization process that is stabilizing gamete formation and genetic/epigenetic regulatory mechanisms [10, 19]. Thus, *R. cassubicifolius* plants are assumed to have overcome the bottleneck of currently polyploidized plants like in our natural hybrid samples [10]. The analysis of sporogenesis in male organs of F_1 *Ranunculus* hybrids has shown an increase in errors comparing di- and polyploid samples, which is consistent with the rest of this study but in contrast to the data gathered by Hojsgaard *et al.* [32]. There, microsporogenesis was described as “regularly and normally proceeding”. These discrepancies could be explained by the smaller sample size of the previous study.

Results raise the old question whether polyploidization, hybridization or a combination of both is the reason for the switch and fixation of apomixis. In order to draw an elusive picture of meiotic progression in aposporous hybrid *Ranunculus* samples, the female side of reproduction was compared to male data. Female development in the parental plant and F_1 generation were analysed previously by Hojsgaard *et al.* [32] and the situation in F_2 *Ranunculus* plants by Barke *et al. [*39]. These experiments have exclusively shown sexual ES formation for parental individuals, while in F_1 and F_2 hybrids apospory was detected [Table 4, 32, 39]. In this study, recently collected data for megasporogenesis of polyploid *Ranunculus* F_2 plants was amended with results of synthetic diploid F_2 hybrids39]. This analysis revealed similar frequencies for occurrence of apospory in di- and polyploid ovaries (Table 4). However, an overall comparison of female and male sporogenesis resulted in significantly higher error rate in female organs rather than on the male side (Fig. 3c). Monosporous development in *Ranunculus* increases the risk of

meiotic errors, as always just one of the megaspores is left to continue ES development. No tendencies towards polysporic embryo sac development were observed, as reported for other apomictic plants [50]. In *Ranunculus,* male sporogenesis leads to four haploid microspores within each one pollen grain. Therefore, reduced male fertility, accomplished by abnormal meiotic behaviour and disturbed microsporogenesis and -gametogenesis, has not as serious consequences as in female ovaries. The remaining intact pollen grains with functional gametes are numerous enough for successful fertilizations. Pseudogamous apomicts like *R. auricomus* plants need pollen for fertilization of polar nuclei for proper endosperm formation. Hence, selection will favour the maintenance of a male function even in apomictic plants [51]. In contrast, ovules are much less numerous, the pollen-ovule ratio ranges in *R. auricomus* from 652 to 1684 [41]. Unlike the situation in pollen, the death of the functional megaspore (whole germ line) easily jeopardizes the female reproduction success of the whole plant. Thus, selection pressure for an alternative apomeiotic developmental pathway is acting much harder on female than on male function in a hermaphroditic plant. In this study, less than 50 % of megasporogenesis in polyploid plants followed sexual development, while nearly 40 % of analysed ovules showed abortion and approx. 10 % formation of an aposporous initial (Table 4). Sexual ES formation in diploid hybrid samples made up more than 60 %, 20 % of the germ lines were fully aborted and 16 % developed aposporously [Table 4, 39]. Thus, the onset of apomixis, as already Darlington [22] proposed, really seems to be an escape from hybrid sterility, but only on the female side. Nonetheless, seed formation in Barke *et al.* [39] was only analysed in diploid plants due to mentioned high seed abortion rates. The effective influence of combined hybridization and polyploidization in *Ranunculus* was mainly observed on embryo sac formation.

Diploid hybridization appears to be a less effective trigger for apomixis than allopolyploidy. This hypothesis is in line with the general scarcity of diploid hybrids expressing apomixis in natural systems [23] and it is supported by the present study, showing significantly higher frequency of meiotic errors in polyploid hybrids than in homoploids (Fig. 3b; Table 3). The most prominent exception is found in the genus *Boechera,* where apomixis is fully functional in diploid hybrids [52]. But, in this genus dramatic chromosomal rearrangements were observed in diploid apomicts [52], and the apomictic diploid hybrid lineages originated from combinations of strongly disparate genomes [53]. Otherwise, apomictic seed formation in natural populations appears in very low frequencies [reviewed by 23] and could be also due to environmentally induced disturbance of sexual development [54]. To which extent meiotic irregularities in diploid hybrids are responsible for the establishment of apomixis, however, needs to be studied. Other potential reasons for emergence of apomixis may be genetic and epigenetic dislocations in angiosperm genomes provoked by hybridization or allopolyploidization, respectively [10, 50, 55]. This hypothesis is supported by several studies that observed heterochronic alterations in female development of synthetic *R. auricomus* hybrids [32, 39], which could be due to reversible epigenetic silencing [56]. Nonetheless, such alterations could be also a consequence of previous karyotypic changes after chromosome loss, rearrangements or missegration. More substantive proofs are required to test this hypothesis.

Conclusions

This study shed new light on cytological processes that happen in young allopolyploids and diploid *Ranunculus* hybrids and their role in apomictic reproduction. Results suggest that polyploidization has a much stronger detrimental effect on male meiosis than homoploid hybridization. Meiotic irregularities are much more frequent in female than in male development, even in the same plant. The correlation of failure of megasporogenesis to the appearance of apospory suggests indeed that disturbed megasporogenesis could be a functional trigger for apomixis. It was concluded that differential selective pressures act on male and female meiosis: While female development is constrained to circumvent meiosis to produce any functional embryo sac, male development can continue with a disturbed meiotic pathway, with selection acting on the huge mass of pollen that is still produced.

Material And Methods

Plant material

In this study, three generations of wild and hybrid plants were used. The parent plants were natural, diploid allogamous *R. carpaticola* and *R. notabilis;* and natural, tetraploid, allogamous *R. cassubicifolius* that all have been collected from wild populations (Table 1, 2) and were determined to reproduce sexually [32]. Homo- and heteroploid hybrid plants had been generated by manual crossings in 2006, which resulted in diploid F_1 hybrids (F, J plants; Table 1) obtained from *R. carpaticola* * *R. notabilis* crosses and triploid F_1 individuals (G plants; Table 1) gained by crossing *R. cassubicifolius* * *R. notabilis* [32]. Additionally, between 2010 and 2012, a second hybrid generation was produced using F_1 plants that have shown apospory [32]. F_2 individuals with F and/or J parents were found to be diploid and aposporous [39, Table 1], while hybrids descending from G parents were determined to be tri- and tetraploid [39, Table 1]. Since the original parental plants were no longer alive, we collected individuals from the same populations between 2011 and 2018 for the study here. In addition, tetraploid *R. notabilis* hybrid plants from another population that was previously described as diploid [49, Table 1]. We regard these plants as recently formed backcrosses with pollen from 4*x R. variabilis,* a species, which occurs at the same location [49]. All analysed plants in this study are grown outdoors in the old botanical garden of the Albrecht-von-Haller Institute for plant science at the University of Goettingen, Germany under the same climatic conditions.

Determination of ploidy and mode of reproduction

Ploidy and mode of reproduction of the hybrids are documented in Hojsgaard *et al.* [32] for the F_1 and in Barke *et al.* [39] for the F_2 generation. The newly collected individuals of the parental species were checked for ploidy and mode of reproduction by flow cytometry following protocols of Barke *et al. [*39]. Flow cytometric seed screening confirmed sexual reproduction for the *R. notabilis* and *R. cassubicifolius* individuals (Supplementary Data Table S2; Supplementary Data Fig. S1).

Flower bud fixations

For studying male meiosis in natural and artificial hybrid *Ranunculus* plants, small flower buds with a maximal diameter of 5 mm were harvested in spring and were directly fixed in ethanol: acetic acid (3: 1) and stored until usage at 4°C. Flower buds fixed with this method were used for orecin staining and chromosome spreads.

For the analysis of megasporogenesis, flower buds of a minimal diameter of 5 mm were collected and fixed in FAA solution (formaldehyde: acetic acid: ethanol: dH_2O; 2:1:10:3.5). After an incubation period of 48 h at room temperature the fixative solution was carefully exchanged by 70 % ethanol and stored at room temperature until analysis of female development [39].

Pollen mother cell orcein staining

Male sporogenesis was analysed by dissecting stamina from fixed flower buds on a microscopic slide, while adding a droplet of 2 % (w/v) lactopropionic orcein solution to the plant tissue. After installing the cover slip, mild thumb pressure was applied to the sample in order to release and stain the pollen mother cells (PMCs).

Chromosome spreads

The behaviour of chromosomes during male meiosis was investigated using the widely known chromosome spreading technique [44, 57] with several minor modifications. Fixed flower buds were washed twice in ddH_2O and once in citrate buffer (pH 4.8) until no “clouds” of fixative were detected. Plant tissue digestion was accomplished by incubation of the buds in an enzyme mixture made from 5 % (w/v) pectinase (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and 5 % cellulase (Onozuka R10; SERVA Electrophoresis GmbH, Heidelberg, Germany) in citrate buffer at 37°C in a moisture chamber for 5 h. After digestion enzyme mixture was carefully exchanged by citrate buffer and samples were stored for 1 h at 4°C. A single flower bud was transferred to a microscopic slide, containing one droplet of 60 % acetic acid, in which the plant tissue was squashed using a bent dissecting needle. Subsequently, the microscopic slide was heated on a hotplate at 45°C and plant tissue was uniformly spread across the warm slide. Therefore, the sample was submerged with freshly made, ice-cold ethanol: acetic acid (3: 1) fixative and then air-dried. Chromosome staining was achieved by adding 20 µl DAPI (1 µl/ ml; 4',6-diamidino–2-phenylindole; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) in VECTASHIELD® antifade mounting medium (VECTOR LABORATORIES, INC., Burlingame, CA, USA) and a cover slip to the sample. Finally, the sample was incubated overnight in the dark at 4°C to develop fully stained chromosomes.

Female development

The female megasporogenesis study of polyploid *Ranunculus* F_2 hybrids was performed using the well-documented differential interference contrast (DIC) microscopy [32, 39]. Prefixed flower buds were dehydrated by incubation for 30 min in 95 % and 100 % ethanol. In a subsequent treatment with an increasing dilution series of methyl salicylate (25; 50; 85; 100 %; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) in ethanol the flower bud tissue was cleared [39, 58]. For microscopy entire *Ranunculus* ovaries

were dissected from the cleared plant tissue and mounted on a microscopic slide in a droplet of pure methyl salicylate.

Microscopy

Visualization of male meiotic chromosomes and of female development in *Ranunculus* samples was carried out with a Leica microscope DM5500B. Images of the orcein-stained PMCs and the cleared ovaries were taken with a DFC 450C camera and LAS V41 software (Leica Microsystems, Wetzlar, Germany). For fluorescent picture imaging, the same microscope equipped with the DFC 365FX camera, the FLUO-filter cube A4 and the LAS AF 3.1.0 software was applied (Leica Microsystems CMS GmbH, Wetzlar, Germany).

Statistical Analyses

Percentages of abnormal male and female meiosis and sporogenesis were calculated for each individual, and shown with boxplots. Descriptive statistical analyses and tests for significant differences of two groups (either diploid versus polyploid PMCs /ovules or female versus male meiosis) were done by applying a Mann-Whitney-U test, due to not normally distributed data, using IBM SPSS Statistics 24 (IBM Deutschland GmbH, Ehningen, Germany).

To investigate the influence of sex, ploidy level and generation on sporogenesis, generalized linear mixed effect model (GLMM) analyses were performed using R package *lme4* v1.1–20 [59]. Sporogenesis was defined as response variable and determined as a binominal state; either normal (0) or abnormal (1). Binominal character distribution in the response variable enabled the application of GLMM analyses from the binomial error structure family [60]. The explanatory variables *sex, ploidy level* and *generation* were defined as categorical, occupying exactly one of a set of non-overlapping options; *sex:* male or female, *ploidy level:* diploid or polyploid, *generation:* P, F_1 or F_2. Interactions were allowed between explanatory variables within each GLMM analysis. The three sampled *Ranunculus* species (Table 1) were defined as random factor within GLMM analyses to control for interspecific effects. The data for natural allopolyploid *R. notabilis* * *R. variabilis* specimens was excluded from further analyses to prevent introduction of a potential bias regarding origin of polyploidy.

In order to test whether *ploidy level, generation* or both influence the course of microsporogenesis, GLMM analyses were executed for each categorical factor and in F_2 *Ranunculus* material, the impact of *ploidy level* on female sporogenesis was analysed. To infer whether male and female sporogenesis were differently affected in F_2 hybrids, the data of Barke *et al.* [39] on embryo sac development were combined with those herein to produce a total F_2 dataset. In addition, verification of adverse developmental effects on sporogenesis, caused by *ploidy levels, sex* or a combination of both, was done by consecutive GLMM analyses of the total F_2 dataset. A Laplace approximation was employed to fit the GLMMs to the data using the R function *glmer()* [59]. Chi-squared tests were performed to test for different effects of

explanatory variables. Results of GLMM analysis and Chi-squared tests were plotted to bar graphs using R v3.5.2 (R Foundation for Statistical Computing 2018).

Abbreviations

Differential interference contrast, DIC; embryo sac, ES; functional megaspore, FM; generalized linear mixed effect model, GLMM; pollen mother cell, PMC; standard deviation, STD.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files]. Additional microscopic images (original resolution) are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the German Research Fund “Deutsche Forschungsgemeinschaft DFG” (project Ho 4395/4–1 and 4395/10–1 within the priority program SPP 1991 TaxonOMICS), both to E. H.

Authors’ contributions

B. H. B. performed research, analyzed and interpreted data. B. H. B. and E. H. wrote the manuscript and designed the research. M. D. performed FCSS experiment and K. K. conducted the R analysis.

Acknowledgements

We thank the anonymous reviewers for valuable comments on the manuscript. We also thank Ladislav Hodač for collecting plants, Silvia Friedrichs for taking good care of the plants and Marius Eilerts for ploidy determination of leaf material.

Supplementary data

Supplementary data comprise the following information. Table S1: More detailed information on the generalized mixed-effect model (GLMM) analyses. These analyses observed effects changing the error frequency of micro- and megasporogenesis in *Ranunculus* with regard to ploidy, generation and sex. Calculations were based on 115 *Ranunculus* plants and more than 13,000 individual data points. R calculation output is visualized including standard error and z value. Regression estimate and p value are calculated by GLMM analysis and the tested factor is referred to the test and base line categories. Table S2: Mean peak indices of reproductive mode of different *Ranunculus* populations. Figure S1: Representative flow cytometry histograms of *Ranunculus* seeds. Figure S2: Chi-squared analyses of erroneous mega- and microsporogenesis in natural and hybrid *Ranunculus* plants.

References

1. Brandeis M. New-age ideas about age-old sex: separating meiosis from mating could solve a century-old conundrum. Biol Rev. 2018;93:801–10. doi:10.1111/brv.12367.
2. Hamant O, Ma H, Cande WZ. Genetics of meiotic prophase I in plants. Annu Rev Plant Biol. 2006;57:267–302. doi:10.1146/annurev.arplant.57.032905.105255.
3. Harrison CJ, Alvey E, Henderson IR. Meiosis in flowering plants and other green organisms. J Exp Bot. 2010;61:2863–75. doi: 10.1093/jxb/erq191.
4. Cifuentes M, Grandont L, Moore G, Marie A, Jenczewski E. On the production and recognition of vowels.pdf. 2010;29–36. doi: 10.1111/j.1469–8137.2009.03084.x.
5. Arnold ML. Natural Hybridization and Evolution. Oxford, UK: Oxford University Press; 1997. https://books.google.de/books?id = Jh8jVjEuDfUC.
6. Stebbins GL. The Role of Hybridization in Evolution. Proc Am Philos Soc. 1959;103:231–51. http://www.jstor.org/stable/985151.
7. Rieseberg LH, Willis JH. Plant Speciation. Science (80-). 2007;317:910 LP–914. doi:10.1126/science.1137729.
8. Mallet J. Hybrid speciation. Nature. 2007;446:279. doi:10.0.4.14/nature05706.
9. Hegarty MJ, Barker GL, Brennan AC, Edwards KJ, Abbott RJ, Hiscock SJ. Extreme changes to gene expression associated with homoploid hybrid speciation. Mol Ecol. 2009;18:877–89. doi:10.1111/j.1365–294X.2008.04054.x.
10. Comai L. The advantages and disadvantages of being polyploid. Nat Rev Genet. 2005;6:836–46. doi: 10.1038/nrg.
11. Zielinski M-L, Mittelsten Scheid O. Meiosis in Polyploid Plants. In: Soltis PS, Soltis DE, editors. Polyploidy and Genome Evolution. Berlin: Springer-Verlag; 2012. p. 33–55.
12. Soltis PS, Soltis DE. The Role of Hybridization in Plant Speciation. Annu Rev Plant Biol. 2009;60:561–88. doi:10.1146/annurev.arplant.043008.092039.
13. Schumer M, Rosenthal GG, Andolfatto P. How common is homoploid hybrid speciation? Evolution (N Y). 2014;68:1553–60. doi:10.1111/evo.12399.

14. Gross BL, Rieseberg LH. The Ecological Genetics of Homoploid Hybrid Speciation. J Hered. 2005;96:241–52. doi:10.1093/jhered/esi026.
15. Arnold ML, Ballerini ES, Brothers AN. Hybrid fitness, adaptation and evolutionary diversification: lessons learned from Louisiana Irises. Heredity (Edinb). 2012;108:159. doi:10.1038/hdy.2011.65.
16. Abbott RJ, James JK, Irwin J a, Comes HP. Hybrid origin of Oxford Ragwort, Senecio squalidus L. Watsonia. 2000;23:123–38.
17. Peruzzi L, Bedini G, Andreucci A. Homoploid hybrid speciation in Doronicum L. (Asteraceae)? Morphological, karyological and molecular evidences. Plant Biosyst. 2012;146:867–77. doi: 10.1080/11263504.2011.634445.
18. Van De Peer Y, Mizrachi E, Marchal K. The evolutionary significance of polyploidy. Nat Rev Genet. 2017;18:411–24. doi:10.1038/nrg.2017.26.
19. Ramsey J, Schemske DW. Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu Rev Ecol Syst. 1998;29:467–501. doi:10.1146/annurev.ecolsys.29.1.467.
20. Asker SE, Jerling L. Apomixis in Plants. Boca Raton, FL: CRC Press; 1992.
21. Nogler GA. Gametophytic Apomixis. In: Johri BM, editor. Embryology of Angiosperms. Berlin: Springer-Verlag; 1984. p. 475–518.
22. Darlington CD. The Evolution of Genetic Systems. Cambridge, UK: Cambridge University Press; 1939.
23. Hojsgaard D, Hörandl E. The Rise of Apomixis in Natural Plant Populations. Front Plant Sci. 2019;10:1–13. doi: 10.3389/fpls.2019.00358.
24. Kaul MLH, Murthy TGK. Mutant genes affecting higher plant meiosis. Theor Appl Genet. 1985;70:449–66. doi:10.1007/BF00305977.
25. Murphy SP, Bass HW. Genetics and Cytology of Meiotic Chromosome Behavior in Plants. In: Bass HW, Birchler JA, editors. Plant Cytogenetics - Genome Structure and Chromosome Function. New York, NY: Springer Science+Business Media, LLC; 2012. p. 193–229.
26. Koltunow AMG, Johnson SD, Rodrigues JCM, Okada T, Hu Y, Tsuchiya T, et al. Sexual reproduction is the default mode in apomictic Hieracium subgenus Pilosella, in which two dominant loci function to enable apomixis. Plant J. 2011;66:890–902. doi: 10.1111/j.1365–313X.2011.04556.x.
27. Hojsgaard D, Martínez EJ, Quarin CL. Competition between meiotic and apomictic pathways during ovule and seed development results in clonality. New Phytol. 2013;197:336–47. doi: 10.1111/j.1469–8137.2012.04381.x.
28. Hörandl E, Greilhuber J, Klímová K, Paun O, Temsch E, Emadzade K, et al. Reticulate evolution and taxonomic concepts in the Ranunculus auricomus complex (Ranunculaceae): Insights from analysis of morphological, karyological and molecular data. Taxon. 2009;58:1194–215. doi: 10.1002/tax.584012.
29. Hörandl E, Dobeš C, Lambrou M. Chromosomen- und Pollenuntersuchungen an österreichischen Arten des apomiktischen Ranunculus auricomus-Komplexes. Bot Helv. 1997;107:195–209. http://doi.org/10.5169/seals–72644.

30. Hörandl E, Greilhuber J. Diploid and autotetraploid sexuals and their relationships to apomicts in the Ranunculus cassubicus group: insights from DNA content and isozyme variation. Plant Syst Evol. 2002;234:85–100. doi:10.1007/s00606–002–0209-x.
31. Paun O, Greilhuber J, Temsch EM, Hörandl E. Patterns, sources and ecological implications of clonal diversity in apomictic Ranunculus carpaticola (Ranunculus auricomus complex, Ranunculaceae). Mol Ecol. 2006;15:897–910. doi: 10.1111/j.1365–294X.2006.02800.x.
32. Hojsgaard D, Greilhuber J, Pellino M, Paun O, Sharbel TF, Hörandl E. Emergence of apospory and bypass of meiosis via apomixis after sexual hybridisation and polyploidisation. New Phytol. 2014;204:1000–12. doi: 10.1111/nph.12954.
33. Dunkel FG, Gregor T, Paule J. New diploid species in the Ranunculus auricomus complex (Ranunculaceae) from W and SE Europe. Willdenowia. 2018;48:227–57. doi: 10.3372/wi.48.48205.
34. Hörandl E. Evolutionary implications of self-compatibility and reproductive fitness in the apomictic Ranunculus auricomus polyploid complex (Ranunculaceae). Int J Plant Sci. 2008;169:1219–28. doi: 10.1086/591980.EVOLUTIONARY.
35. Pellino M, Hojsgaard D, Schmutzer T, Scholz U, Hörandl E, Vogel H, et al. Asexual genome evolution in the apomictic Ranunculus auricomus complex: examining the effects of hybridization and mutation accumulation. Mol Ecol. 2013;22:5908–21. doi:10.1111/mec.12533.
36. Hodač L, Barke BH, Hörandl E. Mendelian segregation of leaf phenotypes in experimental F2 hybrids elucidates origin of morphological diversity of the apomictic Ranunculus auricomus complex. Taxon. 2018;67:1082–92. doi:10.12705/676.6.
37. Hörandl E. Comparative Analysis of Genetic Divergence among Sexual Ancestors of Apomictic Complexes Using Isozyme Data. 2004. doi: 10.1086/386557.
38. Hörandl E, Paun O, Johansson JT, Lehnebach C, Armstrong T, Chen L, et al. Phylogenetic relationships and evolutionary traits in Ranunculus s.l. (Ranunculaceae) inferred from ITS sequence analysis. Mol Phylogenet Evol. 2005;36:305–27. doi: 10.1016/j.ympev.2005.02.009.
39. Barke BH, Daubert M, Hörandl E. Establishment of Apomixis in Diploid F2 Hybrids and Inheritance of Apospory From F1 to F2 Hybrids of the Ranunculus auricomus Complex. Front Plant Sci. 2018;9 August:1–12. doi:10.3389/fpls.2018.01111.
40. Nogler GA. Genetics of apospory in apomictic Ranunculus Genetics of apospory in apomictic. Bot Helv. 1984;94:411–22.
41. Izmailow R. Reproductive strategy in the Ranunculus auricomus complex (Ranunculaceae). Acta Soc Bot Pol. 1996;65:167–170. doi: 10.5586/asbp.1996.029.
42. Arnold ML, Hodges SA. Are natural hybrids fit or unfit relative to their parents? Trends Ecol Evol. 1995;10:67–71. doi:10.1016/S0169–5347(00)88979-X.
43. Grandont L, Jenczewski E, Lloyd A. Meiosis and Its Deviations in Polyploid Plants. Cytogenet Genome Res. 2013;140:171–84. doi:10.1159/000351730.
44. De Storme N, Geelen D. The Arabidopsis Mutant jason Produces Unreduced First Division Restitution Male Gametes through a Parallel/Fused Spindle Mechanism in Meiosis II. Plant Physiol.

2011;155:1403–15. doi:10.1104/pp.110.170415.

45. Schinkel CCF, Kirchheimer B, Dullinger S, Geelen D, De Storme N, Hörandl E. Pathways to polyploidy: indications of a female triploid bridge in the alpine species Ranunculus kuepferi (Ranunculaceae). Plant Syst Evol. 2017;303:1093–108. doi: 10.1007/s00606–017–1435–6.
46. Kumar S, Jeelani SM, Rani S, Gupta RC, Kumari S. Meiotic analysis in some species of Ranunculus (Ranunculaceae) from the Western Himalayas. J Japanese Bot. 2013;88:46–53. doi: 10.1508/cytologia.73.137.
47. Körnicke M. Über Ortsveränderung von Zellkarnern. SB Niederrhein Ges Nat Heilk Bonn. 1901;14–25.
48. Guan JZ, Wang JJ, Cheng ZH, Liu Y, Li ZY. Cytomixis and meiotic abnormalities during microsporogenesis are responsible for male sterility and chromosome variations in Houttuynia cordata. Genet Mol Res. 2012;11:121–30. doi: 10.4238/2012.January.17.2.
49. Hörandl E, Greilhuber J, Dobeš C. Isozyme Variation and Ploidy Levels within the Apomictic Ranunculus auricomus Complex: Evidence for a Sexual Progenitor Species in Southeastern Austria. Plant Biol. 2000;2:53–62. doi:10.1055/s–2000–9148.
50. Carman JG. Asynchronous expression of duplicate genes in angiosperms may cause apomixis, bispory, tetraspory, and polyembryony. Biol J Linn Soc. 1997;61:51–94. doi: 10.1111/j.1095–8312.1997.tb01778.x.
51. Mogie M. Evolution of Asexual Reproduction in Plants. London, UK: Chapman and Hall; 1992.
52. Kantama L, Sharbel TF, Schranz ME, Mitchell-Olds T, de Vries S, de Jong H. Diploid apomicts of the Boechera holboellii complex display large-scale chromosome substitutions and aberrant chromosomes. Proc Natl Acad Sci U S A. 2007;104:14026–31. doi: 10.1073/pnas.0706647104.
53. Beck JB, Alexander PJ, Allphin L, Al-Shehbaz IA, Rushworth C, Bailey CD, et al. Does hybridization drive the transition to asexuality in diploid Boechera? Evolution (N Y). 2012;66:985–95. doi:10.1111/j.1558–5646.2011.01507.x.
54. Klatt S, Schinkel CCF, Kirchheimer B, Dullinger S, Hörandl E. Effects of cold treatments on fitness and mode of reproduction in the diploid and polyploid alpine plant Ranunculus kuepferi (Ranunculaceae). Ann Bot. 2018;121:1287–98. doi: 10.1093/aob/mcy017.
55. Rieseberg LH, Whitton J, Gardner K. Hybrid Zones and the Genetic Architecture of a Barrier to Gene Flow Between Two Sun ower Species. Biotechnology. 1999;152:713–27. doi: 10.1038/hdy.1981.98.
56. Hand ML, Koltunow AMG. The genetic control of apomixis: Asexual seed formation. Genetics. 2014;197:441–50. doi: 10.1534/genetics.114.163105.
57. Jones GH, Heslop-Harrison JS. Classical and molecular cytogenetics of Arabidopsis. In: Wilson ZA, editor. Arabidopsis - A Practicle Approach. Oxford: Oxford University Press; 1996. p. 105–24.
58. Young BA, Sherwood RT, Bashaw EC. Cleared-pistil and thick-sectioning techniques for detecting aposporous apomixis in grasses. Can J Bot. 1979;57:1668–72. doi:10.1139/b79–204.
59. Bates D, Mächler M, Bolker B, Walker S. lme4: Linear Mixed-Effects Models using “Eigen” and S4. 2019. doi:10.2307/2533043>.

60. Crawley MJ. Statistics: An Introduction Using R (2nd Edition). 2nd edition. Chichester: John Wiley & Sons, Ltd; 2015.

Tables

Table 1: Natural plants and synthetic hybrids of the *Ranunculus auricomus* complex analysed in this study.

Generation	*Ranunculus* Plants	Reproduction Mode	Plant ID	Ploidy	Reference
Parent Plants	*R. carpaticola*	Sexual	8483, LH040	2*x*	[32] Supplementary Data Table S2, Supplementary Data Fig. S1
	R. notabilis	Sexual	10137, 9609	2*x*	[49]
	R. cassubicifolius	Sexual	LH008, LH009	4*x*	Supplementary Data Table S2, Supplementary Data Fig. S1
F_1 Hybrids	*R. carp.* * *R. not.*	Sexual	F, J	2*x*	[32]
	R. cassu. * *R. not.*	Facultative apomictic	G	3*x*	
F_2 Hybrids	*R. carp.* * *R. not.* * *R. carp.* * *R. not.*	Facultative apomictic	F * F, F * J, J * F, J * J	2*x*	[39]
	R. cassu. * *R. not.* * *R. cassu.* * *R. not.*	Facultative apomictic	G * G	3*x*, 4*x*	
Natural Hybrids	*R. not.* * *R. variabilis (?)*	unknown	10136	4*x*	[49]

Table 2: List of wild collected natural Ranunculus plants analysed in this study incl. Herbarium voucher depositories - GOET (Herbarium University Goettingen) and WU (Herbarium University of Vienna). No permits were requested for the collection of these Ranunculus samples.

Ranunculus Plants	Plant ID	Localities (Collector, Date)	Plant Identification (Herbarium)
R. notabilis	9609, 10137	Austria, Burgenland, Strem valley, Moschendorfer forest (Hörandl, 8 May 2011)	Hörandl (WU)
*R. not. * R. var.* (?)	10136	Austria, Burgenland, Strem valley, Moschendorfer forest (Hörandl, 8 May 2011)	Hörandl (WU)
R. carpaticola	8483	Slovakia, Slovenské rudohorie, Revúca, hill Skalka (Hörandl, 5 May 1998)	Hörandl (WU)
R. carpaticola	LH040	Slovakia, Slovenské rudohorie, Banskobystrický kraj (Hodač, 3 May 2018)	Hörandl (GOET)
R. cassubicifolius	LH008	Austria, Lower Austria, Ybbs valley, Eisenwurzen (Hodač, 1 May 2017)	Hörandl (GOET)
R. cassubicifolius	LH009	Austria, Lower Austria, Ybbs valley, Eisenwurzen (Hodač, 1 May 2017)	Hörandl (GOET)

Table 3: Analysis of male development in di- and polyploid Ranunculus gametes during sporogenesis. Mean percentages of normal and abnormal sporogenesis were determined by orcein staining and bright field microscopy.

Taxa	Ploidy	Plant ID	n	normal sporogenesis (range)	abnormal sporogenesis (range)
Parent species					
R. notabilis	2*x*	10137, 9609	923	0.99 (0.50 - 0.99)	0.02 (0.01 - 0.50)
R. carpaticola	2*x*	8483, LH040	369	0.97 (0.94 - 0.99)	0.03 (0.01 - 0.05)
R. cassubicifolius	4*x*	LH008, LH009	324	0.97 (0.96 - 0.98)	0.03 (0.02 - 0.04)
Synthetic F_1 Hybrids					
*R. carpaticola * R. notabilis*	2*x*	J, F	3154	0.99 (0.99 - 1.00)	0.01 (0.00 - 0.01)
*R. cassubicifolius * R. notabilis*	3*x*	G	645	0.89 (0.79 - 0.99)	0.11 (0.11 - 0.21)
Synthetic F_2 Hybrids					
*R. car. * R. not. * R. car. * R. not.*	2*x*	F x F, F x J, J x F, J x J	3653	0.98 (0.95 - 1.00)	0.03 (0.00 - 0.17)
*R. cas. * R. not. * R. cas. * R. not.*	3*x*, 4*x*	G x G	211	0.86 (0.76 - 0.95)	0.14 (0.05 - 0.24)
Natural Hybrids					
*R. notabilis * R. variabilis (?)*	4*x*	10136	1001	0.82 (0.50 - 0.99)	0.18 (0.01 - 0.50)
Diploid Samples			8099	0.98 (0.83 - 1.00)	0.02 (0.00 - 0.17)
Polyploid Samples			2181	0.87 (0.50 - 0.99)	0.13 (0.01 - 0.50)
Total			10280	94.58 %	5.42 %

Table 4: Analysis of female development in di- and polyploid Ranunculus plants. Mean percentages of normal meiotic cell division, abnormal meiosis and full ovule abortion were investigated by DIC microscopy.

Taxa	Ploidy	Plant ID	n	normal meiosis (range)	abnormal meiosis (range)	aborted meiosis (range)
Parent species [32]						
R. notabilis	2*x*		86	0.96 (0.94 – 1.00)	0.00	0.04 (0.00 - 0.05)
R. carpaticola	2*x*		135	0.84 (0.83 - 0.90)	0.00	0.16 (0.10 - 0.18)
R. cassubicifolius	4*x*		98	0.95 (0.94 - 0.90)	0.00	0.05 (0.00 - 0.06)
Synthetic F_1 Hybrids [32]						
*R. carpaticola * R. notabilis*	2*x*	J, F	257	0.67 (0.44 - 1.00)	0.11 (0.00 - 0.33)	0.22 (0.00 - 0.56)
*R. cassubicifolius * R. notabilis*	3*x*	G	191	0.69 (0.54 - 0.87)	0.15 (0.07 - 0.32)	0.15 (0.00 - 0.29)
Synthetic F_2 Hybrids						
*R. car. * R. not. * R. car. * R. not.* [39]	2*x*	F * F, F * J, J * F, J * J	4811	0.63 (0.45 - 0.82)	0.16 (0.08 - 0.26)	0.21 (0.00 - 0.39)
*R. cas. * R. not. * R. cas. * R. not.*	3*x*, 4*x*	G * G	186	0.49 (0.06 - 0.66)	0.12 (0.06 - 0.15)	0.39 (0.19 - 0.88)

Table 5: Generalized mixed-effect model (GLMM) analyses discovering manipulating effects influencing the error rate of male and female meiosis and sporogenesis in Ranunculus with regard to ploidy level, generation and sex. Calculations were based on 115 Ranunculus plants and more than 13,000 individual data points. Statistical computation procedure in R is depicted. Regression estimate and p value are calculated by GLMM analysis as the tested factor is referred to the test and base line categories. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for statistical significance of the test. For more detailed statistical info see Supplementary Data Table S1.

Subset	n	Tested factor(s)	Base line categories	Test categories	GLMM Regression Estimate	p value
Male	9193	*ploidy level*	2*x*	4*x*	2.19	***
		generation	F_2	P	- 0.77	*
				F_1	- 0.63	**
		combinedeffect	2*x*, F_2	4*x*, P	- 0.88	0.08
				4*x*, F_1	- 0.60	0.09
Female	3660	*ploidy level*	2*x*	4*x*	0.17	0.46
Male/ Female	7438	*ploidy level*	2*x*	4*x*	0.17	0.46
		sex	female	male	- 2.44	***
		combined effect	2*x*, female	4*x*, male	2.02	***

Figures

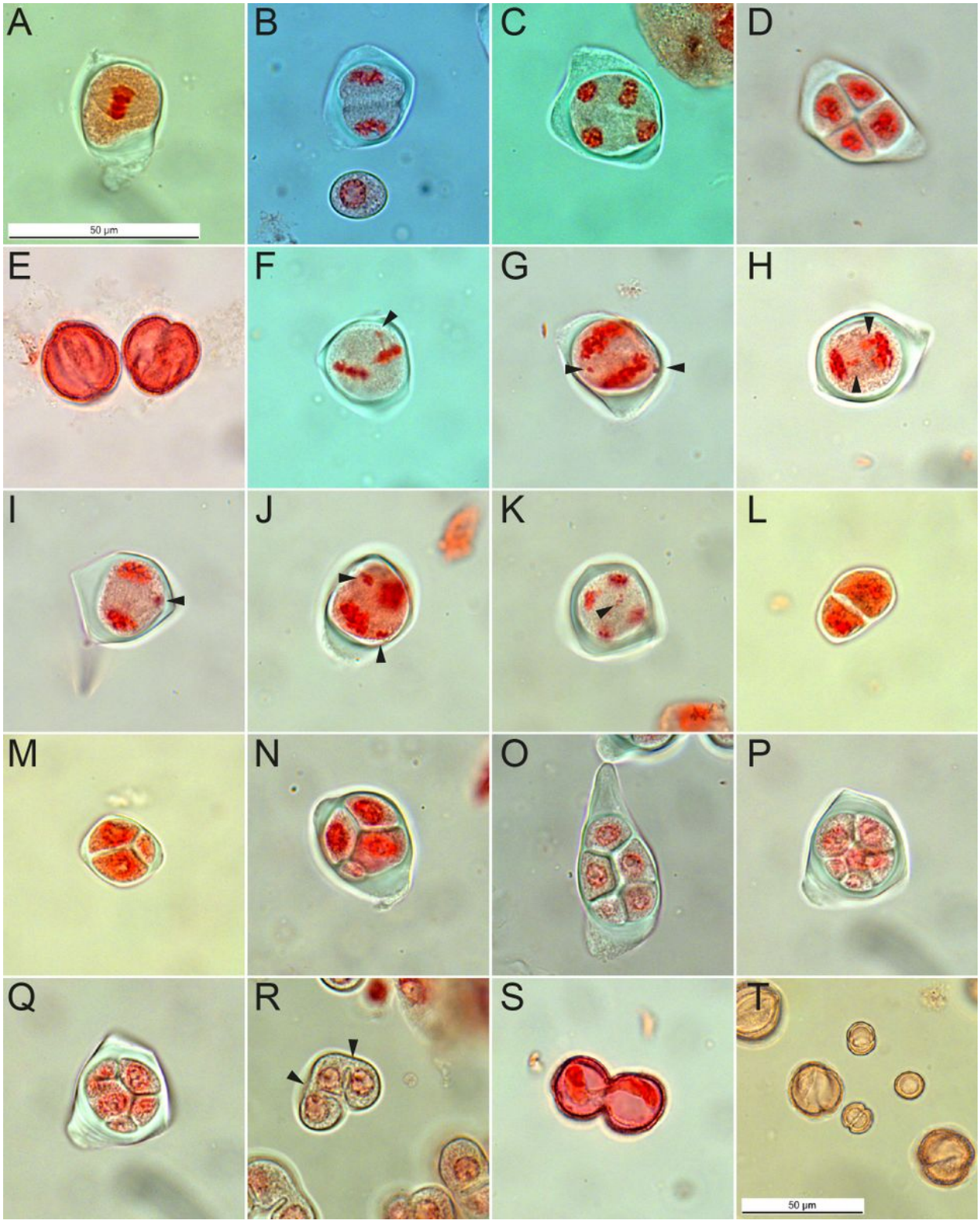

Figure 1

Development of male gametes in Ranunculus plants. a. – e.) Regular meiosis of PMCs, a.) PMC at metaphase I, b.) PMC at telophase I during cell plate formation, c.) PCM at the end of anaphase II, d.) Meiotically developed tetrad of microspores, e.) Homogeneous-sized pol-len grains, f. – p.) Various developmental failures in Ranunculus PMCs, f.) PMC at meta-phase II showing a sticky out-of-plate chromosome (arrowhead), g. + h.) PMCs with lagging chromosomes at anaphase I (arrowhead), i. + j.)

PCMs with irregular spindle activity (ar-rowhead), resulting in abnormal chromosome segregation at anaphase II, k.) PMC at ana-phase II with several lagging chromosomes, l.) A Dyad, m.) A Triad, n.) Tetrad with three normally sized microspores and one miniature microspore, o.) Polyad of five uniformly sized microspores, p. + q.) Figure of the same sporad at different levels. Polyad with seven microspores at different sizes, r.) Incompletely separated microspores. Arrowheads point to connections between the three nuclei-containing microspores, s.) Dyad pollen grain, t.) Het-erogeneously-sized micropollen grains. Genotypes: a.) F3 * J6 (22); b.) J9A; c., d., g., j.) 10136 (15); e.) 10137 (08); f.) J6 * F7 (14); h., i., k.) G5A; l., m., r., s.) F10 * F7 (04); n., t.) 10136 (08); o.) 10136 (02); p., q.) G16A. Scale bars = 50 µm.

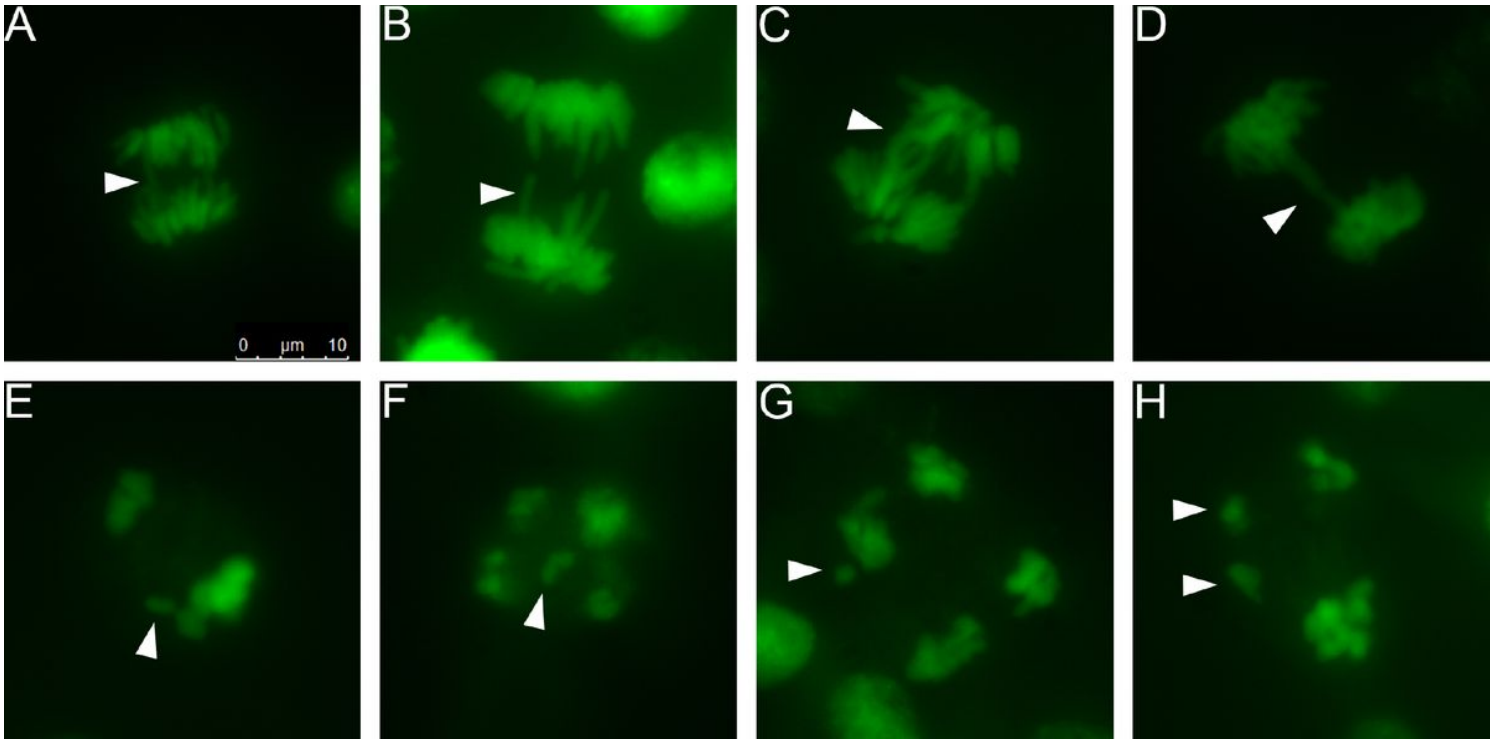

Figure 2

DAPI staining of abnormal chromosome configurations during microsporogenesis of Ranun-culus plants. a. – d.) Sticky chromosomes in PMCs during anaphase I (arrowheads), e. – h.) PMCs display stickiness due to clumped chromosomes, e.) PCM with laggard at anaphase I (arrowhead), f. + g.) PMCs at anaphase II with lagging chromosomes (arrowheads), h.) Er-ratically separated bivalents at anaphase II (arrowheads). Genotypes: a.) F3 * J6 (18); b. - d.) F3 * J6 (09); e., h.) F3 * J6 (30); f., g.) F3 * J6 (03). Scale bar = 10 µm.

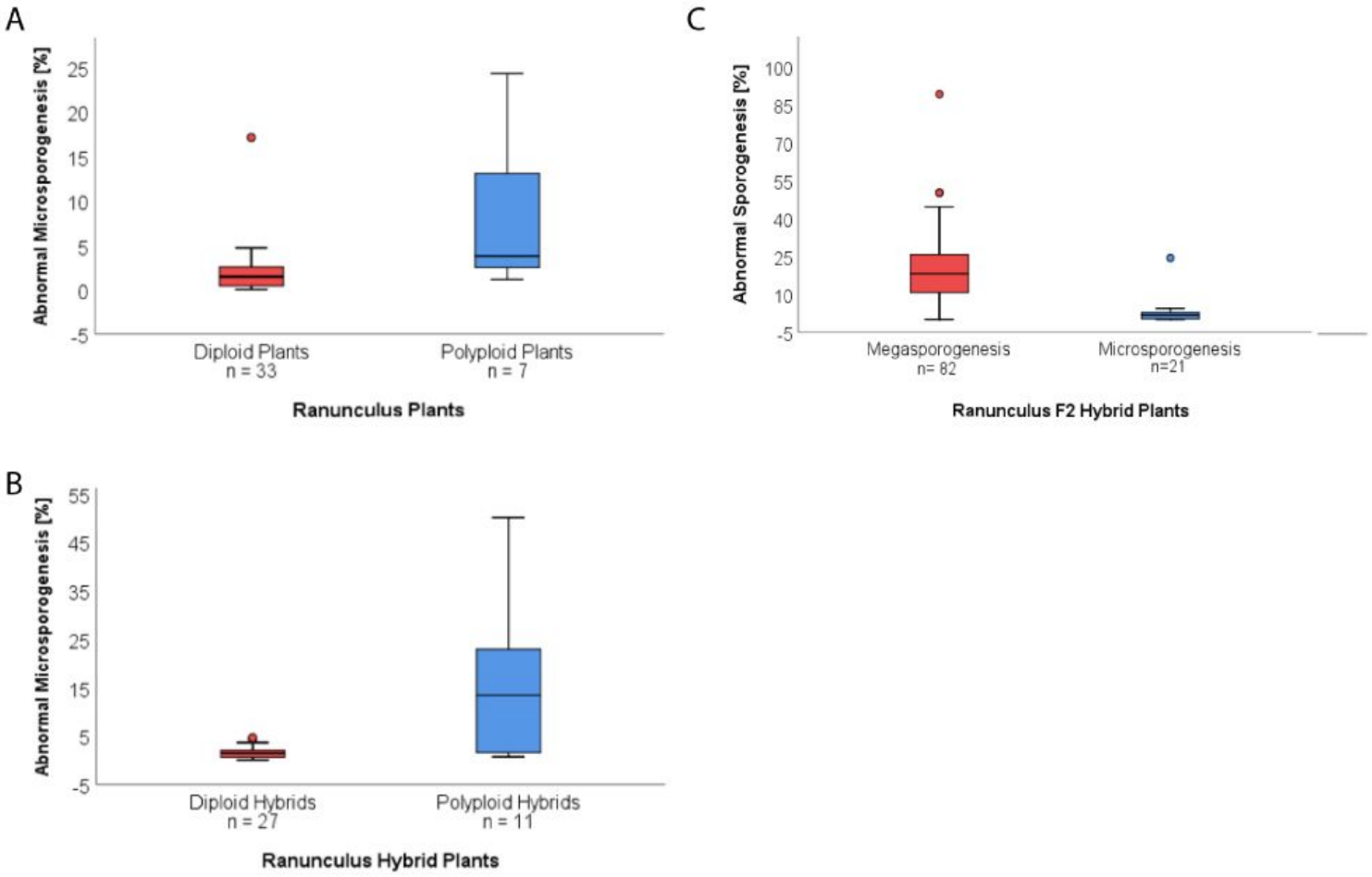

Figure 3

Analysis of irregular male and female sporogenesis in natural and hybrid Ranunculus plants. a.) Boxplot analysis of percentages of erroneous male meiosis of all three generations. Comparison of diploid and polyploid PMCs revealed a significantly increased frequency of abnormal sporogenesis in polyploid-derived samples ($p = 0.012$, Mann-Whitney-U test). b.) Abnormal microsporogenesis depicted for all di- and polyploid hybrid plants, of which allo-polyploids showed significantly more irregularities during development than homoploid in-dividuals ($p = 0.006$, Mann-Whitney-U test).c.) F2 hybrid plants showed different percent-ages of irregular sporogenesis depending on the sex and ploidy. Statistical comparison of male and female failure in sporogenesis irrespective of ploidy showed a significantly higher frequency of developmental error in female development ($p < 0.001$, Mann-Whitney-U test). Outliers are marked as filled circles, the box represents the interquartile range and in the boxplots the median is displayed.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS2SupplementaryData.doc
- FigureS1SupplementaryData.doc

- TableS2SupplementaryData20190821.doc
- TableS1SupplementaryData20190821.doc