The Evolutionary Establishment of Apomixis in Hybrids of the *Ranunculus auricomus* Complex: Developmental and Cytogenetic Studies

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Declaration of Independency

Herewith I ensure, that this doctoral thesis "The Evolutionary Establishment of Apomixis in Hybrids of the *Ranunculus auricomus* Complex: Developmental and Cytogenetic Studies" has been written independently and with no other sources and aids than quoted. Apart from the publications below, the thesis has not been published so far. I also declare that I have not previously applied for a doctoral degree at another university.

_____ Göttingen, April 30th, 2019

Birthe Hilkka Barke

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List of Abbreviations

°C	Degree centigrade
µl	Microliter
µm	Micrometer
ac	Antipodal cell
AGO	ARGONAUTE protein
AIC	Aposporous initial cell
cc	Central cell
dH_2O	Demineralized water
DAPI	4′,6-diamidino-2-phenylindole
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
e.g.	*exempli gratia*
ec	Egg cell
ES	Embryo sac
et al.	*et alia*
F_1	First hybrid generation
F_2	Second hybrid generation
F_3	Third hybrid generation
FAA solution	Fixative solution
FAM	6-carboxyfluorescein dye
FCSS	Flow cytometric seed screen
Fig.	Figure(s)

FM	Functional megaspore
G_2	Gap 2 phase
GLMM	Generalized linear mixed effect model
h	Hour(s)
HEX	Hexachloro-fluorescein
Hz	Hertz
i.a.	*inter alia*
ii	Inner integuments
LOA	LOSS OF APOMEIOSIS
LOP	LOSS OF PARTHENOGENESIS
m	Maternal
min	Minutes
ml	Milliliter
mm	Millimeter
MMC	Megaspore mother cell
N	Drop out
p	Paternal
p	Probability value
P	Parental generation
PCR	Polymerase chain reaction
PI	Peak index/ Peak indices
PMC	Pollen mother cell
R.	*Ranunculus*

RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RT	Room temperature
s	Seconds
S	Supplementary
sc	Synergid cell
SSR	Simple sequence repeat
STD	Standard deviation
vs.	*Versus*

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Summary

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1 Summary

In plant evolution, polyploidization and hybridization are important forces that both effectively contribute to developmental alterations. Asexual seed formation, known as apomixis, is mainly found in polyploid and/ or hybrid species but how these factors trigger the functional activation of apomictic reproduction remains elusive. The *Ranunculus auricomus* complex is worldwide distributed and consists almost exclusively of polyploid apomictic and a very small number of diploid and tetraploid sexual species. Due to the presence of sexual and apomictic species, this complex is an adequate model system to study the establishment and evolution of apomixis in natural plant populations.

In this study, synthetically derived young diploid and polyploid *Ranunculus* hybrids, including their parental species were used for detailed microscopic investigations of their flow of male and female gametophyte development. In both, micro- and megasporogenesis multiple abnormalities were discovered. Female development in diploid F_2 hybrids showed significantly enhanced frequencies of aposporous initial cell formation, which is associated with strong genomic dosage effects. These effects are considered to be the consequence of both F_1 parent plants being aposporous. Analyses of seed formation revealed beside sexually formed seeds, several intermediate B_{III} seeds as well as a few completely apomictic ones. In agree with this, male development showed severe irregularities in meiotic cell division and in sporogenesis. Especially, allopolyploid plants performed significantly more abnormal than homoploids. The error frequency in diploid F_2 *Ranunculus* hybrids was significantly higher as in the F_1 or in the parental generation. Furthermore, meiosis in female plant organs was significantly more prone to severe alterations than in male.

All these results indicate that hybridization, rather than polyploidization, is the apomixis-triggering factor in synthetic *Ranunculus* plants. In addition, the disturbed course of meiosis in micro- and megasporogenesis caused major implications in the female development, while on the male side only minor consequences were observed. These observations indicate strong, selective pressure acting on female development, whereas male gametophyte formation appears to be more or less unaffected. The switch to asexual seed formation in young, diploid *Ranunculus* hybrids is a rescue from imminent hybrid sterility, which is assumed to be caused by disturbed female meiosis.

2   General Introduction

2.1   Sexual Reproduction in Angiosperms

Flowers are the reproductive units of angiosperm plants, containing male or female sexual/ reproductive organs, or both (hermaphroditic flowers). The anthers of flowering plants represent male reproductive organs, while the corresponding female tissue is the carpel-embedded ovule. Each anther is capable of developing hundreds or even thousands of pollen grains, whereas each ovule only forms one single embryo sac (ES). The vast majority of angiosperms, more than 70%, produce the *Polygonum*-type ES, which was first described in *Polygonum divaricatum* (Strasburger, 1879). In the ovule, a diploid megaspore mother cell (MMC) divides by meiosis and forms four haploid megaspores. Only a single spore of this tetrad, usually the one closest to the chalazal pole, is designated to be the functional megaspore (FM), while all others start to abort. The FM starts to perform three successive rounds of mitosis without cytokinesis. These divisions result in a reduced coenoyte, also termed ES, which consist of one egg cell that is flanked on both sides by each one synergid cell. Due to these cells this ES region is called synergid pole. The area on the opposite side is known as chalazal pole, which harbors three antipodal cells. In the center of the ES two polar cells are formed (Maheswari, 1950). Either shortly before or right upon fertilization, these cells fuse without karyogamy and are named central cell (Yadegari and Drews, 2004). At this step megagametogenesis is complete and shows a mature, reduced ES.

The process in male reproductive tissue is relatively similar. A pollen mother cell (PMC) performs meiotic cell division and generates four microspores. In contrast to female gamete formation, in microsporogenesis none of the four haploid microspores degenerates. Subsequent mitosis leads to the formation of a haploid, mature pollen grain equipped with a vegetative and a generative cell that are enclosed by a double layered cell wall. The vegetative cell forms the pollen tube after pollination, whereas the generative cell produces two sperm cells (McCormick, 1993, 2004; Yadegari and Drews, 2004). Upon pollination of the plant's stigma by pollen grain, a pollen tube grows towards the synergid pole of the ES, guiding the two sperm cells. At its destination, the pollen tube grows inside one synergid and triggers its abortion. The sperm cells enter the ES and one of them fuses with the egg cell, forming a diploid embryo and the second male gamete fertilizes the central cell and develops into the triploid endosperm tissue. Seed for-

mation is complete after final development of the protective seed coat by the ovule's inner integuments (Maheswari, 1950).

2.2   Apomixis - Asexual Seed Formation

In angiosperm plants, seed formation is usually achieved by sexual reproduction but in about 220 genera a second, asexual pathway is described (Carman, 1997; Hojsgaard, Klatt, *et al.*, 2014). This developmental mode is termed "apomixis" and results in clonal, maternal seeds (Nogler 1984a). Apomixis research has a long tradition in botany and was documented for the first time in the early 19th century by James Smith, who discovered an "abnormality" in *Alchornea ilicifolia* (Smith, 1841; Asker and Jerling, 1992). The actual term "apomixis" was primarily introduced by Winkler (1908). In ensuing decades, this phenomenon was extensively described in plants with specific focus on female gametogenesis, yielding an impressive collection of different apomictic types (Gustafsson, 1946; León-Martínez and Vielle-Calzada, 2019).

In the early years of research, apomixis was considered as an abnormal trait that prevents sexual recombination (Darlington, 1939). It was assumed that a loss of meiosis inescapably results in the loss of heterogenity and thus, in an evolutionary dead end (Darlington, 1939). The majority of apomictically reproducing species belongs to three large plant families: Rosaceae, Asteraceae and Poaceae (Richards, 1997; Hojsgaard, Klatt, *et al.*, 2014). Developmental characteristics such as dioecy (O'Connell and Eckert, 1999), self-incompatibility (Bicknell *et al.*, 2003) and heterosis (Asker and Jerling, 1992) as well as special habitat preferences are tightly linked to apomixis. Another typical feature of most apomicts is that they are perennials and often combine apomixis with vegetative development, which make the plants capable of establishing large populations of clonal individuals (Bicknell and Koltunow, 2004). Privileged habitats of many apomicts are usually described as "hostile" because they either exhibit short growth phases or various other limitations such as crossing barriers (Asker and Jerling, 1992). For instance, the apomict, *Ranunculus kuepferi*, grows in high altitudes in the alps, where plants are confronted with poor soil and extreme meteorological conditions, including a short vegetation periods regularly interrupted by cold snaps (Körner, 2003; Klatt *et al.*, 2018). These alpine plants have occupied ecological niches that were shaped by Pleistocene glaciations (Kirchheimer *et al.*, 2018). Detailed phylogenetic studies on the positioning and occurrence of apomixis in angiosperms estimate that this reproductive mode evolved several times independently (Carman, 1997; Hörandl and Hojsgaard,

General Introduction

2012). Since a few decades, apomixis arouses new interest among scientists, aiming to improve the economic and agricultural value of commonly grown crop plants by introduction of apomixis. Apomictically reproducing crops, such as maize, rice or wheat, would hold several advantages. The most important would be the retention of heterosis (breeding enhancement) in hybrid offspring (Bicknell and Koltunow 2004; Spillane *et al.* 2004), followed by economic reasons like efficient time and cost reduction for breeding activities (Savidan, 2000b; Spillane *et al.*, 2004). Nonetheless, apomixis is not observed in economically important crop species, yet (Spillane *et al.*, 2004). The only exceptions are documented in *Citrus*, *Rubus*, *Mangifera* as well as in tropical forage grasses (Bicknell and Koltunow, 2004). Research projects on the generation of apomictic crops are executed since many years but until today, only little achievements have been made, due to economically unsatisfactory yield of asexual seeds (Savidan, 2000a, 2001; Spillane *et al.*, 2004). The process of gametophytic apomixis is composed of three essential developmental phases: Most importantly apomeiosis, which is ES formation by avoidance of meiosis. It is directly followed by fertilization-independent development of the plant embryo (parthenogenesis) and endosperm formation with or without pollen contribution (Koltunow and Grossniklaus, 2003).

Apomixis in flowering plants can be separated into two groups that differ in their mode of seed development. The first one is either called adventious embryony or, more often, sporophytic apomixis (Asker and Jerling, 1992). The asexual process involves embryo formation entirely detached from meiosis and parthenogenesis. Meaning, in sporophytic apomixis a somatic nucellar cell immediately develops into a diploid plant embryo (Gustafsson, 1947a; b; Grossniklaus *et al.*, 2001). However, this thesis focuses exclusively on gametophytic apomixis, which is mainly found in herbaceous plants and can be further subdivided into two modes: Diplospory and apospory (Bicknell and Koltunow, 2004). Both mechanisms are relatively similar, except for the origin of the seven-celled, *Polygonum*-type ES. In diplospory, the diploid MMC does not divide meiotically but rather directly starts with three rounds of mitosis, forming an unreduced ES. This type of gametophytic apomixis was identified in several plant species *i.a.* in *Taraxacum* and *Boechera* (Gustafsson, 1946; Araújo *et al.*, 2000). By contrast, in aposporous ovules the MMC does perform meiosis and develops a FM like in sexually reproducing plants. In the meantime, a somatic nucellar cell, called aposporous initial cell (AIC), changes its cell fate. This AIC takes control of the germ line, divides three times mitotically, irrespective

of cytokinesis, and forms an unreduced ES, while the sexual FM stalls. ES development is a permanent race between sexuality and apospory in facultative apomicts. It is commonly won by the more successful aposporous pathway (Hojsgaard *et al.*, 2013). In every ovule of a plant, another reproductive trait can be active and sometimes, sexual and apospory embryos can be observed in the very same plant ovule (polyembryony; *e.g.* Koltunow, 1993). Apospory occurs for example in *Pennisetum* and *Ranunculus* species (Nogler, 1971; Peel *et al.*, 1997). This asexual reproduction mode is heritable not only from one generation to the next, but can also be transplanted by usage of apomictic pollen as donor (Carman, 1997; Ozias-Akins and van Dijk, 2007). In plant species, the penetrance of asexual seed formation varies, ranging from obligate to facultative apomixis (Asker and Jerling, 1992). Obligate apomicts, which reproduce exclusively via clonal seed formation, were regarded as quite frequent but more recent observations seem to disprove this assumption (Savidan *et al.*, 2000). Nowadays, the existence of obligate apomixis is under debate because seed screenings revealed remnant sexuality even in plants, that were previously claimed to be exclusive apomicts (Hörandl and Paun, 2007; Hojsgaard and Hörandl, 2019). An example for such a proposed obligate apomict are *Hieracium* plants, which turned out to be facultative apomicts with only residual traces of sexuality (Hand *et al.*, 2015). Almost all apomicts reproduce facultatively, exhibiting different frequencies of sexuality, which result in completely mixed seed sets. According to this, a single plant individual is able to form sexual, apomictic and intermediate forms of seeds (Nogler 1984a). There are two intermediate seed types known to occur in gametophytic apomicts that represent incomplete frequencies of asexual reproduction either in female or in male gametes. The first type, B_{III} hybrids, is derived from an aposporous ES, of which egg and central cell, were fertilized by one reduced sperm nuclei each. Therefore, this reproductive pathway results in increased ploidy levels of the offspring (Rutishauser, 1948; Matzk *et al.*, 2000). Polyhaploids are the second type of seeds. They originate from meiotically formed egg cells that develop independently by parthenogenesis to functional, but rarely found seeds (Nogler 1984a). Beside the mentioned agricultural and economic aspects, natural apomictic plants offer great potential to gather fundamental knowledge on developmental and especially evolutionary processes of this asexual trait (Koltunow and Grossniklaus, 2003; Grimanelli, 2012). One conspicuous feature of most gametophytic apomicts is their polyploid genome, whereas sexual relatives tent to be diploids (Asker and Jerling, 1992). This is not absolute because a small num-

ber of diploid apomicts exists (*e.g.* Asker and Jerling, 1992; Naumova *et al.*, 1999; Schinkel *et al.*, 2016). It was assumed by Carman (1997) that apomictic seed formation is achieved by preceding events of hybridization and/ or genome duplication. Both, hybridization and polyploidization are powerful factors in plant evolution and development. Therefore, it is assumed that they play significant roles in the emergence and maintenance of apomixis (Carman, 1997).

2.3 Polyploidization

In flowering plants, polyploidization is an important evolutionary driving force, broadening species diversity (Wendel and Doyle, 2005) by establishment of new phenotypes that opens novel ecological niches for these plants for invasion (Adams, 2007). Multiple sets of chromosomes are very common in plants. It is even accepted that each plant species has experienced at least one event of whole genome duplication (Jiao *et al.*, 2011). Nonetheless, polyploidy in plants seems to be a success story by mistake rather than by norm because it was revealed that neopolyploids usually suffer from low diversification frequencies and are more prone to extinction than young diploids (Mayrose *et al.*, 2011; Grandont *et al.*, 2013). Polyploids that escaped extinction adapt their genomes by reducing redundant genomic information (Comai, 2005). This evolutionary process is called diploidization and is responsible for severe changes in the genomes such as depletion of gene duplicates or, in case of maintaining duplicates, they are commonly subject to sub- and neofunctionalization (Adams and Wendel, 2005; Comai, 2005). There are two distinct types of polyploids, strongly depending on the composition of their chromosome sets as well as their origin. Autopolyploids are the result of restitutional meiotic cell division and the formation of unreduced gametes, whereas allopolyploids are derived from various scenarios. For example, allopolyploids can be established by crossing of either two diploid, divergent species subsequently followed by genome duplication or by crossing of two heteroploid species resulting in a ploidy increase. In particular, adapted polyploids are evolutionary fitter than newly evolved ones (Comai, 2005). Polyploidization, irrespective of the mode, has profound influence on the plant, for example on gene expression and, most importantly, on its meiotic cell division (Quarin *et al.*, 2001; Comai, 2005; Zielinski and Mittelsten Scheid, 2012). Other well-described effects of polyploidization are the enlargement of nuclei and of the plant cell itself. Furthermore, the process has also fundamental consequences for DNA methylation patterns (Adams, 2007) and is suspected to affect sexual reproduction (Comai, 2005). Due to the fact that most apo-

micts are polyploids, polyploidization is believed to be somehow involved in the occurrence or maintenance of apomixis (Asker and Jerling, 1992). Discoveries of fully functional diploid apomicts like in *Paspalum* and *R. kuepferi*, indicate that a polyploid genome is not an essential requirement for the switch from sexual to apomictic reproduction (Ortiz *et al.*, 2013; Schinkel *et al.*, 2016, 2017). A reasonable hypothesis to explain the tight connection between apomixis and polyploidy was developed by Nogler (1982, 1984a; b), who proposed the presence of dosage effects in apomictic *Ranunculus* plants, assuming powerful genetic factors that control apomictic reproduction. He further speculated that these factors have strong recessive lethal effects on all haploid gametes, which is likely, considering the small number of diploid apomicts. Another possible role of polyploidization could be its proven ability to influence gene expression by amplification and/ or stabilization of apomixis-related gene clusters (Quarin *et al.*, 2001; Bicknell, 2004; Comai, 2005). In early years of genetic and molecular research on apomixis, it was thought that apomixis has to be a single, dominant gene, which is responsible for the inheritance of the trait (Nogler, 1984b; Savidan, 1992), but more recent studies indicate that the essential key components of gametophytic apomixis, namely apomeiosis and parthenogenesis are located on diverse, independent genetic loci (Catanach *et al.*, 2006; Koltunow *et al.*, 2011; Ogawa *et al.*, 2013). Pollination experiments revealed that these two loci can be inherited and additionally, the establishment of several mutant lines in *Hieracium* showed that apomictic plants are able to return to sexual ES formation, when lacking the locus for apospory (Catanach *et al.*, 2006; Koltunow *et al.*, 2011, 2013; Hand *et al.*, 2015). Furthermore, a feature of these apomixis loci is their genomic infrastructure, which is often heterochromatin and transposon rich and possesses not seldom extensive stretches of repetitive sequences, which all together may be responsible for the observed reduced or even suppressed recombination in these genomic regions (Akiyama *et al.*, 2004, 2005; Okada *et al.*, 2011). As described, polyploidization does lead to many genetic and developmental alterations but experiments indicate that polyploidy alone is not responsible for the emergence of apomixis (*e.g.* Hojsgaard, Greilhuber, *et al.*, 2014). However, it could act together with hybridization in order to trigger apomictic reproduction in plants (Asker and Jerling, 1992; Hand and Koltunow, 2014; Hojsgaard and Hörandl, 2019).

2.4 Interspecific Hybridization

Another frequently occurring evolutionary effect in plant speciation and development is interspecific hybridization (Arnold, 1997; Soltis and Soltis, 2009; Nolte and Tautz, 2010). As in polyploidization two different categories are known – homoploid and allopolyploid hybrids. The latter ones double their chromosome number right after hybridization, whereas homoploids remain diploid (Stebbins, 1959; Rieseberg and Willis, 2007). Hybridization forces the combination of two divergent chromosome sets, which can have catastrophic outcome for the hybrid progeny (Arnold, 1997). The efficiency of *i.a.* meiosis relies very much on chromosomal homology. Thus, successful chromosome paring and segregation, the two most sensitive processes of meiosis I, highly depend on sequence similarity (Comai, 2005; Mallet, 2007). Disturbed meiosis leads to abnormal sporogenesis, which in turn, negatively influences other essential developmental mechanisms like gametogenesis and gamete formation (Mallet, 2007; Rieseberg and Willis, 2007; Zielinski and Mittelsten Scheid, 2012). The sum of defective development can have variable effects in newly evolved hybrid plants, especially in the first generation (F_1; Hegarty *et al.*, 2009). Neohybrids are often less viable than their parents and either show a reduced fitness or are completely sterile (Arnold, 1997; Mallet, 2007; Hegarty *et al.*, 2009). Other consequences of unbalanced gamete formation caused by chromosome missegregation can be aneuploidy or even cell death (Cifuentes *et al.*, 2010). Observations like this led to negative hypotheses, calling hybrids "hopeful monsters" as done by Mallet (2007) or claiming that they suffer from genomic shocks (McClintock, 1984). Beside erroneous meiosis, there are several other accepted outcomes of hybridization. The clash of genomes can either perish adapted gene complexes by meiotic cell division (Nolte and Tautz, 2010) or can cause serious changes in gene expression or DNA methylation (Carman, 1997; Adams, 2007). However, many hybrid plants have different frequencies of fitness because hybridization produces certain genotypic and phenotypic varieties that are not per se less fit than their relatives. They rather represent multiple degrees of fitness, ranging from less fit to comparable fit as the parent generation (Arnold and Hodges, 1995; Arnold, 1997). As mentioned earlier, hybridization events have enormous influence on meiosis and other important processes in plant development and speciation such as gene expression and epigenetic silencing (Carman, 1997; Comai, 2005; Adams, 2007; Sailer *et al.*, 2016). For example, the performance of megasporogenesis and –gametogenesis in natural *Ranunculus* hybrids and in synthetic *Ranunculus* F_1 hybrids were analyzed by Hojsgaard *et al.* (2014). These experiments

showed massive developmental changes during ES formation, especially in cell fate and time flow of the synthetic hybrids. Furthermore, in the first generation of synthetic hybrids spontaneous AIC occurrence was documented (Hojsgaard, Greilhuber, *et al.*, 2014). Together with results from different gene expression studies, comparing apomicts to sexual reproducing plants, Hojsgaard *et al.* (2014) inferred that the developmental failures could originate from hybridization-caused heterochronic expression of sex-related genes that may be responsible for the detected spontaneous emergence of AIC in *Ranunculus* hybrids (Sharbel *et al.*, 2009, 2010; Pellino *et al.*, 2013). Besides temporal changes, the steric expression of important developmental genes can be altered as well (Grimanelli, 2012). Furthermore, epigenetic changes in gene expression can be induced by hybridization and these reversible alterations are also known to be heritable from one generation to the next (Grimanelli, 2012; Hand and Koltunow, 2014). All these characteristics make epigenetic silencing a powerful mechanism to rewrite developmental programs of plants upon hybridization and therefore, a potential trigger of apomixis (Grimanelli, 2012). Due to the numerous and diverse genetic and developmental problems that commonly negatively influence the viability and fitness of young hybrid plants, this evolutionary force is regarded as potential elicitor of apomictic seed production (*e.g.* Ernst, 1918; Asker and Jerling, 1992; Carman, 1997; Hand and Koltunow, 2014; Hojsgaard and Hörandl, 2019). The assumption is experimentally supported by findings of Paun *et al.* (2006), who supposed that hybridization serves as generator of apomictic species in the *Ranunculus cassubicus* complex, because the apomictic hexaploid *R. carpaticola* was found to be hybrid progeny derived from a sexual, diploid *R. carpaticola* and a sexual, autotetraploid *R. cassubicifolius*. This observation led to the presumption that the switch to apomixis in this complex demands both, hybridization and poly-ploidization (Paun, Stuessy, *et al.*, 2006). Based on this, Paun *et al.* (2006) concluded that primarily a polyploid genome of a sexual species could serve as starting point, but essentially requires an additional hybridization event in order to change from sexual to apomictic seed formation. Other recent studies have confirmed the hybrid origin of many other apomicts like the microsatellite approaches of Beck *et al.* (2012) and Šarhanová *et al.* (2017). As described, hybridization is also known to strongly affect the course of meiosis, which represents another source of reduced fitness and cell death (Arnold, 1997; Comai, 2005). These and other consequences of hybridization inspired Darlington (1939) to his famous hypothesis that the switch to apomictic seed formation seems to be

forced after hybridization in order to escape from these negative implications. All these obtained facts elevate hybridization rather than polyploidization to the top candidate for triggering gametophytic apomixis in angiosperm plants.

2.5 The *Ranunculus auricomus* Complex

The *R. auricomus* complex is part of *Ranunculus* sect. *auricomus* and consists of about 800 species (Hörandl and Gutermann, 1998; Hörandl *et al.*, 2009). The complex grows all over Europe, ranging from Mediterranean to arctic regions. These herbaceous, perennial plants are also common colonizers in Alaska, Greenland as well as in western Serbia (Jalas and Suominen, 1989; Hörandl *et al.*, 2009; Dunkel, 2015; Dunkel *et al.*, 2018). Due to their yellow-golden flowers these plants are also known as goldilocks. *Ranunculus* species that are abundant in central Europe are able to occupy various habitats such as forests, meadows, wetlands as well as cultivated land (Hörandl and Paun, 2007; Hörandl *et al.*, 2009). The complex comprises mainly polyploid species that reproduce via gametophytic apomixis (Nogler, 1984b; Jalas and Suominen, 1989). Apomictic seed formation in *R. auricomus* species requires aposporous ES development, parthenogenesis as well as central cell fertilization in order to ensure endosperm formation (Rutishauser, 1954; Izmailow, 1967; Nogler, 1984a). These agamospecies were determined as mainly tetraploid (2n = 32), although tri-, penta- and hexaploid species are documented as well (Jalas and Suominen, 1989; Hörandl *et al.*, 1997). In addition to the polyploid apomicts, a small number of sexual reproducing *Ranunculus* species were detected with either a diploid (2n = 16) or a tetraploid chromosome set (Hörandl *et al.*, 1997; Paun and Hörandl, 2006; Dunkel *et al.*, 2018). In this complex apomixis is associated with polyploidy and hybridization, whereas diploid or autopolyploid genomes are exclusively found in sexual reproducing plants (Nogler, 1984a; Hörandl *et al.*, 1997; Paun, Stuessy, *et al.*, 2006). The rare sexual species are geographically limited to a small distribution area, while the apomictic species have colonized habitats all over Europe (Hörandl and Paun, 2007). Evolutionary, the three sexual outcrossers *R. notabilis*, *R. cassubicifolius* and *R. carpaticola* have been verified as ancestor species of all polyploids and it has been hypothesized by Paun *et al.* (2006) that autopolyploidization, as found in tetraploid *R. cassubicifolius*, subsequently accompanied with hybridization could result in asexual seed formation (Hörandl *et al.*, 2009; Pellino *et al.*, 2013; Hodač *et al.*, 2018).

3   Research Objectives

In the past decades the *R. auricomus* complex developed into a widely accepted and used model system to study the emergence and evolution of apomixis in natural plant populations (*e.g.* Hörandl, 2008; Hojsgaard, Greilhuber, *et al.*, 2014). Nevertheless, there are still many unanswered questions. One of the most interesting questions deals with the onset of gametophytic apomixis in *Ranunculus* and how it is connected to evolutionary factors like polyploidization and hybridization. Therefore, the thesis focuses on the elucidation of this connection using synthetic homo- and heteroploid *Ranunculus* hybrid generations as well as their sexual parent plants. Functional gametophytic apomixis in *Ranunculus* demands a tight interaction of apomeiosis, parthenogenesis and pseudogamous endosperm formation (Nogler, 1984a; b). In order to identify and to evaluate developmental changes associated with the switch to apomixis were investigated in detail. The following chapters answer research questions closely related to the main goal of this thesis: The clarification of how apomixis is triggered in the *R. auricomus* complex and whether hybridization, polyploidization or both contribute to it.

3.1   Chapter 1:

Female Gametophyte Formation in *Ranunculus* F$_2$ Hybrids

The first chapter of this thesis concentrates on interspecific hybridization as potential elicitor of gametophytic apomixis, because historically this functional driver of evolution was suspected of provoking asexual seed formation (*e.g.* Ernst, 1918). As mentioned, most apomictic plants comprise polyploid genomes but polyploidy is not an essential prerequisite as proven by a small number of diploid apomicts (*e.g.* Sharbel *et al.*, 2009; Ortiz *et al.*, 2013; Schinkel *et al.*, 2017). The importance and frequency of polyploidy can neither be ignored nor denied and will therefore be addressed in the second chapter.

Due to the fact that apomixis is most abundantly noted in allopolyploids and rarely in diploid hybrids, hybridization seems to have a role in the emergence of apomixis (Asker and Jerling, 1992). The assumption is strongly supported by observation that hybridization events are able to result in major implications, influencing genetic and epigenetic constitution of hybrid offspring (Carman, 1997; Rieseberg *et al.*, 1999; Grimanelli, 2012). A possible scenario, caused by the clash of two divergent plant genomes, could be severe changes in the temporal and/ or spatial expression of genes that are associated with

sexual reproduction, which might end in the developmental switch to apomixis (Carman, 1997; Sharbel *et al.*, 2009, 2010). Profound disturbances like heterochronic gene expressions and spontaneous AIC formation were documented by Hojsgaard *et al.* (2014) in synthetic F_1 *Ranunculus* hybrids. These plants were derived from manual crossings of sexual reproducing diploid *R. notabilis* and *R. carpaticola* individuals and autotetraploid *R. cassubicifolius* plants (Paun, Stuessy, *et al.*, 2006; Hojsgaard, Greilhuber, *et al.*, 2014). In addition to (epi-) genetic alterations, interspecific hybridization of sexual plant individuals can have negative effects on the progression and outcome of meiotic cell division, which will be discussed in the second chapter as well (Comai, 2005). All these hybridization-caused effects are well-known and found to be responsible for diminished hybrid fitness or even for hybrid sterility (Arnold, 1997; Rieseberg and Willis, 2007). Therefore, Darlington (1939) captioned apomixis as "savior" from hybrid sterility. In order to gain more precise information on whether the hypothesis of Darlington applies to plants of the *R. auricomus* complex, diploid F_2 hybrid plants originated from F_1 *Ranunculus* plants of Hojsgaard *et al.* (2014) that have shown aposporous ES formation, were analyzed for their frequency of apospory, developmental alterations as well as for apomictic seed formation in the first chapter of this thesis. Before these experiments were performed the sexual origin of the diploid F_2 plants was demonstrated by SSR genotyping. These analyses allow the exclusion of all other modes of reproduction such as selfing or apomixis, which was essential in this study. Apomictic reproduction in *Ranunculus* starts with the formation of an AIC adjacent to the meiotically derived FM and therefore, the progression of megasporogenesis was analyzed with the intention to detect and quantify irregular course of megasporogenesis and especially the frequency of spontaneous AIC formation by DIC microscopy. Furthermore, the study wants to test the widely held belief of Nogler (1984a; b) that the frequency of apospory is positively correlated to genetic dosage effects, which will be done by statistical comparison of the frequencies of aposporous ES development in *Ranunculus* hybrids with one or two aposporous parents. With Flow cytometric seed screens the establishment and efficiency of all three essential key elements of gametophytic apomixis were investigated by determining the reproductive mode of hand-made F_3 *Ranunculus* seeds and finally, quality and viability of these seeds were tested by germination experiments.

3.2 Chapter 2:

The Influence of Hybridization, Polyploidization and Apomixis on Meiotic Cell Division

The second chapter of this thesis is centered on the sequence of meiotic cell division in the light of several consequential evolutionary factors like hybridization, polyploidization and apomixis. Beside the prevalent hybrid character of apomictic plants, they are almost invariably polyploid as well, but the function of polyploidy in conjunction with the establishment of apomixis is not fully elucidated yet (Asker and Jerling, 1992). Numerous different hypotheses exist that try to explain this connection. For example, hybridization was replaced/ accompanied by polyploidization as apomixis activator (*e.g.* Quarin *et al.*, 2001) but in most cases, polyploidy is believed to act as genomic stabilizer by *e.g.* restoring homologous chromosome pairs or as promoter of genetic isolation during plant speciation (Alix *et al.*, 2017). Due to all these variable opinions, the second chapter aims to shed light on this long lasting problem by describing and evaluating the course of meiosis in three different *Ranunculus* generations.

As outlined above, meiosis is a highly essential developmental process required for gamete formation in eukaryotic organisms, including angiosperm plants (Hamant *et al.*, 2006; Brandeis, 2018). It is already known that evolutionary events like hybridization and polyploidization can disturb the normal progression of sexual gamete formation, which can lead to reduced plant viability and sterility (De Storme and Mason, 2014). Meiosis is a research topic of high interest and therefore, a lot is already known about its behavior, when faced with *e.g.* hybridization. Typical meiotic outcomes of hybridization in plants are severe limitations and alterations during chromosome segregation *i.a.* laggards, which might result in meiotic restitution and therefore the establishment of allopolyploids (De Storme and Mason, 2014). Other possible consequences can be reduced fitness, aneuploidy or apoptosis of gametes (Zielinski and Mittelsten Scheid, 2012). In addition, the onset of gametophytic apomixis is essentially depending on apospory, which is the circumvention of meiotic gamete formation (Nogler, 1984a). Therefore, the onset of apomixis could save hybrid plants from sterility by skipping the hybridization-caused erroneous meiotic cell division (*e.g.* Darlington, 1939; Asker and Jerling, 1992; Carman, 1997). However, the functional linkage of meiotic failure upon hybridization, polyploidization or both and the switch to the complete bypass of meiosis needs to be

studied in more detail. This goal is achieved by cytogenetic experiments of micro-sporogenesis in chapter two. As sample material manually crossed homo- and hetero-ploid *Ranunculus* F_1 and F_2 hybrids, their di- and tetraploid parental plants and a natural, young tetraploid *Ranunculus* hybrid were used. In order to assess the consequences of polyploidization and hybridization a list of all meiotic abnormalities were made, based on more than 10,000 examined microspores, including meiotic stages. This is the first large-scale attempt to disentangle the influence of the evolutionary forces on male sporogenesis and therefore, on the onset of apomixis in *Ranunculus* by microscopy and statistical applications, calculating generalized linear mixed effect models.

4   Chapter 1

Establishment of Apomixis in Diploid F₂ Hybrids and Inheritance of Apospory From F₁ to F₂ Hybrids of the *Ranunculus auricomus* Complex

Birthe Hilkka Barke, Mareike Daubert, Elvira Hörandl

4.1   Abstract

Hybridization and polyploidization play important roles in plant evolution but it is still not fully clarified how these evolutionary forces contribute to the establishment of apomicts. Apomixis, the asexual reproduction via seed formation, comprises several essential alterations in development compared to the sexual pathway. Furthermore, most natural apomicts were found to be polyploids and/ or hybrids. The *Ranunculus auricomus* complex comprises diploid sexual and polyploid apomictic species and represents an excellent model system to gain knowledge on origin and evolution of apomixis in natural plant populations. In this study, the second generation of synthetically produced homoploid ($2x$) and heteroploid ($3x$) hybrids derived from sexual *R. auricomus* species was analyzed for aposporous initial cell formation by DIC microscopy. Complete manifestation of apomixis was determined by measuring single mature seeds by flow cytometric seed screen. Microscopic analysis of the female gametophyte formation indicated spontaneous occurrence of aposporous initial cells and several developmental irregularities. The frequency of apospory was found to depend on dosage effects since a significant increase in apospory was observed, when both F₁ parents, rather than just one, were aposporous. Other than in the F₁ generation, diploid *Ranunculus* F₂ hybrids formed B_III seeds and fully apomictic seeds. The results indicate that hybridization rather than polyploidization seems to be the functional activator of apomictic reproduction in the synthetic *Ranunculus* hybrids. In turn, at least two hybrid generations are required to establish apomictic seed formation.

4.2   Introduction

Apomixis in Angiosperm plants is, by definition, seed formation via asexual reproduction, resulting in clonal, maternal offspring (Nogler, 1984a). Gametophytic apomixis, which is the focus of our study, combines two steps: (1) apomeiosis, *i.e.* the formation of an unreduced embryo sac, and (2) parthenogenesis, *i.e.* the development of an unfertilized egg cell into an embryo. Almost all apomictic plants are polyploids and/ or hybrids

but the role of these processes for establishment of apomixis is still not well-understood. There is evidence that the functional establishment of apomixis is not exclusively ploidy-dependent but an important factor in increasing and optimizing related gene expression (Quarin *et al.*, 2001; Bicknell and Koltunow, 2004; Comai, 2005). A reason for the importance of polyploidy in apomictic plants can be conjectured by gene dosage effects, which state that haploid gametophytes abort due to recessive lethal effects of apomixis-controlling genetic factors (Nogler, 1982, 1984a; b). This assumption is supported by the rarity of diploid apomicts but a few exceptions are Scandinavian *Potentilla argentea* biotypes, diplosporous *Boechera* species (Müntzing, 1928; Böcher, 1951; Sharbel *et al.*, 2009), *Paspalum* and *Ranunculus kuepferi* individuals (Ortiz *et al.*, 2013; Schinkel *et al.*, 2016, 2017). However, emergence of apomixis is not only achieved by ploidy but could be also an effect of hybridization (Asker and Jerling, 1992). Often hybridization of sexual plants leads to severe disturbances influencing genetic and epigenetic composition or meiotic cell division that can result in progeny with reduced fitness (Carman, 1997; Rieseberg *et al.*, 1999; Comai, 2005). Disturbances are thought to be attenuated by the mentioned allopolyploidization, which in turn might lead to asynchronous gene expression due to stabilization and inheritance of genomic changes (Mogie, 1992; Carman, 1997). One possibility to get away from hybrid sterility is the switch to apomictic reproduction as hypothesized by Darlington (1939).

This switch is still not well-understood but many hypotheses have been developed, which involve several different molecular scenarios like genetic control mechanisms or epigenetic regulation. One popular hypothesis claims that heterochronic expression of sexual reproduction genes, which is caused by hybridization, is the trigger for apomictic seed formation (Carman, 1997; Sharbel *et al.*, 2009, 2010). This idea is supported by recent findings of Hojsgaard *et al.* (2014), who discovered severe changes in the timing of megagametogenesis in synthetic *Ranunculus auricomus* F$_1$ hybrids. In early studies, it was assumed that apomixis is inherited as single dominant trait and maybe as only one gene (*e.g.* Nogler, 1984a; Savidan, 1992). More recent studies have shown that important apomictic characteristics such as apomeiosis, parthenogenesis and fertilization-independent endosperm formation seem to be controlled by several independent loci (*e.g.* Schallau *et al.*, 2010; Ogawa *et al.*, 2013). The developmental pathways of *Hieracium* apomicts support these findings because mutant plants were able to return to sexuality, when lacking the apospory locus (Catanach *et al.*, 2006; Koltunow *et al.*, 2013). Although,

gene expression studies were carried out, no connection between apomixis and certain gene clusters were identified, but it was determined that apomixis often co-segregates with a block of gene-poor heterochromatin (Huo *et al.*, 2009; Ochogavía *et al.*, 2011; Grimanelli, 2012). Apomictic reproduction in angiosperm plants is a heritable and facultative process probably regulated by differently expressed genes responsible for controlling sexual development or it might be the result of reversible, epigenetic silencing (Hand and Koltunow, 2014). Amongst others, Carman (1997) proposed that the switch to asexual seed formation is triggered by gene duplication subsequently followed by changes in epigenetic gene expression (*e.g.* Koltunow, 1993). Today, it is verified that hybridization and polyploidization can result in altered epigenetic regulations as well as genetic changes in plants (Comai, 2005). DNA modifications such as methylations or RNA interference are heritable and do not affect DNA sequences (Jaenisch and Bird, 2003) but such dosage effects might be the activator of apomictic development after hybridization or polyploidization events (Ozias-Akins and van Dijk, 2007). Thus, epigenetic regulation and reprogramming of plant development can be important factors for apomixis activation (Grimanelli, 2012). Identification of apomixis loci is difficult because recombination is often suppressed in these regions, which might be caused by allelic divergence (Hand and Koltunow, 2014).

The *R. auricomus* complex consists of mainly apomictic polyploid species but additionally a few di- and tetraploid obligate sexual species (*R. carpaticola, R. cassubicifolius*, and *R. notabilis*) are known (Hörandl and Gutermann, 1998; Paun, Greilhuber, *et al.*, 2006; Hörandl *et al.*, 2009; Hojsgaard, Greilhuber, *et al.*, 2014). Sexually reproducing species were found to be self-incompatible, while the apomicts, like typical allopolyploids, were characterized as self-fertile (Hörandl, 2008). In *R. auricomus* plants gametophytic apomixis was described already by Nogler (1984a; b), starting with aposporous formation of an unreduced embryo sac from a somatic nucellar cell in short proximity to a meiotically developed megaspore tetrad or embryo sac that subsequently aborts. The embryo is formed parthenogenetically, whereas successful endosperm development usually requires fertilization of the polar nuclei (pseudogamy; Koltunow and Grossniklaus, 2003; Koltunow *et al.*, 2011). Asexual *Ranunculus* taxa are not obligate apomicts because they still comprise, to some extent, the capacity to reproduce sexually (Nogler, 1984a; b; Hojsgaard, Greilhuber, *et al.*, 2014; Klatt *et al.*, 2016).

Chapter 1

Although apomixis has been studied for more than 100 years now, it is still unclear, how the effective switch toward apomixis in natural plant populations is achieved. More specifically, the specific effects of hybridity vs. polyploidy on developmental pathways are unclear and difficult to entangle in natural allopolyploid apomicts. This study wants to shed light on the developmental events right upon hybridization vs. polyploidy in synthetic F_2 plants of the *R. auricomus* complex as a potential cause of apomixis. Hojsgaard *et al.* (2014) analyzed the corresponding parental F_1 hybrid generation to the plants used in this study and described first evidence of spontaneous apospory and developmental asynchrony in diploid and triploid hybrid *Ranunculus* gametophytes. However, functional apomictic seeds were only produced in polyploids, at very low frequencies. Here, we investigate F_2 hybrid plants generated by manual crossing, where either both parents or one parent had apospory before (Hojsgaard, Greilhuber, *et al.*, 2014). Since hybridization often is connected to allopolyploidization, which was also shown for natural hybrids of the *R. auricomus* complex (Paun, Stuessy, *et al.*, 2006; Pellino *et al.*, 2013), the determination of potential ploidy shifts in the F_2 plants was checked by flow cytometry. According to Carman (1997) theory, we expected that allopolyploid F_2 hybrids would have higher frequencies of apospory and apomictic seed formation than diploid ones due to asynchrony of gene expression. We expect an increase of apospory, not only from the first hybrid generation to the next, but also higher frequencies in F_2 plants descending from both aposporic parents, due to (epi)allelic dosage effects (Nogler, 1984b). Apomictic reproduction can be passed on to the next plant generation by male pollen (Nogler, 1984a; Van Dijk *et al.*, 1999), which led to the assumption that maternal, aposporous plants pollinated by an aposporous paternal plant will result in an accumulation of apomictic dosage effects in the offspring. Furthermore, we carefully analyzed female development to test whether similar severe alterations and temporal irregularities during gametogenesis occur as previously observed by Hojsgaard *et al.* (2014). To get insights into abortion rates during seed development we analyzed seed set of the F_2 plants. To test the hypothesis that diploid hybrid plants are also capable of producing apomictic seeds, the well-developed seeds were analyzed by flow cytometric seed screening. This step is depending on successful coupling of apospory to parthenogenesis and proper endosperm formation. Finally, by generating manual crosses of the F_2 plants and by raising F_3 seedlings, we have experimentally proven the viability of the next hybrid generation.

4.3 Materials and Methods

4.3.1 Plant Materials

Two hundred synthetic F_2 hybrid plants were generated from crossing diploid F, J, and triploid G plants that had shown apospory before (Table S1; Hojsgaard, Greilhuber, *et al.*, 2014). Since the F_1 had formed almost no apomictic seed (Hojsgaard, Greilhuber, *et al.*, 2014), the F_2 is expected to have maternal and paternal genome contributions. All plants were grown under equal outdoor conditions in the old botanical garden of the Albrecht-von-Haller Institute for Plant Sciences at the University of Goettingen, Germany. First flowering of the plants occurred after 2–3 years of cultivation.

4.3.2 Ploidy Determination

The ploidy of the F_2 plants was determined by analyzing leaf material by flow cytometry (Matzk *et al.*, 2000; Hojsgaard, Greilhuber, *et al.*, 2014). Small Silica gel-dried leaf pieces of ~5 mm^2 were chopped in 200 µl Otto I buffer (Otto, 1990) with a razor blade and filtered through a CellTrics® filter (30 µm mesh, Sysmex Partec GmbH, Görlitz, Germany) into a flow cytometry sample tube (3.5 ml, 55 × 12 mm, Sarstedt, Nümbrecht, Germany). DNA in the filtrate was stained by adding 800 µl DAPI-containing Otto II buffer (Otto, 1990). The fluorescence intensity of stained leaf nuclei were performed with a CyFlow®; Space flow cytometer (Sysmex Partec GmbH) at a gain of 416 nm. As ploidy references di- and polyploid F_1 hybrid plants analyzed by Hojsgaard *et al.* (2014) were used. For all samples a minimum of 3,000 nuclei was counted and data analyses were done with the FloMax software version 2.81 (Sysmex Partec GmbH).

4.3.3 Genotyping of F_2 Plants

A simple sequence repeat (SSR) genotyping approach was conducted to verify the parentage of plants. In order to exclude spontaneous selfing, unintended cross-contaminations during manual pollination as well as clonal, apomictic origin of the F_2 *Ranunculus* generation, six loci (Table 1) were used to verify the hybrid origin of the plants following the genotyping protocol of Klatt *et al.* (2016). Genomic DNA was extracted from dried leaf samples using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. Polymerase chain reactions (PCR) were performed with a final sample volume of 25 µl, containing 1 µl template DNA, 12.5 µl BIOMIX (Eurofins Genomics, Ebersberg, Germany), 0.2 µl Forward primer (10 µM), 1.0 µl Reverse primer (10 µM), 1 µl CAG-primer (FAM or HEX labeled). PCR reactions were achieved in a Bio-Rad TM100™ Thermal Cycler with the following parameters: 94°C for 10 min, then 14 ×

(denaturation at 94°C for 60 s, annealing at 62°C + 0.5°C per cycle for 90 s, extension at 72°C for 60 s), followed subsequently by 35 × (denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s), last extension step at 72°C for 60 s and final storage conditions at 4°C. PCR sample concentrations were adjusted before 85 µl formamide (HiDi) were added. This mixture was run in an automatic capillary sequencer Genetic Analyzer 3130 (Applied Biosystems, Forster City, CA, USA) using GeneScan 500 Rox (Applied Biosystems) as size standard after a denaturing pretreatment for 3 min at 92°C. Scoring of the electropherograms was done using GeneMarker V2.4.2 (SoftGenetics LLC, State College, PA, USA) a binary presence/ absence matrix of alleles was exported for genotype characterization because of the presence of several "null" alleles, which may be due to the hybrid origin of the parent plants. The SSR profiles were analyzed in FAMD applying the Jaccard similarity index and generating neighbor joining trees (Schlueter and Harris, 2006). The visualization of trees was done in FigTree v1.4.2 (Rambaut, 2009; Figures S4–S14). The data confirmed non-maternal offspring and parental combinations in the F_2 generation (Tables S2-S12).

Table 1: Characteristics of the six SSR markers used for F_2 hybrid genotyping. T_a (annealing temperature).

Locus	Primer Sequences (5' – 3')	T_a [°C]	Repeat motif	References
LH08	F: GGAGGATATGAGCGGTTCAGA	54	$(CA)_8(TA)_7$	Klatt *et al.* 2016
	R: TATGATGCGTATGGGCGGAG	55		
LH09	F: TTATACGTGACCATCCGCCG	55	$(TG)_6(CG)_4$	Klatt *et al.* 2016
	R: CATTTTCAATGGTGCGAATACGA	53		
R84	F: CATCCGAAGTTAGGGTTGGA	60	$(CAA)_9$	Here
	R: GAGAAAGGTGTGAGCTTGGG	60		
LH11	F: CCAACGGACACTGCTCTTCT	55	$(TC)_{18}$	Klatt *et al.* 2016
	R: TGCTACTCAACCTTGAACTCGA	54		
R2562	F: TACCGCAACAACAATGAAGG	60	$(TC)_{22}$	Here
	R: ATCTCACAAATTTGCCGTCC	60		
R2477	F: CACCTGGTTCTGGTCCTGTT	60	$(TC)_{16}$	Here
	R: GAGCGTGTGCAACAACTCAT	60		

4.4 Female Development

To evaluate the frequency of aposporous initial cell formation in contrast to the occurrence of sexually derived functional megaspores in ovules of *R. auricomus* hybrids, differential interference contrast (DIC) microscopy was applied (Hojsgaard, Greilhuber, *et al.*, 2014).

Ranunculus flower buds with a minimal diameter of 5 mm were harvested and directly fixed in FAA solution (formaldehyde: acetic acid: ethanol: dH_2O; 2:1:10:3.5) for 48 h at

room temperature. The fixative was replaced with 70% ethanol, in which samples were stored until further treatments. Thereafter, plant tissue was dehydrated using 95% and 100% ethanol for each 30 min, before the flower buds were cleared in an increasing dilution series of methyl salicylate (25; 50; 85; 100%; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) in ethanol (Young *et al.*, 1979; Hojsgaard, Greilhuber, *et al.*, 2014). Complete ovaries were dissected from the flower buds and mounted in pure methyl salicylate on object slides. DIC microscopy analysis was performed with Leica DM5500B microscope equipped with a DFC 450 C camera and LAS V41 software (Leica Microsystems, Wetzlar, Germany).

Discrimination of sexual and aposporic cells was accomplished by evaluation of the location of the two cell types. While sexual megaspores usually occurred at the chalazal site of the degraded germ line, asexual initial cells were found close to the sexual megaspores but obviously in somatic ovule tissue. In some ovules a temporal coexistence of functional megaspore and potential aposporous initial cell was observed (Figure 1). Percentages of sexual functional megaspores (FMs), functional aposporous initial cells (AICs) and aborted ovules are given in Table 2.

Statistical analyses and test for significant differences of the two groups (one parent vs. both parents aposporous) were done by applying an arcsin transformation and one-way ANOVA using IBM SPSS Statistics 24 (IBM Deutschland GmbH, Ehningen, Germany).

4.5 Seed Set

To determine the reproductive fitness of the *Ranunculus* F_2 hybrids by seed formation, the plants were transferred from the botanical garden to a YORK® climate chamber (18°C, humidity of 60%, day: night regime of 16 h: 8 h; Johnson Controls, Milwaukee, WI, USA) to prevent unwanted pollination events *e.g.*, by bees or other insects. At least three flowers per plant were manually cross pollinated and subsequently packed in plastic Crispac bags (2 mm Ø holes, Baumann Saatzuchtbedarf, Waldenburg, Germany) to collect ripe seeds. Harvested *Ranunculus* seeds were visually assessed and mature, brown achenes were counted and separated from aborted, yellow ones. Furthermore, full endosperm development was tested by shortly applying thumb-pressure to each achene (Klatt *et al.*, 2016). Based on these numbers, the seed set was calculated for single collective fruits, for individual plants as well as for each hybrid cross after Hörandl (2008). Seeds were stored at 4°C until usage.

4.6 Flow Cytometric Seed Screen (FCSS)

The unique development pathways of single *Ranunculus* hybrid seeds were comprehended by flow cytometric measurements and data analysis (Matzk *et al.*, 2000). Single seeds were ground by two small steel beads (4 mm Ø, Qiagen, Hilden, Germany) in a 2 ml SafeSeal micro tube (Sarstedt) using a TissueLyser II (Qiagen) for 7 s at 30 Hz s^{-1}. DNA extraction started with inverting the seed powder for 30 s after adding 200 µl of Otto I buffer (Otto, 1990). Subsequent procedures such as sample filtration, nuclei staining and sample measurements were identical to the ploidy determination protocol (*incl.* gain settings). In FCSS, the ploidy of endosperm and embryo in seeds (C values) were determined by calculating means of DNA content for each peak by using the FloMax software. Based on these data the "peak index" (PI) was calculated (mean peak value of endosperm/ mean peak value of embryo DNA content), which allowed, together with the peak positions, the identification of the specific reproduction pathway of every single seed (Table 3; after Klatt *et al.*, 2016; modified).

Earlier *R. auricomus* studies (*e.g.* Hojsgaard, Greilhuber, *et al.*, 2014; Klatt *et al.*, 2016) had revealed an eight-nucleate *Polygonum* type embryo sac, and hence a peak index of 1.5 is characteristic for sexually formed seeds. These consist of a reduced embryo sac, in which fertilization of the egg cell by one sperm nucleus results in a zygotic embryo ($n + n$) while the two fused reduced central cell nuclei both were fertilized by the other reduced male gamete ($2n + n$) (Table 3, pathway A). A classical apomictic *R. auricomus* seed is considered to exhibit peak indices of 2.0 – 4.0 which is due to the unreduced embryo sac nuclei, the parthenogenetic development of the embryo ($2n + 0$) and either autonomous endosperm ($4n + 0$; PI = 2.0, Table 3, pathway D) or the pseudogamous formation of the endosperm by central cell fertilization by two unreduced pollen nuclei ($4n + 4n$; Table 3, pathway E, peak index 4.0). We regard an interpretation of pathway D as G2 peak of the embryo as unlikely as *Ranunculus* seeds always form a rapidly growing endosperm, with endosperm peaks usually being higher than embryo peaks (while G2 peaks are always much smaller). Pathway E could also result from endosperm endopolyploidy following pathway D, but nevertheless is a case of an asexual seed. The other cases of central cell fertilization by one reduced pollen nucleus ($4n + n$; peak index 2.5) or by two reduced pollen nuclei ($4n + 2n$; peak index 3.0) as typical for established *Ranunculus* apomicts (Hojsgaard, Greilhuber, *et al.*, 2014; Klatt *et al.*, 2016) were not detected in our study. An intermediate case between sex and apomixis is the occur-

rence of so-called maternal B_{III} hybrids (Table 3, pathway C). Here, an asexually formed, unreduced egg cell is fertilized by a reduced male gamete and the endosperm is developed after fertilization of the central cell by a reduced male pollen nucleus as well. This combination results in a ploidy shift of the embryo ($2n + n$) and endosperm peaks ($4n + n$) and a unique peak index of 1.7. One single case of a paternal B_{III} hybrid was found. Here, egg cell and central cell of a reduced embryo sac were fertilized by each one unreduced pollen nucleus forming a triploid embryo ($n + 2n$) and tetraploid endosperm ($2n + 2n$; peak index = 1.3; Table 3, pathway B).

4.6.1 Germination Rates

In order to determine the viability of seeds formed by F_2 plants, up to ten seeds per plant from all 13 genotypes (Table 5) were sown onto sterilized Fruhstorfer soil (type P mixed with 1/ 3 sand), covered with quartz gravel and incubated in a YORK® climate chamber (16°C, humidity of 60%, day: night regime of 16 h: 8 h; Johnson Controls) for 10 weeks. In spring, the pots were transferred to the old botanical garden (University of Göttingen) to ensure natural sprouting conditions. Germination was checked weekly. The final germination rates were calculated after 23 weeks.

4.7 Results

4.7.1 Ploidy Determination and Genotyping of F_2 Hybrids

Most of the *Ranunculus* F_2 plants from diploid parents (F, J crosses) were found to be diploid with one exception (one new triploid plant). The individuals with a "G" in the name descended from crosses of *R. cassubicifolius* ($4x$) with *R. notabilis* ($2x$) and were previously determined as triploid. As expected from the aneuploidy of the $3x$ parent plants, the F_2 offspring was determined as $3x$, $4x$, and $6x$ (Table S1).

4.7.2 Female Development

About 4,900 ovules, from ten different synthetic *Ranunculus* F_2 hybrid crosses, corresponding to 79 plant individuals, were examined for the mode of female development. All analyzed ovules belong exclusively to diploid *Ranunculus* plants because polyploid individuals in general formed only a very small number of flower buds. The fraction of these buds which showed the informative stage of development was too small to be statistically analyzable. The same was true for the crossings J9A × J20A, F10 × J33 and F7A × J9. Altogether, 4,811 ovules from 79 diploid plants were interpretable. Ovules showed disturbed megasporogenesis indicated by persistence of meiotic germ cell proliferation at manifold time points. The normal, sexual trait, in which the germ line cell located

closest to the chalazal pole developed further into a functional megaspore, was observed in 63.08% (mean of all *Ranunculus*samples, Table 2; Figure S3a). An overall mean percentage of 16.08% of all analyzed hybrid ovules was found to develop aposporously (Figure S1; Table 2). Apospory was indicated by the occurrence of AIC in close proximity to a sexual functional megaspore, for instance one cell layer below (Figure 1). AICs occurred in two hybrid classes with an aposporous father, in five with an aposporous mother, and in three classes where both parents were aposporous (Table 2). The proportion of apospory in the analyzed hybrids derived from both aposporous parents (mean 21.18% ± 11.83 STD, median 19%) was higher in comparison to F_2 plants that originate from parents, of which only one formed aposporic embryo sacs (mean 13.98% ± 13.94 STD, median 11%). The difference was statistically weakly significant (P= 0.012, Figure 2).
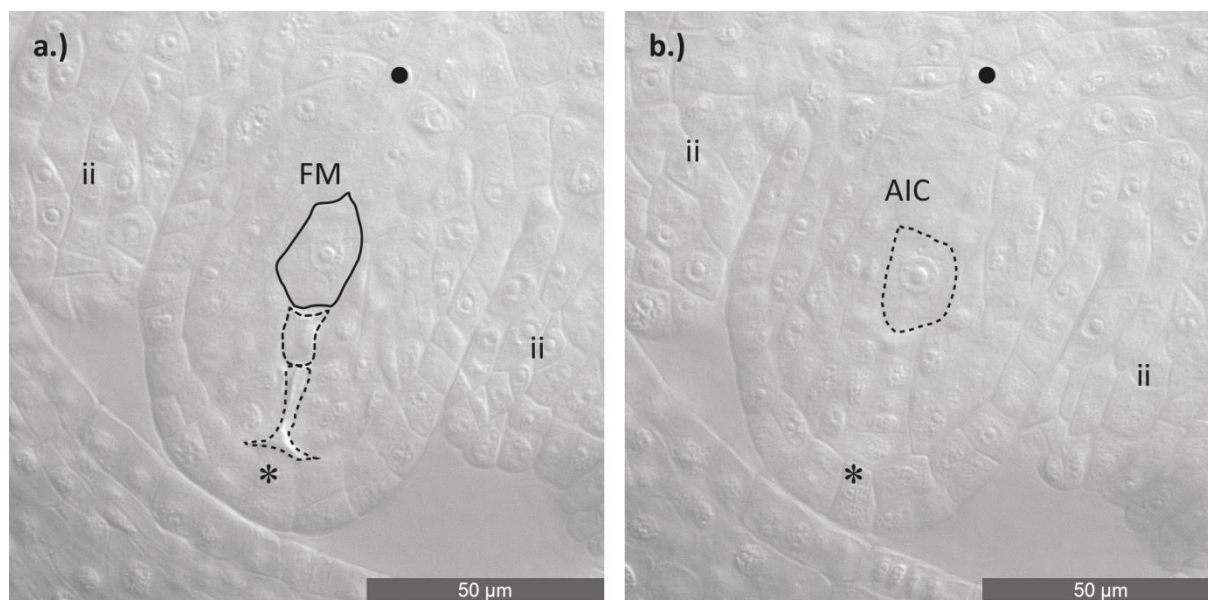
Figure 1: Asexual embryo sac development in an ovule of a diploid *Ranunculus* F_2 hybrid. (a) Ovule during functional megaspore formation. The germ line with the four meiotic products is visible, of which three cells are aborted and only the one near the chalazal pole survived and developed into a functional megaspore. (b) Identical ovule as in (a). but this image displays one cell layer above the germ line, showing an aposporous initial cell. Plant individual: J10 × J30 (12). FM, functional megaspore; AIC, aposporous initial cell; ii, inner integuments; *, micropylar pole; •, chalazal pole. Scale bar: 50 µm.
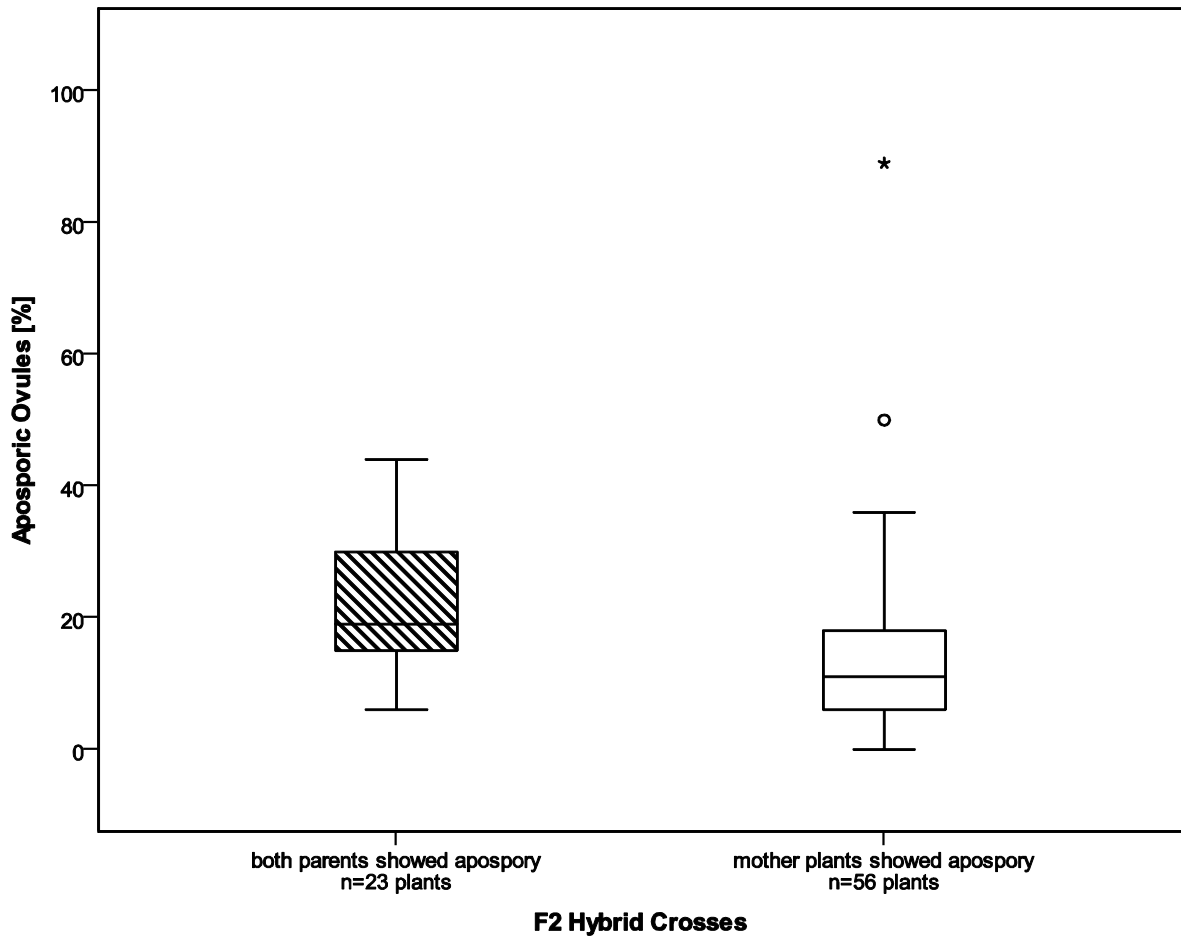
Figure 2: Boxplots of percentages of aposporous ovules for diploid F_2hybrids. Hybrid plants descending from parents that both have shown apospory before (left) formed significantly more aposporous ovules than the plants with only an aposporous mother plant (P = 0.012). Outliers are marked as stars and open circles, the box represents the interquartile range and in the boxplots the median is displayed.

4.7.3 Seed Set, Flow Cytometric Seed Screen, Germination Rates

The *R. auricomus* F_2 hybrids were used to create seed by hand-pollination between individuals in 2016. This seed set revealed a mean of 22.49% well-developed, mature *Ranunculus* seeds, while the remaining 77.51% were identified as aborted (Figure S2; Table 3). None of the polyploid F_2 plants was able to form mature, living seeds for analysis.

In the FCSS analysis only plants were taken into account that produced at least three mature, viable seeds that displayed both an embryo and an endosperm peak in FCSS histograms. Overall, 600 mature F_3 hybrid seeds were analyzed by single-seed FCSS to elucidate their individual mode of development. The measurements showed that seven out of twelve *Ranunculus* crosses had exclusively formed sexual seeds, while the others developed B_{III} and apomictic seeds as well (Figure 3; Tables 3, 4). In total, fourteen non-sexual seeds were detected, which equals 2.33% of the 600 investigated seeds. Eleven

(78.57%) of these seeds were classified as maternal B_{III} hybrids, one as paternal B_{III} hybrid and the other two apomictic seeds developed either as shown in pathway "D" or as in pathway "E" (Figure 3; Table 3).

In total, nearly 280 *Ranunculus* seeds were sown in February 2017 and cultivated in a climate chamber and afterwards outside in the botanical garden under natural conditions. The overall germination rate of all 13 different tested genotypes was determined after 23 weeks and was in the mean 36.96% (Table 5).
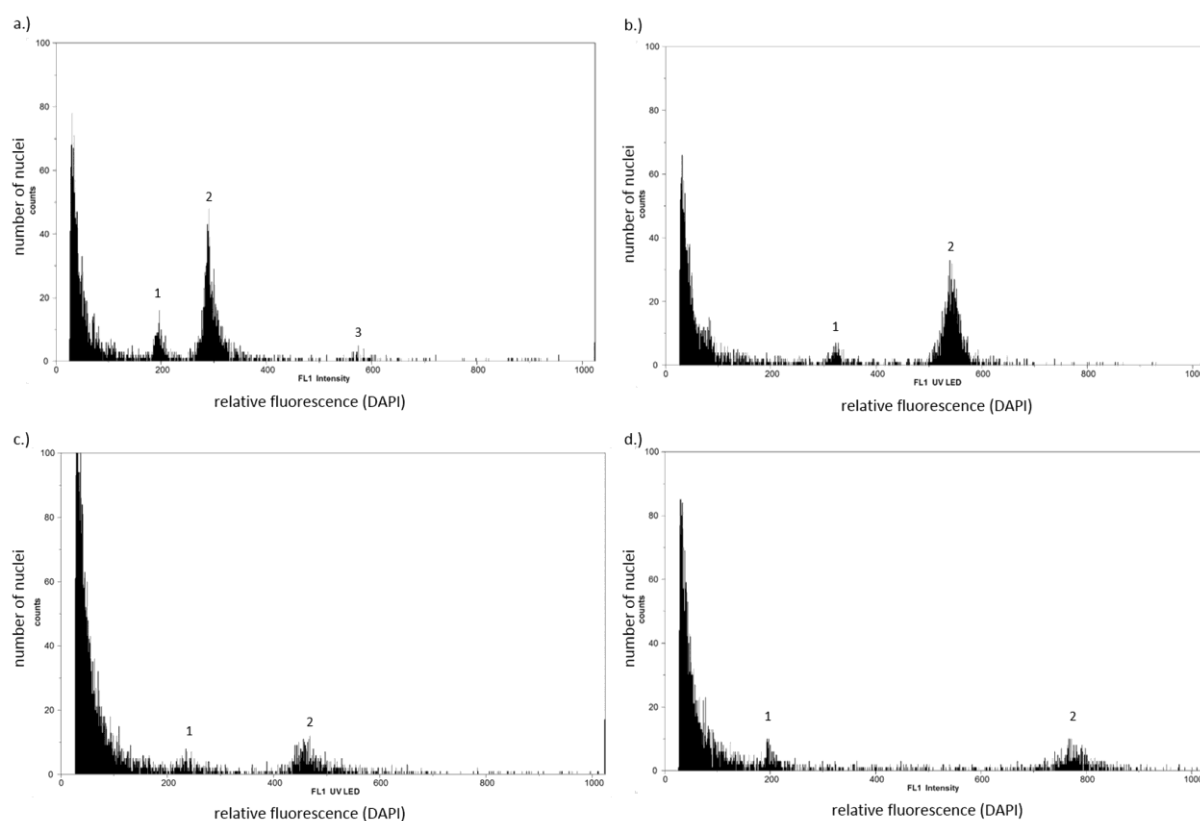
Figure 3: Flow cytometry histograms of *Ranunculus* F_3 hybrid seeds formed from diploid F_2 parents (A–D). General peak labeling: 1 embryo peak, 2 endosperm peak, 3 peak of endosperm cells in the G_2 phase of the cell cycle. (A) Sexual seed with a diploid embryo and triploid endosperm (pathway A). (B) Maternal B_{III} hybrid seed with a triploid embryo and a pentaploid endosperm tissue. The embryo sac was formed by apospory, which led to a diploid embryo and a tetraploid endosperm, but both the embryo and the central cell got fertilized by each one reduced male gamete (pathway C). (C) Asexual seed with a diploid embryo and a tetraploid endosperm (pathway D). The embryo, derived from an unreduced embryo sac, as well as the endosperm developed without fertilization. (D)Asexual *Ranunculus* seed with a diploid embryo and a near octoploid endosperm (pathway E). From the unreduced embryo sac, the embryo developed parthenogenetically into a diploid embryo and the unreduced polar nuclei got both fertilized by two unreduced pollen nuclei. Genotypes: (A,D) J30 × J18 (01) X J10 × J30 (04), (B) F10 × J33 (12) X F10 × J33 (07), (C) J20 × J2 (14) X J20 × J2 (18).

Table 2: Analysis of female development in diploid *Ranunculus* F$_2$ hybrid ovules at the end of sporogenesis and beginning of gametogenesis. Mean percentages of sexual functional megaspore (FM) formation, aposporous initial cell (AIC) formation and ovule abortion were determined by DIC microscopy. "Type" designates whether only the maternal (m) or the paternal (p) parent or both (mp) of the hybrid class was aposporous (see Table S1).

Hybrids	Type	No.	No. of	FM (range)	potential AIC	aborted ovule
F10 x F7	mp	4	159	0.45 (0.20 - 0.71)	0.23 (0.14 - 0.30)	0.32 (0.00 - 0.60)
J10 x J30	mp	8	710	0.68 (0.27 - 0.94)	0.14 (0.06 - 0.21)	0.18 (0.00 - 0.63)
J24 x J22	mp	11	360	0.72 (0.54 - 0.94)	0.26 (0.06 - 0.44)	0.02 (0.00 - 0.27)
F3 x J6	m	11	838	0.45 (0.11 - 0.85)	0.24 (0.01 - 0.89)	0.31 (0.00 - 0.64)
F7 x J9	m	3	169	0.61 (0.18 - 0.84)	0.14 (0.10 - 0.18)	0.25 (0.00 - 0.72)
J6 x F3	p	14	992	0.60 (0.21 - 0.98)	0.08 (0.00 - 0.19)	0.32 (0.00 - 0.79)
J6 x F7	p	8	427	0.78 (0.50 - 0.93)	0.18 (0.05 - 0.50)	0.04 (0.00 - 0.31)
J10 x J14	m	3	278	0.50 (0.25 - 0.67)	0.10 (0.07 - 0.16)	0.39 (0.16 - 0.68)
J20 x J2	m	14	795	0.67 (0.06 - 1.00)	0.09 (0.00 - 0.20)	0.24 (0.00 - 0.94)
J30 x J18	m	3	83	0.82 (0.80 - 0.85)	0.18 (0.15 - 0.20)	0.00
Total		**79**	**4811**	**63.08 %**	**16.08 %**	**20.85 %**

Table 3: Reproductive pathways of seed development of F$_3$ hybrids seeds of the *Ranunculus auricomus* complex identified by Flow Cytometric Seed Screen (FCSS).

Embryo Sac (ES)	Embryo (Em)	Endosperm (End)	Male Gametes		Em C + (End C)	PI	End ratio (m: p)
			Egg Cell	Central Cell			
Reduced	Zygotic	Fertilized	1 n	1 n	2 C + (3 C)	1.5	2: 1
Reduced	Zygotic	Fertilized	2 n	2 n	3 C + (4 C)	1.3	2: 2
Aposporous	Zygotic	Pseudogamous	1 n	1 n	3 C + (5 C)	1.7	4: 1
Aposporous	Parthenogenetic	Autonomous Endosperm	0	0	2 C + (4 C)	2.0	4: 0
Aposporous	Parthenogenetic	Pseudogamous	0	2x 2 n	2 C + (8 C)	4.0	4: 4

Table 4: Reproductive pathways of seed development of F_3 hybrids seeds of the *Ranunculus auricomus* complex identified by Flow Cytometric Seed Screen (FCSS).

Hybrid parent	No. of Seeds	PI (Range)	Reproductive pathways (Table 1)	Non-Sexual Seeds [%]
F10 x F7	38	1.47 (1.40 – 1.59)	A	0.0
J10 x J30	43	1.49 (1.42 – 1.59)	A	0.0
J24 x J22	35	1.50 (1.43 – 1.69)	A	0.0
F3 x J6	111	1.48 (1.23 – 1.70)	A	5.13
	6	1.65 (1.65 – 1.66)	C	
F7A x J6	9	1.52 (1.44 – 1.71)	A	0.0
F7 x J9	8	1.48 (1.24 – 1.55)	A	0.0
F10 x J33	59	1.49 (1.41 – 1.64)	A	3.28
	2	1.67 (1.66 - 1.69)	C	
J6 x F3	107	1.48 (1.30 – 1.70)	A	1.82
	1	1.32	B	
	2	1.70 (1.67 – 1.73)	C	
J6 x F7	95	1.48 (1.33 – 1.58)	A	0.0
J9A x J20A	6	1.48 (1.44 – 1.51)	A	0.0
J10 x J14	4	1.50 (1.49 – 1.51)	A	0.0
J20 x J2	62	1.49 (1.38 – 1.61)	A	3.13
	1	1.59	C	
	1	1.93	D	
J30 x J18	9	1.46 (1.43 – 1.51)	A	10.00
	1	3.92	E	
Total	**600**	**-**		**2.17 %**

Table 5: Percentage of reproductive mode found in *R. auricomus* hybrid seeds harvested from synthetic F_2 plants.

Genotype (2017)	Maternal Genotype	No. of Seeds	Germination Rate [%]
F_3-A	F10 x F7	5	60.00
F_3-B	F10 x J33	50	36.00
F_3-C	F3 x J6	20	30.00
F_3-E	F6A x J15B	10	30.00
F_3-F	F7 x J9	3	100.00
F_3-G	F7A x J6	10	10.00
F_3-K	J15 x F6A	10	10.00
F_3-L	J20 x J2	36	41.67
F_3-M	J24 x J22	36	55.56
F_3-N	J30 x J18	5	40.00
F_3-O	J6 x F3	72	31.94
F_3-P	J6 x F7	9	66.67
F_3-Q	J9A x J20A	10	10.00
Total:	**13**	**276**	**36.96 %**

4.8 Discussion

Gametophytic apomixis is a long studied topic in developmental and evolutionary botany a (*e.g.* Winkler, 1908; Gustafsson, 1946; Nogler, 1984a). Its functional causes, however, are still unclear and under extreme debate because manifold hypothesis and ideas circulate in order to explain this phenomenon. The most important potential natural triggers are hybridization (Ernst, 1918; Mogie, 1992), polyploidization (*e.g.* Sober, 1984) or a combination of both (*e.g.* Bierzychudek, 1985; Asker and Jerling, 1992). This specific type of reproduction demands three synchronized and balanced phases to ensure growing of viable, apomictic seeds (Grimanelli *et al.*, 2001). First, the effective circumvention of meiotic cell division (*e.g.* via apospory), then the parthenogenetic establishment of an embryo and finally, the successful endosperm development. In the present study synthetic *R. auricomus* hybrid plants of the second generation were analyzed. To common knowledge sexual *Ranunculus* plants follow the *Polygonum* type of female development (Nogler, 1973) but evidently this important process was heavily altered, indicated by persistence or abortion of embryo sac formation. Similar but more severe developmental disturbances have been described by Hojsgaard *et al.* (2014), who analyzed the parental generation of the plants in focus here.

4.8.1 Frequencies and Genomic Dosage Effects on Apospory

All analyzed *Ranunculus* plants of the second hybrid generation were invariably identified as diploid, non-maternal genotypes, which means that these plants were sexually formed without any spontaneous ploidy shift. In the grand mean, 16.08% of the investigated F_2 ovules showed aposporous initial cell formation, while only a mean of 11% of the diploid F_1 hybrid ovules had aposporous development (Hojsgaard, Greilhuber, *et al.*, 2014). In addition, apospory in F_2 hybrids seems to be dependent on dosage of heritable genetic control factors, because plants that originated from parents that both had shown apomeiotic embryo sac development, displayed a significantly enhanced percentage of asexual ovules (21.18% ± 11.83 STD) compared to individuals with only one aposporous parent (13.98% ± 13.94 STD, P = 0.012). Since all plants were kept under equal conditions, we can rule out differential stress influence on frequencies of apospory (Klatt *et al.*, 2016; Rodrigo *et al.*, 2017). The influence of genomic dosage of control factors on frequencies of aposporous ovule formation was already shown previously in crossing experiments of polyploid *Ranunculus* by Nogler (1984b). However, other than assumed by Nogler (1984a), our results suggest that also haploid male and female gametes can

carry apospory-controlling heritable control factors. *Ranunculus* F_2 hybrids illustrate developmental disturbances during megasporogenesis and -gametogenesis that are often thought to result in either whole ovule abortion or in reduced fertility due to failures during megagametophyte formation (Figures S3b-d). Similar, but more drastic irregularities were observed by Hojsgaard *et al.* (2014) when analyzing the temporal and developmental processes during female development of the F_1 generation. In contrast, natural *Ranunculus* hybrids show milder discrepancies in embryo sac and seed formation (Nogler, 1971, 1972; Hojsgaard, Greilhuber, *et al.*, 2014).

However, it is still unresolved which apomeiosis-provoking factor triggers reprogramming of a somatic nucellar cell. Since an effect of polyploidy can be ruled out in our F_2 plants, it is assumed that all these alterations are due to previous hybridization, which consequences are known to be the strongest and most perceptible in the first few hybrid generations, especially in diploid plants (Barton, 2001). Hybridization is a powerful driving force in plant speciation and evolution that can result in genomic shocks (Rieseberg *et al.*, 2003). Hybridization can cause dramatic chromosomal rearrangements which were shown to be associated to apomixis in diploid, diplosporous *Boechera* (Kantama *et al.*, 2007). It is further supposed that hybridization events dislocate timing and pattern of gene expression of sexual reproduction controlling genes by changing their genomic constitution or epigenetic regulation (Koltunow, 1993; Carman, 1997; Hand and Koltunow, 2014; Shah *et al.*, 2016). Epigenetics is altered upon hybridization in plants and such reversible changes like DNA methylation or RNA interference are thought to be able to cause apomixis, without affecting the plants' genome sequence (Comai, 2005; Ozias-Akins and van Dijk, 2007; Grimanelli, 2012). In Ha *et al.* (2009) speculated that genomic shocks can be prevented by specific small RNAs formed during hybridization or polyploidization, providing improved genome stability to hybrid plants. Furthermore, it was shown that the onset of reproductive actions in mutant *Arabidopsis* ovules were mainly caused by small RNA silencing pathways involving the AGO9 protein (Olmedo-Monfil *et al.*, 2010). Cell-to-cell signaling is a feature of double-stranded small RNAs, where they tend to silence their target genes (Molnar *et al.*, 2010). The assumption of cell-to-cell signaling is reasonable because aposporous initials always emerge in the direct neighborhood of the megaspore tetrad (*e.g.* Figure 1). Small RNAs commonly interact with proteins of the ARGONAUTE family and form together the RNA-induced silencing complex (RISC), which is an essential component during tran-

scriptional and posttranscriptional gene silencing (Bourc'his and Voinnet, 2010; Feng *et al.*, 2010; Mallory and Vaucheret, 2010). Thus, it seems reasonable that heritable epigenetic processes are responsible for functional silencing of the sexual reproduction pathway in favor of apomixis (Grimanelli, 2012; Hand and Koltunow, 2014).

Our results confirm that apospory is a facultative mechanism, which includes parallel existence of sexual and apomictic development and thus finally in a mixture of sexual and asexual seeds (Nogler, 1984a). The facultative character of gametophytic apomixis could be the result of the ability to maintain the epigenetically unsilenced genomic state (Hand and Koltunow, 2014).

4.8.2 Diploid Hybrids are Able to Reproduce via Apomictic Seed Formation

During seed formation, further developmental processes come into play and influence proportions of sexually *vs.* apomictically formed seed. Successful seed formation in sexual *Ranunculus* species is highly dependent on fertilization of the egg and the central cell nuclei. In pseudogamous apomicts, fertilization of the central cell and endosperm development is important for seed formation as well. In angiosperms, the optimal ratio of maternal (m) to paternal (p) genome contributions in the endosperm was determined to be 2:1 due to genomic imprinting. Deviations from this ratio have deleterious effects leading to heavy disturbances or even to seed abortions (Spielman *et al.*, 2003; Vinkenoog *et al.*, 2003). *Ranunculus* species were characterized as very sensitive to endosperm imbalances (Hörandl and Temsch, 2009). Failure of endosperm development likely explains high seed abortion rates of our F_2 hybrids. More than three-fourth of all seeds harvested from diploid *Ranunculus* F_2 hybrids were found to be dead, either due to abortion at early stages of development or due to mal-developed endosperm tissue (Figure S2). Only a mean of 22.49% of achenes were intact. Conversely, polyploid F_2 hybrids failed completely to produce mature seeds. Even well-formed achenes showed no embryo peak in FCSS analyses. Therefore, no evaluation on the reproductive mode from polyploid plants could be made. Extreme seed abortion rates in the diploids appeared to be at expense of apomictic development as finally almost only functional sexual seeds were formed. This differs fundamentally from natural *Ranunculus* apomicts that produce higher proportions of apomictic than sexual seeds (Hojsgaard, Greilhuber, *et al.*, 2014; Klatt *et al.*, 2016).

In other apomictic plant genera the assertiveness of the sexual pathway seem to mainly depend on the survival rate of functional meiotic cells that possibly can be influenced by pollination timing (Espinoza *et al.*, 2002; Hojsgaard *et al.*, 2013). Natural apomicts have found various strategies to circumvent seed failure *e.g.* by sustaining the optimal conditions or by tolerating variations in the paternal contribution to the endosperm (*e.g.* Savidan, 2007; Dobeš *et al.*, 2013). In our synthetic F_2 plants, seed formation trod various paths. Most of the diploid plants developed sexual seeds and maintained the favored conditions of 2m: 1p ratio, while the apomictic seeds showed several types of genomic imbalance based on an unreduced embryo sac. B_{III} hybrid seeds revealed a different endosperm contribution of 4m: 1p or 2m: 2p (Table 3). The seed that developed fully autonomously without any fertilization showed an extreme change to 4m: 0p (Table 3) and the seed that followed reproductive pathway D showed a modified genomic imbalance in the endosperm of 4m: 4p (Table 3). A comparable tolerance was also previously observed in polyploid F_1 plants, which showed alterations in genome dosage as well (Hojsgaard, Greilhuber, *et al.*, 2014). Autonomous endosperm development was before reported for apomictic *R. auricomus* (Klatt *et al.*, 2016), and here analysis of the FCSS histogram verifies this rare observation (Figure 3c). Also in apomictic *R. kuepferi*, autonomous endosperm occurred very rarely (Schinkel *et al.*, 2016).

However, the two most common modes of seed formation in natural apomictic *Ranunculus* were not detected in this study. Typical apomictic *Ranunculus* seeds are composed of an unreduced embryo sac that parthenogenetically developed into an embryo plus a pseudogamously developed endosperm formed by fertilization of one unreduced or two reduced male gametes (PI = 2.5 and 3.0 respectively, *e.g.* Klatt *et al.*, 2016). Notably, these cases restore the optimal 2m: 1p ratio in the endosperm and usually represent the most frequent case of functional apomictic seeds in *Ranunculus* (Hojsgaard, Greilhuber, *et al.*, 2014). It is likely that none of these "standard" cases was found in the F_2 hybrids due to various reasons: (1) in many cases, premature embryo sac formation during the bud stage was observed, before pollen is available (Figures S3e, f); (2) the pollen quality of hybrid plants could have been low, as it is typical for *Ranunculus* apomicts (Izmailow, 1996; Hörandl *et al.*, 1997; Schinkel *et al.*, 2017); in both cases, failure of endosperm development could have caused seed abortion. Alternatively, several crosses (F × F and J × J genotypes) could have resulted in sibling cross incompatibility.

The formation of several B_{III} hybrids by diploid F_2 hybrids indicates an insufficient coupling of apospory and parthenogenesis, which are both essential for the functional establishment of gametophytic apomixis. This circumstance could be due to the early onset of apospory in F_1 hybrids as described by Hojsgaard *et al.* (2014) and again verified in the F_2 plants by appearance of fully mature, seven-nucleic embryo sacs already in flower bud stage (Figures S3e, f). It is assumed that apospory is connected to extreme long time periods of egg cell receptivity, which reduces the degree of parthenogenetically formed embryos and in turn increases the number of B_{III} seeds as found in the F_2 hybrids (Martinez *et al.*, 1994; Nogler, 1995). Apospory and parthenogenesis are under different genetic control mechanisms (Nogler, 1984a; Ozias-Akins and van Dijk, 2007). The coupling of these processes is obviously not yet established in the majority of F_2 hybrids studied here, except for two apomictically formed seeds.

4.8.3 The Role of Polyploidy for Expression of Apomixis

Our results shed a new light on the role of polyploidy. In almost all plants, natural apomictic reproduction occurs together with polyploidization, which led to the conclusion that polyploidy is an essential necessity for apomixis rather than an option (*e.g.* Bierzychudek, 1985; Carman, 1997; Koltunow and Grossniklaus, 2003). Nonetheless, a few reports on natural diploid apomictic plants are known *e.g.* in *Boechera* (Dobeš *et al.*, 2006; Aliyu *et al.*, 2010), *Paspalum* (Siena *et al.*, 2008), and *R. kuepferi* (Schinkel *et al.*, 2016). Thus, the switch to gametophytic apomixis in the F_2 generation analyzed here is maintained by the hybrid character of the plants and not by polyploidization. Nevertheless, the identified B_{III} hybrid seeds, derived from diploid parents, would result in triploid neopolyploids with a high potential for apospory because of maternal gene dosage effects. Fertilization of the unreduced triploid egg cell by an aposporous pollen donor will result in tetraploids with increased dosages for apospory. So-called female triploid bridges are described in mediating polyploid apomicts (*e.g.* Schinkel *et al.*, 2017). In this aspect our results support the hypothesis by Schinkel *et al.* (2017) that apomixis would be rather a cause than a consequence of polyploidy.

Flow cytometric observations of apomictically developed diploid *Ranunculus* seeds do not support the hypothesis by Nogler (1984a) that inheritance of apospory-controlling factors would require unreduced gametes because of recessive lethal effects in the haploid genome. Mature diploid, asexual *Ranunculus* seeds show that they do not suffer from recessive lethal effects during seed formation. Since hybridization events are

known to cause disturbances in epigenetic regulation, the observed irregularities and temporal alterations are likely to be due to changes of epigenetic genome modulation (*e.g.* Grimanelli, 2012). In order to get a complete picture of the establishment of apomixis in *Ranunculus* hybrid plants, the viability of harvested seeds, derived from synthetic F_2 hybrids, was analyzed by determination of their germination rate. Sexual *Ranunculus* species are known to have a higher fitness than apomictic species in terms of seed set (Izmailow, 1996; Lohwasser, 2001; Hörandl, 2008). However, germination rates between diploid F_1 hybrids, hexaploid apomicts, and diploid sexual species did not differ significantly from each other (Hörandl, 2008). The observed mean germination rate of c. 37% in the F_2 hybrids studied here falls within the range of means of c. 35–54% of the previous study. The germination process is obviously not significantly disturbed in F_2 hybrids, which means that further hybrid generations can be formed.

4.9 Conclusion

The success of apospory in diploid *Ranunculus* F_2 hybrids was found to be based on irregularities during female development, triggered by interspecific hybridization that strongly interfered with temporal and developmental course of action. The frequency of unreduced embryo sac formation depended on the dosage of genetic control factors passed on by the parent generation. However, the connection of apospory, parthenogenesis and pseudogamous endosperm formation is not yet reliably installed in synthetic F_2 hybrids, which is indicated by a high rate of aborted seeds, probably suffering from unbalanced maternal: paternal genome contributions to the endosperm. Nevertheless, a small but not negligible number of apomictic and B_{III} seeds was obtained, meaning that the establishment of apomixis in *Ranunculus* hybrids potentially can continue in further generations. On the one hand, B_{III} hybrid plants are assumed to be the next step toward stabilization and extension of apomictic potential in a polyploid background. On the other hand, polyploid F_2 hybrids in this study, only formed a small number of mature but not analyzable seeds without detectable embryo tissue. Thus, for the next plant generation several triploid individuals are expected that would be highly aposporous but mostly seed-sterile. In a larger, evolutionary timescale, rare successful polyploid apomictic seed formation would be favored by natural selection and increase in frequency. This process could result in the establishment of a functional apomictic, polyploid new *Ranunculus* lineage.

4.10 Supplementary Material
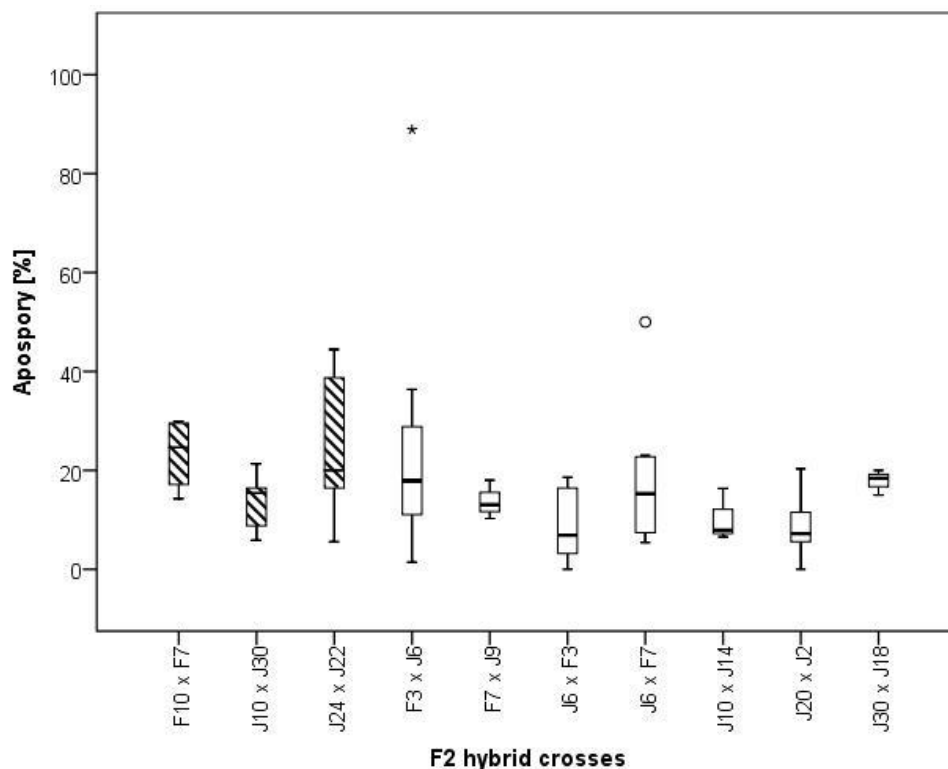
Figure S1: Mean percentage of apospory found in ovules of *Ranunculus* F_2 hybrids. The percentage of apospory varies between 7 and 25 % in *Ranunculus* F_2 hybrid ovules. Striped box plots correspond to F_2 crosses that descend from parents, which both have shown apospory before. Hybrid crosses depicted as white box plots have only an aposporous mother or father plant. N – Numbers are listed in Table 3. Outliers are marked as stars and open circles and in the boxplots the median is displayed.
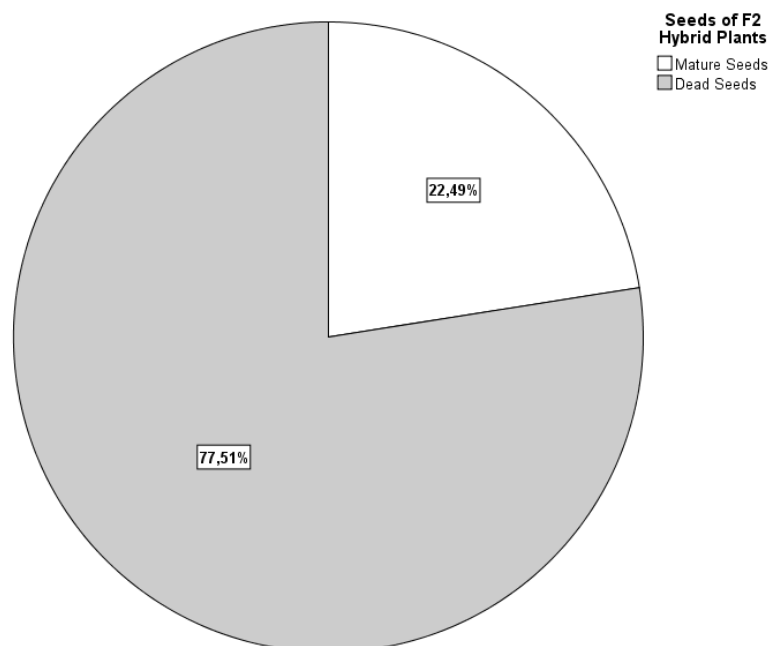
Figure S2: Mean seed-set of diploid *Ranunculus auricomus* hybrids. The F_3 seeds were produced by hand-pollination of the synthetic F_2 hybrids and visually and mechanically analyzed for proper development (n=8681). Turning out that only 22.49 % of the harvested seeds were mature and alive, while the rest was maldeveloped and aborted (77.51 %).
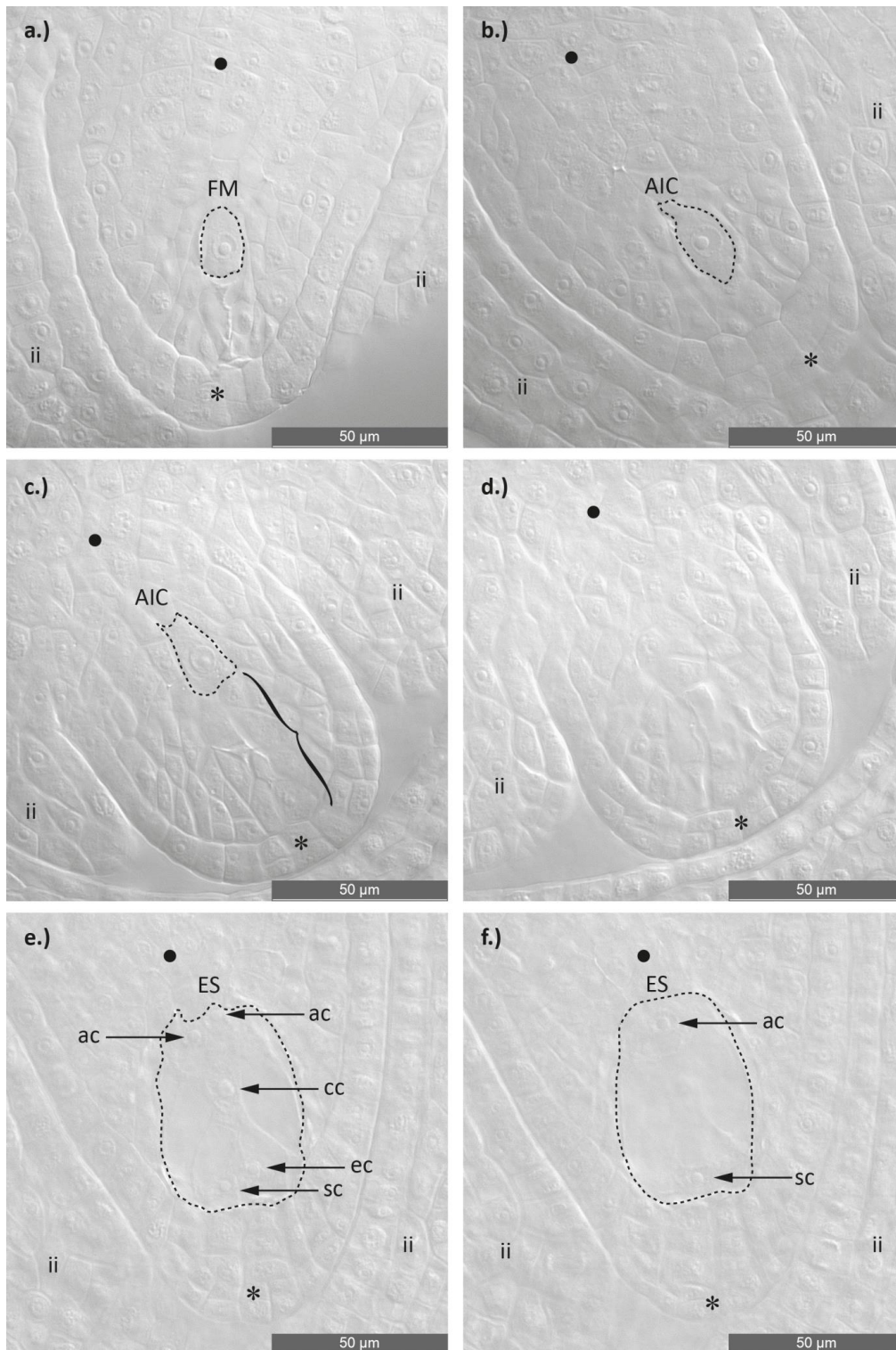
Figure S3: Different stages of ovule development in diploid *Ranunculus* F₂ hybrids in flower buds. a.) Regular, sexual ovule showing a functional megaspore after meiosis completion. b.) Aposporous initial cell, indicated by its position in the ovule and missing functional megaspore as well as aborted megaspores. c.) Aposporous initial cell, all four meiotic products are aborted. d.) Completely aborted germ line without AIC formation. e.+ f.) Precocious ovule development, mature, seven nucleic embryo sac at flower bud stage. Both figures show the same embryo sac at different cell layers. Plant individuals: a.+ b.) J10xJ30 (05); c.)+ d.) F3xJ6 (24); e.+ f.) J10xJ30 (04). FM, functional megaspore; AIC, aposporous initial cell; ES, embryo sac; ac, antipodal cell; cc, central cell both polar nuclei are already fused; ec, egg cell; ii, inner integuments; sc, synergid cell; *, micropylar pole; ●, chalazal pole; }, four aborted megaspores. Scale bar: 50 µm.

Chapter 1

Table S1: Natural *Ranunculus auricomus* species and their synthetic hybrid offspring. The F₁ and the F₂ generation were generated by manual crossing of the relative parent generation in 2006 and 2010, respectively. The letters F and J function as abbreviations for plants descending from *R. carpaticola* x *R. notabilis* crosses and the letters I and G belong to hybrids originating from *R. cassubicifolius* x *R. notabilis* crosses. # describes the quantity of plant individuals of a genotype, * describes uncertain ploidy cases in this genotype: G16A x I2A, J6 x F7 (03), J6 x F7 (12), J20 x J2 (04) and ' indicates a ploidy shift: J24 x J22 (21) is triploid.

Parental Generation (Natural Plants)		F₁ Hybrid Generation (2006)			F₂ Hybrid Generation (2010)	
			Sexual Plants	Apo. Plants	Crosses of apo. mat. Plants (No.)	Ploidy of F₂ Plants
Ranunculus carpaticola (Soó)	2x sexual (allogamous)	*R. carpaticola* x *R. notabilis* (≈ J, F plants)	J2, J6, J9, J14, J15, J18, J18A, J20A, J33	F3, F7, F7A, F10, J9A, J10, J20, J22, J24, J30, J30A	F3 x J6 (#31), F7 x J9 (#4), F7A x J6 (#4), F10 x J33 (#19), J9A x J20A (#1), J10 x J14 (#20), J20 x J2* (#28), J30 x J18 (#4), J30A x J18A (#3), G12 x G7A (#1), G16A x GI2A*(#1)	2x, 2x, 2x, 2x, 2x, 2x, 2x, 2x, 2x, 6x, 4x
Ranunculus notabilis (Hörandl & Gutermann)	2x sexual (allogamous)	*R. cassubicifolius* x *R. notabilis* (≈ G, I plants)	G1, G7A, I2, I2A	G9, G12, G16A, G19	**Crosses of apo. pat. Plants (No.)** / J6 x F3* (#37), J6 x F7* (#16), G1 x G9 (#2)	**Ploidy of F₂ Plants** / 2x, 2x, 3x, 4x
					Crosses of apo. mat. & pat. Plants (No.)	**Ploidy of F₂ Plants**
Ranunculus cassubicifolius (W. Koch)	4x sexual (allogamous)				F10 x F7 (#6), J10 x J30 (#14), J24 x J22' (#29), G19 x G9 (#1)	2x, 2x, 2x, 3x, 3x

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Table S2: Selected SSR data verifying the non-clonal origin of synthetic *Ranunculus* F$_2$ hybrids by depicting the presence of paternal private alleles. m, maternal; p, paternal; N, drop out. The total matrix comprises six loci with altogether 33 alleles (coded as binary presence/absence data).

	LH08_176	R84_174	R2562_375
f1_F10A_m	0	0	0
f1_F7A_p	1	1	1
f2_F10xF7_1	1	N	0
f2_F10xF7_2	1	1	0
f2_F10xF7_3	1	1	N
f2_F10xF7_4	1	0	0
f2_F10xF7_5	1	1	0

Table S3: Selected SSR data verifying the non-clonal origin of synthetic *Ranunculus* F$_2$ hybrids by depicting the presence of paternal private alleles. m, maternal; p, paternal; N, drop out. The total matrix comprises six loci with altogether 33 alleles (coded as binary presence/absence data).

	LH08_162	LH08_176	R84_162	LH11_254	R2562_369	R2562_385	R2477_291	R2477_299
f1_F10A_m	0	0	0	0	0	0	0	0
f1_J33_p	1	1	1	1	1	1	1	1
f2_F10xJ33_1	0	1	N	1	0	0	0	1
f2_F10xJ33_10	N	N	1	1	N	N	0	0
f2_F10xJ33_11	1	0	0	N	0	0	1	0
f2_F10xJ33_12	1	0	0	N	N	N	N	N
f2_F10xJ33_13	1	0	0	0	0	0	0	0
f2_F10xJ33_14	N	N	0	0	0	0	0	0
f2_F10xJ33_15	N	N	1	N	N	N	N	N
f2_F10xJ33_16	1	0	1	0	1	0	0	0
f2_F10xJ33_18	1	0	0	0	0	1	N	N
f2_F10xJ33_19	0	1	0	0	0	0	0	1
f2_F10xJ33_2	1	1	1	1	1	0	1	0
f2_F10xJ33_3	1	1	0	N	N	N	N	N
f2_F10xJ33_4	1	0	1	0	1	1	0	0
f2_F10xJ33_5	N	N	1	1	0	0	0	0
f2_F10xJ33_6	0	1	0	0	1	0	0	0
f2_F10xJ33_7	0	1	1	1	1	0	1	0
f2_F10xJ33_8	1	0	0	N	N	N	N	N
f2_F10xJ33_9	1	0	N	1	1	0	1	0

Table S4: Selected SSR data verifying the non-clonal origin of synthetic *Ranunculus* F_2 hybrids by depicting the presence of paternal private alleles. m, maternal; p, paternal; N, drop out. The total matrix comprises six loci with altogether 33 alleles (coded as binary presence/absence data).

	LH09_206	LH08_176	R84_162	LH11_254	R2562_367	R2562_405	R2477_265	R2477_291
f1_F3A_m	0	0	0	0	0	0	0	0
f1_J6_p	1	1	1	1	1	1	1	1
f2_F3xJ6_1	0	0	0	0	1	0	0	0
f2_F3xJ6_10	1	1	1	0	1	0	1	1
f2_F3xJ6_11	1	1	1	1	0	0	N	N
f2_F3xJ6_12	0	N	1	0	0	1	1	1
f2_F3xJ6_13	1	1	0	1	0	0	0	0
f2_F3xJ6_14	1	1	1	0	0	1	1	1
f2_F3xJ6_15	0	1	0	1	0	1	0	0
f2_F3xJ6_16	1	0	1	0	N	N	1	0
f2_F3xJ6_17	0	1	0	1	0	1	N	N
f2_F3xJ6_18	0	0	0	1	0	0	0	1
f2_F3xJ6_19	0	N	0	1	0	1	0	0
f2_F3xJ6_2	0	N	0	1	0	1	N	N
f2_F3xJ6_20	0	0	1	0	0	0	1	1
f2_F3xJ6_21	1	0	0	1	1	0	0	0
f2_F3xJ6_22	1	1	1	1	1	0	0	1
f2_F3xJ6_23	1	1	0	1	0	0	N	N
f2_F3xJ6_24	0	N	1	0	1	1	1	0
f2_F3xJ6_25	0	1	0	1	1	0	1	0
f2_F3xJ6_26	1	0	0	0	0	0	1	0
f2_F3xJ6_27	1	1	1	0	0	0	1	1
f2_F3xJ6_28	0	1	0	1	N	N	N	N
f2_F3xJ6_29	0	1	1	0	0	1	1	0
f2_F3xJ6_3	0	0	1	0	1	1	1	1
f2_F3xJ6_30	0	0	0	1	1	0	N	N
f2_F3xJ6_31	0	1	0	1	0	1	0	0
f2_F3xJ6_4	0	0	1	0	0	0	1	0
f2_F3xJ6_5	1	N	0	1	0	0	0	0
f2_F3xJ6_6	0	1	1	1	0	1	N	N
f2_F3xJ6_7	0	0	0	1	0	1	0	0
f2_F3xJ6_9	0	0	1	1	0	0	N	N

Table S5: Selected SSR data verifying the non-clonal origin of synthetic *Ranunculus* F₂ hybrids by depicting the presence of paternal private alleles. m, maternal; p, paternal; N, drop out. The total matrix comprises six loci with altogether 33 alleles (coded as binary presence/absence data).

	R84_162	LH11_254	R2562_405	R2477_265	R2477_291
f1_F7A_m	0	0	0	0	0
f1_J6_p	1	1	1	1	1
f2_F7AxJ6_1	0	1	0	0	0
f2_F7AxJ6_2	0	0	1	1	0
f2_F7AxJ6_3	0	1	1	0	0
f2_F7AxJ6_4	1	0	0	1	0

Table S6: Selected SSR data verifying the non-clonal origin of synthetic *Ranunculus* F₂ hybrids by depicting the presence of paternal private alleles. m, maternal; p, paternal; N, drop out. The total matrix comprises six loci with altogether 33 alleles (coded as binary presence/absence data).

	LH09_206	LH11_218	R2562_367	R2562_385	R2477_285
f1_J10A_m	0	0	0	0	0
f1_J14A_p	1	1	1	1	1
f2_J10xJ14_1	1	1	1	0	0
f2_J10xJ14_10	0	0	0	1	N
f2_J10xJ14_11	0	1	1	0	1
f2_J10xJ14_12	0	0	1	0	0
f2_J10xJ14_13	0	0	1	0	1
f2_J10xJ14_14	0	1	0	0	0
f2_J10xJ14_15	1	0	1	0	0
f2_J10xJ14_16	1	1	0	1	1
f2_J10xJ14_17	0	1	0	0	0
f2_J10xJ14_18	0	0	1	0	0
f2_J10xJ14_2	1	1	1	0	0
f2_J10xJ14_3	0	0	N	N	N
f2_J10xJ14_4	N	0	N	N	N
f2_J10xJ14_5	0	0	1	0	N
f2_J10xJ14_6	1	0	N	N	N
f2_J10xJ14_7	1	1	1	0	N
f2_J10xJ14_8	1	0	N	N	0
f2_J10xJ14_9	1	0	1	0	0

Table S7: Selected SSR data verifying the non-clonal origin of synthetic *Ranunculus* F$_2$ hybrids by depicting the presence of paternal private alleles. m, maternal; p, paternal; N, drop out. The total matrix comprises six loci with altogether 33 alleles (coded as binary presence/absence data).

	LH08_162	LH11_218	R2562_385	R2477_285
f1_J10A_m	0	0	0	0
f1_J30A_p	1	1	1	1
f2_J10xJ30_10	N	N	0	1
f2_J10xJ30_11	N	N	0	1
f2_J10xJ30_12	N	N	0	1
f2_J10xJ30_13	N	N	0	1
f2_J10xJ30_14	N	N	0	1
f2_J10xJ30_3	1	0	0	0
f2_J10xJ30_4	0	0	0	0
f2_J10xJ30_5	N	N	0	1
f2_J10xJ30_6	N	N	0	0
f2_J10xJ30_7	N	N	1	1

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Table S8: Selected SSR data verifying the non-clonal origin of synthetic *Ranunculus* F_2 hybrids by depicting the presence of paternal private alleles. m, maternal; p, paternal; N, drop out. The total matrix comprises six loci with altogether 33 alleles (coded as binary presence/absence data).

	LH11_218	LH11_242	R2562_367	R2562_405
f1_J20A_m	0	0	0	0
f1_J2A_p	1	1	1	1
f2_J20xJ2_1	N	N	N	N
f2_J20xJ2_10	0	1	1	0
f2_J20xJ2_11	0	1	1	0
f2_J20xJ2_12	0	1	1	0
f2_J20xJ2_13	1	0	1	0
f2_J20xJ2_14	N	N	N	N
f2_J20xJ2_16	1	0	0	1
f2_J20xJ2_17	1	0	0	0
f2_J20xJ2_18	1	0	0	0
f2_J20xJ2_19	0	1	0	1
f2_J20xJ2_2	N	N	0	1
f2_J20xJ2_20	0	1	1	0
f2_J20xJ2_21	0	1	1	0
f2_J20xJ2_22	N	N	1	1
f2_J20xJ2_23	1	0	1	0
f2_J20xJ2_24	N	N	N	N
f2_J20xJ2_25	0	1	N	N
f2_J20xJ2_26	N	N	N	N
f2_J20xJ2_27	N	N	N	N
f2_J20xJ2_28	1	0	1	0
f2_J20xJ2_3	N	N	1	0
f2_J20xJ2_4	N	N	1	0
f2_J20xJ2_5	N	N	0	1
f2_J20xJ2_6	N	N	N	N
f2_J20xJ2_7	1	0	N	N
f2_J20xJ2_8	1	0	1	0
f2_J20xJ2_9	1	0	1	0

Table S9: Selected SSR data verifying the non-clonal origin of synthetic *Ranunculus* F_2 hybrids by depicting the presence of paternal private alleles. m, maternal; p, paternal; N, drop out. The total matrix comprises six loci with altogether 33 alleles (coded as binary presence/absence data).

	LH08_164	R84_171	LH11_218	LH11_242	R2562_367	R2562_405	R2477_265
f1_J24_m	0	0	0	0	0	0	0
f1_J22A_p	1	1	1	1	1	1	1
f2_J24xJ22_1	0	0	0	1	0	1	0
f2_J24xJ22_10	0	1	1	0	1	0	1
f2_J24xJ22_11	0	0	N	N	N	N	N
f2_J24xJ22_12	N	1	1	1	N	N	0
f2_J24xJ22_13	0	1	N	N	N	N	N
f2_J24xJ22_14	N	0	1	0	0	1	1
f2_J24xJ22_15	0	0	0	1	0	1	1
f2_J24xJ22_16	0	N	1	1	0	0	0
f2_J24xJ22_17	0	1	0	1	0	0	1
f2_J24xJ22_18	0	1	1	1	N	N	0
f2_J24xJ22_19	0	1	0	0	1	1	1
f2_J24xJ22_2	0	1	0	1	N	N	1
f2_J24xJ22_20	0	1	N	N	N	N	N
f2_J24xJ22_21	N	N	0	0	0	0	1
f2_J24xJ22_22	0	0	0	1	1	0	0
f2_J24xJ22_23	0	1	N	N	N	N	N
f2_J24xJ22_24	N	N	1	0	0	0	1
f2_J24xJ22_25	0	N	0	1	1	0	1
f2_J24xJ22_3	0	1	1	0	0	1	1
f2_J24xJ22_4	0	0	N	N	N	N	N
f2_J24xJ22_5	N	1	1	0	0	0	1
f2_J24xJ22_6	0	1	N	N	N	N	N
f2_J24xJ22_7	0	1	0	1	0	0	1
f2_J24xJ22_8	0	1	0	1	0	0	1
f2_J24xJ22_9	0	0	1	0	0	0	1

Table S10: Selected SSR data verifying the non-clonal origin of synthetic *Ranunculus* F$_2$ hybrids by depicting the presence of paternal private alleles. m, maternal; p, paternal; N, drop out. The total matrix comprises six loci with altogether 33 alleles (coded as binary presence/absence data).

	LH09_206	R84_162	R2562_367	R2562_405	R2477_299
f1_J30A_m	0	0	0	0	0
f1_J18B_p	1	1	1	1	1
f2_J30AxJ18A_1	0	1	1	0	0
f2_J30AxJ18A_2	0	1	1	0	1
f2_J30AxJ18A_3	0	1	0	0	0
f2_J30xJ18_1	0	0	1	1	0
f2_J30xJ18_2	0	1	1	0	N
f2_J30xJ18_3	1	1	0	0	N

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Table S11: Selected SSR data verifying the non-clonal origin of synthetic *Ranunculus* F_2 hybrids by depicting the presence of paternal private alleles. m, maternal; p, paternal; N, drop out. The total matrix comprises six loci with altogether 33 alleles (coded as binary presence/absence data).

	LH08_180	R84_171	LH11_224	R2562_357	R2562_375	R2477_285
f1_J6_m	0	0	0	0	0	0
f1_F3A_p	1	1	1	1	1	1
f2_J6xF3_1	0	1	0	1	0	0
f2_J6xF3_10	1	0	0	0	1	0
f2_J6xF3_11	0	1	0	1	0	0
f2_J6xF3_12	1	1	N	N	N	0
f2_J6xF3_13	0	0	0	1	0	0
f2_J6xF3_14	0	1	0	1	0	1
f2_J6xF3_15	1	0	0	0	0	0
f2_J6xF3_18	0	1	0	0	0	0
f2_J6xF3_19	1	1	0	0	0	1
f2_J6xF3_20	1	0	0	0	1	1
f2_J6xF3_21	0	0	0	0	0	0
f2_J6xF3_22	0	1	0	1	0	1
f2_J6xF3_23	1	1	1	0	0	0
f2_J6xF3_24	1	0	0	N	N	0
f2_J6xF3_25	1	1	1	0	0	0
f2_J6xF3_27	0	0	0	1	0	0
f2_J6xF3_28	0	1	1	1	0	N
f2_J6xF3_29	0	1	0	1	0	N
f2_J6xF3_3	0	1	0	0	0	0
f2_J6xF3_30	1	0	0	1	0	0
f2_J6xF3_4	0	1	0	1	0	1
f2_J6xF3_5	0	1	1	1	0	1
f2_J6xF3_6	0	1	0	0	0	0
f2_J6xF3_7	0	1	0	N	N	N
f2_J6xF3_8	1	1	0	0	1	N
f2_J6xF3_9	0	1	0	N	N	1

Table S12: Selected SSR data verifying the non-clonal origin of synthetic *Ranunculus* F$_2$ hybrids by depicting the presence of paternal private alleles. m, maternal; p, paternal; N, drop out. The total matrix comprises six loci with altogether 33 alleles (coded as binary presence/absence data).

	R84_171	R84_174	LH11_230	R2562_357	R2562_375	R2477_285
f1_J6_m	0	0	0	0	0	0
f1_F7A_p	1	1	1	1	1	1
f2_J6xF7_1	0	1	0	1	0	0
f2_J6xF7_10	1	0	1	1	0	N
f2_J6xF7_11	1	0	1	1	0	0
f2_J6xF7_12	1	0	1	1	0	0
f2_J6xF7_13	N	N	1	1	0	0
f2_J6xF7_15	1	0	1	1	0	0
f2_J6xF7_2	1	0	1	1	0	N
f2_J6xF7_3	1	0	1	1	0	0
f2_J6xF7_4	N	N	N	1	0	N
f2_J6xF7_5	1	1	0	1	0	N
f2_J6xF7_6	0	1	0	N	N	0
f2_J6xF7_7	1	1	0	N	N	0
f2_J6xF7_8	1	0	0	1	0	0
f2_J6xF7_9	1	1	0	1	0	N

Figure S4: Neighbor joining trees derived from SSR data (all loci and alleles). Each two synthetic *Ranunculus* F₁ hybrids and their sexually formed offspring were analyzed. F₁ parent plants are depicted in red.

Figure S5: Neighbor joining trees derived from SSR data (all loci and alleles). Each two synthetic *Ranunculus* F₁ hybrids and their sexually formed offspring were analyzed. F₁ parent plants are depicted in red.

Figure S6: Neighbor joining trees derived from SSR data (all loci and alleles). Each two synthetic *Ranunculus* F$_1$ hybrids and their sexually formed offspring were analyzed. F$_1$ parent plants are depicted in red.

Figure S7: Neighbor joining trees derived from SSR data (all loci and alleles). Each two synthetic *Ranunculus* F₁ hybrids and their sexually formed offspring were analyzed. F₁ parent plants are depicted in red.
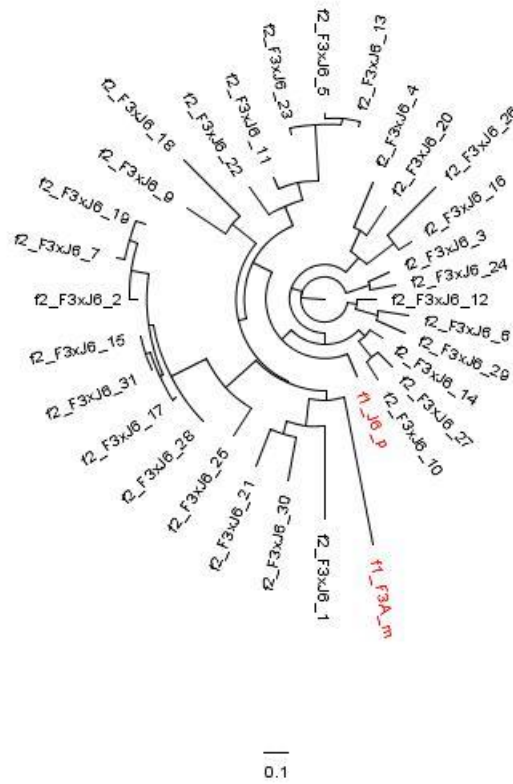
Figure S8: Neighbor joining trees derived from SSR data (all loci and alleles). Each two synthetic *Ranunculus* F₁ hybrids and their sexually formed offspring were analyzed. F₁ parent plants are depicted in red.
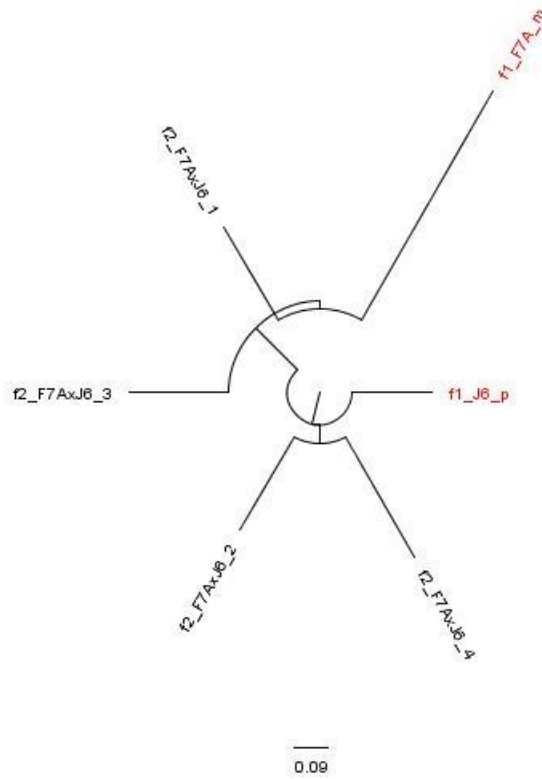
Figure S9: Neighbor joining trees derived from SSR data (all loci and alleles). Each two synthetic *Ranunculus* F₁ hybrids and their sexually formed offspring were analyzed. F₁ parent plants are depicted in red.

Figure S10: Neighbor joining trees derived from SSR data (all loci and alleles). Each two synthetic *Ranunculus* F_1 hybrids and their sexually formed offspring were analyzed. F_1 parent plants are depicted in red.
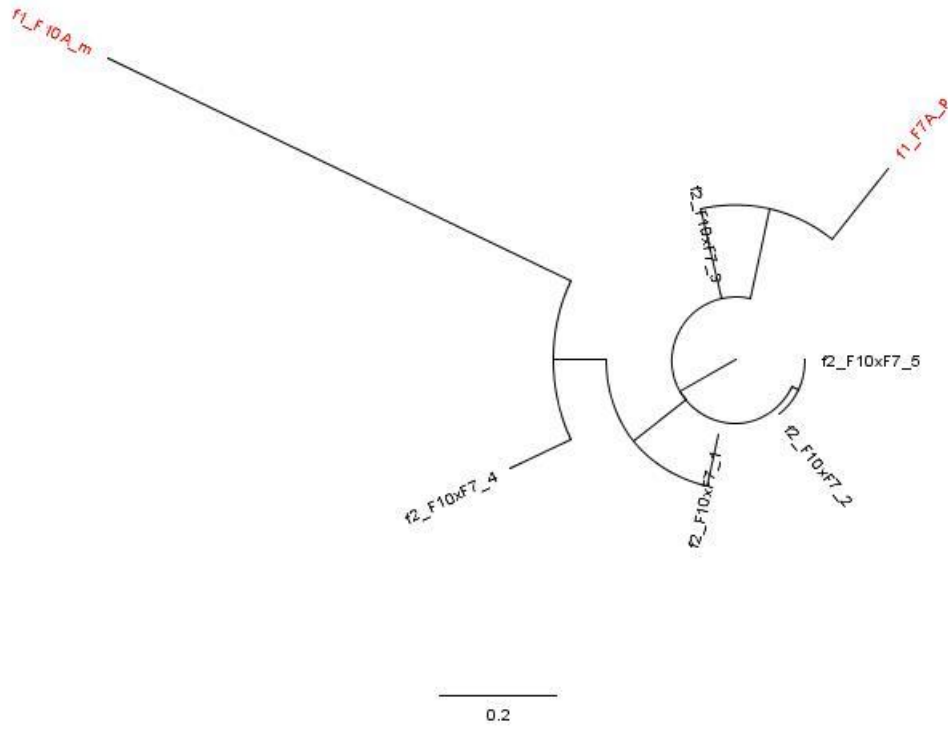
Figure S11: Neighbor joining trees derived from SSR data (all loci and alleles). Each two synthetic *Ranunculus* F₁ hybrids and their sexually formed offspring were analyzed. F₁ parent plants are depicted in red.

Figure S12: Neighbor joining trees derived from SSR data (all loci and alleles). Each two synthetic *Ranunculus* F$_1$ hybrids and their sexually formed offspring were analyzed. F$_1$ parent plants are depicted in red.
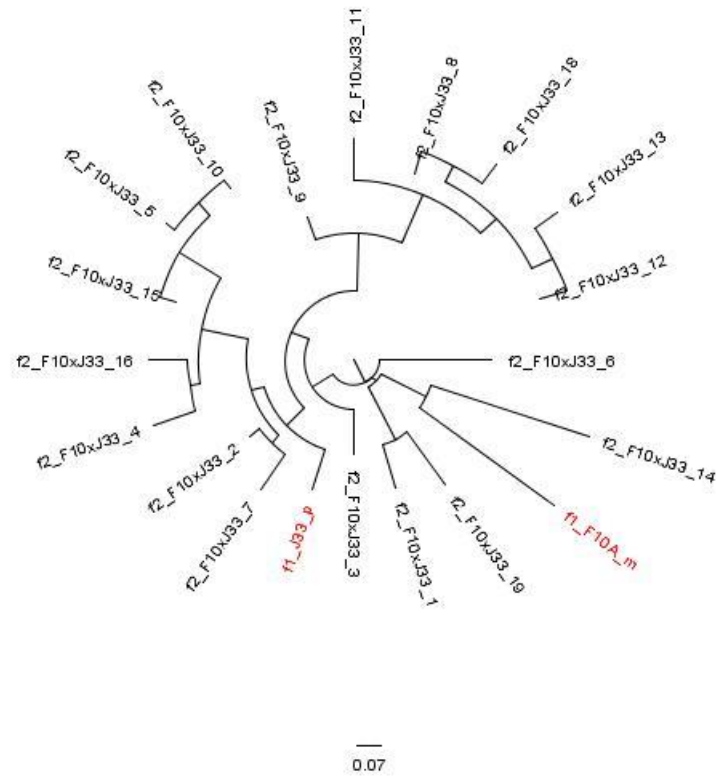
Figure S13: Neighbor joining trees derived from SSR data (all loci and alleles). Each two synthetic *Ranunculus* F$_1$ hybrids and their sexually formed offspring were analyzed. F$_1$ parent plants are depicted in red.
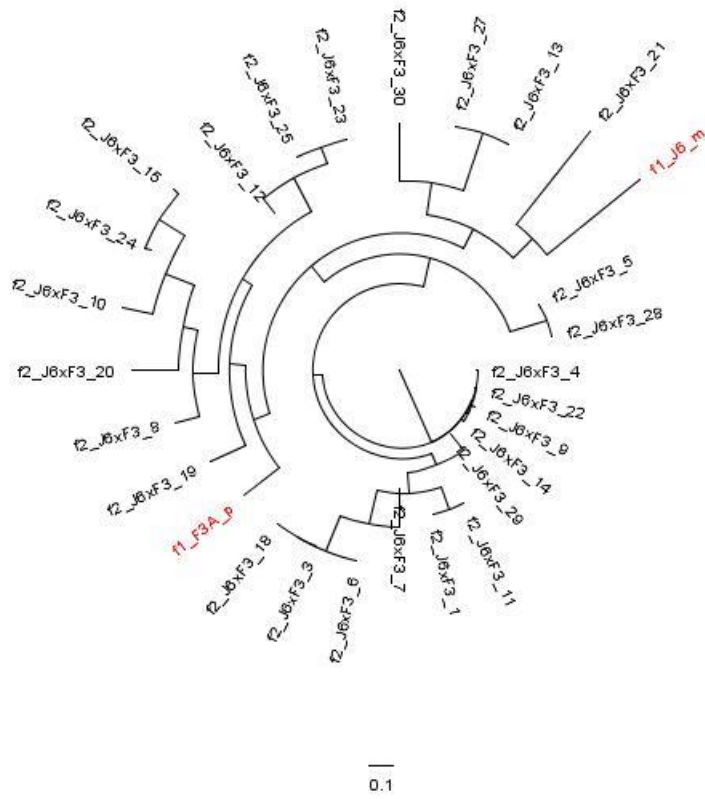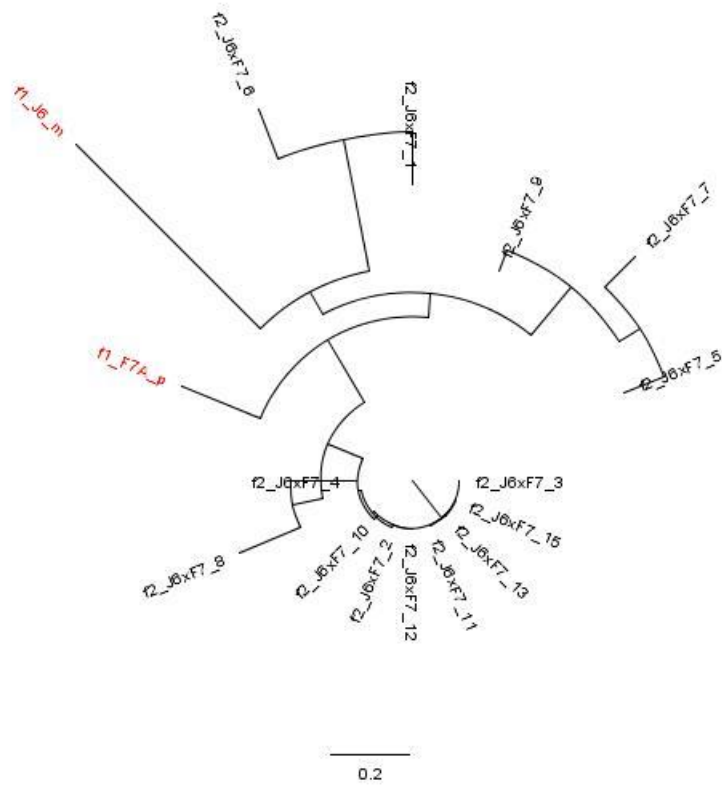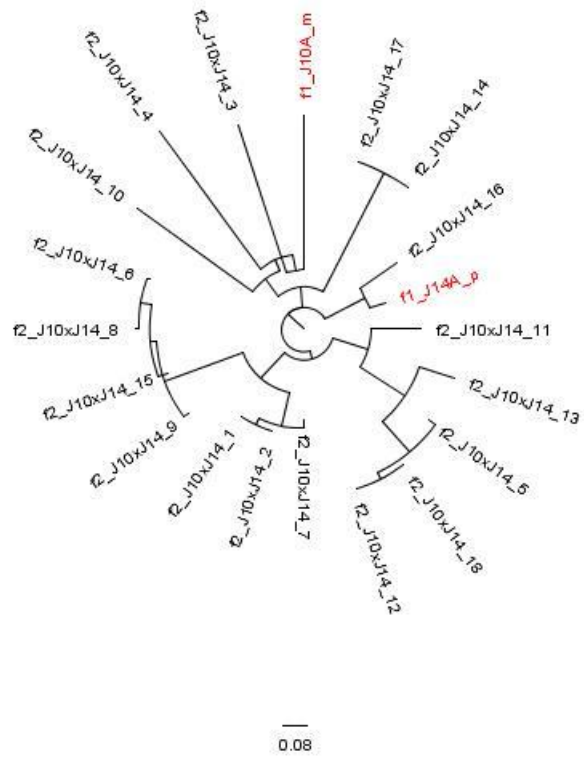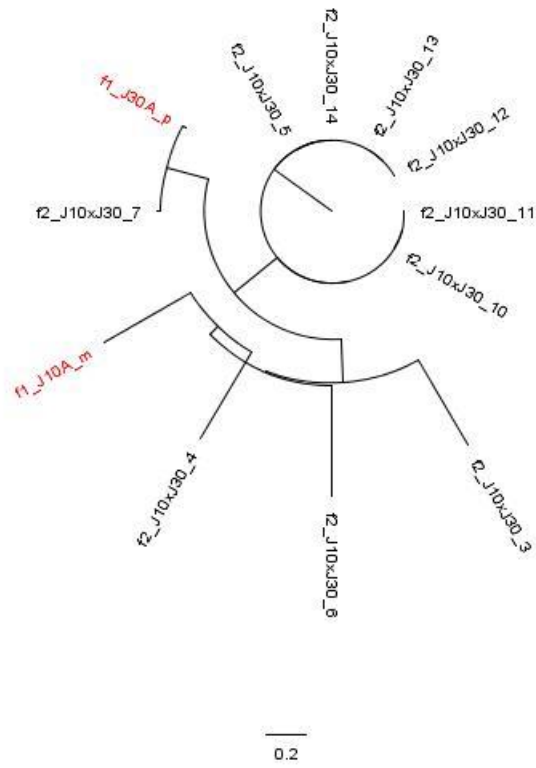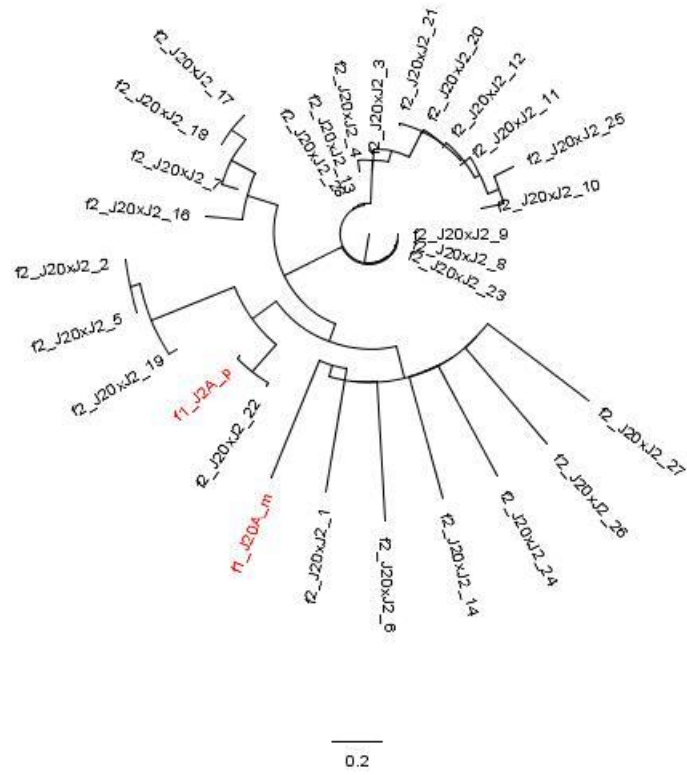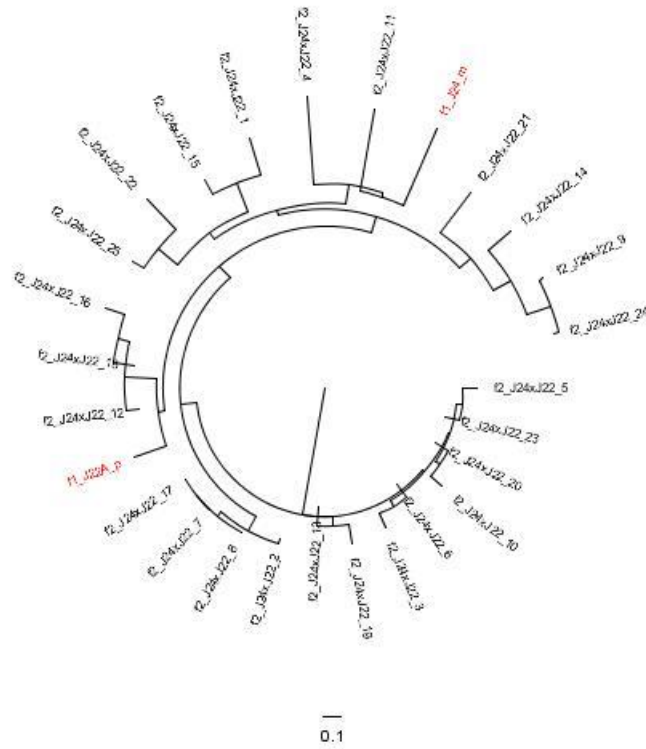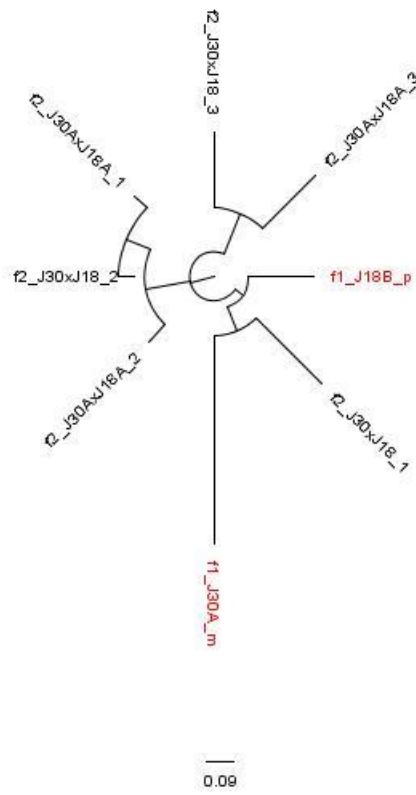
Figure S14: Neighbor joining trees derived from SSR data (all loci and alleles). Each two synthetic *Ranunculus* F$_1$ hybrids and their sexually formed offspring were analyzed. F$_1$ parent plants are depicted in red.

5   Chapter 2

The Relation of Meiotic Behavior to Hybridity, Polyploidy and Apomixis in the *Ranunculus auricomus* Complex (Ranunculaceae)

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5.1   Abstract

Background and Aims

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.

Methods

Recently formed di- and polyploid *Ranunculus* hybrids as well as their parental species were analyzed 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.

Key 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 favor alternatives to the female meiotic pathway. Relation of investigated meiotic errors with the observed occurrence of apospory in *Ranunculus* hybrids identi-

fies disturbed female meiosis as potential elicitor of apomixis in order to rescue these plants from hybrid sterility. Meiotic disturbance appears to be stronger in neo-polyploids than in homoploid hybrids, which may contribute to the prevalence of apomixis in polyploid plants.

5.2 Introduction

In all eukaryotic organisms meiosis is the core of sexual reproduction, which ensures recombination and thus evolution and speciation (*e.g.* Brandeis, 2018). 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; Hamant *et al.*, 2006). 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 (Harrison *et al.*, 2010). Exact chromosome segregation is strictly required since unbalanced gamete formation can lead to cell death, sterility or aneuploidy (Cifuentes *et al.*, 2010).

Interspecific hybridization is a frequently phenomenon in plants (Arnold, 1997), which results either in offspring with a doubled chromosome number (allopolyploids) or in diploid hybrids (homoploids; Stebbins, 1959; Rieseberg and Willis, 2007). Hybridization creates a versatile range of hybrids with each different genotypes and divergent fitness (Arnold, 1997). Therefore, plant evolution is highly influenced by hybridization (Soltis and Soltis, 2009). Nonetheless, hybrids generally have a negative connotation and are even termed as "hopeful monsters" because of their reduced fitness (Mallet, 2007). 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 (Arnold, 1997; Mallet, 2007). The strongest effects of hybridization on plant fertility are usually found in F_1 hybrids (Mallet, 2007; Hegarty *et al.*, 2009). 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 (Comai, 2005; Rieseberg and Willis, 2007; Zielinski and Mittelsten Scheid, 2012). Another potential cause for poor hybrid fitness may be the rupture of important gene clusters by meiotic recombination that can lead to profound alterations in gene expression (Dobzhansky, 1941; Hegarty *et al.*, 2009).

However, fertility of plant hybrids is highly variable, and eventually subsequent generations can establish novel evolutionary lineages (Arnold, 1997). Historically, homoploid hybrid speciation was assumed as rarely arising phenomenon (*e.g.* Arnold, 1997; Soltis and Soltis, 2009), because of missing concrete identification evidences (Rieseberg and Willis, 2007). In face of recent reconsideration, the importance of this topic among evolutionary biologists grew, while only a few cases of homoploid hybrid plants are known (Schumer *et al.*, 2014). Best documented and described natural homoploid hybrids belong to the taxa *Helianthus* and *Iris* (*e.g.* Gross and Rieseberg, 2005; Arnold *et al.*, 2012). 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 (Mallet, 2007; Soltis and Soltis, 2009) but reproduction isolation can be achieved by spatial isolation, karyotype and/ or ecological divergence (Rieseberg and Willis, 2007).

The situation in polyploid plants is complicated as well, since they 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 (Comai, 2005). Such errors get less over generations because the polyploid character becomes stabilized by cytological diploidization that acts on gamete formation. 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; Comai, 2005; Cifuentes *et al.*, 2010). Although diploidization is found in polyploids, several differences in this process are documented between auto- and allopolyploid plants (Cifuentes *et al.*, 2010). Autopolyploids are the result of restitutional meiosis, gaining unreduced gametes that develop into plants with increased ploidy, often via a triploid bridge (Ramsey and Schemske, 1998). In contrast, allopolyploids are not only caused by unreduced gamete formation, but additionally by a hybridization event of two species. The course of meiosis in autopolyploids is disturbed due to fact that such plants are equipped with more than two copies of each chromosome, which favors the emergence of homologous multi- and univalents, while allopolyploids are able to develop regular bivalents during prophase I (Zielinski and Mittelsten Scheid, 2012). Indeed, the frequency and likelihood of allopolyploids recognizing one or more homeologous pairing partners fundamentally depends on sequence divergences of the parental genomes. Difficul-

ties in chromosome alignment and synapsis still occur on regular basis in young diploid hybrids due to the forced pairing of even homeologous partners. In turn, these mismatches can lead to generation of uni- and multivalents and thus to missegregation; ending up in reduced fertility (Comai, 2005; Grandont *et al.*, 2013). Overall, polyploid plants with hybrid origin tend to behave during meiosis as diploids, because the homologs derived from the same parent can form bivalents (Comai, 2005; Cifuentes *et al.*, 2010). This way, the problems of homeolog pairing can be avoided.

Apomixis, which is asexual seed formation, is able to circumvent meiotic cell division in various different developmental pathways (Asker and Jerling, 1992). One common form of apomixis involves mitotic embryo sac (ES) development out of a somatic nucellar cell (apospory), resulting in clonal, maternal egg cells (Nogler, 1984a). 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 (Darlington, 1939; Asker and Jerling, 1992; Comai, 2005). Indeed, most apomicts are polyploids and/ or hybrids but how apomixis is triggered in natural plant populations is still under debate (Hojsgaard and Hörandl, 2019).

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. Research in this field concentrates on the identification of apomictic reproduction in plant species or on molecular control mechanisms responsible for triggering the switch from sexuality to apomixis for potential applications in agriculture (Spillane *et al.*, 2004; Ozias-Akins and van Dijk, 2007; Kumar *et al.*, 2013; Hand and Koltunow, 2014; Kumar, 2017). Apomixis affects in plants only 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 (Kaul and Murthy, 1985; Hamant *et al.*, 2006; Murphy and Bass, 2012). Due to these technical reasons, only a few empirical studies are available on a possible correlation of meiosis behavior and expression of apomixis (Koltunow *et al.*, 2011; Hojsgaard *et al.*, 2013). 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 comprises about 800 described species and is colloquially known as goldilocks (Hörandl *et al.*, 2009). The vast majority of these species are apomictically reproducing polyploids, while a small number of *Ranunculus* species are diploid (2n = 19) and tetraploid (2n = 32) sexuals (Hörandl *et al.*, 1997; Hörandl and Greilhuber, 2002; Paun, Greilhuber, *et al.*, 2006; Hojsgaard, Greilhuber, *et al.*, 2014; Dunkel *et al.*, 2018). The sexual species, *R. notabilis*, *R. carpaticola* and *R. cassubicifolius* are obligate outcrossers (Hörandl, 2008; Hojsgaard, Greilhuber, *et al.*, 2014) and can be regarded as progenitor species of the whole polyploid complex (Hörandl *et al.* 2009; Pellino *et al.* 2013; Hodač *et al.* 2018). Functional apomixis in *Ranunculus* demands effective coupling of apomeiosis and parthenogenetic egg cell generation (Nogler, 1984a). Unsuccessful linkage of these two crucial steps towards apomictic reproduction can result in increased offspring ploidy (Nogler, 1984a; Barke *et al.*, 2018).

In fact, cytological analysis in the *R. auricomus* complex have been performed on either female or male development focusing on gametogenesis and following processes such as pollen quality determination (Nogler, 1984a; b; Izmailow, 1996; Hörandl *et al.*, 1997). Reduced female fertility of F_1 hybrids has been observed by Hörandl (2008) and Hojsgaard *et al.* (2014). The present study provides an analysis of chromosomal behavior 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 (Barke *et al.*, 2018). 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 behavior in diploid versus polyploid plants, and also different selective constraints for female and male development.

5.3 Materials and Methods

5.3.1 Plant material

In this *Ranunculus* 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 6) and were determined to reproduce sexually (Hojsgaard, Greilhuber, *et al.*, 2014). Homo- and heteroploid hybrid plants had been generated by manual crossings in 2006, which resulted in diploid F_1 hybrids (F, J plants; Table 6) obtained from *R. carpaticola* x *R. notabilis* crosses and triploid F_1 individuals (G plants; Table 6) gained by crossing *R. cassubicifolius* x *R. notabilis* (Hojsgaard, Greilhuber, *et al.*, 2014). Additionally, between 2010 and 2012, a second hybrid generation was produced using F_1 plants that have shown apospory (Hojsgaard, Greilhuber, *et al.*, 2014). F_2 individuals with F and/ or J parents were found to be diploid and aposporous (Barke *et al.*, 2018; Table 6), while hybrids descending from G parents were determined to be tri- and tetraploid (Barke *et al.*, 2018; Table 6). 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 (Hörandl *et al.*, 2000; Table 6). We regard these plants as recently formed backcrosses with pollen from 4x *R. variabilis*, a species, which occurs at the same location (Hörandl *et al.*, 2000). All analyzed 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.

5.3.2 Determination of ploidy and mode of reproduction

Ploidy and mode of reproduction of the hybrids are documented in Hojsgaard *et al.* (2014) for the F_1 and in Barke *et al.* (2018) 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.* (2018). Flow cytometric seed screening confirmed sexual reproduction for the *R. notabilis* and *R. cassubicifolius* individuals (Supplementary Data Table S14; Supplementary Data Fig. S15).

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Table 6: Natural plants and synthetic hybrids of the *Ranunculus auricomus* complex analyzed in this study.

Generation	*Ranunculus* Plants	Reproduction Mode	Plant ID	Ploidy	Reference
Parent Plants	*R. carpaticola*	Sexual	8483, LH040	2x	Hojsgaard *et al.*, 2014, Suppl. Table 14, Suppl. Fig. 15
	R. notabilis	Sexual	10137, 9609	2x	Hörandl *et al.*, 2000
	R. cassubicifolius	Sexual	LH008, LH009	4x	Suppl. Table 14, Suppl. Fig. 15
F$_1$ Hybrids	*R. carp.* x *R. not.*	Sexual	F, J	2x	Hojsgaard *et al.*, 2014
	R. cassu. x *R. not.*	Facultative apomictic	G	3x	
F$_2$ Hybrids	*R. carp.* x *R. not.* X *R. carp.* x *R. not.*	Facultative apomictic	F x F, F x J, J x F, J x J	2x	Barke *et al.*, 2018
	R. cassu. x *R. not.* X *R. cassu.* x *R.not.*	Facultative apomictic	G x G	3x, 4x	
Natural Hybrids	*R. not.* x *R. variabilis (?)*	Facultative apomictic (?)	10136	4x	Hörandl *et al.*, 2000

5.3.3 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$_2$O; 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 (Barke *et al.*, 2018).

5.3.4 Pollen mother cell orcein staining

Male sporogenesis was analyzed 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).

5.3.5 Chromosome spreads

The behavior of chromosomes during male meiosis was investigated using the widely known chromosome spreading technique (Jones and Heslop-Harrison, 1996; De Storme and Geelen, 2011) with several minor modifications. Fixed flower buds were washed twice in ddH$_2$O 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.

5.3.6 Female development

The female megasporogenesis study of polyploid *Ranunculus* F$_2$ hybrids was performed using the well-documented differential interference contrast (DIC) microscopy (Hojsgaard, Greilhuber, *et al.*, 2014; Barke *et al.*, 2018). 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 (Young *et al.*, 1979; Barke *et al.*, 2018). 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.

5.3.7 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).

5.3.8 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 and generation on sporogenesis, generalized linear mixed effect model (GLMM) analyses were performed using R package *lme4* v1.1-20 (Bates *et al.*, 2019). 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 (Crawley, 2015). The explanatory variables *sex*, *ploidy* and *generation* were defined as categorical, occupying exactly one of a set of non-overlapping options; *sex*: male or female, *ploidy*: 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 6) were defined as random factor within GLMM analyses to control for interspecific effects. The data for natural allopolyploid *R. notabilis* x *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*, *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* on female sporogenesis was analyzed. To infer whether male and female sporogenesis were differently affected in F_2 hybrids, the data of Barke *et al.* (2018) 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*, *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()* (Bates *et al.*, 2019). 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).

5.4 Results

5.4.1 Male development

In order to determine whether the hybrid character or the ploidy of *Ranunculus* plants has an influence on the male gametes during meiotic division, more than 10,000 PMCs were analyzed for abnormalities (Table 7). 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 7, Fig. 4d). 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 2, Fig. 6a). In addition, erroneous male gamete formation in all hybrid plants was analyzed, 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 7; Fig. 6b).

Various abnormalities at different meiotic stages were identified in male gametes of all *Ranunculus* hybrid generations independently of ploidies (Table 7). Irregularities included lagging chromosomes and chromatin bridges at metaphase I (Fig. 4f). At anaphase I laggards and sticky chromosomes and disoriented spindle activities were detected (Fig. 4g, h, i, Fig. 5a - e). Disoriented spindle activity as well as scattered chromosomes occurred during anaphase II (Fig. 4j). In addition, micronuclei were formed during telophase II (Fig. 4k, Fig. 5f - 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. 4l – 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. 4r - t).

5.4.2 Female development

Female meiosis of three polyploid *Ranunculus* F_2 hybrid individuals, derived from two different crosses (G1 x G9, G16A x I2A), was analyzed 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 8). 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 37% of the analyzed F_2 ovules (Table 8). 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 analyzed ovules were found to be dead (Table 8). 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).

5.4.3 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. 6c, Table 7, 8). Therefore, the frequencies of abnormal male and female sporogenesis were analyzed for differences, revealing a significantly stronger defective meiosis on the female than on the male side of development (Fig. 6c).

5.4.4 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 9; Supplementary Data Table S13). 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* 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 poly-

ploidy on developmental behavior 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* 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. S14b). 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. S14c).

5.5 Discussion

Hybridization and polyploidization are known to have substantial effects on male and female reproductive programs in angiosperm plants (*e.g.* Comai, 2005). Although hybridization was recently shown to play an important role in the onset of apospory in diploid *Ranunculus* plants, its interaction with meiotic behavior remained unclear (Hojsgaard, Greilhuber, *et al.*, 2014; Barke *et al.*, 2018). The investigation of both, male and female sporogenesis in *Ranunculus*, including cytological investigations, 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 analyzed 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 7). Limited viability and fertility of young hybrids are extensively described and therefore, poor hybrid fitness is often taken for granted in case

Table 7: 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	2x	10137, 9609	923	0.99 (0.50 - 0.99)	0.02 (0.01 - 0.50)
R. carpaticola	2x	8483, LH040	369	0.97 (0.94 - 0.99)	0.03 (0.01 - 0.05)
R. cassubicifolius	4x	LH008, LH009	324	0.97 (0.96 - 0.98)	0.03 (0.02 - 0.04)
Synthetic F_1 Hybrids					
R. car. x R. not.	2x	J, F	3154	0.99 (0.99 - 1.00)	0.01 (0.00 - 0.01)
R. cas. x R. not.	3x	G	645	0.89 (0.79 - 0.99)	0.11 (0.11 - 0.21)
Synthetic F_2 Hybrids					
R. car. x R. not. X R. car. x R. not.	2x	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. x R. not. X R. cas. x R. not.	3x, 4x	G x G	211	0.86 (0.76 - 0.95)	0.14 (0.05 - 0.24)
Natural Hybrids					
R. notabilis x R. variabilis (?)	4x	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 %**

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Table 8: Analysis of female meiosis 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 (Hojsgaard *et al.,* 2014)						
R. notabilis	2x		86	0.96 (0.94 – 1.00)	0.00	0.04 (0.00 - 0.05)
R. carpaticola	2x		135	0.84 (0.83 - 0.90)	0.00	0.16 (0.10 - 0.18)
R. cassubicifolius	4x		98	0.95 (0.94 - 0.90)	0.00	0.05 (0.00 - 0.06)
Synthetic F₁ Hybrids (Hojsgaard *et al.,* 2014)						
R. car. x R. not.	2x	J, F	257	0.67 (0.44 - 1.00)	0.11 (0.00 - 0.33)	0.22 (0.00 - 0.56)
R. cas. x R. not.	3x	G	191	0.69 (0.54 - 0.87)	0.15 (0.07 - 0.32)	0.15 (0.00 - 0.29)
Synthetic F₂ Hybrids						
R. car. x R. not. *X R. car. x R. not.* (Barke *et al.,* 2018)	2x	F x F, F x J, J x F, J x J	4811	0.63 (0.45 - 0.82)	0.16 (0.08 - 0.26)	0.21 (0.00 - 0.39)
R. cas. x R. not. *X R. cas. x R. not.*	3x, 4x	G x G	186	0.49 (0.06 - 0.66)	0.12 (0.06 - 0.15)	0.39 (0.19 - 0.88)
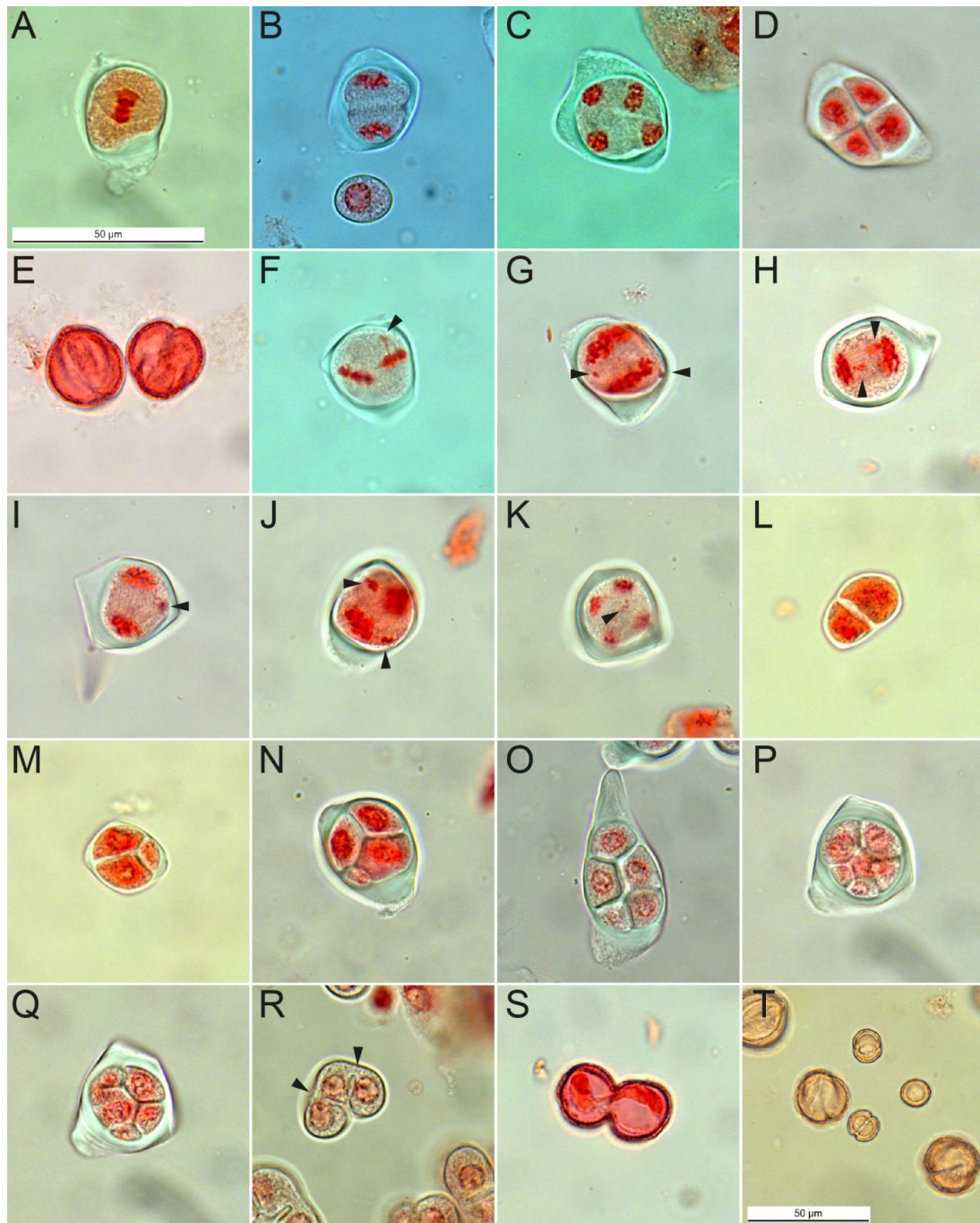
Figure 4: Meiosis in male gametes of *Ranunculus* plants. a. – e.) Regular meiosis of PMCs, a.) PMC at metaphase I, b.) PMC at telophase I during cell plate formation, c.) PCM at the end of anaphase II, d.) Meiotically developed tetrad of microspores, e.) Homogeneous-sized pollen grains, f. – p.) Various failures of meiosis in *Ranunculus* PMCs, f.) PMC at metaphase 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 (arrowhead), resulting in abnormal chromosome segregation at anaphase II, k.) PMC at anaphase 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.) Heterogeneously-sized micropollen grains. Genotypes: a.) F3 x J6 (22); b.) J9A; c., d., g., j.) 10136 (15); e.) 2 10137 (08); f.) J6 x F7 (14); h., i., k.) G5A; l., m., r., s.) F10 x F7 (04); n., t.) 10136 (08); o.) 3 10136 (02); p., q.) G16A. Scale bars = 50 µm.
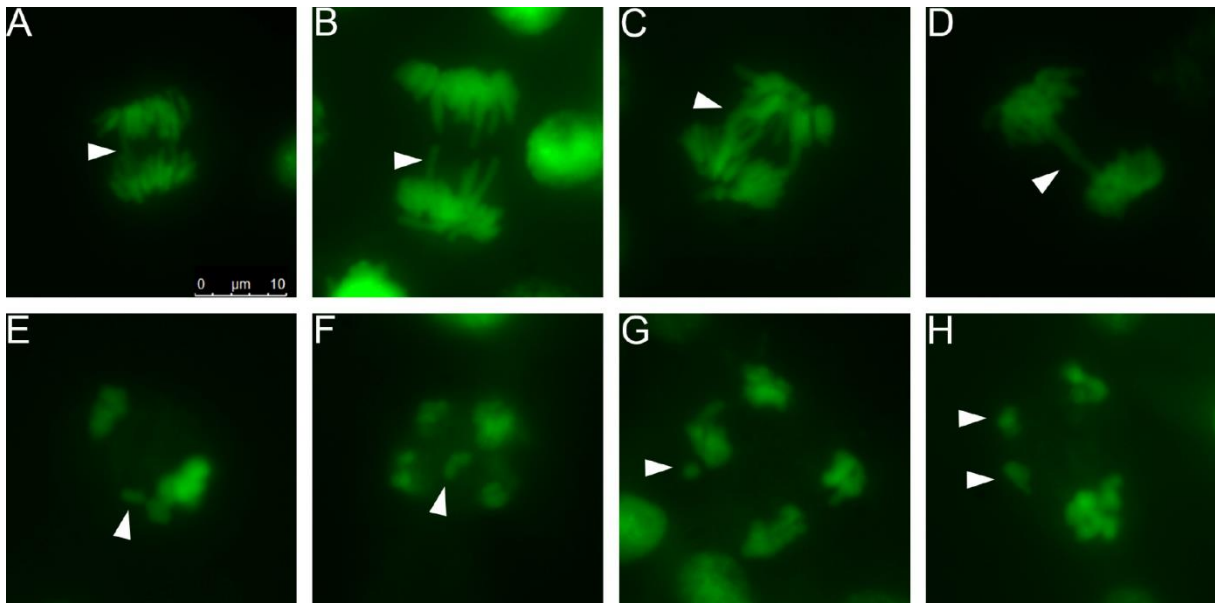
Figure 5: DAPI staining of abnormal chromosome configurations during microsporogenesis of *Ranunculus* plants. a. – d.) Sticky chromosomes in PMCs during anaphase I (arrowheads), e. – h.) PMCs display stickiness due to clumped chromosomes, e.) PCM with laggard at anaphase I (arrowhead), f. + g.) PMCs at anaphase II with lagging chromosomes (arrowheads), h.) Erratically separated bivalents at anaphase II (arrowheads). Genotypes: a.) F3 x J6 (18); b. - d.) F3 x J6 (09); e., h.) F3 x J6 (30); f., g.) F3 x J6 (03). Scale bar = 10 μm.

Table 9: Generalized mixed-effect model (GLMM) analyses discovering manipulating effects influencing the error rate of male and female sporogenesis in *Ranunculus* with regard to ploidy, 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. Slightly significant (0.1 > p > 0.05) and significant (p < 0.05) p values of tested factors are marked in bold.

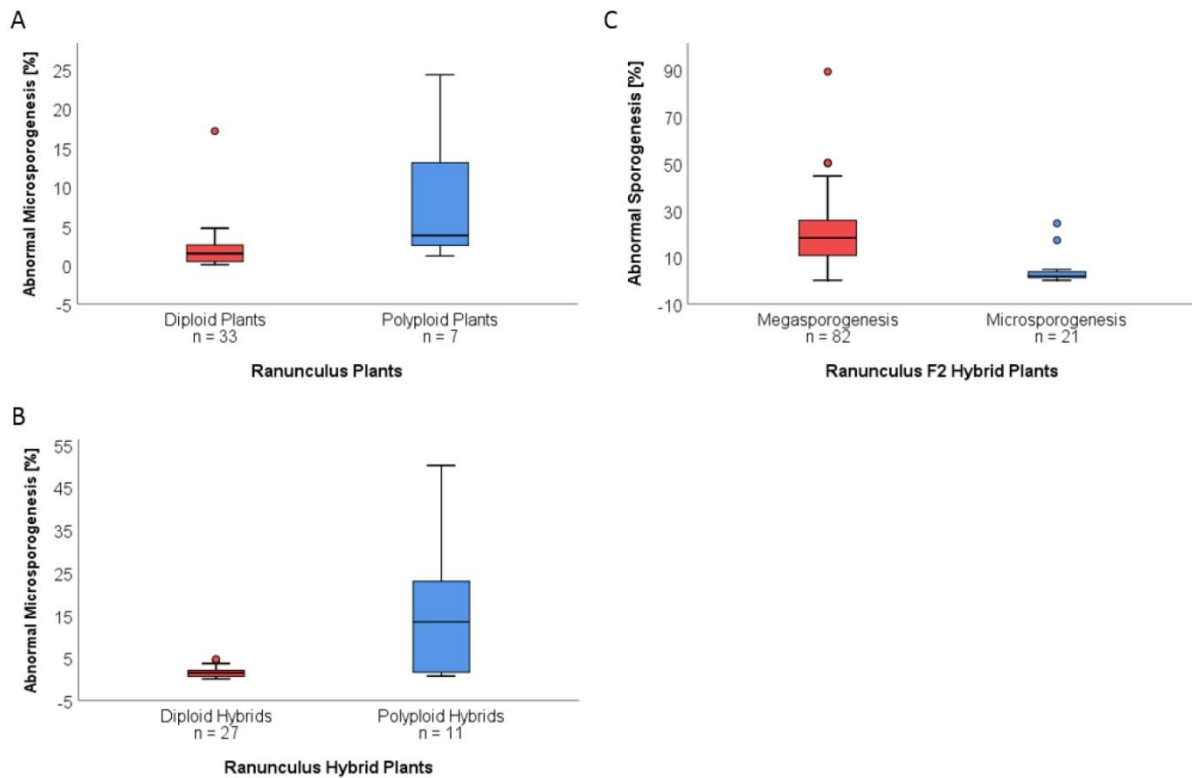| Subset	n	tested factor(s)	base line categories	test categories	GLMM Regression Estimate	p value
Male	9193	*ploidy*	2x	4x	2.19	***
		generation	F_2	P	- 0.77	*
				F_1	- 0.63	**
		combined effect	2x, F_2	4x, P	- 0.88	**0.08**
				4x, F_1	- 0.60	**0.09**
Female	3660	*ploidy*	2x	4x	0.17	0.46
Male/ Female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

Figure 6: 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.) F_2 hybrid plants showed different percentages of irregular meiosis depending on the sex and ploidy. Statistical comparison of male and female failure in meiosis irrespective of ploidy showed a significantly higher frequency of meiosis error in female development (p < 0.001, Mann-Whitney-U test). c.) Abnormal microsporogenesis depicted for all di- and polyploid hybrid plants, of which allopolyploids showed significantly more irregularities during develpment than homoploid individuals (p = 0.006, Mann-Whitney-U test). Outliers are marked as filled circles, the box represents the interquartile range and in the boxplots the median is displayed.

of natural hybrid progeny (Soltis and Soltis, 2009; Arnold *et al.*, 2012), 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 (Arnold and Hodges, 1995; Hegarty *et al.*, 2009). 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 9, Fig. 6c). DNA methylation alterations are widely associated with both, hybridization and polyploidization, while showing non-additive gene expression in di- and polyploid hybrid plants (Wang *et al.*, 2006; Hegarty *et al.*, 2008, 2009).

Chapter 2

The current study found that overall 5.42% of all analyzed samples showed an altered course of male sporogenesis (Table 7) 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. 4l - 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 favors malfunctions in male reproductive development rather than hybridization alone (Fig. 6a). 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 (Comai, 2005; Grandont *et al.*, 2013). This assumption is supported by the observation of anaphases with an odd number of spindle poles (Fig. 6i). Nevertheless, chromosome mispairing cannot be ruled out because unbalanced chromosome segregation was regularly detected as well (Fig. 4j). In rare cases, the plants showed incomplete cell plate assembly, forming unseparated aggregations of poly-nucleated microspores and in consequence, dyad pollen grains (Fig. 6r - s). Sporads, equipped with more than the normal quantity of four meiotic products, were believed to originate from 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 (De Storme and Geelen, 2011). However, this link was not yet demonstrated in *Ranunculus auricomus* (Hörandl *et al.* 1997). Quantitative pollen analyses in apomictic *Ranunculus kuepferi* found a great variation in pollen size, and dwarf pollen in tetraploids to be inviable (Schinkel *et al.*, 2017). The observed abnormalities during male gamete development seem to be relatively common phenomena in polyploid Ranunculaceae. Kumar *et al.* (2013) 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 (Kumar *et al.*, 2013). In cytomixis, the transfer of chromatin or even whole chromosomes via cytoplasmic channels or intercellular bridges can be achieved, presuming adjacent microspores (Körnicke, 1901; Guan *et*

al., 2012). For example the disoriented chromosome in Figure 4g 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. 4t; Kumar *et al.*, 2013). To estimate whether the obtained results are the con sequences of synthetically generated polyploid *Ranunculus* hybrids, additionally, an adapted tetraploid (*R. cassubicifolius*, parent species) and a potential young, natural allopolyploid (Table 6) 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 (Hörandl *et al.*, 2000). 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 6; Supplementary Data Table S13). 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 6, Fig. 6a, b).

Furthermore, the age and degree of diploidization seems to play a crucial role for meiosis function, because the tetraploid *R. cassubicifolius*, which are at least 80,000 years old (Pellino *et al.* 2013), displayed very low frequencies of abnormal male gamete development that are similar to that of diploid *Ranunculus* material (Table 6). 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 (Ramsey and Schemske, 1998; Comai, 2005). Thus, *R. cassubicifolius* plants in this study are assumed to have overcome the proposed bottleneck of currently polyploidized plants like in our natural hybrid samples (Comai, 2005). 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.* (2014). There, microsporogenesis was described as "regularly and normally preceding". 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 meiosis in the parent plant and

F_1 generation were analyzed previously by Hojsgaard *et al.* (2014) and the situation in F_2 *Ranunculus* plants by Barke *et al.* (2018). These experiments have exclusively shown sexual ES formation for parent individuals, while in F_1 and F_2 hybrids apospory was detected (Table 8; Hojsgaard, Greilhuber, *et al.*, 2014; Barke *et al.*, 2018). In this study, recently collected data for megasporogenesis of polyploid *Ranunculus* F_2 plants was amended with results of synthetic diploid F_2 hybrids, published in Barke *et al.* (2018). This analysis revealed similar frequencies for occurrence of apospory in di- and polyploid ovaries (Table 8). 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. 6c). The product of female meiosis is usually a tetrade of haploid megaspores, of which only the one closest to the chalazal pole survives. This spore is called functional megaspore and develops further into a reduced ES. We did not observe tendencies towards polysporic embryo sac development, as reported for other apomictic plants (Carman, 1997). In contrast, the male process leads in *Ranunculus* to four haploid microspores within each one pollen grain. Therefore, reduced male fertility, accomplished by abnormal meiotic behavior and disturbed microsporogenesis and -gametogenesis, has not as serious consequences as in female ovaries, because the remaining intact pollen grains with functional gametes are numerous enough for successful fertilizations. Pseudogamous apomicts, like *Ranunculus auricomus* plants, need pollen for fertilization of polar nuclei for proper endosperm formation. Hence, selection will favor the maintenance of a male function even in apomictic plants (Mogie, 1992). In contrast, ovules are much less numerous, the pollen-ovule ratio ranges in *Ranunculus auricomus* from 652 to 1684 (Izmailow, 1996). 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 hermaph-roditic plant. In this study, less than 50% of megasporogenesis in polyploid plants followed sexual development, while nearly 40% of analyzed ovules showed abortion and approx. 10% formation of an aposporous initial (Table 8). 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 8; Barke *et al.*, 2018). Thus, the onset of apomixis, as Darlington (1939) proposed, really seems to be an escape from hybrid sterility, but only on the female side. Nonetheless, seed formation in Barke *et al.* (2018) was only analyzed 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 (Hojsgaard and Hörandl, 2019) and it is supported by the present study, showing significantly higher frequency of meiotic errors in polyploid hybrids than in homoploids (Fig. 6b; Table 7). The most prominent exception is found in the genus *Boechera*, where apomixis is fully functional in diploid hybrids (Kantama *et al.*, 2007). But, in this genus dramatic chromosomal rearrangements were observed in diploid apomicts (Kantama *et al.*, 2007), and the apomictic diploid hybrid lineages originated from combinations of strongly disparate genomes (Beck *et al.*, 2012). Otherwise, apomictic seed formation in natural populations appears in very low frequencies (reviewed by Hojsgaard and Hörandl, 2019) and could be also due to environmentally induced disturbance of sexual development (Klatt *et al.*, 2018). 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 (Carman, 1997; Rieseberg *et al.*, 1999; Comai, 2005). This hypothesis is supported by several studies that observed heterochronic alterations in female development of synthetic *R. auricomus* hybrids (Hojsgaard, Greilhuber, *et al.*, 2014; Barke *et al.*, 2018), which could be due to reversible epigenetic silencing (Hand and Koltunow, 2014). Besides that, apomixis-related changes in gene expression could not be nailed down to certain gene clusters (Grimanelli, 2012). Moreover, it was observed that apomictic key components (apomeiosis, parthenogenesis and independent endosperm formation) are genetically controlled by independent loci (Ogawa *et al.*, 2013). Thus, epigenetic regulation and reprogramming of plant reproduction are considered as powerful factors on the way to functional apomixis. Nonetheless, such alterations could be also a consequence of previous karyotypic changes after loss of chromosomes, chromosomal rearrangements or missegration. More substantive proofs are required to test this hypothesis.

Chapter 2

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.

5.6 Supplementary Material
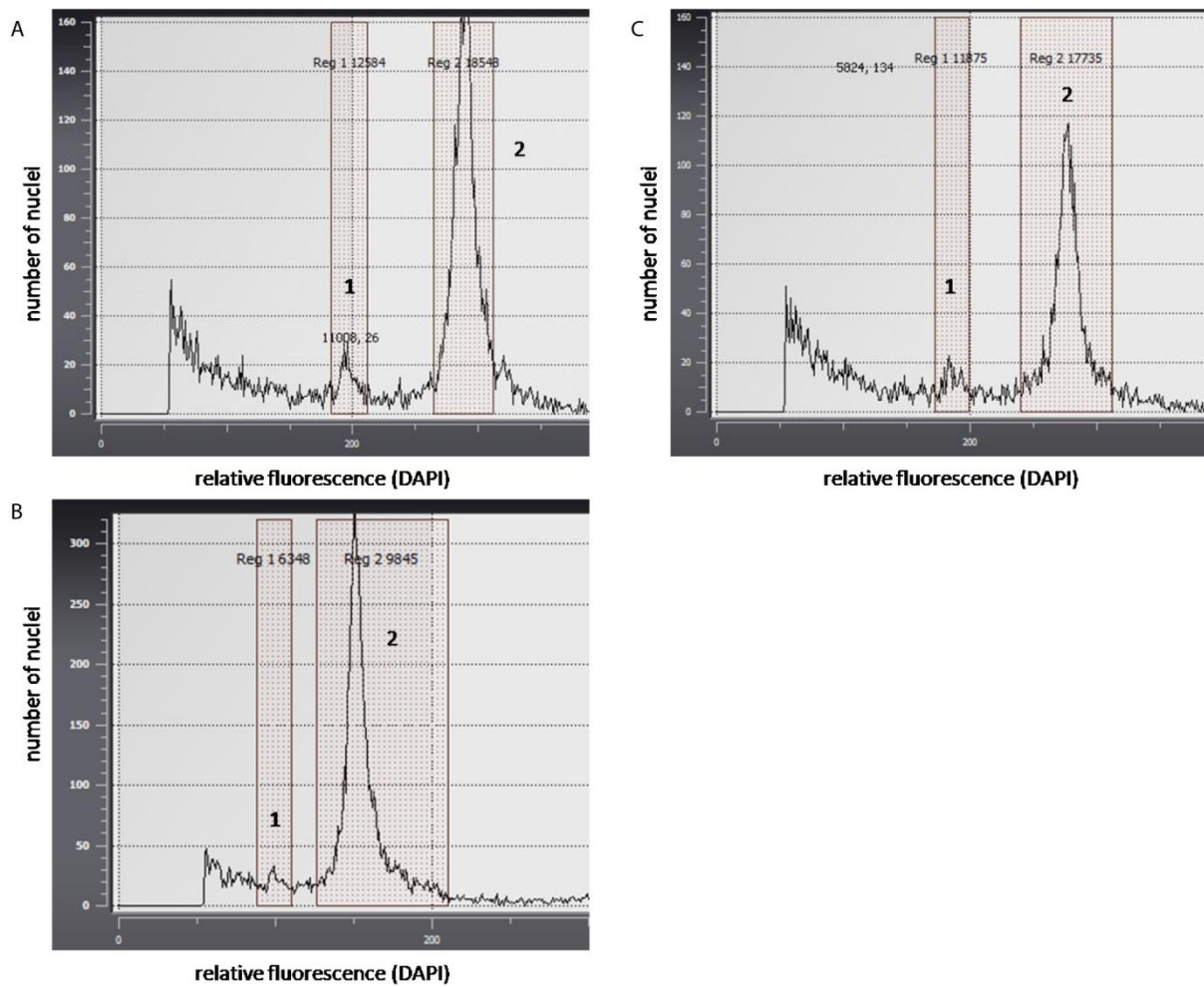
Figure S15: Representative flow cytometry histograms of *Ranunculus* seeds (a.–c.). General peak labeling: 1 embryo peak and 2 endosperm peak. a.) *R. cassubicifolius* sexual seed with a tetraploid embryo and a hexaploid endosperm. b.) *R. carpaticola* sexual seed with a diploid embryo and a triploid endosperm. c.) *R. cassubicifolius* sexual seed with a tetraploid embryo and a hexaploid endosperm. Populations: a.) LH008, b.) LH040, c.) LH009.
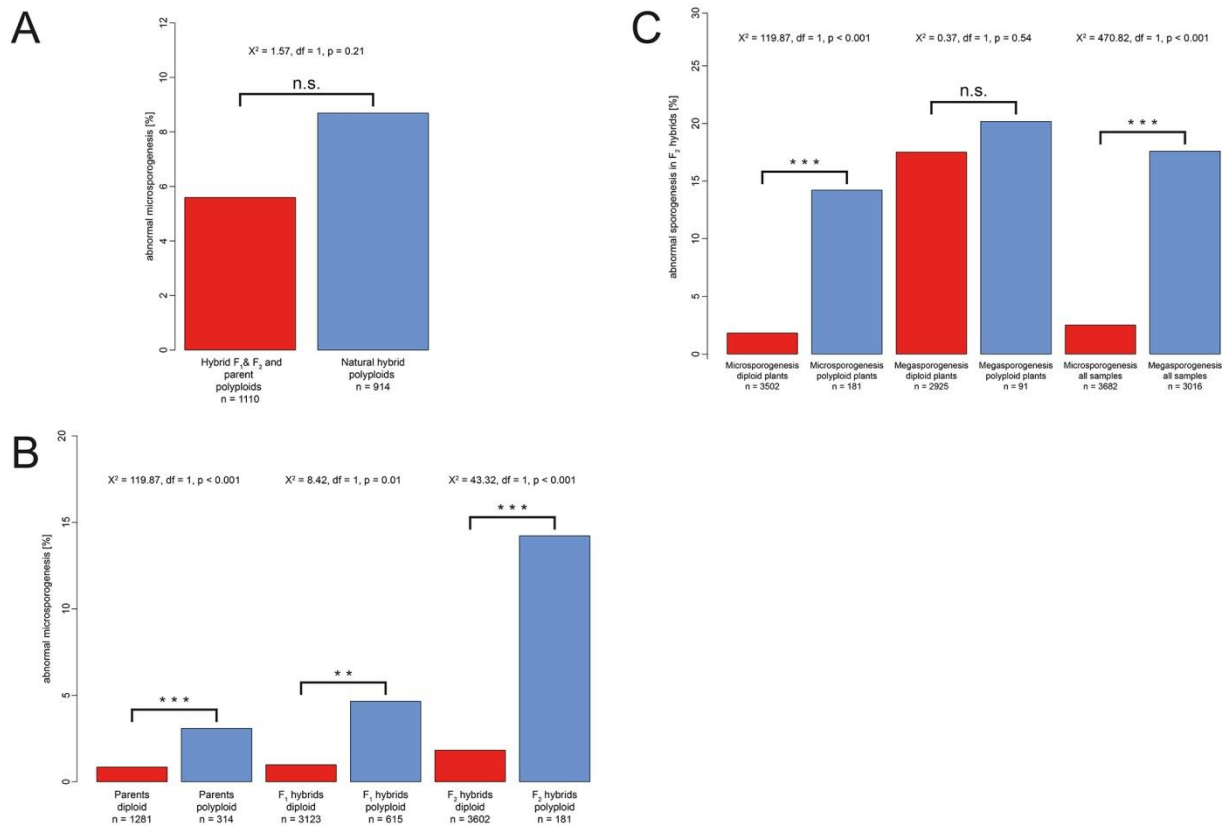
Figure S16: *Chi*-squared analyses of erroneous mega- and microsporogenesis in natural and hybrid *Ranunculus* plants. a.) Bar graph depicting percentages of sporogenetic failures in *R. cassubicifolius*, polyploid F_1 and F_2 hybrids as well as in natural polyploid hybrids, showing no significant difference ($X^2 = 1.57$, df = 1, p = 0.21). b.) Highly significant differences 5 between di- and polyploid parent plants ($X^2 = 119.87$, df = 1, p < 0.001), F_1 hybrids ($X^2 = 8.42$. df = 1, p = 0.01) and F_2 hybrids were found ($X^2 = 43.32$, df = 1, p < 0.001). c.) Comparison of megasporogenesis in di- and polyploid F_2 hybrids revealed no significant difference ($X^2 = 0.37$, df = 1, p = 0.54), whereas highly significantly more errors in microsporogenesis were found in polyploid F_2 plants compared to diploid F_2 individuals ($X^2 = 119.87$, df = 1, p < 0.001). Overall, significantly more abnormal sporogenesis was observed in female than in male gamete development ($X^2 = 470.82$, df = 1, p < 0.001).

Table S13: Generalized mixed-effect model (GLMM) analyses observing 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. Significant (p < 0.05) p values of tested factors are marked in bold.

Subset	n	Tested Factor(s)	Base Line	Test Category	GLMM Regression Estimate	Standard Error	z Value	p Value
Male	9193	ploidy		(intercept)	-3.99	0.13	-31.85	< 0.001
			2x	4x	2.19	0.23	9.38	**< 0.001**
		generation	**F$_2$**	**P**	**-0.77**	**0.33**	**-2.35**	**< 0.05**
			F$_2$	**F$_1$**	**-0.63**	**0.22**	**-2.85**	**< 0.01**
		combined effect	2x, F$_2$	4x, P	-0.88	0.50	-1.76	0.08
			F$_2$	4x, F$_1$	-0.60	0.35	-1.71	0.09
Female	3660	ploidy	2x	(Intercept)	-1.55	0.04	-35.08	< 0.001
				4x	0.17	0.24	0.73	0.46
Male/ Female	7438	ploidy		(Intercept)	-1.55	0.04	-35.08	< 0.001
			2x	4x	0.17	0.24	0.73	0.46
		sex	**female**	**male**	**-2.44**	**0.13**	**-18.36**	**< 0.001**
		combined effect	**2x, female**	**4x, male**	**2.02**	**0.33**	**6.05**	**< 0.001**

Table S14: Mean peak indices (PI) of reproductive mode of different *Ranunculus* populations. The peak index (endosperm DNA content / embryo DNA content) indicates a seed formed by sexual double fertilization, resulting in a 2n embryo and a 3n endosperm.

Population	Species	Ploidy	No. of Seeds	PI (Range)	Reproduction Mode
LH008	*R. cassubicifolius*	4x	13	1.51 (1.46 - 1.56)	sexual
LH009	*R. cassubicifolius*	4x	12	1.46 (1.34 - 1.51)	sexual
LH040	*R. carpaticola*	2x	14	1.53 (1.44 - 1.60)	sexual

6   General Discussion

In angiosperm plants, two distinct pathways are described that lead to reproduction via seeds – the sexual and the asexual pathway. The asexual one is also referred to as apomixis and produces clonal, maternal offspring (Asker and Jerling, 1992). This ability is of enormous agricultural and economic interest, but none of the important crop plants are capable of apomictic reproduction (Carman, 1997). The transfer of the apomictic trait to crops like maize and rice is considered as non-satisfying (Savidan, 2000b; Spillane *et al.*, 2004). There are several mechanisms of apomixis documented, however, this thesis focused on gametophytic apomixis, more precisely the aposporous variant. It comprises three essential events that are required in the following order to generate full apomictic seeds: apospory, parthenogenesis and endosperm formation (Nogler, 1984a; Asker and Jerling, 1992; Grimanelli *et al.*, 2001). In defiance of intensive research on apomixis during the last decades, the main question, how the plant's reproductive system is able to switch from sexuality to apomixis still remains unanswered. However, it is a matter of fact that almost all known apomicts share two stunning characteristics. First, most of them were determined to be polyploid, with only a few, rare exceptions to that rule and secondly, apomicts were identified to be of hybrid origin (Nogler, 1984a; Asker and Jerling, 1992; Grossniklaus *et al.*, 2001; Hand and Koltunow, 2014; Hojsgaard and Hörandl, 2019). Based on these two evidence, scientists want to identify and understand genetic, epigenetic and cytogenetic changes in apomictic plant individuals with the overall goal of explaining and simulating the emergence of functional apomixis in agricultural breeding (reviewed by Asker and Jerling, 1992; Hand and Koltunow, 2014; Hojsgaard and Hörandl, 2019), but the actual impact of polyploidization and hybridization on the onset of apomictic seed formation in natural plant populations is not yet settled. Thus, this thesis aims at filling the particular knowledge gap by using three different *Ranunculus* generations, including natural diploids and tetraploids and two synthetic hybrid generations, comprising homo- and heteroploid individuals were used as sample material. In order to verify the establishment of apomixis in hybrid *Ranunculus* samples, the functionality of apospory and pseudogamous endosperm formation, as two of three key processes of gametophytic apomixis, were checked for successful collaboration. In addition to that, the importance of polyploidiy for the reproductive change was checked by studying the progression of male meiosis and sporogenesis.

General Discussion

Hybridization is one of the most abundant and potent evolutionary drivers in the plant kingdom, with major influence on speciation and reproduction (Mallet, 2008; Nolte and Tautz, 2010). Additionally, natural apomicts are often of hybrid origin, which makes hybridization a potential trigger of functional apomixis (Ernst, 1918; Asker and Jerling, 1992). Therefore, to investigate whether this mechanism is the activator of gametophytic apomixis in plants of the *R. auricomus* complex, Hojsgaard *et al.* (2014) have generated a first hybrid generation by controlled, manual crossing of di- and tetraploid sexual *Ranunculus* species. Microscopic analyses have shown spontaneous occurrence of AICs in di- and triploid hybrids at low frequencies, which was regarded as first step towards apomictic seed formation (Hojsgaard, Greilhuber, *et al.*, 2014). However, only one asexual seed, derived from a triploid F_1, was documented (Hojsgaard, Greilhuber, *et al.*, 2014) and therefore, the present follow-up study was designed with F_2 hybrids (Chapters 1, 2). The true hybrid character of the second plant generation was genetically verified by SSRs to exclude a clonal or a selfing origin of the plants.

Almost 4900 diploid F_2 ovules were individually screened by DIC microscopy for enhanced frequencies of apospory, which was confirmed. About 16% of the analyzed *Ranunculus* samples showed apospory, representing an increase in apospory compared to the F_1 plants (Hojsgaard, Greilhuber, *et al.*, 2014). The observed spontaneous emergence of aposporous ES development held remarkable features and experimentally verified that apomixis is transferrable through male pollen from one plant generation to the next as previously shown by Nogler (1984a) and Van Dijk *et al.* (1999). Furthermore, a significant increase in apospory in diploid *Ranunculus* F_2 (21%), originating from parental plants that both have shown spontaneous AIC formation in contrast to F_2 plants (14%) with only one aposporous parent was found. These results of chapter 1 are in line with observations of Nogler (1984b), who discovered the influence and importance of genomic dosage effects on gamete formation in polyploid *Ranunculus* species. In turn, the data argue against Nogler's (1984b) speculation that apomixis-inheritance in *Ranunculus* is mediated only by one single dominant allele that occupies recessive lethal effects in haploid gametophytes. Although, similar single genomic control factors are found in other apomictically reproducing plants such as in *Tripsacum* (Grimanelli *et al.*, 1998) and *Pennisetum* (Roche *et al.*, 2001), Nogler's (1984b) hypothesis could not be perpetuated by the confirmed presence of diploid, aposporous F_2 hybrids. Apospory was additionally observed in polyploid F_2 plants but at a lower frequency as in the ancestral gen-

General Discussion

eration (Chapter 1, 2). As consequence, AIC formation in *Ranunculus* seems to be activated by hybridization and further enhanced by the accumulation of genomic dosage effects, whereas polyploidization is not considered as necessary activator. This assumption was made, because the entire *Ranunculus* samples in chapter 1 were diploid hybrids. In addition to apospory, other abnormalities in female sporogenesis and gametogenesis were identified. Heavily disturbed sequence of female gametophyte formation was observed in F_2 hybrids, sometimes including whole germ line abortion and thus, sterility. Similar, corresponding developmental problems were already mentioned by Hojsgaard *et al.* (2014) for F_1 individuals, which are considered as common feature of young hybrid generations (Arnold and Hodges, 1995). Such drastic effects are associated by disturbed meiotic cell division, which progression is known to be negatively influenced by interspecific hybridization (Comai, 2005). Therefore, hybrids are usually regarded as less fit and maladaptive, especially in the first few generations, which was nicely, experimentally demonstrated for F_1 and F_2 *Ranunculus* hybrids (Arnold, 1997; Mallet, 2007; Hojsgaard, Greilhuber, *et al.*, 2014; Chapter 1). Both, temporal and spatial performance of megasporogenesis and megagametogenesis were affected in F_2 plants, which confirmed the strong influence of hybridism on apospory occurrence in *Ranunculus* plants (Chapter 1).

In order to gain more informative data on the role of hybridization, the functionality of male sporogenesis in natural, sexual *Ranunculus* plants (parental plants) was compared to natural hybrids as well as to the synthetic F_1 and F_2 hybrid plants (Chapter 2). With these cytogenetic experiments the proposed impact of hybridization on the sensitive mode of meiosis was revised by analyzing more than 10,000 PMCs. Although, several abnormalities during male meiosis and sporogenesis were determined in diploid F_1 and F_2 individuals, no significant increase in erroneous development was documented compared to the natural, parental plants. Meiosis is one of the most strictly controlled processes in eukaryotic organisms and its functionality strongly depends on accurate pairing and segregation of homologous chromosomes (Hamant *et al.*, 2006; Harrison *et al.*, 2010). Smallest imperfections, as observed in the present thesis, of spindle orientation, synapsis, cytokinesis or complete skipping of meiosis I or II lead to unbalanced gamete formation and therefore, to aneuploidy, sterility or cell abortion (Bretagnolle and Thompson, 1995; Cifuentes *et al.*, 2010; De Storme and Geelen, 2011). In addition to the investigation of cytogenetic progression in diploid *Ranunculus* plants during micro-

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sporogenesis, the same analyses were performed in polyploid sexual and hybrid plants. Again various abnormalities were observed, *e.g.* chromosome mispairing, erroneous spindle formation as well as unusual numbers and shapes of microspores. In hybrid plants, two divergent genomes come together and are supposed to from functional gametophytes but, as mentioned, functional meiosis demands perfect pairing of homologous chromosomes to establish bivalents (Hamant *et al.*, 2006), which was determined to be stunted in these *Ranunculus* hybrids (Chapter 1, 2). However, depending on how closely the hybrid parents were related, homolog pairing is relatively rare (Comai, 2005; Zielinski and Mittelsten Scheid, 2012). Instead, homeologs are forced to form pairs, which tents to result in uni- and multivalents rather than bivalent chromosomes (Comai, 2005; Cifuentes *et al.*, 2010). Hindered bivalent formation seems to be applicable in these analyses, due to the frequent observation of lagging chromosomes. If possible, the subsequent segregation of faulty chromosome pairs was assumed to lead to unbalanced meiotic products and thus, to differentially viable and sized microspores (Comai, 2005; De Storme *et al.*, 2012; Zielinski and Mittelsten Scheid, 2012). The observed broad range of pollen sizes and viabilities in *Ranunculus* samples confirmed fundamental problems in regular bivalent formation. Additionally, to normally sized and shaped gametes, very small dwarf-microspores were detected as well. A proposed correlation, of increased or decreased ploidy level to the size of pollen grains, is not yet proven in *Ranunculus* but in other angiosperms like *Arabidopsis*, which strongly suggests the same dependency for pollen in this study (Bretagnolle and Thompson, 1995; Hörandl *et al.*, 1997; De Storme and Geelen, 2011). Similar small microspores were also found in *R. kuepferi* plants and all of them were determined as invariable non-viable/ sterile (Schinkel *et al.*, 2016). Thus, a similar, deadly fate is assumed for the described *R. auricomus* dwarf-spores.

Additionally, to the homoploids, male sporogenesis was analyzed in polyploid *Ranunculus* hybrids (Chapter 2), because polyploidization as the second important factor, driving plant speciation and evolution, is assumed to have impact on male gamete formation as well (Soltis and Soltis, 2009). A polyploid genome can be achieved for example by genome doubling, caused by unreduced gametes, or in combination with preceded hybridization results in polyploid plants (Carman, 1997; Paun *et al.*, 2007). In all allopolyploids in this thesis, including the natural ones, the frequency of developmental irregularities was significantly more abundant than in diploid individuals. Therefore, it was assumed that the combination of hybridization and polyploidization significantly enhances the

described malfunctions during pollen grain formation. These facts of chapter 2 are supported by Carman (1997), who claimed that hybridization and polyploidization together have disturbing effects on gamete formation, which are supposed to result in apomixis. This is likely, because most apomicts appear to be allopolyploids (Asker and Jerling, 1992; Carman, 1997), which also applies to *Ranunculus* individuals in this study. The reason for the switch to apomixis could be that neopolyploids have been determined to be less fit than adapted polyploid plants, because recently formed polyploids often suffer from genomic and developmental errors (Comai, 2005), whereas apomictic reproduction serves as rescue mechanism. These statements are fully supported by the cytogenetic data determined by this thesis. In general, diploidization is known to have calming and stabilizing effects on disturbed developmental programs of young polyploid plants. During this process dispensable genes or whole chromosomes are deleted from polyploid genomes (Adams and Wendel, 2005; Comai, 2005). Chromosome deletions and laggards were numerously observed and other significantly enhanced errors of male sporogenesis were identified in chapter 2, in which PMCs of natural, young allopolyploids were compared to PMCs of the tetraploid, parental species *R. cassubicifolius*. Interestingly, these natural polyploids revealed surprisingly low frequencies of developmental irregularities (3%) compared to the young, natural *Ranunculus* hybrids (18%). The finding strongly argues towards the reduced viability and fitness of young neopolyploids in comparison with older, well-adapted polyploid plants. Regular meiosis in *Ranunculus* results in a tetrad, which consists of four haploid, same sized microspores but in the "defective" *Ranunculus* hybrids, especially in the polyploid ones, multiple numbers and various sizes of sporads were found (Chapter 2). The generation of dyads, triads and polyads is assumed to be caused by abnormal spindle function as described by d'Erfurth *et al.* (2008) and by De Storme and Geelen (2011). Despite of the mechanism, the outcome of dyad formation is supposed to be a certain percentage of unreduced gametophytes in the analyzed *Ranunculus* samples, but definite clarification is required. By contrast, microspores of documented *Ranunculus* triads and polyads have very likely an aneuploid character and may abort during further development (Cifuentes *et al.*, 2010; De Storme and Geelen, 2011). Male gametogenesis is highly susceptible to temperature stresses such as heat and cold, which act on meiotic recombination *e.g.* on spindle orientation and polarity (De Storme and Geelen, 2011, 2014). In this thesis, frequencies of enhanced irregularities in allopolyploid plants cannot be attributed to such abiotic influ-

ences because all analyzed *Ranunculus* individuals were grown side by side under identical, natural outdoor conditions of the old botanical garden of the University of Goettingen. According to the present data, hybridization together with poly-ploidization acts intensely on all important meiotic checkpoints, including chromosomal pairing, synapsis, spindle organization, chromosome segregation as well as on cytokinesis by establishing aberration of sporads and pollen grains. Another possible explanation for the here documented problems during gamete formation, including micro-sporogenesis and micro-gametogenesis, is the feasible appearance of cytomixis. This prophase I–associated procedure is known to have influence on the genomic constitution of meiotic products, because genomic information, in terms of chromatin stretches or chromosomes, can be exchanged through cytoplasmic channels or chromatin bridges of spatially close meiocytes (Körnicke, 1901; Heslop-Harrison, 1966; Guan *et al.*, 2012). Therefore, cytomixis leads to the formation of unbalanced microspores and is described as most abundant in plants with anyway faulty development as in hybrids, polyploids as well as in apomicts (Boldrini *et al.*, 2006). Forage grasses, like *Brachiaria* are prone to cytomixis (Boldrini *et al.*, 2006). Analog meiotic failures were found in microspores of Himalayan *Ranunculus*, including heterogeneous-sized male gametophytes (Kumar *et al.*, 2013), as observed in Chapter 2. The parallels suspect the occurrence of cytomixis in homo- and allopolyploids in this study as well. Although, several observations of odd positioned chromatin were made in chapter 2, the actual taking place of cytomixis was not experimentally confirmed. The verification of this kind of genomic exchanges requires a different experimental setup, including sample embedding and microtome sectioning as done by Kumar *et al.* (2013). Nevertheless, it still considered as another plausible explanation for *i.a.* sterile dwarf-microspores, because the analyzed *Ranunculus* individuals comprise at least two of three confirmed properties of cytomixis (Boldrini *et al.*, 2006). Even though a multitude of developmental errors was found, the overall frequency in *Ranunculus* samples remained relatively low (max. 18%), ensuring more than enough mature and functional material for successful pollination. Concluding, disturbed male meiosis has only minor evolutionary impact on the affected *Ranunculus* individuals, whereas even a mild perturbation of female gamete development has fatal and deadly consequences (Chapters 1, 2). In the majority of sexually reproducing angiosperms, each ovule contains a single MMC that develops meiotically into a tetrad of megaspores, of which three directly undergo programmed cell death (apoptosis), leaving one FM. In comparison,

products of erroneous meiotic cell division are often non-viable and are prone to abortion and regularly result in fully sterile ovules (Cifuentes *et al.*, 2010). This statement by Cifuentes *et al.* (2010) was confirmed, because both, male and female development, showed dysplastic or entirely dead gametes (Chapter 1, 2).

Rare cases of spontaneous apomictic seed formation do exist, *i.a.* in diploid *R. kuepferi* plants (Schinkel *et al.*, 2017). Based on such unexpected occurrences of apomixis, it appears obvious that the onset is somehow related to changes of the sexual pathway, rather than being an independent developmental trait (Ozias-Akins and van Dijk, 2007). Another supporting indication is the facultative character of most apomicts (Asker and Jerling, 1992). In the past 20-30 years, genetic and epigenetic changes upon hybridization and/ or polyploidization became the center of apomixis research (*e.g.* Carman, 1997; Ozias-Akins and van Dijk, 2007; Grimanelli, 2012; Fei *et al.*, 2019). Today, numerous genomic loci were found and associated to apomictic reproduction and in most of these cases apomictic key processes were found to be controlled by several separate loci (reviewed by Fei *et al.*, 2019). One example for apomixis-related genetic control is found in *Hieracium pilosella* and is associated with two loci, called LOSS OF APOMEIOSIS (LOA) and LOSS OF PARTHENOGENESIS (LOP; Feng *et al.*, 2010; Singh *et al.*, 2011; Sailer *et al.*, 2016). As the names imply, these loci are responsible for, either apospory or parthenogenesis induction, and only in combination they do result in apomictic seed formation (Feng *et al.*, 2010). Loci with similar purposes were identified in other asexually reproducing plants but epigenetic regulation seems to play a more important role in the functional establishment of apomixis (Ozias-Akins and van Dijk, 2007; Grimanelli, 2012; Fei *et al.*, 2019). This hypothesis arose from certain *Arabidopsis* mutants that revealed apomixis-like phenotypes. As cause, the protein ARGONAUTE9 (AGO9) attributed to small RNA silencing pathway was identified as specific genetic marker for apomixis (Olmedo-Monfil *et al.*, 2010). Experimentally this correlation was supported by maize mutants, which showed the involvement of an AGO9 homolog (Singh *et al.*, 2011). The small RNA silencing pathways are important directors of epigenetic modulations and members of the ARGONAUTE family are known to act in RNA interference by effecting small RNA molecules (Mallory and Vaucheret, 2010; Molnar *et al.*, 2010). Additionally, Bourc'his and Voinnet (2010) described the action of small RNA silencing on important developmental processes such as gametogenesis, which, to some extent, support the ideas of Carman (1997). He claimed a tightly linked connection of apomixis establishment and

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fundamental changes on gene expression (heterogenic expression) and heritable epige-netic gene regulation triggered either by polyploidization, hybridization or both (Sharbel *et al.*, 2009, 2010; Grimanelli, 2012). Examples for the linkage of reversible gene silencing and apomixis are rare. Regardless of this, DNA methylation pattern were determined to be diverse in sexuals and apomicts, which is interesting because this type of modifications are known to be involved in gamete formation and other important de-velopmental processes of plants (Spielman *et al.*, 2003; Garcia-Aguilar *et al.*, 2010; Fei *et al.*, 2019) Epigenetic DNA modifications are reversible and reprogrammable by events like hybridization and modification patterns can be inherited, while they do not directly alter genomic sequences (Jaenisch and Bird, 2003; Ozias-Akins and van Dijk, 2007). Alt-hough, changes in temporal and spatial gene expression were shown to be inducible by hybridization and polyploidization, Carman's idea is not yet completely proven. Never-theless, it appears to be another reasonable explanatory approach for the cryptic occur-rence of apomictic seed formation in this study. In defiance of these promising indica-tions, an involvement of epigenetic silencing during the onset of apomixis in analyzed *Ranunculus* hybrids can neither be confirmed nor denied, due to the non-molecular character of the present study. Knowledge about the proposed connection of genetics/ epigenetics and the change from sexual to apomictic development is important for fu-ture research and therefore, demands to be mentioned here as well.

In order to confirm the successful connection of apospory, parthenogenesis and pseudogamy, mature seeds, derived from manual crossing of diploid F_2 *Ranunculus* hy-brids, were analyzed by single seed FCSS (Chapter 1). Seed formation in flowering plants is a very sensitive process and therefore, strictly controlled, especially endosperm for-mation is considered as a weak point. Mature seeds request a specific 2 : 1 ratio of ma-ternal (m) to paternal (p) genome to ensure genomic imprinting (Vinkenoog *et al.*, 2003). This genome contribution is common in sexual reproducing anigosperms, be-cause endosperm formation is based on fertilization of the maternal central cell. This ES enclosed cell is equipped with two haploid polar nuclei and gets usually fertilized by a haploid male pollen nucleus, resulting in the desired, necessary genomic endosperm ra-tio. Alternative genome contributions commonly have negative effects on the whole seed, ranging from developmental disturbances to complete abortion (Spielman *et al.*, 2003; Vinkenoog *et al.*, 2003). In contrast to sexually formed seeds, apomictic develop-ment in species of the *R. auricomus* complex possess unreduced embryo sacs that get

fertilized by male pollen (Nogler, 1984a). Depending on whether the pollen nucleus is reduced or unreduced, different types of genome imbalances are obtained that are invariably deleterious to *Ranunculus* seed, as they are susceptible (Hörandl and Temsch, 2009). This fact was confirmed by almost 80% aborted diploid F_2 seeds and absolute failure of polyploid seed formation in this study (Chapter 1). The remaining mature, diploid F_2 derived seeds were analyzed by single seed FCSS in order to determine their mode of reproduction. To do so, only seeds that obtained clear embryo and endosperm peaks were considered for evaluation, which revealed miscellaneous modes of reproduction. However, most of these seeds were determined to be of sexual origin, including the described endosperm genome contribution (2m : 1p). The penetrance of asexual reproduction in all kinds of facultative apomicts on meiotic product survival in ovules, which is supposed to be assessable by the timing of flower pollination (Espinoza *et al.*, 2002; Hojsgaard *et al.*, 2013). Furthermore, Nogler (1971, 1973) discovered an early prophase-linked initiation of AIC formation in highly apomictic *Ranunculus* plants. Assertiveness of sexual ES establishment determined as strongly depending on the temporal onset of apospory, because chances of sexual FM success were rapidly dwindling with an early switch to apospory (Christoff and Papasova, 1943). Crossing experiments of apomictic *Ranunculus* plants with sexual or parental individuals (backcrosses), interestingly showed deferred apospory initiation, which boosted the success rate of sexual seed (Nogler, 1971, 1973).

However, beside sexually formed seeds, many different types of asexual *Ranunculus* F_2 derived seeds were identified in the present study by FCSS. In defiance of the reported intense dependence of genomic endosperm ration retention in sexual *Ranunculus* seeds, apomictic fruits are capable of accepting minor abnormal contributions (Savidan, 2007; Hörandl and Temsch, 2009; Dobeš *et al.*, 2013). Among the asexual seeds, several intermediate cases of B_{III} seeds were documented, which endosperms showed genomic contributions of 4m : 1p and 2m : 2p. In addition, diploid F_2 hybrids were able to reproduce fully apomictic, revealing either complete autonomous endosperm formation (4m : 0p) or double fertilization of the unreduced central cell by two unreduced nuclei (4m : 4p). Remarkably, until these analyses formation of autonomous endosperm was only rarely observed in apomictic *R. kuepferi* seeds but never before in *R. auricomus* (Schinkel *et al.*, 2016). Although, results are largely consistent with tolerated genomic imbalances of F_1 *Ranunculus* hybrids, none of the reproduction modes, considered as characteristic in

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asexual *R. auricomus*, was detected (Hojsgaard, Greilhuber, *et al.*, 2014; Klatt *et al.*, 2016). Previous studies showed apomictic endosperm development mainly maintains the demanded genomic ratio by pseudogamy, in which the central cell gets fertilized by either one unreduced male nucleus or by two reduced ones (Hojsgaard, Klatt, *et al.*, 2014; Klatt *et al.*, 2016). Absence of these two modes is most probably due to the observed high frequency of erroneous pollen grains in di- and polyploid *Ranunculus* hybrids. Unbalanced gametes have in extreme cases no chance to further develop into mature pollen grains *e.g.* the dwarf-microspores, whereas milder disturbances are tolerated. For example, chromosome counts by Hojsgaard *et al.* (2014) confirm this assumption, because individuals have shown single chromosome gains or losses that did not impair plant development, viability or fitness. Nonetheless, seed formation is a sensitive process due to a very narrow tolerance range in plants of the *R. auricomus* complex, which is buttressed by bad pollen quality of apomictic plants and a high sensitivity to endosperm imbalances (Izmailow, 1996; Hörandl *et al.*, 1997; Hörandl and Temsch, 2009). Furthermore, some of the aborted F_2 plant derived seeds might have fell prey to sibling cross incompatibilies. Failure of F_2 polyploids to form mature seeds is therefore subject to one of the mentioned enhanced disturbances in male meiosis in allopolyploids and thus, to negative effects during endosperm formation. Several diploid F_2 *Ranunculus* hybrids generated maternal B_{III} seeds, which are widely regarded as an intermediate step between sexuality and apomixis. This is, although an aposporous egg cell was established, double fertilization of egg and central cell occurred, as described during sexual reproduction (Nogler, 1984a; Asker and Jerling, 1992). Emerging plants from B_{III} seeds show an increase in ploidy (Rutishauser, 1948; Matzk *et al.*, 2000). In case of the present seed set, triploid F_3 plants are expected with an elevated frequency of apospory due to created genomic dosage effects. Emanating from these B_{III} hybrid seeds, a triploid bridge, serving as spring board to the formation of tetraploid apomicts is established, as documented in *R. kuepferi* populations studied by Schinkel *et al.* (2017). The present results argue towards a hybridization caused, polyploidization enhanced and changed pattern of reversible, epigenetic DNA modulation. This conclusion is on the one hand based on the overall strong developmental alterations in both, male and female gametophyte formation as well as in the high percentage of aborted or mal-developed *Ranunculus* seeds. In each of these two important key elements of plant reproduction, negative effects were particularly heightened by the combination of hybridity and polyploidy. Addi-

tionally, the formation of apomictic seeds by the F_2 homoploids demonstrates that functional apomictic reproduction in *Ranunculus* does not rely on polyploidy but on hybridization. On the other hand, the high number of aborted seeds and the formation of B_{III} seeds indicate that fully functional apomixis is not yet completely achieved in diploid F_2 plants. The result suggests increasing penetrance of apomixis in the following plant generations. Although, hybridization was identified as an important factor in the emergence of gametophytic apomixis in hybrid plants of the *R. auricomus* complex, the role of polyploidization remains elusive. Schinkel *et al.* (2017) speculated that polyploidization is not responsible for the switch to apomixis rather a consequence with calming and stabilizing properties (Comai, 2005). The assumption could apply and be considered as true but only in the long evolutionary context. Allopolyploid plants in this study, extremely suffered from all kinds of disturbances and failures, provoked by hybridization but immensely forced by polyploidization. This is a long known phenomenon in neopolyploids, which tend to struggle in the first generations with strong implications like disturbed development and especially with faulty meiosis and therefore, by a reduced viability and fitness (Comai, 2005; Soltis and Soltis, 2009). Comai (2005) has described the first polyploid generations of a plant as a "bottleneck" of evolution, because he claims that, although almost all plant genera have experienced polyploidization in their evolutionary history. This speciation driver is only successful more by accident than by default (Grandont *et al.*, 2013). However, older, adapted polyploids are obviously successful and widely distributed, because during their first generations these plants were prone to heavy genome alterations such as deletion of futile genomic information in terms of genes or chromosomes and remodeling processes like neofunctionalization or restructuring (Adams and Wendel, 2005; Comai, 2005). Afterwards adapted polyploids are regarded as evolutionary fitter as diploid plants due to buffering effects on negative occurrences such as deleterious mutations (Grandont *et al.*, 2013; Alix *et al.*, 2017). In addition, allopolyploids have advantages over homoploids. However, both hybrid versions are excellent colonizers, conquering habitats divergent to the one of their parents, diploid hybrids often not satisfactorily isolated from their parents (Arnold *et al.*, 2012). This is founded in steady gene flow between hybrid and their parents, while missing genome isolation by ploidy (Arnold, 1997).

During the early years of apomixis research, this mode of asexual seed formation, was considered as an evolutionary dead end (Darlington, 1939; Stebbins, 1950) due to the

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lack of genome variation as achieved by sexual reproduction (Muller, 1964; Richards, 2003). Nowadays, the opinion has changed. Molecular and developmental studies have shown that genetic diversity is established in apomictic species (Hörandl, 2009; Hojsgaard and Hörandl, 2015). Gametophytic apomicts often harbor residual amounts of sexuality, which becomes apparent in the generation of facultative seed sets, containing a mixture of sexual and apomictic fruits, which was demonstrated again in the present thesis (Asker and Jerling, 1992; Chapter 1). Furthermore, apomixis was found to be often linked to heterozygosity, because a large proportion of these plants were determined to be allopolyploids (Hörandl and Paun, 2007). Hybridization, as an important topic in apomixis research, is, on the one hand, a potential elicitor of asexual reproduction as experimentally demonstrated in this thesis, but on the other hand, hybridization and backcrossings are considered to be sources of genetic diversity in clonal apomicts (Hörandl and Paun, 2007; Mogie *et al.*, 2007). Together with advantages like the anticipated meiotic destruction of beneficial gene combinations and the excellent ability to found new populations at extreme habitats, apomictically reproducing plants, *e.g. R. auricomus*, are evolutionary fit and highly capable of establishing new, successful lineages (Bierzychudek, 1985; Hörandl and Paun, 2007; Hörandl and Temsch, 2009), which is assumed to happen with the natural polyploid hybrid and to some extent with the diploid F_2 hybrids in this thesis (Chapter 2). However, suspicion of superseded, sexual *Ranunculus* species is assumed to be without any reason, because sexual individuals are known to have higher fitness than apomicts. Nonetheless, apomictic plants are much broader distributed on the globe, whereas sexual plants are more or less restricted to small habitats like sexuals of the *R. auricomus* complex or of *R. kuepferi* (Hörandl, 2008; Hörandl and Temsch, 2009; Schinkel *et al.*, 2016). Since apomixis can be transmitted via male pollen, one could speculate that sexual plants could become apomictic via introgression of the asexual trait but this was determined as rather unlikely due to seasonal developmental differences like flowering periods and spatial separated habitats (Hörandl *et al.*, 2000; Hörandl, 2008; Hörandl and Temsch, 2009). This can be confirmed by personal observations: In spring, the F_2 hybrid plants were found to be in full bloom two to three weeks earlier than the F_1 and parental plants. This indicates ongoing speciation by flowering time isolation in diploid hybrids. However, natural hybridization of *Ranunculus* happens frequently, as demonstrated by the observed natural allopolyploid in study. All these characteristics not only make the *R. auricomus* an interesting complex to study the

emergence of apomixis in natural populations, based on cytological and developmental studies. Additionally, the complex offers the opportunity to shed light on the evolution on such interesting plant species and their establishment.

7 Final Conclusion

In conclusion, this thesis offers valuable clues on how apomixis is triggered in plants of the *R. auricomus* complex. It seems to be a complicated linkage of several events upon hybridization, rather than just one. Definitely, experimental data contain a reference to the high importance of hybridization in this context, because not only spontaneous apospory was observed in diploid *Ranunculus* hybrids but, more interestingly, these F_2 plants were able to form fully apomictic seeds (Chapter 1). Furthermore, it was proven that the frequency of, at least, aposporous ES development strongly depends on genomic dosage effects. In order to draw a complete cytological picture of gametophyte formation in *Ranunculus* plants additive analyses were performed on the male side, revealing increasing frequencies of developmental errors in diploid F_1 and F_2 hybrids but the highest frequencies of abnormal meiosis and sporogenesis were found in polyploid hybrids (Chapter 2). The evidence is that the developmental irregularities, as on the female side, are caused by interspecific hybridization but subsequent polyploidization enhanced the effect drastically. The cumulative impact of polyploidization on faulty gamete development could to be the same on apomixis. Since most apomicts were polyploids and hybrids, it is very likely that hybridization is the activator of severe developmental errors in the *R. auricomus* complex and polyploidization the intensifier, whereas apomixis can be regarded as savior from impending hybrid sterility. Thus, hybridization could be acknowledged as the elicitor of apomixis in *R. auricomus*. However, its impact on the genetic and epigenetic endowment of the analyzed plants remains elusive, since this thesis clearly focused on cytological and developmental diversifications, rather than on molecular changes. Finally, the obtained data represent a big step forward in the illumination of gametophytic apomixis emergence in natural plant population.

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9 Appendices

9.1 Acknowledgements

Appendices

9.2 Publication List in Peer-Reviewed Journals

Barke BH, Daubert M, Hörandl E. **2018**. Establishment of Apomixis in Diploid F_2 Hybrids and Inheritance of Apospory From F_1 to F_2 Hybrids of the *Ranunculus auricomus* Complex. *Frontiers in Plant Science* **9**: 1–12. DOI: 10.3389/fpls.2018.01111.

Barke BH, Karbstein K, Daubert M, Hörandl E. The Relation of Meiotic Behavior to Hybridity, Polyploidy and Apomixis in the *Ranunculus auricomus* Complex (Ranunculaceae). Submitted to: *Annals of Botany*.

9.2.1 Contribution to Publications

Declaration of the author's own contribution to manuscripts with multiple authors

Chapter 1:

Establishment of Apomixis in Diploid F_2 Hybrids and Inheritance of Apospory From F_1 to F_2 Hybrids of the *Ranunculus auricomus* Complex. B.H.B. performed research, analyzed and interpreted data. B.H.B. and E.H. wrote the manuscript. E.H. designed the research. M.D. performed some FCSS experiments.

Chapter 2:

The Relation of Meiotic Behavior to Hybridity, Polyploidy and Apomixis in the *Ranunculus auricomus* Complex (Ranunculaceae). 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 experiments. K.K. did the R analyses.