Evidence for reproductive interference between sexual and apomictic populations of the Easter Daisy (*Townsendia hookeri*, Asteraceae)

by

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Abstract

*Townsendia hookeri* (Asteraceae) plants can reproduce sexually or via apomixis (i.e. asexual reproduction through seeds), and the breeding system is tightly linked with ploidy level, so that sexual outcrossers are exclusively diploid while apomictic plants are polyploid. The species grows from central Colorado to British Columbia, with a disjunct distribution in Yukon Territory. Outside Yukon, sexual populations are restricted to the southernmost portion of the range (Colorado and southern Wyoming) and apomictic populations occur from Wyoming to Canada, a pattern consistent with geographical parthenogenesis.

The major objective of this study was to expand our understanding of the factors that have shaped and maintain this distribution, conferring an apparent advantage to apomictic lineages over their sexual progenitors. Having documented that polyploid plants retain the ability to produce some functional pollen, I hypothesized that if sexual forms spread into an apomictic population, and thus receive mostly heterospecific pollen, they would have reduced reproductive success, because the progeny sired in sexual-asexual crosses are predicted to be weak or inviable, and to include hybrid apomicts. This asymmetric reproductive interference could help explain why diploids have failed to spread into the territory dominated by apomicts.

To test this hypothesis I performed a cross-pollination experiment in the field. I showed that diploid and polyploid cytotypes have comparable reproductive success (at least, in the area where the experiment was conducted), and I also confirmed that diploids are outcrossers while polyploids are apomictic. The crossing experiment indicates that, despite its low viability, pollen produced by apomicts can fertilize and negatively affect diploid seed parents. As predicted, when sexual plants received heterospecific rather than conspecific pollen, seed set and germination rate were reduced, and seedlings had a lower survival rate. Flow cytometric analysis of offspring from sexual-asexual crosses revealed the presence of putative euploid (diploid, triploid and tetraploid) as well as aneuploid cytotypes. Based on ploidy level it is reasonable to expect that polyploid hybrids inherited apomictic genes and that at least some of them might be fully apomictic.
Preface

The research question and methodological design for this thesis were developed collaboratively by my supervisor, Dr. Jeannette Whitton, and me. I directed and carried out most of the field work for this project; however some plant materials were collected in the field by Adam Wilkinson. Whitton and Wilkinson assisted me with the crossing experiment in 2012, and Jamie Leathem assisted in 2013. Chris Lee and A. Wilkinson helped with seed collection. I also carried out the experiments in the growth chamber and the flow cytometric analysis. The pollen study was conducted in collaboration with C. Lee, Erica Li-Leger and Alberto Ruiz-Larrea. I completed the data analysis and the writing of the thesis. Dr. Jeannette Whitton, Dr. Roy Turkington and Dr. Sally Aitken assisted with interpretation of the results and provided helpful feedback.
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Chapter 1: Introduction

The establishment, expansion and persistence of a population depend in part on the ability of its members to respond to spatial and temporal environmental variation. Understanding how individuals respond to such variation is especially challenging for taxa that include both apomictic (i.e., clonal) and outcrossing sexual plants (a so-called ‘polyploid agamic complex’; Babcock and Stebbins, 1938; Grant, 1981), because it is unlikely that the factors that promote clonality versus outcrossing would diverge among closely related forms over short distances.

The complexity of these systems has intrigued researchers for many decades as reflected in the ongoing debate on their taxonomic treatment (e.g., Dickinson, 1998; Hörandl, 1998) and in our poor understanding of the factors that determine the appearance and proliferation of most agamic complexes (e.g., Hörandl and Hojsgaard, 2012).

*Townsendia hookeri* (the Easter Daisy) is a well-documented North American agamic complex (Beaman, 1957; Thompson and Whitton, 2006) that provides a model system for studying the origins and spread of apomixis. An experimental investigation of reproductive interactions between extant sexual and apomictic populations of the Easter Daisy could shed light on the circumstances that allow the persistence of sexual individuals and successful parthenogenetic counterparts in nature.

1.1 The Easter Daisy (*Townsendia hookeri*)

Named by William Jackson Hooker in honour of his friend, the American botanist David Townsend (Larsen, 1927), the genus *Townsendia* (Asteraceae) includes approximately thirty annual, biennial or perennial herbs native to western North America. The species of *Townsendia* tend to have limited distributions, to the point that "narrow endemism may be regarded as a common feature of
the genus” (Beaman, 1957). *Townsendia hookeri* occupies a relatively wide range, occurring from the base of the Front Range, in Colorado, to Yukon Territory in northwestern Canada. The species is also known as Townsend’s Easter Daisy, because its flowering time is in early spring. *Townsendia hookeri* is a diminutive, densely rosulate, perennial species characterized by short stems that end with tufts of narrow, linear leaves and sessile, showy inflorescences (also called flower heads or capitula) (Figure 1.1). Ray florets are pistillate with white to pinkish corollas, whereas disk florets are hermaphroditic with yellow corollas. Pollen is released when the style elongates pushing out through the anthers. Fruits are achenes (single-seeded, indehiscent, oblanceolate, compressed and pubescent) with a large pappus that facilitates wind-dispersal (Beaman, 1957). The achenes of a mature inflorescence form a round cluster, referred to as ‘seed head’ (Figure 1.2), and tend to tumble away from the mother plant as a unit until they gradually detach and disperse individually (personal observation). *Townsendia hookeri* populations typically grow about 2,000 m above sea level in the southernmost portion of the range, and their elevation declines with latitude to a minimum of approximately 600 m a. s. l. in Yukon (Garani et al., unpublished data). The species thrives on xeric, nutrient poor, sandy soils, along road cuts, trails, and in areas of natural disturbance (e.g., steep hill slopes) with sparse and usually low vegetation cover, and patches of bare soil.

### 1.2 Apomixis

In roughly half of the taxa within *Townsendia* individuals can reproduce either sexually or asexually through apomixis (Beaman, 1957). Here used as a synonym of agamospermy, apomixis describes a suite of processes whereby seeds are produced from unfertilized ovules (Richards, 1997). When the development of the embryo presupposes the formation of an unreduced megagametophyte, the process is termed gametophytic apomixis (Stebbins, 1950; Nogler, 1984). In turn, gametophytic
apomixis can be further subdivided into diplospory and apospory, depending on the cell from which the embryo arises. In diplosporous plants, the unreduced embryo sac originates from the megasporocyte, which undergoes mitotic or mitotic-like divisions, instead of a normal reductional meiosis. In aposporous plants the unreduced megagametophyte arises by somatic divisions of a cell of the nucellus (Nogler, 1984; Asker and Jerling, 1992; Bicknell and Koltunow, 2004; Whitton et al., 2008). In both cases, the unfertilized and unreduced egg cell develops into an embryo through parthenogenesis; thus, the resulting seed generally has the same ploidy level and genetic content as the maternal parent. Based on endosperm initiation, it is also possible to distinguish between “autonomous” and “pseudogamous” apomicts. In autonomous apomixis, endosperm formation is independent of pollination, while in pseudogamous forms the fertilization of the polar nuclei by a pollen nucleus is essential for the formation of viable progeny (Nogler, 1984; Asker and Jerling, 1992; Bicknell and Koltunow, 2004; Whitton et al., 2008). Autonomous endosperm development is common only in the Asteraceae (Nogler, 1984; Mogie, 1992; Noyes, 2007).

Through his embryological studies on Townsendia, Beaman discovered that diplospory prevails in the genus, and that embryo and seed development are independent of pollination (Beaman, 1957). For T. hookeri in particular, apomixis is described as obligate and diplosporous, with autonomous endosperm formation. Gametophytic apomixis in plants is commonly linked with polyploidy (Asker and Jerling, 1992; Whitton et al., 2008), and available evidence indicates that this is so in T. hookeri (Beaman, 1957; Thompson and Whitton, 2006). This means that apomictic individuals are exclusively autopolyploid, whereas their sexual conspecifics are strict outcrossing diploids, with a basic chromosome number of 9 (thus, 2n=2x=18) (Beaman, 1954; Beaman, 1957).

Despite being obligate and autonomous apomicts, polyploid individuals of the Easter Daisy show no apparent reduction in inflorescence or flower size compared to their sexual progenitors, and the male function is generally maintained, as observed in most plants showing apospory or diplospory
Apomixis only affects the formation and development of the female gametophyte, without any direct influence on the male sporogenesis. Nonetheless, low quality pollen is often produced, especially in autonomous apomicts, where disturbed and irregular microsporogenesis can generate highly unbalanced pollen nuclei and sometime leads to male sterility (Nogler, 1984; Asker and Jerling, 1992). Disruptive effects of polyploidy on male meiosis are likely to be a major cause of this failure. Beaman (1954; 1957) observed that male meiosis is normal in diploid members of the genus, where nine bivalents are found at metaphase I. In contrast, meiotic irregularities are frequent in polyploid apomicts, where both bivalents and univalent are found at metaphase I, and “giant” multi-nucleate pollen grains are occasionally formed, together with a large number of aborted grains of different sizes (Beaman, 1954; Beaman, 1957). Thus, as observed in most apomicts with only rare exceptions (for instance, Meirmans et al., 2006), most polyploid members of *T. hookeri* retain the ability to produce some functional pollen.

### 1.3 Assessment of breeding system: pollen analysis and flow cytometry (FCM)

The existence of clear cytological differences between the pollen of sexual and apomictic plants has been exploited to discriminate between diploid and polyploid cytotypes of the Easter Daisy. Indeed, with the exception of pollen features, sexual and apomictic plants do not possess any distinctive morphological character. Pollen size and stainability (an indicator of viability) can be used to attribute individual samples to a specific breeding type (Beaman, 1957; Thompson and Whitton, 2006). Using these methods, we have conducted a range wide survey of populations, examining 58 pollen samples obtained from herbarium and personal collections, including desiccated and fresh material (Garani et al., unpublished; Appendix A). In accordance with previous results from Thompson and Whitton (2006), this survey revealed a striking contrast between the small (mean diameter and standard error: 22.31 ± 0.25 µm) and highly viable (82.81 ± 2.63 % stainability)
pollen typical of diploid populations, and the large, irregular (34.75 ± 0.86 µm) and mainly inviable (25.73 ± 2.3 %) pollen of apomictic populations (Appendix A).

Given the tight association between ploidy and breeding system that characterizes *T. hookeri*, it is also possible to determine the mating strategy of a plant by measuring its genome size. Doležel and Bartoš (2005) showed that flow cytometry (FCM) is a powerful and reliable method to estimate the nuclear genome size of plants. This technique indirectly assesses the nuclear DNA content of a tissue by measuring the degree of fluorescence emitted by a suspension of nuclei stained with a DNA-specific fluorochrome. This approach is based on the assumption that the amount of light emitted by the stained nuclei has a linear relationship with the genome size (Doležel and Bartoš, 2005; Doležel et al., 2007). The nuclear DNA content of non-replicated somatic genome measured with FCM is generally termed 2C-value, following Greilhuber et al. (2005). FCM is more practical than chromosome counting because it can be applied to desiccated leaf tissue (Suda and Trávníček 2006; Sears, 2011), sample preparation is significantly easier and a large number of nuclei can be measured in a short time (Sears, 2011). The range wide survey carried out by Garani et al. (unpublished) also included flow cytometrical analysis of 68 samples collected from 25 populations across the range, excluding Yukon (described in Appendix B). Flow cytometry data matched the expected ploidy levels estimated via pollen analysis, but also revealed that nuclear DNA contents of polyploids form two distinct clusters, suggesting that apomicts can be either triploid or tetraploid (Appendix B). Combined with pollen studies, FCM of leaf material proved to be a powerful tool in our investigation of the geographical distribution of sexual and apomictic populations of the Easter Daisy.
1.4 Geographical parthenogenesis

According to Beaman (1957), apomixis must represent an adaptive advantage in some of the species of *Townsendia*, because the range of sexual individuals is often significantly less extensive than that of their apomictic counterparts. This is especially true for the Easter Daisy, where sexual diploid populations are restricted to the southern, historically unglaciated areas of the range, namely Colorado and Wyoming, with a few sexual populations also found at the northern limit, in Yukon. In contrast, apomictic populations seem to be excluded from the territory occupied by sexual diploids, but have established further north, and have colonized formerly glaciated portions of the species’ range (Beaman, 1957; Thompson and Whitton, 2006; Thompson et al., 2008).

Moreover, available evidence suggests that sexual and asexual forms do not occur in the same population. The geographical distribution of sexual and apomictic populations of *T. hookeri* (excluding Yukon) has been recently reassessed using pollen cytology and FCM (Garani et al., unpublished; Appendices A and B). The outcome of this work was consistent with previous findings, but it also showed that triploid populations occur east of the Rockies, while tetraploids thrive only west of the Great Continental Divide.

The non-overlapping distribution of sexuals and apomicts is consistent with a pattern known as geographical parthenogenesis, wherein asexual forms, compared to their sexual relatives, tend to have broader ranges, colonize higher latitudes and altitudes, and generally occupy areas of the range opened by glacial retreat (Bierzychudek, 1985).

At present, no data exist to explain why the geographic distribution of apomicts significantly exceeds that of diploid populations in the Easter Daisy. Likewise, the factors that allow the two forms to persist across the species’ range, and that limit their co-occurrence at local scales are unknown. It has been hypothesized that sexual diploids formerly occupied a broad range that was disrupted by the last period of glaciation, which confined some relic populations to the southern
limit of the original species’ range. Following glacial retreat, apomictic plants were more successful than their sexual relatives in recolonizing the postglacial landscape (Thompson and Whitton, 2006).

1.5 Hypotheses for the occurrence of sexual and apomictic populations in *Townsendia hookeri*

Although probably transient over evolutionary time scales (e.g., Stebbins, 1950; Maynard Smith, 1978; Van Dijk, 2003), the success of apomictic lineages over their sexual progenitors is remarkable and could be the result of different factors. One clear advantage of apomixis is uniparental reproduction, which guarantees no dependence on mating partners or pollinators, and decreases inbreeding depression through maintenance of heterozygosity. Reproductive assurance (Stebbins, 1950) would facilitate the establishment of apomicts via long-distance dispersal of seeds in newly opened territory, conferring an initial benefit to polyploid cytotypes over their diploid progenitors (e.g., Hörandl, 2006). Moreover, asexual polyploids could rely on a more plastic genotype, characterized by broad tolerance ranges, which allow them to occupy a wider spectrum of habitats (“general purpose genotype hypothesis”; Lynch, 1984). According to Lynch (1984), the most successful clone lineages, in the long term, are those with a highly generalized genetic makeup, because specialized clones will disappear as soon as the narrow habitats in which they thrive are no longer available. Broader ecological tolerances (such as, for instance a greater resistance to pathogens, drought or cold) could also be the result of the phenotypic and genetic effects associated with a polyploid condition (Levin, 1983; Otto and Whitton, 2000), which is typical of apomictic forms.

These arguments might explain why apomictic polyploids are able to establish in a site but do not clarify why they would not be invaded by sexuals in the long term. An alternative explanation is that the distribution of related sexual and asexual breeding types could have been shaped by the
disruptive effects of pollen produced by apomicts on the reproduction of their outcrosser relatives.

In this scenario, whenever sexual individuals disperse into an area occupied by their apomictic
descendants they receive primarily pollen of polyploid origin. Despite its low viability, such pollen
can function in crosses with sexual seed parents, as shown in many experimental studies (e.g.,
Sterk, 1987; Morita et al., 1990b; Tas and Van Dijk, 1999; Noyes, 2000; Hörandl and Temsch, 2009).
Sexual-asexual crosses can have many effects on the reproductive performance of diploids. For
instance, pollen from apomicts can produce a decreased seed set on sexual mothers, as described
for plants of *Taraxacum japonicum* that grow in the presence of the asexual congener *T. officinale*
(Takakura et al., 2009; Matsumoto et al., 2010). The F1 generation can also have reduced fitness
owing to “mentor effects”, a phenomenon that determines the breakdown of self-incompatibility
systems in outcrossing sexuals when the receptive stigma is saturated with a mixture of apomictic
and self-pollen (Richards, 1997). Lastly, viable pollen produced by asexual cytotypes can mediate
the transfer of apomictic genes to the offspring of sexual conspecifics through the generation of
fertile, apomictically reproducing hybrids. The inheritance of apomixis via hybridization has been
established in a number of artificial crosses, for example, in *Erigeron* (Noyes and Rieseberg, 2000)
and *Taraxacum* (Tas and Van Dijk, 1999), and was also observed in natural mixed sexual-apomictic
populations (Van Dijk, 2007). As a result, there is a high chance that the reproductive success of
sexual plants receiving pollen from surrounding apomicts would be greatly reduced and that,
evolvedually, outcrossers would be eliminated entirely from the local mixed-population (Mogie, 1992;
Mogie et al., 2007). Therefore, this unidirectional interaction (autonomous apomicts can serve as
pollen parents of progeny of outcrossers, but not vice versa) can be seen as a form of reproductive
interference (Kuno, 1992) that leads to the creation of an impenetrable front that blocks the
northward migration of sexual diploids into the territory occupied by their polyploid descendants.

Uniparental reproduction, the general purpose genotype hypothesis, polyploidy and reproductive
interference could explain why apomictic polyploid individuals prevail in most of the species’
range, but these hypotheses do not clarify why apomicts fail to thrive in areas where the diploids are found and where they originated. A possible explanation is that outcrossing diploids are restricted to a portion of the range where they hold a selective advantage over their asexual counterparts. Indeed, the two cytotypes could differ in ecological traits and, in particular, they could have divergent edaphic tolerances. Beaman (1957) recognized the importance of edaphic factors in influencing the distribution of the species of Townsendia and claimed that “especially within the range of a species, the occurrence of plants on localized edaphic situations is frequently striking.”

The analysis of soil samples collected at different localities across the range showed that the concentration of some minerals (especially Se, Pb, Al, Cu, Ni and Mg) varies among different habitats (Thompson and Whitton, unpublished), and it has been suggested that diploids may occur on substrates with unusual concentrations of specific elements (Whitton et al., 2008).

1.6 Study objectives

The overall objective of this study was to expand our understanding of the factors that have shaped and maintain the geographical distribution of sexual and apomictic populations of Townsendia hookeri across the species’ range. The following broad questions were addressed:

I. Why are sexual populations restricted to the southern, unglaciated portions of the species range?

II. What factors limit the co-occurrence of sexual and apomictic forms at local scales?

The focus of this study was to test the hypothesis that reproductive interference might prevent the coexistence of different breeding types within a population and hinder the range expansion of sexuals into the territory occupied by their apomictic descendants. To test this hypothesis, I cross-pollinated sexual and apomictic populations in the field, I assessed the reproductive success of seed paternal plants and I determined whether sexual-asexual crosses produced F1 polyploid hybrids.
**Figure 1.1** Flowering plant of *Townsendia hookeri*. The picture was taken in the Medicine Bow National Forest (Wyoming, U.S.) in April of 2012.

**Figure 1.2** Plant of *Townsendia hookeri* at fruiting stage, showing a typical seed head. The picture was taken in the Medicine Bow National Forest (Wyoming, U.S.) in June of 2012.
Chapter 2: Could reproductive interference limit the range expansion of sexual populations of *Townsendia hookeri* into the territory occupied by their polyploid descendants?

2.1 Introduction

*Townsendia hookeri* (Asteraceae) is a diminutive, perennial herbaceous species that thrives on the foothills of the Rocky Mountains, from central Colorado to British Columbia, with a disjunct distribution in Yukon Territory (Beaman, 1957; Thompson and Whitton, 2006). Outside Yukon, sexual populations are only found in the southernmost portion of the species range (Colorado and southern Wyoming), whereas apomictic populations occur from southern Wyoming to Alberta and British Columbia. Moreover, in *T. hookeri* the reproductive strategy has been shown to be tightly coupled with ploidy level, where diploid plants are invariably outcrossers and polyploidy is always associated with asexuality in the form of apomixis (Beaman, 1954; Beaman, 1957). Apomictic forms retain the ability to produce showy flowers and some functional pollen despite both endosperm and embryo development are completely independent of pollination (Beaman, 1954; Beaman, 1957).

No data exist to explain why sexual populations fail to invade the territory occupied by apomictic populations, and why the two forms do not co-occur at local scales. This study focused on the hypothesis that the occurrence of asymmetric reproductive interference between apomictic polyploid and sexual diploid cytotypes might have the potential to hinder the coexistence of different breeding types at local scales and could limit the northward migration of sexuals. To test this hypothesis, I conducted a cross-pollination experiment between extant sexual and apomictic populations in the field. To properly interpret my results, I collected data on the reproductive success of sexual and asexual plants under natural conditions, and I confirmed apomixis and sexuality using a pollinator exclusion test. Reproductive success of seed mother plants was
assessed based on seed set, achenes mass, germination rate and seedling survival. Ploidy of seed and pollen parents was confirmed using flow cytometry on silica-dried leaf tissue and was used to infer breeding system. The same technique was applied to the progeny of sexual-asexual crosses to verify the occurrence of F1 polyploid hybrids.

2.2 Methods

2.2.1 Study site

In his monograph, Beaman (1957) describes *Townsendia hookeri* as a promising but also challenging study system, in particular because, to his knowledge, no one had succeeded in getting greenhouse-grown plants to flower and set seed. After over fifty years, we have not yet been able to overcome this major obstacle, therefore I did my experiment in the field, rather than working in a more conventional and controlled environment, such as a greenhouse or growth chamber.

To conduct the field experiment, it was necessary to identify six natural populations (two apomicts and four sexuals) each with at least fifty flowering individuals, that were all within a two hour-drive (less than 80 km apart) so that I could collect pollen and complete crosses within a single day. Six populations with compatible characteristics were located in southern Wyoming (Albany county) based on previous work (Thompson and Whitton, 2006; Lee and Whitton, unpublished data). These populations were at least 1 km apart from each other and they were all found in the Medicine Bow National Forest, with the exception of one population that was located on private land outside the city of Laramie (Figure 2.1). The ploidy level and breeding system of each population was subsequently confirmed using pollen analysis (following the methods described in Appendix A) and flow cytometry on silica-dried leaf tissue (described in section 2.2.4). A summary of the main features of each population, including geographical locations, IDs and ploidy estimates are provided in Table 2.1.
2.2.2 Experimental design and field work

The six populations of *T. hookeri* were split into two subsets, corresponding to two replicate experiments (R1 and R2). Each subset included: one apomictic pollen donor population (AD), one sexual pollen donor population (SD), and one sexual pollen recipient population (SR). Within a population, individuals were randomly assigned to a specific treatment, and only one flower head per plant was used. This approach avoided pseudoreplication, while reducing the impact to individual plants. Flower heads were enclosed with semi-transparent fine-mesh organza bags that prevented achene dispersal and hampered animal and wind pollination (Cosendai *et al.*, 2013). Flower heads were exposed to one of four different treatments: control (C), isolation (I), sexual-sexual crossing (SS) or sexual-apomictic crossing (SA), as shown in Figure 2.2. The control was used to assess seed production under open pollination. Flower heads, with withered ray florets and developing infructescences, were bagged to prevent wind dispersal of achenes. The control was performed on ten plants in both apomictic (AD) and sexual (SR) populations, for a total of forty flower heads. The isolation treatment was used to confirm that polyploids are apomicts and diploids are self-incompatible outcrossers. In a closely related species, *Townsendia aprica*, Tepedino *et al.* (2004) compared the effects of autogamy (isolated and unmanipulated flower heads) versus geitogamy (receptive stigmas treated with fresh pollen from other florets on the same plant) and found no significant difference between the two treatments, which both produced low seed sets (<2.5% of disk florets produced filled achenes). Based on these results, very low seed sets in isolated sexual plants are considered an indication of self-incompatibility, although a definitive test would require the application of self-pollen. As with the controls, the isolation treatment was carried out on ten plants in both the two AD and the two SR populations (forty individuals in total), and involved bagging flower heads at the bud stage to prevent them from receiving any foreign pollen. It should be noted that self-pollen was not removed because emasculation of florets has not been accomplished in this system (in the Asteraceae, this is sometimes done by cutting off the top of
each inflorescence when in bud; Richards, 1997), and it was also not pivotal to the isolation test. The same method has been successfully applied to other Compositae, where the removal of anthers was similarly not practical (e.g., Morita et al., 1990b; Noyes, 2000; Brock, 2004). The goal of sexual-sexual and sexual-apomictic crosses was to compare the effects of conspecific (from diploids) versus heterospecific (from polyploids) pollen grains on reproductive success of sexual seed parents under hand-pollination. For this reason, the two treatments only differed in the source of pollen grains, which was either sexual (from SD population, in SS) or apomictic (from AD population, in SA). The crosses were performed by gently rubbing two flower heads from a donor population on the flower head of a sexual recipient during anthesis. Each donor inflorescence was collected from a different plant and used only once to avoid pollen contamination between crosses. In each recipient population, fifty flower heads (one hundred in total) were bagged before the onset of anthesis and assigned to one of the two treatments, so that twenty-five individuals received pollen from a diploid donor and the other twenty-five received pollen from a polyploid donor. Bags were opened only to perform the artificial pollination. Flower heads from pollen donor populations were collected, kept in paper bags and stored at about 4°C for less than two hours before the hand-pollination was completed.

The treatments were conducted in April 2012 and achenes were collected in June 2012, with the exception of the isolation treatment on apomicts, which was conducted in 2013, because I could not find a sufficient number of buds during the first year. Moreover, population 2027 was severely affected by a wildfire during the summer of 2012 and it was necessary to conduct the isolation treatment on a different population. Population 2031, previously described by Lee and Whitton (unpublished data), was determined to be an adequate replacement based on its location (roughly 20 km north of Laramie) and ploidy level (polyploid). Subsequent analyses confirmed that pollen viability and ploidy level of members of this population are comparable to those of population 2027 (Table 2.1, Figure 2.1). Hand-pollination was repeated twice over a three-day period in population
2030. This was not achieved for population 2033, due to weather conditions (snow covering the plants); therefore recipients in this population received pollen only once. An attempt to repeat the entire experiment in 2013 was impeded by adverse weather conditions and consecutive snow storms. Thus, the two experiments are not considered replicates as originally intended, and were analyzed separately, comparing the effects of two versus one day of cross-pollination. Achenes were retrieved from 176 of the 180 plants bagged under one of the four treatments. Only four individuals could not be relocated, thus the organza bags proved to be highly effective at preventing seed dispersal, without hindering the survival of the plant. A summary of the work completed in the field is provided in Table 2.2.

### 2.2.3 Estimation of reproductive success

The reproductive success of seed mother plants under the four treatments was assessed by evaluating the cumulative effects of four different parameters associated with achenes quantity and quality, namely: seed set, average achene mass, germination rate and seedling survival.

The main goal of the cross-pollination experiment was to test the potential effects of pollen produced by apomicts on the reproductive success of sexual seed parents. To understand the magnitude of this interaction, I first needed to gather information on the reproductive success of both cytotypes under natural conditions, i.e. the control treatment. I also had to show that the absence of external pollen (in the isolation treatment) would not affect reproductive success of polyploids (consistent with their being apomictic), but would drastically impact diploids, demonstrating that the latter are strictly sexual outcrossers. For this reason, seed set, mass of achenes, germination and seedling survival rate were initially measured and compared among cytotypes under control and isolation treatments, and secondly among cross-pollination treatments in sexual populations.
2.2.3.1 Seed set

Seed heads (capitula) retrieved from the field were kept in paper bags and stored at 4°C inside a box containing silica gel. The total number of achenes collected from each seed head was recorded, and achenes were classified as “filled”, when plump and brown, and as “empty”, when flat and unpigmented. Fruits with intermediate colorations were scored as filled if fleshy and as empty if clearly undeveloped (Figure 2.3).

Seed set was calculated as the proportion of filled achenes in a given seed head, and it was expressed as a percentage:

\[
\text{Seed set (\%)} = \left( \frac{\text{no. filled achenes}}{\text{no. filled + no. empty achenes}} \right) \times 100
\]

2.2.3.2 Average achene mass (mg)

The average mass of filled achenes was calculated for individual seed heads by averaging the weight of the entire set of fruits. The estimates are reported in milligrams.

2.2.3.3 Germination rate

Germination tests were conducted on a random subset of filled and empty achenes obtained from every bagged plant that produced fruits under one of the four treatments. During preliminary trials, I noticed that undeveloped fruits are more susceptible to fungal and bacterial infections, therefore I decided to germinate the two achene types following the same protocol, but separately.

The seeds of *T. hookeri* do not possess dormancy, therefore no stratification or any other form of conditioning is necessary to induce germination (personal observation). Ten achenes (or all the achenes if fewer were available) were randomly selected from each maternal plant. After the pappus was carefully cut off, achenes were surface sterilized with a 10% (v:v) bleach solution for 5
minutes and rinsed three times with distilled water. A 1% (w:v) agar solution was autoclaved and poured into sterile 100 × 15 mm Petri dishes in a laminar flow hood. Once the medium solidified, the sterilized achenes from each maternal plant were sown in two different Petri plates, so that the insurgence of any infection inside a dish would affect and potentially kill only half or less of the fruits of a single plant, reducing the risk of losing entire subsets. Petri plates were sealed with parafilm and transferred to a growth chamber (Adaptis A1000, Conviron, Winnipeg, MB, Canada), where achenes were incubated at a constant temperature of 23°C, with 12 hours of light and 12 hours of dark per day. Achenes were inspected every two days over the course of three weeks, and recorded as germinated once the radicle was at least 5 mm long.

The germination rate was calculated as the proportion of sown fruits (filled or empty) that successfully germinated, and it was reported as a percentage. For instance, the germination rate of filled achenes for a given plant was calculated as:

\[
\text{Germination rate per filled achene (\%) = \left( \frac{\text{no. filled achenes germinated}}{\text{no. filled achenes sown}} \right) \times 100}
\]

2.2.3.4 Survival rate per transplanted seedling

All seedlings obtained from C and SA treatments were transferred from agar to soil, whereas only a fraction of the seedlings obtained from SS treatment (representing eight mother plants per population) and none from the I treatment were potted because of space constraints.

The seedlings were removed from the plate together with a small amount of agar still surrounding the tap root in order to minimize the risk of damaging or breaking the radicles. After the transplant, seedlings were kept in a growth chamber (Bigfoot series, model GC-20, BioChambers Inc., Winnipeg, MB, Canada) and exposed to a daily regime of 12 hours of light at 20°C followed by 12 hours of dark at 10°C.
I observed that, at the stage of four to five true leaves, seedlings had already developed an extensive tap root (Figure 2.4), and could be considered fully established plants. Based on this observation, the survival rate was calculated as the percentage of transplanted seedlings that developed a minimum of four true leaves:

\[
\text{Survival rate per transplanted seedling (\%)} = \left( \frac{\text{no. established plants}}{\text{no. transplanted seedlings}} \right) \times 100
\]

### 2.2.3.5 Statistical analysis

Seed set, achene mass, germination and survival rate were computed for each seed mother plant, as described above.

Comparisons of seed set, germination rate and survival rate between treatments and breeding systems were evaluated using a generalized linear model (GLM) by performing an Analysis of Deviance with binomial errors (and a logit link function). This method was selected following Crawley (2007), who showed that the ‘glm’ function represents an adequate tool for the analysis of proportion data, and can replace more traditional approaches, such as arcsine transformation or non-parametric statistics. The differences in average mass of filled achenes between treatments and breeding systems were tested using Student's t-tests.

The statistical analysis was carried out in R (version 3.0.3; R Core Development Team, 2014) using the "lattice" package for data visualization (Sarkar, 2008) and the "plyr" and "car" packages for data manipulation (Fox and Weisberg, 2011; Wickham, 2011).
2.2.4 Flow cytometry (FCM)

2.2.4.1 Plant material

Flow cytometry was used to confirm the ploidy and breeding system of pollen and seed parental plants, previously inferred via pollen analysis (following the methods reported in Appendix A), and to assess ploidy level of the progeny generated in sexual-asexual crosses (SA treatment).

The ploidy level of putative apomictic pollen donor and diploid pollen recipient plants was estimated from field-collected tissue (samples from sixteen individuals harvested in June 2012) as well as from material grown from seeds (samples from seventeen individuals). The latter was obtained from achenes germinated and grown in a growth chamber, following the protocol described previously. These achenes were harvested from control plants, therefore they are representative of the breeding system of their population of provenance. Leaf material of both origins (field-collected and grown from seed) was stored at ambient temperature in paper envelopes containing silica gel. Within one to four weeks of collection, the dehydrated tissue was removed from the silica, transferred to 2 ml screw cap microtubes and stored in a -80°C freezer.

The rationale for analyzing desiccated material instead of fresh leaves was to ensure that the results were comparable to the ploidy assessments carried out by Garani et al. (unpublished) on field-collected, silica-dried leaf tissue gathered from populations across most of T. hookeri's range (Appendix B).

Achenes produced by seed parents under the SA treatment were germinated in February 2013 and seedlings were grown as described above until large enough to allow sampling of leaf material. Between December 2013 and January 2014, 20 to 40 mg of fresh tissue (ten to twenty leaves) were collected from mature plants. Leaf material was harvested only from large individuals that had at least twice as many leaves (177 in total) to avoid killing the plants. This method ensured that material could be collected multiple times from a single plant, allowing for a reassessment of low
quality results. Leaf tissue was desiccated in silica gel and stored in a deep freezer (-80°C), as previously described for pollen and seed parents.

2.2.4.2 Ploidy-level determination

For sample preparation, 10 mg of dried leaf tissue was placed in a 2 ml microcentrifuge tube containing a 6.35 mm ceramic sphere (Qbiogene, catalog no. 6540-424) and 600 µl of nuclear isolation buffer (pH 7.0) of the following composition: 15 mM HEPES, 1 mM EDTA, 0.2% (v:v) Triton X-100, 80 mM KCl, 20 mM NaCl, 300 mM sucrose, 0.5 mM spermine, 0.25 mM PVP-40 (modified from De Laat and Blaas, 1984), plus 1 µl ml⁻¹ β-mercaptoethanol. The tubes were placed symmetrically into a test tube rack, modified to attach to a Mastercraft 7.5A reciprocating saw (with a stroke length of 28.6 mm), and the leaf material was ground by oscillating the tubes on the saw at ‘low’ setting for 60 seconds. This method for extraction of plant material by using a reciprocating saw has been shown to be highly effective and reliable (Alexander et al., 2007), as it ensures higher consistency and is less labor intensive compared to chopping samples with a razor blade (Galbraith et al., 1983; Doležel et al., 2007). The resulting homogenate was passed through a Partec CellTrics® columns equipped with a 30 µm filter gauze (Münster, Germany) and collected into 1.5 ml centrifuge tubes. Before and after filtering the homogenate, 50 µl of nuclear isolation buffer was added to each column to pre-wet the filter and then to remove any residual nuclei from the gauze. The final volume of filtrate in each 1.5 ml tube was approximately 700 µl. The nuclei suspensions were stored in ice for 45 to 60 minutes and then moved to a 4°C refrigerator for up to three hours prior to running on the flow cytometer.

*Pisum sativum* cv. Ctírad (2C-value 9.09 pg; Doležel et al., 1998), *Secale cereale* cv. Daňkovské (2C-value 16.19 pg; Doležel et al., 1998), and *Vicia faba* cv. Inovec (2C-value 26.9 pg; Doležel et al., 1992) were chosen as DNA standards. For each species, two to three plants were grown from seed and fully expanded leaves were harvested, desiccated using silica gel and stored in a deep freezer.
(at -80°C), following the same method applied to *T. hookeri* samples. Nuclei suspensions were prepared as described above. When using an internal reference, it is recommended the standard be ground with each sample (Price *et al*., 2000). At the same time, it is necessary to ensure that the genome size of the sample is as close as possible to the genome size of the standard, never exceeding a twofold difference and avoiding overlap (Suda and Trávníček, 2006; Doležel *et al*., 2007). The DNA content of offspring from SA treatment was unknown and could range from diploid (roughly 12 pg) to tetraploid (over 21 pg), thus no internal standard was adequate for the whole range of genome sizes; this is the reason references were prepared separately. Nonetheless, following recommendations of Price *et al*., 2000, a subset of samples of known genome size was ground together with the appropriate internal standard to test for the presence of cytosolic inhibitors and ensure that independently processing target and reference leaf tissue does not alter the outcome of the analysis. The 2C-values obtained in the co-ground trial matched the ploidy level previously estimated for the population of origin of each sample (Appendix C), providing evidence that my method produces accurate results in this study system.

The nuclei suspensions were incubated with RNase A (50 μg ml⁻¹; Invitrogen) for 30 minutes at room temperature prior to being analyzed on a Becton-Dickinson FACScalibur (BD, San Jose, CA, USA) benchtop flow cytometer equipped with a 488 nm laser, following the procedure presented in Sears (2011). Data were acquired and analyzed using BD CellQuest Pro software (BD, 1 Becton Drive, Franklin Lakes, New Jersey, USA 07417), which also allowed adjustment of the settings of the cytometer. At first, an aliquot of each internal standard was stained with 100 μg ml⁻¹ of propidium iodide (PI, Invitrogen) and run on the cytometer on its own to ensure that the sample preparation yielded good quality nuclei extractions. In addition, the voltage of the photomultipliers (FL2) was adjusted so that the peak of the G⁰/G₁ phase nuclei of *P. sativum* occurred at channel number 200 on the FL2-A ("area", a measure of relative florescence) axis. G⁰/G₁ and G₂ populations of single nuclei were gated (i.e., isolated) on the plot that shows pulse width (FL2-W) against area (FL2-A) and the
rate at which the $G_0/G_1$ peak of each standard increased on the histogram of fluorescence intensities (FL2-A histogram) was recorded. Aliquots of each $T.$ hookeri sample were stained and run following the same procedure. This method allowed identification of the proper internal standard for each sample, so that reference and target tissue $G_0/G_1$ populations of nuclei were as close as possible on the FL2-A axis, without overlapping. The ratio of internal standard to sample was determined based on the relative rate of increase of each $G_0/G_1$ peak on the FL2-A histogram previously measured, with the intent to produce two $G_0/G_1$ peaks of similar height. Reference and $T.$ hookeri samples were combined accordingly, co-stained and run twice, until a minimum of 2,000 particles were measured in every individual run. For example, $P.$ sativum always produced a rapidly growing $G_0/G_1$ peak on the FL2-A histogram when run on its own, while diploid $T.$ hookeri samples were generally four times slower. In this case, 20 µl of $P.$ sativum would be mixed with 80 µl of $T.$ hookeri and 5 µl of PI. For the subset of samples of $T.$ hookeri of known genome size that were ground together with the internal standard, 100 µl of the mixed nuclei suspension was stained with 5 µl of PI and run following the same protocol outlined previously.

The files produced in BD CellQuest were analyzed using the computer program FlowJo vX.0.6 (Tree Star Inc., Ashland, Oregon, USA). The fluorescence of the sample relative to the internal standard was determined based on the FL2-W versus FL2-A cytogram obtained for each individual run. On this plot, $G_0/G_1$ populations of nuclei from both the reference and the target tissue were isolated from the debris background using the Autogate feature, which automatically finds subpopulations of particles based upon equal probability distribution (Figure 2.5). FlowJo was also used to calculate the total number of nuclei, the mean position (channel number, CN) and the coefficient of variation (CV, defined as the standard deviation of the peak divided by its mean channel number and expressed as percentage) of each peak on the FL2-A histogram. In accordance with Suda and Trávníček (2006), the outcome of an individual run was included in the analysis when the following quality requirements were met: the fluorescence intensities for at least 2,000 nuclei were collected,
the CV values of the reference and the *T. hookeri* sample were ≤6%, and both peaks on the histogram of fluorescence intensities had a normal shape. The genome size of *T. hookeri* samples (2C-value) were calculated based on the linear relationship between the standard and the sample fluorescence intensities, as follows:

\[
2C_{T. hookeri \ sample} (pg) = \frac{2C_{\text{internal standard}} (pg) \times \text{CN}_{T. hookeri \ sample}}{\text{CN}_{\text{internal standard}}}
\]

Flow cytometry was completed between December 2013 and March 2014. Calculations and graphical analyses of the data were conducted using the “lattice” package (Sarkar, 2008) in R (version 3.0.3; R Core Development Team, 2014).

### 2.3 Results

#### 2.3.1 Reproductive success of sexual versus apomictic populations under natural conditions (C and I treatments)

For this analysis, populations of the same cytotype were considered replicates and, as a consequence, were grouped together as ‘sexuals’ (2030 and 2033) versus ‘apomicts’ (2019, 2027 and 2031).

The total number of filled and empty achenes per capitulum was consistent across the four populations, with an overall mean and standard error (SE) of 81.21 ± 3.79 achenes per capitulum (Figure 2.6). This suggested that sexual and asexual plants used in this study had flower heads of similar sizes, based on the assumption that the total number of achenes can be used as a proxy of the inflorescence size because each floret produces only one ovule, and thus one fruit.

Under the control treatment sexuals and apomicts had similar percentage seed set, with average values and SE of 48.96 ± 3.15% and 52.63 ± 2.2%, respectively (Figure 2.7). In contrast, the
isolation treatment drastically decreased the percentage of developed achenes per seed head produced by sexuals (0.84 ± 0.34%), but had no significant impact on apomicts (46.15 ± 2.34%) relative to controls (Analysis of Deviance, P<2.2e-16; Table 2.3). All apomictic plants in this experiment (n=38) set seed, regardless of treatment, and the same was true for open-pollinated sexual diploids (n=20). Conversely, twelve out of nineteen diploid mothers set no seed in the isolation treatment, and the remaining seven produced only between one and four filled achenes. Although polyploids had a slightly lower seed set when external pollen was removed compared to the control treatment, most of the offspring produced by polyploids under isolation treatment were viable (215 of 217 seeds from twenty-two parents sown in agar germinated).

As observed for seed set, the average mass of filled achenes did not differ significantly between the two mating types under the control treatment, with a mean and SE of 0.69 ± 0.033 mg for sexuals and 0.77 ± 0.027 mg for apomicts (t=1.8393, df=36, P=0.0741). As expected, all the 1560 empty achenes tested (representing 156 mother plants) failed to germinate. The average germination rate per filled achene was significantly lower in polyploid controls (75.98 ± 5.66%) compared to diploid controls (90.05 ± 3.48%; Analysis of Deviance, P=0.0421; Figure 2.8; Table 2.4). Conversely, seedling survival rate was significantly higher for apomictic controls (91.11 ± 2.53%) than for sexuals (81.38 ± 5.53%; Analysis of Deviance, P=0.0428; Figure 2.9; Table 2.5).

### 2.3.2 Experimental crossings (SS and SA treatments)

Data on percentage seed set (Figure 2.10) reveal that both populations were pollen limited in the experimental crosses, with over a 60% decline in seed set between open pollination (C) and crossing with conspecific pollen (SS; mean and standard error of 18.02 ± 2.26% for 2030 and 13.46 ± 2.24% for 2033). Nonetheless, the experimental crosses were effective and most sexual mother plants set seed. In population 2030, the seed set of plants that received pollen from apomictic donors was roughly half of that produced by individuals that received pollen from sexual donors.
(10.34 ± 1.5% in SA and 18.02 ± 2.26% in SS; Analysis of Deviance, P=0.004279; Table 2.6), and only one plant (in the SA treatment) failed to set seed. In population 2033, the average seed set was similar in the two treatments (15.25 ± 3.41% in SA and 13.46 ± 2.24% in SS), but the mean values for seed parents in SA showed a great degree of variation, where some plants failed to set seed entirely (n=8), and others (n=6) had high seed sets, between 31% and 50%.

The average mass of filled achenes was significantly different between the two recipient populations (2030 had larger fruits), but not between treatments within a population (Figure 2.11). Nonetheless, it should be noted that achenes from sexual-asexual crosses in population 2033 were 15% lighter than those from sexual-sexual crosses (0.47 ± 0.03 mg for SA versus 0.557 ± 0.03 mg for SS).

In contrast to seed set, the germination rate was not affected by treatment in population 2030 (Figure 2.12), but in 2033 mean germination was significantly lower when plants received apomictic (76.83 ± 7.29%) rather than sexual pollen (85.87 ± 5.11%) (Analysis of Deviance, P=0.01492; Table 2.7).

Finally, Figure 2.13 shows that seedling survival rate had a similar trend in the two recipient populations in response to treatment. On average, offspring of sexual-apomictic crosses were less likely to survive than seedlings from sexual-sexual crosses and the decline between SS and SA treatment was significant in both populations (Analysis of Deviance, P=0.035 for 2030 and P=0.0129 for 2033; Table 2.8).

### 2.3.3 Flow cytometry (FCM)

Of the 434 achenes from sexual-asexual crosses that germinated (221 from 22 mothers in population 2030 and 213 from 16 mothers in population 2033), 306 became established plants (i.e., survived). It should be noticed that the majority of the seedlings that perished failed to develop
beyond the cotyledon stage once moved from agar to soil. FCM was conducted on a subset of 102 individuals, selected based on their size (smaller plants were not analyzed because they did not provide enough leaf material, as discussed in section 2.2.4.1) and representing the largest number of maternal plants possible (twenty mothers in population 2030 and twelve mothers in population 2033).

Over 76% of the samples met the quality standards set for this study (26/33 for parents and 78/102 for SA treatment progeny), and these requirements were often exceeded. On average, I was able to detect the florescence intensities of roughly 6,000 nuclei per sample, and for most measurements the CV values were consistently below 5% (66.7% of observations for SA progeny and 80.8% for parents). The best results were obtained when the leaf material was dried in silica gel for 21 days or longer, and polyploid samples typically had lower nuclei yields and higher CVs, as previously reported by Suda and Trávníček (2006).

The size ranges of nuclear DNA corresponding to different ploidies of *T. hookeri* were estimated in a previous work by performing flow cytometry on 68 samples collected from 25 populations known to be either diploid or polyploid based on pollen analysis (Garani and Whitton, unpublished; see Appendix B). These estimates largely overlap with the results of an earlier study on populations of *T. hookeri* from Yukon Territory (Thompson *et al.*, 2008). Diploid genome size was between 11 and 14 pg, whereas polyploid nuclear DNA formed two distinct clusters: genomes between 17 and 21 pg were estimated to be triploid and genomes larger than 21 pg were considered to be tetraploid. Based on this method, I was able to confirm that the two seed parent populations (2030 and 2033) were diploid and the two apomictic pollen parent populations (2019 and 2027) were triploid (Figure 2.14). Furthermore, flow cytometry data showed that, as expected, individuals in population 2031 have nuclear DNA sizes comparable to those of plants from population 2027 (Figure 2.14).
The analysis of leaf tissue from the offspring of sexual-apomictic crosses revealed different trends in the two recipient populations (Figure 2.15). Roughly half of the thirty-seven SA offspring from 2033 were diploid (48.7%) and only one plant was clearly a polyploid (likely a triploid). The remaining individuals had intermediate nuclear DNA contents, either smaller than a diploid (2C-value < 11 pg; 13.5% of the samples), or larger than a diploid but smaller than a polyploid genome (14 pg < 2C-value < 17 pg; 35.1% of the samples), suggestive of an aneuploid condition. In contrast, nearly half of the progeny of population 2030 was found to be polyploid (twenty out of forty-one), with a striking predominance of tetraploid individuals. Sixteen plants (39%) were clearly diploid and five (12.2%) were classified as aneuploid (or near-diploid), because their genome sizes slightly exceeded the upper boundary of the diploid range (2C-value > 14 pg).

2.4 Discussion

In this study we showed, for the first time, evidence for the potential existence of reproductive interference between sexual and apomictic populations of the Easter Daisy. In fact, the results from the cross-pollination experiment indicated that pollen produced by apomicts could reduce the reproductive success of sexual seed mothers. In addition, an analysis of ploidy level revealed the presence of polyploid cytotypes within the F1 generation obtained from sexual-asexual crosses. Thus, pollen from apomictic donors certainly sired at least a fraction of these progeny. These findings suggest that unidirectional reproductive interactions mediated by pollen might limit the range expansion of diploid cytotypes into the territory occupied by their polyploid descendants.

My experiment also showed that outcrossing and apomictic cytotypes were found to have comparable reproductive success under open pollination, at least in the southern portion of the species’ range, where the experiment was conducted. As expected, the pollinator exclusion test confirmed apomixis in polyploid populations and sexuality in diploid populations.
2.4.1 Reproductive success of sexual versus apomictic populations under natural conditions (C and I treatments)

The isolation treatment showed that sexual diploids largely failed to set seed when florets were prevented from receiving external pollen. Nonetheless, a few mothers produced a low number of filled achenes, and this is likely due to accidental pollen contamination, as reported in similar pollinator exclusion experiments (O'Connell and Eckert, 1999; Brock, 2004). In fact, organza bags were effective at preventing pollen movement between inflorescences, but, given that flower heads are sessile and cannot be completely isolated from the surrounding rosette or the ground, small insects incidentally carrying pollen from other individuals might sometimes be able to crawl inside the bags and reach the florets. At the same time, the isolation treatment did not hinder the ability of apomicts to set seed. Although their average seed set was lower compared to that of unmanipulated polyploids (control), achenes produced by isolated flower heads of apomicts were usually viable, as inferred by the high germination rate of filled achenes. The difference in seed set could, at least partially, have to do with the year in which the experiment was completed. The isolation treatment on apomictic populations was done in the spring of 2013, a year after all the other treatments. In the spring of 2013, plants were exposed to unusually low temperatures and consecutive snow storms (personal observation), which likely affected their reproductive output. These considerations notwithstanding, the outcome of the isolation treatment confirmed that diploids are outbreeders and polyploids rely on apomixis. In addition, the absence of fruit production observed in unemasculated sexual inflorescences indicated that they are likely to be self-incompatible, a trait observed in sexual relatives of most other agamic complexes (Asker and Jerling, 1992), and described as a characteristic of Townsendia (Beaman, 1957; Tepedino et al., 2004).

The reproductive success of sexual and apomictic populations of the Easter Daisy was estimated by measuring seed set, achene mass, germination rate and seedling survival in open-pollinated mother plants (controls); and the four parameters were subsequently compared among breeding types.
Sexuals and apomicts had similar seed set, as well as inflorescence of comparable size based on the number of both filled and empty achenes produced per capitulum. Apomicts had slightly heavier filled achenes, but the difference was not significant. Polyploid achenes had a lower germination rate than diploids, however apomictic seedlings had a higher chance of surviving and becoming fully established plants. Overall, the cumulative effects of breeding system on the four different parameters measured allows me to conclude that sexual diploids and apomictic polyploids have similar reproductive success in the area where this study was carried out, which represents the only portion of the range where populations of the two cytotypes are found in close proximity. This means that apomicts do not seem to possess a clear advantage over their diploid progenitors in terms of reproductive output, because they do not produce a larger number nor a higher proportion of filled achenes compared to diploid mothers. In addition, the combined results of germination and survival rates, show that apomictic and sexual offspring have the same overall fitness during the early stages of development. Seed dispersal has been shown to be important in the spread of apomictic forms in other taxa, such as Antennaria parlinii, where apomicts produce smaller, lighter and more numerous achenes, with a consequent increase in dispersal potential over their sexual counterparts, especially in disturbed areas (Michaels and Bazzaz, 1986; O’Connell and Eckert, 2001). In T. hookeri I did not observe any major difference in fruit mass or fruit morphology between diploid and polyploid cytotypes. However, preliminary studies of dispersal traits suggest that polyploids may be able to disperse farther from their parental plants, because their fruits, owing to characteristics of the pappus, have lower terminal velocity (i.e., they descend over a known distance in still air at a slower pace) than achenes of sexuals (Ruiz-Larrea and Whitton, unpublished). Based on our current understanding of the system, it appears unlikely that apomicts have been able to re-colonize vast portion of the species range as a consequence of a greater reproductive efficiency resulting from the reallocation of resources toward the female function.
Instead, apomicts might be more successful because of the advantages of uniparental reproduction or due to an enhanced dispersal ability.

2.4.2 **Experimental crossings (SS and SA treatments)**

The cross-pollination protocol was successful, based on the observation that most crosses yielded some filled achenes and it was possible to compare the reproductive success of seed parents receiving pollen from apomictic (SA) versus sexual (SS) donors. The method used to cross-pollinate inevitably produces a mixture of self and cross pollen on the stigma of recipient florets, but the presence of self-pollen should not have compromised the outcome of the crosses, because diploids are likely to be self-incompatible.

Seed set was significantly lower in plants treated with heterospecific (SA) rather than conspecific (SS) pollen in population 2030 (2-day pollination). Reduced fruit production in polyploid-diploid compared to diploid-diploid crosses has been reported in a number of studies on *Taraxacum* (Richards, 1970; Morita *et al*., 1990a; Tas and Van Dijk, 1999), and can be attributed to the low quality pollen produced by apomicts, i.e. to the availability of fewer viable grains compare to the pollen produced by sexuals (Thompson and Whitton, 2006; Garani *et al*., unpublished) or to the early abortion of zygotes sired by aneuploid grains (Richards, 1997; Tas and Van Dijk, 1999). In population 2033 (1-day pollination) the two treatments (SS and SA) produced similar average seed set, but in the SA treatment the seed set of individual seed parents was more widely spread around the mean, with more than 30% of plants failing to set seed entirely and another 24% having high seed set (between 31% and 50%). It is unlikely that this variation was due to pollen amount or quality alone, because each recipient inflorescence was pollinated with two flower heads collected from two distinct pollen donors, but at this stage it is not possible to determine what caused the observed pattern. Moreover, in both populations seed set of crossed individuals (especially in
sexual-sexual crosses) was low compared to that of open-pollinated plants (controls), likely because of pollen limitation. It must be noted that the florets in a flower head are not receptive simultaneously, but open sequentially inward. Because the crosses were performed mainly when the outer disk florets were in anthesis, it is possible that no viable pollen was available by the time the more central florets matured. Brock (2004) encountered a similar issue when crossing dandelions in the field, and attributed the reduced seed set of crossed compare to unmanipulated plants to the use of an insufficient number of donor florets. Repeating the treatment over a longer period of time may have resolved this issue, however achieving full pollination in experimental crosses in the field can be complex.

Pollen from asexual donors did not decrease seed set of parental plants (relative to sexual-sexual crosses) in 2033 as observed in 2030, but achenes from sexual-asexual crosses in population 2033 were slightly lighter and had significantly lower germination rate than achenes sired by diploid pollen parents. None of these differences was significant in population 2030. Survival rate was significantly lower for seedlings from sexual-apomictic crosses compared to offspring from sexual-sexual crosses, and this was true for both recipient populations. The factors causing lower germination rates in 2033 and the decreased survival of progeny derived from sexual-asexual crosses cannot be entirely determined at this stage. Similar results were obtained with Taraxacum, where it was proposed that a decreased germination rate and lower survival could be linked to inbreeding depression in selfed progeny (Tas and Van Dijk, 1999). Alternatively, inviable offspring could result from reproductive incompatibilities between individuals of different ploidy levels, as observed in interploidy crosses between sexually reproducing groups, where progeny are often inviable because of imbalances in ploidy between different tissues of the hybrid seeds (i.e., ‘triploid block’; Marks, 1966; Ramsey and Schemske, 1998).
To summarize, this study shed light on two major potential outcomes of reproductive interactions between sexual diploids and apomictic polyploids in *T. hookeri*, despite the limitations and difficulties imposed by working in the field rather than in a controlled environment. First, the cross-pollination experiment provided evidence that apomicts can successfully fertilize their diploid progenitors, meaning that reproductive barriers among cytotypes are not complete. Second, I found a consistent trend toward a decrease in reproductive success of diploid seed parents when treated with pollen of apomictic origin compared to conspecific pollen. If we consider that each trait analyzed represents a distinct stage of the early offspring development (from seed set to seedling survival), we can conclude that the additive effects of one stage on the next provide substantial support to my initial hypothesis that asymmetric reproductive interference may play a role in preventing the expansion of diploid populations northward, into the apomicts range (e.g., Hörandl, 2006).

### 2.4.3 Flow cytometry (FCM)

After assessing the reproductive success of hand-crossed sexual plants, I used flow cytometry to confirm the ploidy of both seed and pollen parents, and to estimate the ploidy level of the progeny that resulted from sexual-apomictic crosses.

The analysis was conducted using a protocol partially modified from Suda and Trávníček (2006) and Sears (2011), that I initially developed for field-collected silica-dried leaf tissue (Appendix B). It is generally recommended to grind the tissue of the standard species together with each sample, because the presence of specific cytosolic compounds may affect reference and target species differently when the tissues are not co-chopped. This can result in the detection of amplified differences in florescence intensity that do not necessarily reflect real differences in nuclear DNA content (Bennett *et al.*, 2000; Price *et al.*, 2000; Doležel *et al.*, 2007). My co-ground test (Appendix
C), however, showed that it is possible to obtain repeatable 2C-value estimates even when *T. hookeri* and reference leaf tissue are prepared separately. Moreover, a similar method, based on co-staining but independently processing nuclei from standard and target species, was successfully used to assess the DNA amount in samples of *Crataegus* (Talent and Dickinson, 2005). The literature also contains examples of studies where the nuclei of internal standard and species of interest were prepared and stained separately before being mixed and analyzed (e.g., Johnston *et al.*, 1996; O'Brien *et al.*, 1996).

Diploid parental plants that received pollen from polyploid donors produced achenes of varying ploidy, including DNA contents that are consistent with euploid and near-euploid (diploid, triploid and tetraploid) as well as aneuploid cytotypes. This finding is consistent with results from similar experimental crossings performed between diploid mothers and triploid pollen donors in *Taraxacum*, where diploid, triploid and tetraploid progeny were detected (Tas and Van Dijk, 1999; Verduijn *et al.*, 2004b; Mártonfiová, 2006; Meirmans *et al.*, 2006). Some studies also reported the occurrence of aneuploid hybrids (Richards, 1970; Sterk, 1987; Morita *et al.*, 1990b).

The number of individual offspring within each ploidy-category varied dramatically between the two seed parent populations and, notably, putative tetraploids were produced only by mothers in 2030. The inconsistency could be due to differences between the pollen source populations, the recipient populations, or their interaction. Without further experiments it is not possible to make a definitive conclusion, but it seems least likely to be the result of differences between the pollen sources. In fact, populations 2019 and 2027 are both triploid and have similar average pollen viability and size. I did however observe inherent differences between the two recipient populations, which have been shown to partially contrast in their reproductive output (2030 produced significantly more and larger fruits than 2033). Finally, it is important to consider that the results from the flow cytometric analysis reflect only the offspring that germinated and that were
large enough to provide sufficient leaf material for the cytological analysis. Thus, FCM allowed the identification of the presence of different cytotypes, but did not allow quantification of the proportion of the total progeny that fell into each ploidy category. It is unlikely that the most vigorous offspring are a random sample of the seeds that germinated. Further experiments designed to isolate the effect of pollen and maternal sources are needed to resolve the causes of differences in the progeny obtained.

Despite these differences, most mother plants in both populations gave rise to some diploid progeny when pollinated with heterospecific pollen. These diploid F1 offspring can have one of two possible origins: they can arise from self-pollination or they can be true hybrids. Selfed diploids can form when the receptive stigma is saturated with both heterospecific and conspecific pollen, a mixture that can induce the breakdown of self-incompatibility systems, in a phenomenon known as the "mentor-effect" (Richards, 1997). True hybrids would result from the fertilization of a haploid ovule by a haploid sperm nucleus, and thus would carry paternal genes, possibly including genes for apomixis. The molecular examination of progeny formed in analogous crosses revealed that mentor-effects are often responsible for the generation of diploid selfed seed, but that hybrid diploids are also formed (e.g., Hughes and Richards, 1988; Noyes, 2000). The proportion of selfed diploid F1 reported by different authors is highly variable. For instance, more than 85% of the viable offspring obtained in experimental crosses between diploid and triploid Taraxacum were the product of selfing and no diploid F1 hybrids were identified (Morita et al., 1990a; Tas and Van Dijk, 1999). In contrast, a cross between diploid sexual Erigeron strigosus and triploid apomictic Erigeron annuus generated selfed diploids (representing 30.8% of the progeny) as well as true diploid hybrids (Noyes, 2000). An even lower proportion of selfed diploid seeds (1.3% of the progeny) resulted when diploid sexual mother plants of the Ranunculus auricomus complex received pollen from tetra- and hexaploid apomict relatives (Hörandl and Temsch, 2009). Because hand-pollination has been performed by rubbing the parental flower heads against each other without emasculation,
it is possible that mentor-effects took place when seed parents received pollen from apomictic donors (selfing generally does not occur when crosses are performed between outbreeding diploids using the same methods; Morita et al., 1990b; Brock, 2004). The present work did not include an in-depth examination of diploid offspring formation. However, it is possible to speculate that, if diploids are the product of selfing, then they will likely have lower average vegetative fitness than outcrossed progeny due to inbreeding depression, as found by Morita et al. (1990a) and Van Dijk (2007).

Approximately half of the individuals produced by mother plants from population 2030 were polyploid (20 of the 41 samples examined), whereas only one polyploid (likely a triploid) was detected among the 37 samples screened from 2033. Triploid and tetraploid progeny can only have arisen from the fertilization of a haploid ovule by diploid and triploid pollen nuclei, respectively. Consequently, such offspring are always of hybrid origin (assuming unreduced gamete production in diploids is negligible, which I infer based on pollen observations from diploids). This also indicates that triploid fathers are able to produce partially reduced diploid (n=2x) as well as unreduced triploid pollen grains (n=3x). Given the high frequency of tetraploid hybrids, microsporogenesis in polyploids may be biased toward triploid pollen grains (Tas and Van Dijk, 1999). Alternatively, it is possible that a disturbed meiosis, which is characteristic of taxa with autonomous apomixis (Asker and Jerling, 1992; Mogie, 1992) often leads to the formation of unbalanced and thus inviable reduced or partially reduced pollen, a process that would not affect unreduced triploid grains if meiosis was bypassed entirely.

Meiotic irregularities during microsporogenesis could also generate a fraction of aneuploid pollen that retains the ability to function in crosses with diploid seed parents, giving rise to aneuploid offspring. Indeed, it seems unlikely that these progeny resulted from selfing. In contrast to what was observed for polyploids, putative aneuploid hybrids were considerably more frequent in
population 2033, where five plants had a nuclear DNA content slightly smaller than diploids and thirteen had genomes intermediate between diploids and triploids. Only five offspring from 2030 fell in the latter category and none in the former. The underlying determinants of this variation are unknown, but they might be associated with altered chromosome numbers, i.e. the result of the irregular chromosome complement of the pollen of triploid parents (Richards, 1970). Variation in chromosomal copy number has been reported in experimental set-ups analogous to the present work, where numerical aneuploidy has been identified using standard chromosome counts. For instance, the 130 F1 Erigeron hybrids obtained by fertilizing diploid sexuals (2n=2x=18) with pollen from apomictic triploids (2n=3x=27) formed a cytologically complex population that displayed a bimodal distribution of chromosome numbers, with peaks at 2n=18-19 and 2n=25-26 and ranging from diploid to tetraploid (2n=36) (Noyes, 2000). Similarly, in a cytological analysis of 48 Tripsacum dactyloides hybrids, obtained from crossing diploid sexual plants (2n=2x=36) with individuals from an apomictic triploid accession (2n=3x=54), the offspring were found to have variable chromosome numbers spanning nearly the entire range from diploid to triploid (Sherman et al., 1991). Recently, genomic and fluorescence in situ hybridization techniques uncovered extensive and repeated patterns of chromosomal variation in naturally occurring populations of the neoallopolyploid Tragopogon miscellus (Chester et al., 2012).

At this stage, it is not possible to determine exactly how these putative euploid and aneuploid F1 generations formed; and assessing their breeding behavior was equally impracticable because, for reasons that are still unclear, T. hookeri fails to flower when kept in a growth chamber. Based on previous work (Richards, 1970; Morita et al., 1990a; Morita et al., 1990b), the reproductive mode of sexual-apomictic hybrids tends to be predicted by ploidy level, i.e. with diploid hybrids being sexual outcrossers and polyploids being apomictic ; alternatively, plants could be sterile. It is highly improbable that diploid offspring would reproduce asexually given that gametophytic apomixis is strongly correlated with polyploidy and apomictic diploids are extremely rare in nature (Nogler,
In accordance with these predictions, Tas and Van Dijk (1999) reported that one-third of the triploid and all of the tetraploid hybrids reproduced via apomixis, whereas none of their four diploid hybrids was apomictic.

Given these results it is possible to conclude that apomictic individuals have the potential to reduce the reproductive success of their sexual progenitors in experimental crosses. In fact, the product of this unidirectional interaction was an array of diploid, polyploid and aneuploid descendants that might have reduced fitness or might be sterile as a result, for instance, of inbreeding depression (selfed diploids) or chromosomal instability. It is also reasonable to expect that at least a portion of such offspring is fully apomictic or possesses apomixis genes and will pass these traits to their progeny. It should be noted that while apomictic hybrid offspring would contribute to the reproductive fitness of their diploid maternal plants, in nature, these apomicts could shift the relative frequencies of the two mating types.

In conclusion, these findings contribute towards explaining why apomictic and sexual forms have not been observed to co-occur in this taxon and why outcrossing diploids have not been found in the central and northern portion of the range (outside Yukon). In fact, this study showed that the persistence of sexual cytotypes could be imperiled in the presence of a large number of apomictic neighbours, while being surrounded by sexuals will have no impact on apomictic individuals, given that they do not require pollen for embryo or endosperm formation and development. As a result, apomicts might have the ability to wipe out co-occurring diploid individuals at local scale, creating a barrier that prevents sexuals from spreading into territories dominated by asexual lineages. Thus, the presence of diploid sexual populations could be restricted only to areas of the range where they hold a selective advantage over polyploids and represent the large majority of individuals (Mogie, 1992; Mogie et al., 2007; Whitton et al., 2008).
Table 2.1 Identification numbers, site descriptions (with GPS coordinates and altitude), pollen data (stainability, average size and standard deviation of viable grains) and ploidy estimates for the populations of *Townsendia hookeri* selected to conduct the cross-pollination experiment, including population 2031 (described in chapter 3). Pollen stainability and size were measured following the methodology described in Appendix A.

<table>
<thead>
<tr>
<th>ID</th>
<th>Locality</th>
<th>Latitude / Longitude (decimal)</th>
<th>Alt. (m)</th>
<th>Mean pollen stainability (%)</th>
<th>Mean pollen diameter (µm)</th>
<th>Std. Dev. (µm)</th>
<th>Estimated ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>2030</td>
<td>19 km E of Laramie (WY), Medicine Bow National Forest - exit #323 (Lincoln Memorial rest area) on Hwy 80</td>
<td>41.24002 -105.43423</td>
<td>2663</td>
<td>94.13</td>
<td>22.14</td>
<td>0.96</td>
<td>Diploid</td>
</tr>
<tr>
<td>2032</td>
<td>26 km E of Laramie (WY), Medicine Bow National Forest - exit #329 (Vedauwoo Glen Rd); drive ~ 3 km E along Vedauwoo Glen Rd.; plants are found on both side of the road.</td>
<td>41.15272 -105.36605</td>
<td>2510</td>
<td>91.6</td>
<td>21.73</td>
<td>1.28</td>
<td>Diploid</td>
</tr>
<tr>
<td>2033</td>
<td>26 km E of Laramie (WY), Medicine Bow National Forest - exit #329 (Vedauwoo Glen Rd); drive ~ 500 m along US Forest Service Rd 700 G from junction with Vedauwoo Glen Rd. (second dirt road from the exit, on the NW side of the road); on W side of the road.</td>
<td>41.16206 -105.40258</td>
<td>2553</td>
<td>93.65</td>
<td>22.15</td>
<td>1.25</td>
<td>Diploid</td>
</tr>
<tr>
<td>2035</td>
<td>30 km E of Laramie (WY), Medicine Bow National Forest - exit #329 (Vedauwoo Glen Rd); drive ~ 4 km E along Vedauwoo Glen Rd.; on W side of the road.</td>
<td>41.15334 -105.35812</td>
<td>2532</td>
<td>92.36</td>
<td>22.02</td>
<td>1.02</td>
<td>Diploid</td>
</tr>
<tr>
<td>ID</td>
<td>Locality</td>
<td>Latitude / Longitude (decimal)</td>
<td>Alt. (m)</td>
<td>Mean pollen stainability (%)</td>
<td>Mean pollen diameter (µm)</td>
<td>Std. Dev. (µm)</td>
<td>Estimated ploidy</td>
</tr>
<tr>
<td>-----</td>
<td>---------------------------------------------------------------------------</td>
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<td>----------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>2019</td>
<td>E Laramie (WY) - 2 km NW along Old Stone Quarry Rd (dirt road) from junction with E Grand Ave/Hwy 80. The dirt road is 800 m W from the intersection of E Gran Ave and Boulder Dr.</td>
<td>41.30645 -105.52158</td>
<td>2313</td>
<td>33.75</td>
<td>33.02</td>
<td>3.24</td>
<td>Polyploid</td>
</tr>
<tr>
<td>2027</td>
<td>40 km W of Laramie (WY), Medicine Bow National Forest - drive 3.6 km N along Fox Creek Rd/Co Rd 47 from junction with 230. From parking spot on S side of the road (next to river) walk ~ 300 m N along a dry creek, then go through the U.S. Forest Service fence on the right.</td>
<td>41.14187 -106.04396</td>
<td>2433</td>
<td>28.19</td>
<td>33.47</td>
<td>4.2</td>
<td>Polyploid</td>
</tr>
<tr>
<td>2031</td>
<td>17 km NE of Laramie (WY) on Roger Canyon Road - 16.6 road km from junction with Grand Avenue; 13.8km from point where Roger Canyon changes from 9th Street.</td>
<td>41.3864 -105.47349</td>
<td>2359</td>
<td>25.53</td>
<td>33.75</td>
<td>5.45</td>
<td>Polyploid</td>
</tr>
</tbody>
</table>
Table 2.2 Summary of the work completed in the field during the spring of 2012 and 2013. For each population, the table reports: the identification number (ID), function (SR = sexual recipient, SD = sexual donor, AD = apomictic donor), replicate experiment (R1 or R2), treatment(s) done, year when the treatment(s) was conducted and number of days over which the pollination was repeated.

<table>
<thead>
<tr>
<th>ID</th>
<th>Function</th>
<th>Replicate</th>
<th>Treatment</th>
<th>Year</th>
<th>Days of pollination</th>
</tr>
</thead>
<tbody>
<tr>
<td>2030</td>
<td>SR</td>
<td>R1</td>
<td>C, I, SS, SA</td>
<td>2012</td>
<td>2</td>
</tr>
<tr>
<td>2032</td>
<td>SD</td>
<td>R1</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2033</td>
<td>SR</td>
<td>R2</td>
<td>C, I, SS, SA</td>
<td>2012</td>
<td>1</td>
</tr>
<tr>
<td>2035</td>
<td>SD</td>
<td>R2</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2019</td>
<td>AD</td>
<td>R1</td>
<td>C, I</td>
<td>2012/2013</td>
<td>--</td>
</tr>
<tr>
<td>2027</td>
<td>AD</td>
<td>R2</td>
<td>C</td>
<td>2012</td>
<td>--</td>
</tr>
<tr>
<td>2031</td>
<td>--</td>
<td>--</td>
<td>I</td>
<td>2013</td>
<td>--</td>
</tr>
</tbody>
</table>

Table 2.3 Summary of two-way factorial Analysis of Deviance testing for the effects of treatment (control vs. isolation), reproduction (sexual vs. apomictic breeding system) and the interaction term on seed set. The analysis was done using GLM with family = quasibinomial to account for overdispersion (meaning that the variance of the error distribution in the data was greater than that expected under the binomial distribution). The significance of terms was assessed with an F-test. **Bold** values are significant at p<0.05.

<table>
<thead>
<tr>
<th>Seed set</th>
<th>SS</th>
<th>d.f.</th>
<th>F</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>600.98</td>
<td>1</td>
<td>181.14</td>
<td>&lt;2.20e-16</td>
</tr>
<tr>
<td>Reproduction</td>
<td>425.53</td>
<td>1</td>
<td>128.26</td>
<td>&lt;2.20e-16</td>
</tr>
<tr>
<td>Treatment : Reproduction</td>
<td>619.63</td>
<td>1</td>
<td>186.77</td>
<td>&lt;2.20e-16</td>
</tr>
<tr>
<td>Residuals</td>
<td>242.19</td>
<td>73</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.4 Summary of one-way factorial Analysis of Deviance testing for the effects of reproduction (sexual vs. apomictic breeding system) on germination rate. The analysis was done using GLM with family = quasibinomial. The significance of terms was assessed with an F-test. **Bold** value is significant at p<0.05.

<table>
<thead>
<tr>
<th>Germination</th>
<th>SS</th>
<th>d.f.</th>
<th>F</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reproduction</td>
<td>12.66</td>
<td>1</td>
<td>4.4417</td>
<td><strong>0.0421</strong></td>
</tr>
<tr>
<td>Residuals</td>
<td>102.61</td>
<td>36</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.5 Summary of one-way factorial Analysis of Deviance testing for the effects of reproduction (sexual vs. apomictic breeding system) on seedling survival rate. The analysis was done using GLM with family = binomial. The significance of terms was assessed with a Chi-square test. **Bold** value is significant at p<0.05.

<table>
<thead>
<tr>
<th>Survival</th>
<th>d.f.</th>
<th>Deviance</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reproduction</td>
<td>1</td>
<td>4.1017</td>
<td><strong>0.04284</strong></td>
</tr>
<tr>
<td>Residuals</td>
<td>36</td>
<td>46.624</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.6 Summary of one-way factorial Analysis of Deviance testing for the effects of treatment (sexual-sexual crosses vs. sexual-apomictic crosses) on seed set for the two pollen recipient populations. The analysis was done using GLM with family = quasibinomial. The significance of terms was assessed with an F-test. **Bold** value is significant at p<0.05.

**Population 2030**

<table>
<thead>
<tr>
<th>Seed set</th>
<th>SS</th>
<th>d.f.</th>
<th>F</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>66.05</td>
<td>1</td>
<td>9.0154</td>
<td><strong>0.004279</strong></td>
</tr>
<tr>
<td>Residuals</td>
<td>344.34</td>
<td>47</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Population 2033**

<table>
<thead>
<tr>
<th>Seed set</th>
<th>SS</th>
<th>d.f.</th>
<th>F</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>3.16</td>
<td>1</td>
<td>0.2814</td>
<td>0.5982</td>
</tr>
<tr>
<td>Residuals</td>
<td>539.81</td>
<td>48</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.7 Summary of one-way factorial Analysis of Deviance testing for the effects of treatment (sexual-sexual crosses vs. sexual-apomictic crosses) on germination rate for the two pollen recipient populations. The analysis was done using GLM with family = binomial. The significance of terms was assessed with a Chi-square test. **Bold** value is significant at p<0.05.

<table>
<thead>
<tr>
<th>Population 2030</th>
<th>Germination</th>
<th>d.f.</th>
<th>Deviance</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>0.2467</td>
<td>0.6194</td>
<td></td>
</tr>
<tr>
<td>Residuals</td>
<td>46</td>
<td>86.384</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Population 2033</th>
<th>Germination</th>
<th>d.f.</th>
<th>Deviance</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>5.926</td>
<td><strong>0.01492</strong></td>
<td></td>
</tr>
<tr>
<td>Residuals</td>
<td>38</td>
<td>97.149</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.8 Summary of one-way factorial Analysis of Deviance testing for the effects of treatment (sexual-sexual crosses vs. sexual-apomictic crosses) on seedling survival rate for the two pollen recipient populations. The analysis was done using GLM with family = binomial. The significance of terms was assessed with a Chi-square test. **Bold** values are significant at p<0.05.

<table>
<thead>
<tr>
<th>Population 2030</th>
<th>Survival</th>
<th>d.f.</th>
<th>Deviance</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>4.4422</td>
<td><strong>0.03506</strong></td>
<td></td>
</tr>
<tr>
<td>Residuals</td>
<td>28</td>
<td>51.191</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Population 2033</th>
<th>Survival</th>
<th>d.f.</th>
<th>Deviance</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>6.1709</td>
<td><strong>0.01299</strong></td>
<td></td>
</tr>
<tr>
<td>Residuals</td>
<td>21</td>
<td>57.635</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.1 Locations of the six populations of *Townsendia hookeri* used to complete the cross-pollination experiment. Apomictic populations (IDs: 2019 and 2027) are represented with blue circles and sexual populations (IDs: 2030, 2032, 2033 and 2035) are represented with red circles. The figure also shows the location of the apomictic population 2031 (described in section 2.2.2).
Figure 2.2 One replicate block of experimental treatments completed in the field on three natural populations of *Townsendia hookeri*. Treatments are indicated with capital letters: C, control; I, isolation; SS, sexual-sexual cross; SA, sexual-apomictic cross. The number of individuals exposed to each treatment is indicated in parenthesis. Plants are represented as having a single inflorescence to indicate that only one flower head per individual was used in a given treatment. Blue inflorescences represent apomictic plants while red inflorescences represent sexual plants.
Figure 2.3 Achenes of *Townsendia hookeri* were classified as “filled” or “empty” based on the following criteria: “filled” achenes were plump and dark brown (first and second achenes from the left), whereas “empty” achenes were flat and lacked pigmentation (fifth and sixth achenes from the left). Light brown achenes were scored as “filled” when fleshy (third achene from the left) and as “empty” when clearly devoid of an embryo (fourth achene from the left).
Figure 2.4 Example of fully established individual of *Townsendia hookeri*, characterized by a large root system and five true leaves.
Figure 2.5 Cytogram of the transit time of propidium iodide-stained nuclei through the laser interrogation point (FL2-W) versus their total fluorescence (FL2-A), obtained via flow cytometry. This type of plot was created using FlowJo vX.0.6 and it was used to identify $\text{G}_0/\text{G}_1$ populations of nuclei belonging to the internal standard (\textit{Vicia faba} cv. Inovec in this example) and the target tissue (polyploid \textit{Townsendia hookeri}). The two subpopulations of nuclei (in light green) were isolated from the debris background (in blue) using the Autogate function.
Figure 2.6 Total number of filled and empty achenes per seed head (capitulum). Each head (indicated by a circle) represents an apomictic (blue) or sexual (red) seed parental plant under control or isolation treatment. The black circles and bars are means and standard errors. Different shades of color define single populations: 2019 is dark blue, 2027 and 2031 are light blue, 2030 is dark red and 2033 is light red.
Figure 2.7 Percentage of filled achenes per seed head (seed set). Each head (indicated by a circle) represents an apomictic (blue) or sexual (red) seed parental plant under control or isolation treatment. The black circles and bars are means and standard errors. The asterisk (*) represents significantly different values (p<0.05) based on the Analysis of Deviance. Different shades of color define single populations: 2019 is dark blue, 2027 and 2031 are light blue, 2030 is dark red and 2033 is light red.
Figure 2.8 Percentage germination rate per filled achene. Each circle represents the mean value for an apomictic (blue) or sexual (red) seed parental plant under control treatment. The black circles and bars are means and standard errors. The asterisk (*) represents significantly different values (p<0.05) based on the Analysis of Deviance. Different shades of color define single populations: 2019 is dark blue, 2027 is light blue, 2030 is dark red and 2033 is light red.

Figure 2.9 Percentage survival rate per seedling transplanted from agar to soil. Each circle represents the mean value for an apomictic (blue) or sexual (red) seed parental plant under control treatment. The black circles and bars are means and standard errors. The asterisk (*) represents significantly different values (p<0.05) based on the Analysis of Deviance. Different shades of color define single populations: 2019 is dark blue, 2027 is light blue, 2030 is dark red and 2033 is light red.
Figure 2.10 Percentage of filled achenes per seed head (seed set). Each head (indicated by a circle) represents a seed parental plant under sexual-sexual (SS) or sexual-apomictic (SA) crossing treatment. Dark grey circles correspond to population 2030, while light grey circles correspond to population 2033. The red circles and bars are means and standard errors. The asterisk (*) represents significantly different values (p<0.05) based on the Analysis of Deviance done between treatments within each population.
Figure 2.11 Average mass (in mg) of filled achenes per seed head. Each head (indicated by a circle) represents a seed parental plant under sexual-sexual (SS) or sexual-apomictic (SA) crossing treatment. Dark grey circles correspond to population 2030, while light grey circles correspond to population 2033. The red circles and bars are means and standard errors. Differences between treatments within each population are not significant based on the T-test (2030: $t=-0.3347$, df=46, $P=0.7393$; 2033: $t=1.7632$, df=37, $P=0.0861$).
Figure 2.12 Percentage germination rate per filled achene. Each circle represents the mean value for a seed parental plant under sexual-sexual (SS) or sexual-apomictic (SA) crossing treatment. Dark grey circles correspond to population 2030, while light grey circles correspond to population 2033. The red circles and bars are means and standard errors. The asterisk (*) represents significantly different values (p<0.05) based on the Analysis of Deviance done between treatments within each population.
Figure 2.13 Percentage survival rate per seedling transplanted from agar to soil. Each circle represents the mean value for a seed parental plant under sexual-sexual (SS) or sexual-apomictic (SA) crossing treatment. Dark grey circles correspond to population 2030, while light grey circles correspond to population 2033. The red circles and bars are means and standard errors. The asterisks (*) represent significantly different values (p<0.05) based on the Analysis of Deviance done between treatments within each population.
Figure 2.14 Genome size (2C-value) of plants from seed or pollen parental populations (including 2031), calculated performing flow cytometry on silica-dried leaf tissue. The number of samples analyzed for each population (n) is reported in the legend. Dotted lines delimit size ranges associated with diploid (2C-v. < 14 pg; in red) and triploid (17 pg < 2C-v. < 21 pg; in blue) genomes. Data points are spread along the y-axis to better visualize overlapping values.

Figure 2.15 Genome size (2C-value) of offspring from sexual-apomictic crosses in populations 2030 (n=41) and 2033 (n=37), calculated performing flow cytometry on silica-dried leaf tissue. Dotted lines delimit size ranges associated with diploid (11 pg < 2C-v. < 14 pg; in red) and polyploid (2C-v. > 17 pg; in blue) genomes. Orange and brown circles represent nuclear DNA contents consistent with an aneuploid condition. Data points are spread along the y-axis to better visualize overlapping values.
Chapter 3: Conclusions

3.1 General conclusions

This research explored possible factors that have contributed to and maintain the current geographical distribution of sexual diploid and apomictic polyploid populations of the Easter Daisy across the species’ range. The study was conducted on populations located in the southern portion of the range (southern Wyoming), where sexual and asexual populations are found in close proximity, and thus where contact between breeding types is more likely to have occurred in the past or could occur in the future. Working in the field had the benefit that plants were maintained in their natural habitat, but it also imposed some limitations owing to adverse weather conditions (mostly snow covering the plants) and other unpredictable natural events. Consistent with Beaman’s results (1957), the isolation treatment indicated that diploids do require mating partners for successful seed set, whereas polyploids are apomictic and able to sire viable progeny in the absence of external pollen. Diploid and polyploid cytotypes were found to have similar reproductive success under open pollination, suggesting that apomixis does not represent a clear advantage in terms of number or size of achenes produced under natural conditions, at least for these populations during the year of observation. Lastly, this work confirmed the prediction that apomicts retain the ability to fertilize their sexual progenitors through their pollen, and that this unidirectional interaction is disadvantageous for diploids. When sexual seed parents received heterospecific rather than conspecific pollen, seed set decreased, the germination rate of achenes was lower and seedlings were less likely to survive and become fully established plants. In addition, the fraction of the progeny that survived had variable ploidy levels, consistent with diploid, triploid and tetraploid, but also aneuploid cytotypes. The breeding system of F1 hybrids was not tested, but it is expected that at least some polyploid offspring would be fully apomictic (Richards, 1970; Morita et al., 1990b) or at least possess apomixis genes (e.g., Noyes, 2006). Based on these lines of
evidence, reproductive interference might contribute to explaining why sexual forms of Townsendia hookeri are restricted to the unglaciated, southernmost part of the range, and failed to spread northward, into the territory occupied by their apomictic descendants. In a postglacial re-colonization scenario, it is possible that faster moving apomictic lineages have established asexual populations in newly opened portions of the range, and that subsequently these populations have become impenetrable to invasion by slower-moving sexual progenitors thanks to the detrimental effects of their pollen on the reproductive success of low-density co-occurring sexual types (Mogie, 1992; Mogie et al., 2007; Whitton et al., 2008).

3.2 Implications

The occurrence of apomictically reproducing plants characterized by a distinct and widespread geographical distribution make the Easter Daisy an ideal system to investigate the coexistence of apomixis and sexuality within a taxon, as well as to ask questions about the factors that determine geographical parthenogenesis, a pattern described in a number of plant groups but still not well understood (reviewed by Hörandl, 2006).

Although most likely only about 1% of flowering plant species are apomictic (Whitton et al., 2008), including representatives in 28 orders and 33 families (Carman, 1997), apomixis represents a fundamental alternative strategy to the predominant sexual pathways. In recent years the study of this peculiar reproductive strategy has gathered much attention, especially for its potential application in crop production. According to Vielle Calzada et al., "If the process of apomixis can be understood and harnessed, the benefits for agriculture are potentially enormous" (Vielle Calzada et al., 1996). Apomixis allows the perpetuation of a fixed genotype through generations by avoiding meiosis and ovule fertilization by male gametes (Grimanelli et al., 2001). Thus, this trait could make it possible to perpetuate hybrid cultivars and other varieties with desirable gene combinations via
clonal seeds, independently of pollination and fertilization (Koltunow et al., 1995; Spillane et al., 2001). Despite the great interest and the even larger number of related scientific publications, little is known about how apomictic crops might interact with their wild relatives. It has been recognized that domesticated plant species can hybridize with their wild progenitors (Ellstrand, 1992; Ellstrand et al., 1999). It is also known that most apomicts are able to produce some functional pollen, because apomixis affects the female reproductive pathway, but has no direct impacts on microsporogenesis (e.g., Nogler, 1984). As a result, it is reasonable to expect that pollen-mediated gene flow could take place and possibly have detrimental effects on wild relatives found within mating distance of the newly introduced apomictic crop varieties. Research conducted on natural plant populations suggested that when non-indigenous apomictic species are introduced to a new area, they can threaten the persistence of native sexual relatives via genetic assimilation (Brock, 2004) or other forms of reproductive interference (Matsumoto et al., 2010). Similarly, experimental studies (e.g., Morita et al., 1990b; Tas and Van Dijk, 1999; Noyes, 2000) and the work presented here showed how the male function of apomictic plants can interfere with the normal sexual reproduction of diploid related forms. Therefore, the 'harnessing' of apomixis and its application in agriculture will definitely require a greater understanding of the ecological implication of this trait (such as gene flow patterns mediated by male gametes) to properly predict the impact that novel crop varieties will have on natural systems.

A better understanding of the dynamics that regulate the coexistence of sexual and apomictic breeding types (i.e., geographical parthenogenesis) would also promote the implementation of more effective conservation actions. Diploid cytotypes play a crucial evolutionary role in an agamic complex, because: (1) they are the only sexually reproducing individuals (thus, they harbor the potential to generate genetic diversity), and (2) they give rise to all polyploid lineages (Babcock and Stebbins, 1938). Therefore, the persistence of sexual diploids is critical for the long-term survival of the species, including sexual and asexual reproductive types. At the same time, in T. hookeri and
other similar taxa, diploids are much less common than their apomictic descendants, and their distribution is restricted within the overall species’ range. Given that diploid sexuals are both irreplaceable and uncommon, their populations should receive special attention when evaluating the status of the species across its range, and when planning management or conservation programs.

3.3 Future directions

While this research has highlighted the disruptive effects that apomictic polyploids can exert on the reproductive success of sexual progenitors through pollen, the next steps are to confirm hybridity, assess fitness and further characterize the reproductive strategy of the progeny from sexual-apomictic crosses (ideally, by inducing flower development and assessing seed set performing the control and isolation treatments described in this study).

Reproductive interference is likely to be only one of the factors that has influenced the spread of sexual and asexual breeding strategies in the Easter Daisy, and further hypotheses await testing. For instance, it has been suggested that abiotic factors could have influenced the geographic distribution of sexual-asexual species complexes, both at large and small scale (Verduijn et al., 2004a). It follows that the absence of apomictic polyploids within the habitat dominated by diploids could result from the existence of divergent edaphic tolerances between breeding types that confer a selective advantage to sexual over asexual populations in the presence of specific soil conditions.

To test this hypothesis, Garani et al. (unpublished) collected soil samples from 25 populations of T. hookeri scattered throughout most of the species’ range, from Colorado to southern British Columbia. The soil samples were analyzed for their chemical and physical properties, but a careful examination of the data has not yet been completed. A future analysis and interpretation of this large data set, coupled with results from ecological niche modeling (Lee and Whitton, unpublished),
will shed light on the contribution of abiotic factors in shaping the observed geographical distribution of *T. hookeri* cytotypes from Colorado to southern Canada.

In an attempt to test hypotheses of ecological divergence of cytotypes, PhD student E. Hersh is setting up a large common garden experiment that includes eight transplant gardens in three regions within the species’ native range. The gardens will provide a unique opportunity to investigate the existence of differences in fitness-related traits (such as survival and reproductive ability) between sexual and apomictic plants, and also to further test the reproductive interference hypothesis. Thus, this ambitious project promises to fill some of the major gaps in our knowledge of the circumstances that contributed to the great success of asexual lineages in *Townsendia hookeri*, but also in other apomictic complexes within and outside the genus.
References


Appendices

Appendix A: Range wide survey of *T. hookeri* populations using pollen analysis

A.1 Methods

Pollen analysis was performed on both dried and fresh pollen grains. Dried pollen samples where obtained from herbarium mounts and from specimens collected by Ph. D. student Chris Lee between 2008 and 2011, including new sites as well as sites visited based on past collections. From each location, one to four full plants (leaves, flowers, roots and stem) were collected for herbarium vouchers and the GPS coordinates of the site were recorded using a GARMIN GPSmap 62st (Datum WGS 84). Fresh pollen was sampled on April 2012 from six natural populations of *Townsendia hookeri*, in which the ploidy level was known based on previous collections. Each sample consisted of five disk florets that were removed from one flower head, placed into a labeled 1.5 ml microtube and stored at 4°C within less than six hours from collection. Pollen was obtained from four individuals in each population, which had fully open flower heads but un-open central disk florets, in order to avoid collecting older pollen grains.

Pollen viability was tested using cotton blue in lactophenol (Radford *et al.*, 1974), a dye that stains callose with high specificity (Stanley and Linskens, 1974) and has been shown to accurately reflect pollen fertility (Mayer, 1991). Moreover, lactophenol was preferred to other dyes also to ensure consistency with previous experiments conducted on *T. hookeri* (Thompson and Whitton, 2006). The preparation and mounting of the pollen involved removing six to eight disk florets from dried inflorescences, including field-collected plants and herbarium mounts, and placing these into 1.5ml centrifuge tubes. This first step does not apply to fresh pollen samples, which were already kept in 1.5 ml tubes. 20 µL of cotton blue and 10 µL of distilled water were added to each tube and the
tubes were vortexed for one minute to release pollen into suspension. 15 µL of the resultant suspension was placed on a slide. The slide was covered immediately with a cover- slip and the edges were sealed with nail polish. Generally, the pollen was observed within a few hours; however, we noticed that slides viewed immediately, as well as days or weeks after preparation, gave consistent results. At least 300 grains were counted in each slide and pollen grains with fully stained, regularly shaped cytoplasm were scored as viable, while pollen units that were a lighter blue in color, translucent and irregularly shaped were considered inviable. The size of the pollen was assessed by measuring the cytoplasm diameter of twenty viable pollen units per slide. Pollen counts and diameter measurements were obtained using a Nikon Eclipse 80i light microscope, at 10x and 40x magnification, respectively. The proportion of stainable pollen and the mean grain size were recorded and the graphical analysis of the data was conducted using R, version 3.0.3 (R Core Development Team, 2014).
A.2 Results

Figure A.2.1 Plot of mean cytoplasm diameter of viable grains by mean pollen stainability, representing diploid (dark grey circles) and polyploid (white circles) cytotypes. For each ploidy category, mean stainability (±standard error) and mean viable pollen size (±standard error) were found to be equal to: 82.81±2.63 % and 22.31±0.25 µm for diploids (n=24); 25.73±2.3 % and 34.75±0.86 µm for polyploids (n=34).
Appendix B: Range wide survey of *T. hookeri* populations using flow cytometry

B.1 Methods

Twenty-five populations of *T. hookeri* were located based on past collections, namely herbarium specimens and sites described by Chris Lee between 2008 and 2011. The survey was limited the portion of the range outside Yukon (from southern British Columbia to Colorado). All the localities were visited in June 2012 and leaf samples from at least three different individuals were obtained in each population, with the exception of two sites in Wyoming, where only one and two plants were located and sampled. The leaf material was stored at ambient temperature in paper envelopes containing silica gel. Within four to five weeks of collection, the dehydrated tissue was removed from silica, transferred to 2 ml screw cap microtubes and stored in a -80°C freezer. The samples were analyzed via flow cytometry between November 2012 and April 2013 following the protocol describe in section 2.2.4.
B.2 Results

![Graph](image)

**Figure B.2.1** Genome size (2C-value) of 68 samples representing 25 populations of the Easter Daisy (samples from the same population always cluster together). The values were estimated performing flow cytometry on silica-dried leaf tissue. Samples were assigned to three different ploidy levels: diploid (2Cx; red circles), triploid (3Cx; blue circles) and tetraploid (4Cx; light blue circles). For each ploidy category, mean 2C-value (±standard deviation) was found to be equal to: 13.02±0.5 pg for diploids (n=23), 18.34±0.89 pg for triploids (n=33) and 22.58±1.28 pg for tetraploids (n=12).
Appendix C: Results from the co-ground test (FCM)

Results from the co-ground trial (section 2.3.2) including: identification numbers of the populations of origin (ID p.), ploidy of the populations of origin based on the results of pollen analysis (Ploidy p.), ID of the samples tested via flow cytometry (ID s.), average 2C-value, standard deviation (std. dev.) and ploidy (DNA ploidy) obtained via flow cytometry. DNA ploidy categories are defined based on the work presented in Appendix B.

<table>
<thead>
<tr>
<th>ID p.</th>
<th>Ploidy p.</th>
<th>ID s.</th>
<th>Mean 2C-value (pg)</th>
<th>std. dev.</th>
<th>DNA ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>2019</td>
<td>3x</td>
<td>19C4</td>
<td>18.847</td>
<td>0.015</td>
<td>3Cx</td>
</tr>
<tr>
<td>2019</td>
<td>3x</td>
<td>19C6</td>
<td>19.271</td>
<td>0.018</td>
<td>3Cx</td>
</tr>
<tr>
<td>2019</td>
<td>3x</td>
<td>19C8</td>
<td>19.052</td>
<td>0.067</td>
<td>3Cx</td>
</tr>
<tr>
<td>2031</td>
<td>3x</td>
<td>31C1</td>
<td>17.134</td>
<td>0.048</td>
<td>3Cx</td>
</tr>
<tr>
<td>2031</td>
<td>3x</td>
<td>31C2</td>
<td>19.045</td>
<td>0.010</td>
<td>3Cx</td>
</tr>
<tr>
<td>2033</td>
<td>2x</td>
<td>33C1</td>
<td>12.555</td>
<td>0.038</td>
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