Reproductive and Genetic Consequences of Fragmentation in Chokecherry (*Prunus virginiana*, L.)

By

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ABSTRACT

Habitat fragmentation has the potential to negatively affect plant populations by disrupting gene flow, reducing genetic diversity and changing mating dynamics. However, the effect of fragmentation has been poorly investigated in the case of widespread species, some of which are keystone in broader community dynamics. In this research, I explored the effects of habitat disruption on important ecological and genetic features of chokecherry (*Prunus virginiana,* L.), a widespread self-incompatible species from temperate regions, by comparing plants from 2 forest fragments (BP-BH) with those from a continuous forest remnant (GB), and combining field studies (chapter one) with marker-gene analysis (chapter two). In chapter one I explore the extent of pollen limitation and the impact of maternal resources and fungal infection on reproductive output, using natural and supplemented pollinations. In chapter two, I explore different indicators of genetic variability, mating system and pollen dispersal parameters using eight microsatellite primers in adult populations and progeny arrays.

I did not find evidence of either a reduction in genetic diversity or the presence of inbreeding after fragmentation, nor did I find evidence of genetic structure among the adult populations. This is explained by the different life-history traits and ecological characteristics of these chokecherry populations, such as a predominantly outcrossing mating system, high levels of gene flow, and long pollen distance dispersal. However, I found an increase in biparental inbreeding and paternity of correlation after fragmentation, and a reduction in the genetic diversity in the progeny arrays of one of the fragments (BP) compared to GB. The evidence that more matings between related individuals occur in fragmented populations is shown to be potentially attributable to

variation in the spatial distribution of genotypes and variation in the density of individuals among populations, both of which could affect the activity of pollinators and cause increased biparental inbreeding. These results from the genetic analyses are also supported by the analyses of ecological factors affecting the populations. I found a significant reduction in the reproductive output of maternal trees in the fragmented populations compared to the continuous population, due to pollen limitation in the former. Additionally, I found that the presence of fungal infection and the availability of maternal resources showed minor but significant indirect effects on the production of fruits. Thus, the results of the ecological data support those from the genetic analyses and indicate that pollen limitation is the main cause for the observed reduction in reproductive success in the fragments.

Overall my results show that a reduction in pollen donors and higher levels of biparental inbreeding are major factors determining the reduction in reproductive success of individuals in the fragmented populations. This suggests that habitat fragmentation has had negative impacts on reproduction in these chokecherry populations.

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DEDICATION

This thesis is dedicated to my parents, who gave me the freedom of choosing my career and supported me all the way. Love you for encouraged me to work hard for my dreams.

Esta tesis está dedicada a mis papis, quienes me dieron la libertad de escoger mi carrera y me han apoyado constantemente. Los adoro por animarme a trabajar duro por mis sueños.

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ACRONYMS

BP – Beaudry Park

BH – Birds Hill

GB – Grand Beach

PSC – Partially self-compatible

SI – Self-incompatibility

SC – Self-compatibility index

A – Autogamy index

PCA – Principal Components Analysis

PC1 – First axis of the PCA

ANCOVA – Analysis of Covariance

ANOVA – Analysis of Variance

SSR – Single sequence repeats

TPM – Two-phase model

LSR – Least-square residuals

SE – Standard errors

CI – Confidence intervals

HWE – Hardy-Weinberg equilibrium

LD – Linkage disequilibrium

EM – Expectation-maximization method

1.0. BACKGROUND

Forest fragmentation is a process by which large areas of primary habitat are divided into smaller isolated remnants by urban development and agricultural use of land. Increasing urbanization and intensive agricultural practices during the past 120 years have drastically modified the landscape of much of North America (Figure 1.1); reducing extant continuous historical forests into small, separated forest fragments (Ramankutty & Foley 1999). The risks of destruction and fragmentation of habitat by the extensive anthropogenic use of resources has long been recognized, (Curtis 1956), however land use policies have not changed significantly over the past 50 years and agriculture, logging and road construction are still one of the greatest threats to forest ecosystems (Chambers *et al.* 2001). In fact, although the peak of agriculture-related deforestation in western Canada occurred over a century ago, the rate of deforestation quantified in this area between 1966 and 1994 was higher than the world average (Hobson *et al.* 2002).

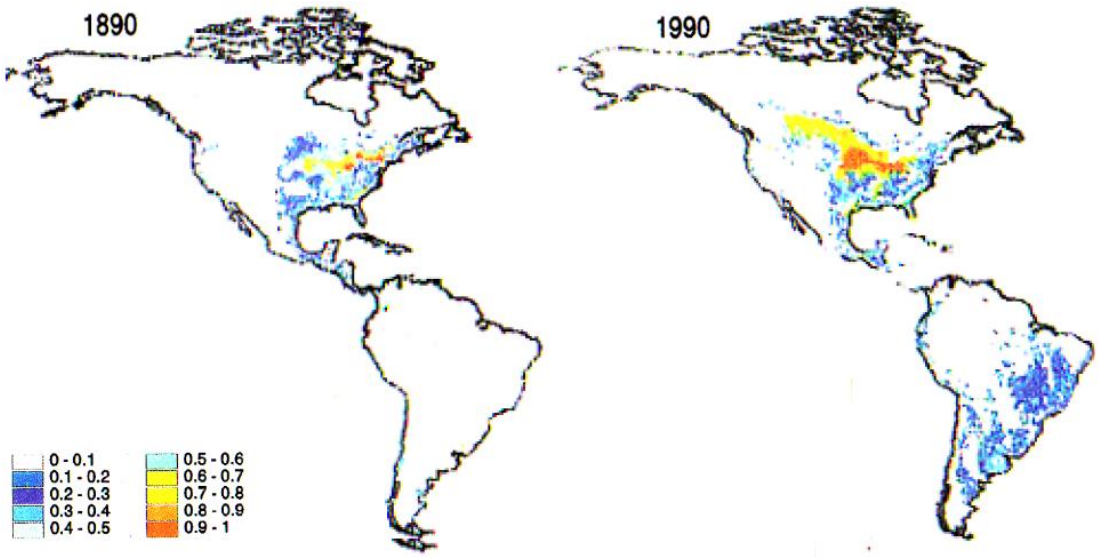

Figure 1. 1. American historical cropland areas from 1890 and 1990. The data set is shown as the percentage of crop cover in a 5-min resolution (in latitude by longitude) grid cell, where zero indicates no crop land. Modified from Ramankutty and Foley (1999).

The development of cities and the implementation of extensive crop fields are effectively permanent processes that may have important impacts on plant populations (Aguilar *et al.* 2008). Habitat loss, and the subsequent alteration of ecosystem processes, have had negative consequences on performance, reproduction and recruitment in several plant species (Young & Clarke 2000; Lienert 2004). The two major factors that determine the negative influence of habitat fragmentation on plant populations are the extent of the reduction in population size, and the degree of isolation of fragments. Small populations may experience increased extinction risks because of the influence of different stochastic events, such as random genetic drift (Lowe *et al.* 2005) or disease, and due to increased levels of inbreeding, which may lead to a reduction in fitness of individuals (Lienert 2004). Isolation, on the other hand, may restrict the exchange of pollen and seeds

between fragments because of the disruption of plant-pollinator and plant-seed disperser interactions (Ghazoul 2005), which collectively can reduce gene flow among populations thereby increasing genetic structure and compounding small population genetic effects such as genetic drift and inbreeding.

In addition to the effect of changes in isolation and size of populations, the effect of habitat fragmentation is also determined by its impact on a variety of ecological processes. For instance, some authors have reported changes in the spatial distribution of individuals (i.e., density) within fragmented plant populations (Fahrig 2003; Bruna & Oli 2005), which may affect the foraging behaviour of pollinators, in animal pollinated species. This may then alter mating dynamics at a local scale and have important potentially negative consequences for plant reproduction (Cunningham 2000; Uchiyama *et al.* 2009).

Studies across a variety of species have revealed that the consequences of fragmentation are not uniform, and in some cases, fragmentation appears to have had no detectable negative effects on plant populations. The response of plant populations to fragmentation has been attributed to different life-history characteristics of species, and to the genetic and/or ecological dynamics of populations within species. Recent reviews on the effects of fragmentation on plant populations have also proposed that if there is a bias against reporting neutral effects of fragmentation on plant populations, the effects of inbreeding and loss of genetic variation may be less common than previously thought (Ghazoul 2005; Kramer *et al.* 2008). These mixed empirical signals are mainly due to the complex set of integrated traits that will determine how plant populations cope with the special conditions associated with habitat fragmentation. For instance, when long-

distance gene dispersal is common, genetic theory of small populations might not apply to forest fragments, since genetic isolation may not occur (Cloutier *et al.* 2007; Byrne *et al.* 2008)**.** It is also possible that reports may fail to detect overall significant effects because of the experimental design employed. For example, in long-lived species such as trees, if most sampled individuals are older adults, the structure of the population may reflect that existing prior to fragmentation (Aguilar *et al.* 2008), underscoring the need to examine both the adult and seed or seedling generations in long-lived species (Honnay *et al.* 2008). Thus, to best address forest fragmentation and its effect on plant species, it is necessary to take into consideration the life history of the species under consideration, and to examine both ecological and genetic processes of the populations studied, coupled with information on the history of the fragmented system.

Empirical evidence suggests that while genetic degradation will occur at a slow pace over subsequent generations after fragmentation, ecological consequences may be more rapid and may also pose an important threat to plant population persistence. A major ecological factor that might be altered by fragmentation is reproductive success, which can have a direct impact on extinction risk (Nason & Hamrick 1997; Lennartsson 2002). A reduction of reproductive output after fragmentation has been documented for several species (Fuchs *et al.* 2003; Aguilar & Galetto 2004; Kolb 2005) and may be caused by a reduction of maternal resources or, more probably, by pollen limitation, in which there is a reduction in the availability of potential mates and/or pollen deposition (Quesada *et al.* 2003; Aguilar *et al.* 2006). Given that the degradation of ancestral habitats forces biota to adapt to new ecological conditions, it is optimal to examine both

the genetic and ecological impacts of fragmentation on plant populations to fully assess the impact of habitat fragmentation on population persistence and dynamics.

OBJECTIVES

In this study I assessed the consequences of forest fragmentation on the reproductive biology, genetic diversity and mating system of chokecherry (*Prunus virginiana*, L.), a widely distributed self-incompatible species from temperate regions, by comparing plants from 2 forest fragments with those from a continuous forest remnant in Southern Manitoba. To explore if a reduction in population size and an increase in habitat disturbance have important ecological and genetic consequences, I combined field studies with marker-gene analysis and tested three main predictions: (i) a reduction of reproductive output in fragmented compared to continuous forest; (ii) a reduction in genetic diversity associated with population differentiation in the fragments; and (iii) alteration of the mating system dynamics following fragmentation. To explore these hypotheses, this study was divided into two research components. The first hypothesis is examined in chapter one, in which different ecological aspects of the reproductive biology of chokecherry are explored, including the extent of pollen limitation and the impact of maternal resources and fungal infection on reproductive output. In chapter two, I investigate the last two hypotheses using DNA markers in adult populations and progeny arrays, and explore different indicators of the genetic variability and parameters of the mating system, and estimate the pollen dispersal curve. The findings from chapter two provide further support for those obtained in chapter one addressing the ecological impacts of fragmentation on reproductive success, and suggest that pollen limitation and

biparental inbreeding may be major factors compromising reproductive success in fragmented populations of chokecherry.

2.0. CHAPTER ONE: Effects of fragmentation on reproductive success on chokecherry (*Prunus virginiana,* L.)

2.1. INTRODUCTION

Extensive anthropogenic use of resources, such as increasing urbanization and intensive agricultural practices, has reduced extant continuous historical forests into small, separated forest fragments. The reduction in size and the increase in isolation between populations coupled with changes in the surrounding environment have been shown to affect ecological and genetic processes in many plant species currently occurring in semi-natural habitats. One of the most important negative effects of human-altered environments on plant populations is the impact on reproductive success, for example the observation of reduced fruit set in disturbed habitats, which is predicted to pose a major threat to plant persistence (Luijten *et al.* 2000; Aizen *et al.* 2002).

Different factors have been proposed to account for the observed decline in fruit set after habitat fragmentation, including pollen limitation and limited maternal resources. Pollen limitation, however, has been advocated to be the most proximate cause of reduced reproductive success in fragmented habitats (Wagenius *et al.* 2007). A vast quantity of empirical evidence suggests that after fragmentation, plant populations can experience changes in the quantity and/or quality of pollen that arrives to plants which, in both cases, may lead to a reduction in fruit set whether it be caused by insufficient pollen receipt, or insufficient receipt of compatible or viable pollen (Quesada *et al.* 2003; Aguilar *et al.* 2006).

For species with self-incompatibility (SI) systems, reductions in pollen quality, which frequently ensue reductions in population size, can be particularly severe. Self-incompatibility systems are genetically based mate recognition systems, controlled by the gene products of the highly variable *S*-locus, in which only individuals that express different *S*-proteins can reproduce (de Nettancourt 1997). In members of the Rosaceae and Solanaceae, self-incompatibility is determined by allele specific interactions between the *S*-proteins expressed in the pistil with those expressed in the haploid pollen grain (so-called gametophytic self-incompatibility-GSI). In these families, the female determinant of the rejection reaction, which is an *S*-RNase, interacts with the gene product of the closely linked male determinant, an *F*-box protein, in an allele manner such that selective degradation of pollen grains carrying the same, cognate *S*-haplotype, are rejected. These mating dynamics mean that self-incompatible individuals are heterozygous at the *S*-locus (because like alleles are rejected), and the probability of finding a compatible mate is dependent on the frequency and number of *S*-alleles in a population which, in turn, are dependent on the population size and structure. If there is a significant decrease in population size, which leads to the loss of *S*-alleles via random genetic drift, theory predicts a reduction in the number of compatible mates in the population (Busch & Schoen 2008). This phenomenon is called mate limitation, and may lower the reproductive output of individuals and increase the probability of population extinction (Wagenius *et al.* 2007). Because of the restriction imposed by the self-incompatibility locus, self-incompatible species generally exhibit greater pollen limitation than self-compatible ones (Larson & Barrett 2000).

Given these limitations of self-incompatibility systems, it has been hypothesized that mutations that weaken the SI response might become advantageous when compatible mates are limited and inbreeding depression is weak (Vallejo-Marin & Uyenoyama 2004; Busch & Schoen 2008). After a severe bottleneck, the so-called "reproductive assurance hypothesis" predicts that small self-incompatible populations might select for quantitative variation in the strength of the SI response, with the occurrence of a 'leaky' SI response (Good-Avila *et al.* 2008). Populations or species that exhibit this 'leaky' SI response are generally called partially self-compatible (PSC), and are characterized by having individuals able to fully recognize self pollen and avoid self-fertilization but also harbour individuals that are partially or fully self-compatible (Good-Avila *et al.* 2008).

In addition to the role of the SI system, other life-history traits may also influence the responses of plant populations to habitat fragmentation. Animal pollinated plants, for example, can be further influenced if fragmentation causes changes in the availability of nesting habitat, behaviour, flight patterns or diversity of pollinators, which may result in a reduction in the quantity and/or quality of pollen delivered to the stigmas (Aizen & Feinsinger 1994; Trant *et al.* 2010). Previous studies have documented changes in plant density after fragmentation which, in turn, may affect pollinator flight patterns. Forest fragmentation may increase the "clumpiness" of plant populations, due to the patchy distribution of suitable habitat or restricted seed dispersal (Ghazoul 2005; Ferrer *et al.* 2009), which may favour shorter intermediate distances and enhance pollen transfer among nearby trees (Garcia *et al.* 2005; Collevatti *et al.* 2010). This foraging behaviour may lead to a reduction in the effective neighbourhood pollination area and an increase in mating between neighbours which are closely related (i.e., biparental inbreeding).

Moderate to high levels of biparental inbreeding have been reported in PSC (Abbott & Forbes 1993; Sun & Ritland 1998) and self-incompatible species, even when SI is expected to limit consanguineous mating (Willi 2009). In these circumstances, it is likely that biparental inbreeding depression will negatively influence reproduction, causing a reduction in fruit set (Hirao 2010) and in the total relative fitness of inbred offspring (Nason & Ellstrand 1995; Vogler *et al.* 1999).

A different proximate limiting factor affecting fruit set is resource limitation, when even after an adequate pollination, only a portion of the flowers develop into fruits (Stephenson 1981). Different factors, such as biotic interactions, can affect the resources available for plant reproduction. If plants become more susceptible to parasites following fragmentation, for example, infected plants may shift the allocation of resources destined for reproduction to defense. This has been documented in a number of natural populations which experienced both an increase in susceptibility to infection after fragmentation, and a concomitant reduction in reproductive output (Lienert 2004; Benítez-Malvido & Arroyo-Rodríguez 2008).

In this chapter, I address the hypothesis that forest fragmentation will reduce reproductive success and increase pollen limitation, by comparing two fragments of forests with one continuous population of chokecherry (*Prunus virginiana*). To detect a reduction in reproductive output, I compare fruit set following natural pollination in the fragmented populations and the continuous site. Pollen limitation was examined by comparing fruit set from natural pollination with that from supplemented pollinations, and by exploring the effect of density on maternal fruit set. I also assess the effect of infection by the fungus *Apiosporina morbosa* as another possible cause for the reduction

in the fruit production after fragmentation, and include tree size as an indirect indicator of maternal resource availability. Finally, I estimate the strength of SI using hand self-pollinations and closed controls, to look for changes in the mating system among populations.

2.2. METHODS

STUDY SPECIES

Chokecherry is a perennial, deciduous, large shrub or small tree in the Rosaceae. Its flowers, which appear from April to July, are perfect, aromatic, 0.6 to 1 cm in diameter, with five white petals that are arranged in cylindrical racemes. The fruits are formed a couple of months following the flowering season, and contain one seed. Chokecherry trees can reproduce vegetatively by root rhizomes which can form networks and extend up to 15m from the base of the tree (Geyer *et al.* 2008), or it can reproduce sexually by animal-pollination. Chokecherry flowers are pollinated by flies, butterflies, early-flying bees, especially in the genera *Andrena, Bombus, Anthophora,* and *Osmi,* and to a lesser degree by hummingbirds (Vicens & Bosch 2000; Geyer *et al.* 2008; Miliczky 2008). Screening for *S*-linked RNase activity in pistils (data not shown) and amplification of *S*-alleles (chapter two) suggests that the species has the self-incompatibility system typical of the Rosaceae family.

Chokecherry is distributed in a large geographic area ranging from Alaska to Newfoundland, throughout southern Canada and much of the US. It grows in many habitat types and plant associations, and occurs naturally in a wide range of soil types and

textures, from soils with almost no depth or fertility, to deep virgin grasslands, with deep profiles and a high level of nutrients (Geyer *et al.* 2008).

STUDY SITES

This research was conducted in three populations located in provincial parks and forests in southeast Manitoba, Canada (Figure 2.1). The first population included an area of approximately 7 km^2 and is located in Beaudry Provincial Park (BP) along the Assiniboine River (lat 49° 52' 3.07" lon 97° 28' 31.45"). The second population is located in Birds Hill Provincial Park (BH) north of Winnipeg, and included approximately 35 km^2 (lat 50° 0' 39.82" lon 96° 54' 54.51"). BP and BH were considered to be fragments since they are small remnants of forest in an intensively used agricultural landscape. The third site (GB) is a continuous population that is located north of Winnipeg, and encompasses two Provincial Parks (Grand Beach and Brighstone Sand Hills), one provincial forest (Belair) and one Provincial wild life management area (Catfish Creek), with a total sampling area of approximately 350 km^2 (lat 50° 33' 43.75" lon 96° 28' 50.60"). The surface area of each sampling site was calculated measuring all the external limits using satellite maps from Google Earth (Google Inc. 2010). Temperature data for the duration of bloom in the populations was obtained from Environment Canada (http://www.climate.weatheroffice.gc.ca) and used to detect variations among locations and between years.

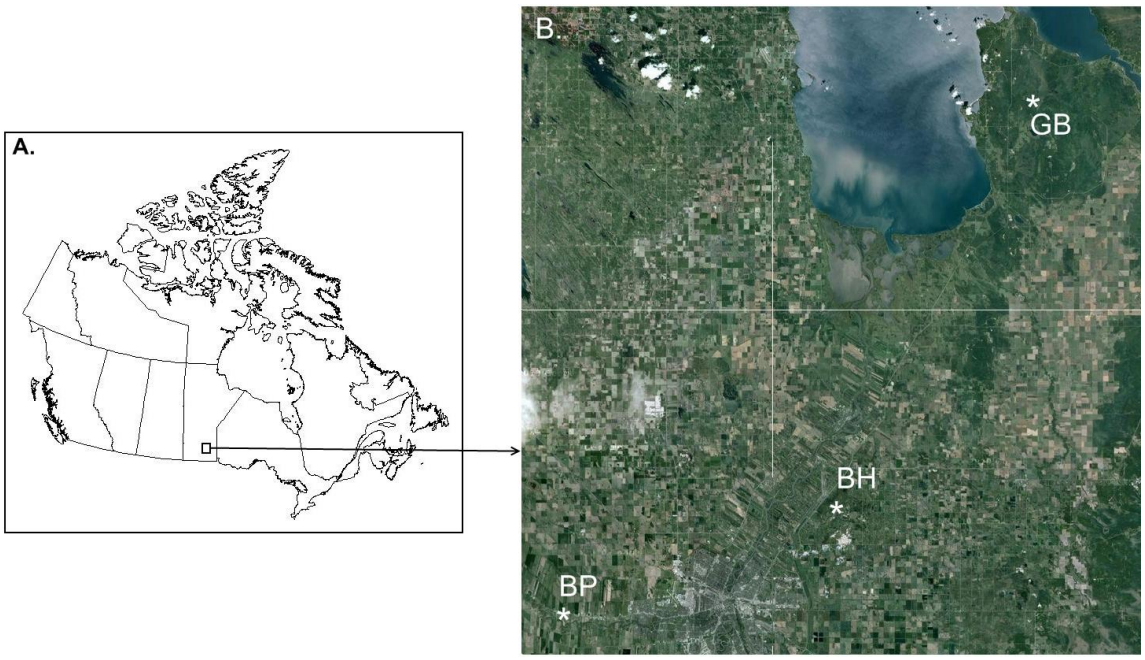

Figure 2. 1. A. Map of Canada showing the location of the study sites; B. Map of the three sampling locations used to explore the effects of fragmentation on chokecherry trees. BP and BH were considered to be fragments since they are small remnants of forest in an intensively used agricultural landscape. GB was considered as a continuous population.

FRUIT SET FROM OPEN POLLINATIONS

To explore the effects of fragmentation on the reproductive output of chokecherry, I selected and tagged 20 trees within each population (60 trees in total) before blooming; however in 2009 only 54 trees produced flowers. Two inflorescences per tree were randomly chosen, tagged and the number of flowers recorded. To avoid seed predation, inflorescences were covered with bridal veil bags three weeks after the flowering season. Since pollination lasts only 3-4 days, bagging the inflorescences did not interfere with the natural pollination process. Two months after the flowering season,

the fruits were collected. To test for significant differences in open fruit set, the data from 2009 and 2010 were analyzed separately using one-way analysis of variance (ANOVA), with population as an explanatory variable. When the main effect of population was significant, Tukey's post-hoc multiple comparison tests were employed to compare population means.

MATERNAL RESOURCES: SIZE, NUMBER OF INFLORESCENCES AND FUNGAL INFECTION

The effect of limited maternal resources on fruit set was explored using four estimates of maternal resource availability: the number of inflorescences per tree and measurements of the height, diameter, and width of trees during the 2010 field season. To eliminate the effect of correlation among size variables, a principal component analysis (PCA) was performed in which the number of inflorescences was also included as an explanatory variable because it was found to have a positive correlation (though not significant) with the three size measurements; bigger trees presented more inflorescences. The first axis of the PCA (*PC1*), which explained 58% of the total variation, was used in further analyses as an indirect indicator of maternal resource availability. All of the variables had a positive contribution to the first axis (Table 2.1), which allowed a straightforward interpretation of the relationships between fruit set and maternal resources; trees with higher *PC1* values were bigger and produced more inflorescences.

Table 2. 1. Contribution of the original size variables to *PC1* (the first axis of the PCA). The first axis explained 58% of the total variation, and all the variables had a positive contribution.

Diameter	Height	Width	No. Inflorescences
0.607	0.571	0.442	0.332

Chokecherry is highly susceptible to attack by the fungus *Apiosporina morbosa* (Zhang *et al.* 2005), an infection characterized by the presence of black misshapen cankers on branches and stem. These swellings are likely to alter the allocation of nutrients with a shift in resources towards defense and away from fruit production (McFadden-Smith *et al.* 2000). To explore the effect of fungal infection on fruit set, the stage of infection was recorded in the trees in which pollination treatments were performed in each population. The stage of infection was classified using 4 categories based on the number of branches affected; (0) for trees without cankers, (1) for trees with cankers in less than half of the branches, (2) for trees with canker in more than half of the branches, and (3) for trees with canker on the main stem. Since an infection on the main stem will cause a rapid decline and will limit the ability of the tree to recover (Snover 2002), individuals were classified in category (3) independently of the number of cankers on the branches. Unfortunately, the number of trees per category was very low; therefore, trees from all categories of infection were pooled to improve statistical power. To estimate significant differences in the occurrence of non-infected and infected individuals in the populations, a chi-square test of independence on the frequency distribution was performed.

An analysis of covariance (ANCOVA) was used to explore the effects of fragmentation, maternal resources and fungal infection on fruit set and their interactions. Fragmentation (populations) and presence of fungus were included in the model as categorical explanatory variables, whereas size (as *PC1*) was a continuous covariate. The full model was reduced to the best fit model by removing and testing non-significant terms, until reaching a minimal adequate model using the Akaike's information criterion (AIC).

POLLEN LIMITATION: HAND-CROSSES AND NEAREST NEIGHBOURS

To explore the presence of pollen limitation in populations, two additional inflorescences were randomly chosen per tagged tree, and covered with bridal veil bags before bloom. During bloom, I applied fresh pollen from one donor located more than 20m away from the focal tree to avoid pollinating within clones (Geyer *et al.* 2008).

A Wilcoxon rank sum test was used to identify significant differences between fruit set after open- (natural) and cross- (supplemented) pollinations within populations. Pollen limitation was assessed using the pollen limitation index (*L*) estimated in each tree as: $1-P_o/P_{c,}$ were P_o is the fruit set from open controls, and P_c fruit set from hand cross-pollinations (Larson & Barrett 2000). One way ANOVA's were performed to detect significant differences in pollen limitation of chokecherry among populations, using *L* as the dependent variable, and population as a fixed main effect. When the main effect of population was significant, Tukey's post-hoc multiple comparison tests were employed to compare population means. One way ANOVA's were also used to detect significant differences in the fruit set after cross-pollinations, using population as an explanatory

variable.

Since the strength of pollen limitation has shown to be correlated with local population density (Ghazoul 2005; Ferrer *et al.* 2009), the approximate density and the average distance of the four nearest neighbours were estimated in each population. The density of plants in populations was estimated as the mean of the total number of individuals recorded in 10 randomly chosen patches of $100m^2$ in each population. The spatial arrangement of individuals within populations was further estimated as the mean distance to the closest four neighbours within a 15 m radius, and the average distance to four neighbours beyond the 15m radius circle. The purpose of this classification was to include clones in the circle as the four nearest neighbours, but to exclude them from the area beyond the circle. Since I was testing limitation in fruit set due to limited available mates, individuals were recorded only if they were flowering. To determine if fruit set is limited when neighbours are further away, two linear regressions were performed; one with the average distances within 15m radius circle, and the other with the distances outside the circle. The average distance to the neighbours was used as the explanatory variable and fruit set as the response variable.

SELF INCOMPATIBILITY

Although chokecherry has a SI system, the production of fruits following both self-pollination (assisted) and closed controls (unassisted), was observed to look for evidence of a partial breakdown in the SI system. To estimate differences in fruit set after self-pollination among populations, the self-compatibility index (*SC*) was calculated as P_s/P_c, where P_s is the fruit set after self-pollination in a plant, and P_c is the fruit set after

cross-pollination in the same plant. In addition, the autogamy index (*A*) was calculated to examine the ability of individuals to set selfed seed autogamously (i.e. in the absence of pollinators), by comparing the production of fruits following unassisted pollination (closed controls) and open controls. The mean fruit set in both reproductive seasons from closed versus open controls and following self- versus cross-pollination were used to estimate the *SC* and *A* index respectively. Individuals were then grouped by their *SC* index into four categories following Ferrer *et al.* (2009): (i) strongly self-incompatible (*SC* index = 0), (ii) self-incompatible (0 < *SC* index < 0.149), (iii) partially self-incompatible (0.15 < *SC* index < 0.49) and (iv) self-compatible (*SC* index > 0.5).

The relationship of *SC* and distance to nearest neighbours was explored using linear regressions, with *SC* as the dependent variable and aggregation of plants within and beyond 15m as independent variables respectively. To detect a relationship between *SC* and open fruit set, and to look for differences in *SC* among populations, an analysis of covariance (ANCOVA) was performed using fruit set as the response variable, population as categorical explanatory variable and *SC* index as continuous explanatory variable. The same models were also employed to assess the effect of *A* on fruit set.

Lastly, to assess variation in the strength of SI, the growth of self- versus cross-pollen tubes in pistils was compared in three individuals per population. Pistils were collected 42h after self- or cross-pollinations, immediately fixed in a solution of 3:1 acetic acid:ethanol for 1h and then stored in 70% ethanol until staining. The visualization of pollen tubes using aniline blue was carried out following the protocol in Myra *et al.* (2006). Additional flowers from tagged trees were emasculated to test for agamospermy. However, there was no evidence of the ability to set seeds without fertilization in any tree

explored.

SUMMARY OF THE STATISTICAL ANALYSES

In Table 2.2, I summarize all the models used to examine the effect of pollen limitation (natural vs. cross-pollinations; *L*), distance to nearest neighbours, fungal infection, *PC1* (indirect indicator of available maternal resources), and *SC* and *A* index, on reproductive output. The fruit set values from natural and cross-pollinations, and *L* values, were transformed using the the arcsine square root of the original values, to meet the ANOVA assumption of normally distributed residuals. All the statistical analyses were conducted in R v. 2.10 (R Development Core Team, http://www.r-project.org)

Table 2. 2. Summary of models used in the analyses of the effect of fragmentation on reproductive success in chokecherry. Response variables were: P_o = fruit set from open controls, P_c = fruit set from hand cross-pollinations, *L*= pollen limitation index and *SC* = self-compatibility index. Location indicates if the analysis was performed per population (PP) or over all populations (P). Year refers to those years in which data were obtained for that analysis. Significance indicates if the analysis showed significant effects (Yes) or not (No). Read methods for details. Asterisks (*) represent analyses also implemented on the autogamy index (*A*).

Test	Explanatory variable	Response variable	Location	Year	Significance
ANOVA Tukey's tests	Populations	P_o	PP	2009/ 2010	Yes
Chi-square	Incidence fungal infection	-	PP	2010	Yes
ANCOVA	Populations *PC1* Fungal infection	P_o	PP	2010	Yes
Wilcoxon rank sum	Type of pollination (P_o - P_c)	-	PP	2009/2010	Yes
ANOVA	Populations	P_c	PP	2009/2010	No
ANOVA Tukey's tests	Populations	*L*	PP	2009/2010	Yes
Linear regressions	Distance to Neighbours within 15m/ outside 15m	P_o	P	2010	No
Chi-square	Categories based on *SC*	-	PP	average 2009-2010	No
*Linear regressions	Distance to Neighbours within 15m/ outside 15m	*SC*	P	average 2009-2010 (for *SC*) 2010 (for Neighbours)	No
*ANCOVA	Populations *SC*	P_o	PP	average 2009-2010	No

FITNESS AND INBREEDING DEPRESSION

Seeds from open and closed controls, and from self- and cross-pollinations, were collected to look for differences in germination rate. Seeds collected in the first season were stored in papers bags, kept dry for 2 months and then subjected to stratification by keeping them at 4°C for 20 weeks in small petri dishes with wet filter paper, following Auger's *et al*. (2002) protocol for chokecherry germination. After 4 weeks, the seeds became infected with fungus suggesting that this protocol needs the application of a pre-fungicide treatment. The seeds were washed with abundant water and dried for one month. A second stratification experiment was implemented but now using soil (W. Dai lab, NDSU, *personal communication*). After 5 months at 4°C, the seeds were transferred to a growth chamber with continuous cycles of 15 hours at 25°C followed by 9 hours at 15°C. After two months in the growth chamber, only 19 from 564 seeds (3.3%) germinated. Unfortunately, this low germination rate did not allow the use of any statistical analysis to draw reliable conclusions about inbreeding depression. The seeds were transferred again to a cold room to start a stratification process, following Morgenson's (1986) protocol for Mongolian cherry (*Prunus fruticosa*) and Amur chokecherry (*Prunus maackii*). Morgenson indicated that 30 days of warm stratification followed by 90-120 days of cold will increase germination in these two hard cherry species. However, after more than 120 days at 4°C none of the seeds germinated. Seeds from the second field season have been in paper bags for 5 months, and will be transferred to a cold room in trays with soil; however germination data from these seeds are not be included in this thesis.

BIPARENTAL INBREEDING IN CROSS POLLINATIONS

To look for the presence of inbreeding depression in fruit set arising from mating among relatives (biparental inbreeding depression), the kinship coefficient between the pollen donors used for the hand cross-pollination and the maternal plant to whom the donor was mated, was estimated and compared to the fruit set produced by the pair. The kinship coefficient was estimated using genetic data from eight microsatellite markers (see chapter two for details) used to genotype trees from one of the fragments (BP) and the continuous population (GB). The kinship coefficient estimates the probability of identity by descent of genes (Ritland & Ritland 1996). In the case of sib-families derived from non-inbred diploid parents, the kinship between sibs is expected to be 0.125 for half-sibs and 0.25 for full-sibs (Lynch & Walsh 1998). The kinship coefficients among pairs of individuals were calculated as described in Loiselle *et al*. 1995 using the software SPAGEDI V. 1.3a (Hardy 2002). A reduction in fruit set when the kinship exceeded 0.125, indicates the presence of biparental inbreeding depression. A regression model was not employed due to the low number of crosses obtained; plots of fruit set as the dependent variable versus kinship as the independent variable, were used instead to explore if there was a reduction in fruit set as a consequence of biparental inbreeding in each population.

2.3. RESULTS

FRUIT SET IN OPEN CONTROLS

During bloom, temperature did not vary among populations within years, but it

did between years. In 2009 the mean and maximum temperature in bloom were 3°C and 6°C higher (20.5°C - 27°C) respectively, compared to 2010 (17.5°C – 21.1°C). The minimum temperatures were similar, being 14°C in 2009 and 13.6°C in 2010. The mean fruit set in all plants from one population, calculated as the percentage of fruits per total number of flowers in an inflorescence, ranged from 0.17(GB) to 0.06 (BP) during the first season, and from 0.20 (GB) to 0.04 (BP) during the second season (Table 2.3). A significant difference in the reproductive output of chokecherries among populations was found during both seasons (Table 2.4). Tukey's tests showed that in 2009, GB displayed a significantly higher fruit set under natural conditions (open control), compared to BP (Tukey's test $p<0.05$). Open fruit set in GB was also higher than in BH, however the difference was not significant (Tukey's test $p=0.19$). Similarly, during the second year GB had the highest open fruit set, and the difference was significant when compared with each fragment (Tukey's test $p<0.001$). There were no significant differences between the fragments in either year (Tukey's test $p>0.05$). These results suggest a population effect on the reproductive output in chokecherry trees.

Table 2. 3. Mean fruit set from open controls of two fragments of forest (BP and BH) and one continuous population (GB) of chokecherry, examined for each year of the study. Standard deviations are in parentheses.

	BP	**BH**	**GB**
2009	0.064 (SD 0.081)	0.111 (SD 0.141)	0.172 (SD 0.127)
2010	0.043 (SD 0.052)	0.114 (SD 0.084)	0.201 (SD 0.108)

Table 2. 4. Analysis of variance of mean open fruit set among three populations of chokecherry for each year of the study.

	2009				2010			
Effect	DF	SS	MS	F	DF	SS	MS	F
Populations	2	0.334	0.162	3.265*	2	0.893	0.446	12.630***
Residuals	51	2.613	0.054		57	2.016	0.035	

* $p<0.05$; *** $p< 0.0001$

MATERNAL RESOURCES: SIZE, NUMBER OF INFLORESCENCES AND FUNGAL INFECTION

To explore the effect of fungal infection on fruit set, the presence of infection was recorded in the trees on which pollinations were performed in each population. The frequency distribution of the number of infected trees differed among populations as shown by chi-square analyses. GB displayed a higher number of healthy trees (without infection) than the overall average in all populations ($\chi^2_{df=1}$ 5.985, $p<0.05$), while BH showed the highest number of infected trees compared to the other populations ($\chi^2_{df=1}$ 8.653, $p<0.005$). BP on the other hand, did not show deviation from expectation and more than half of the trees samples were not infected ($\chi^2_{df=1}$ 1.491, $p=0.225$) (Figure 2.2).

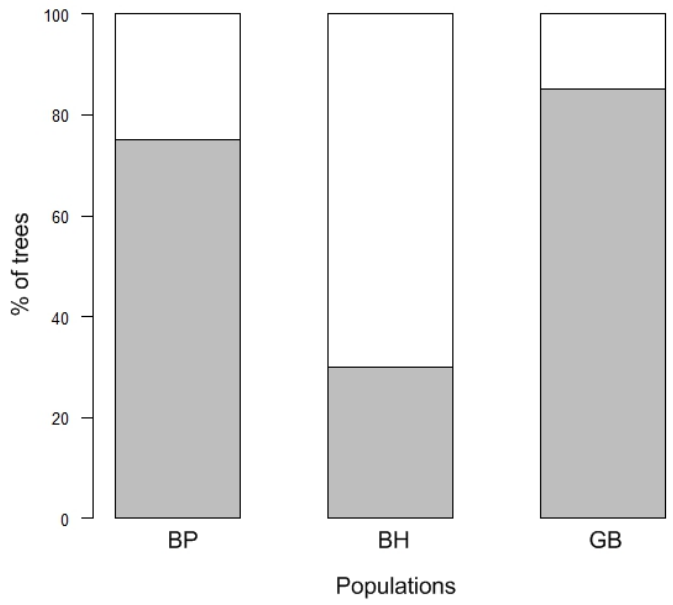

Figure 2. 2. Percentage of healthy (grey) and infected (white) chokecherry trees in two fragments of forest (BP and BH) and one continuous population (GB). There was a significant excess and deficit of infected trees in BH and GB respectively.

Results from the ANCOVA (Table 2.5) were consistent with previous analyses and revealed that population was the only main factor with significant effects on fruit set (F 15.801, p<0.0001). However, the interactions between fungus and *PC1* also significantly affected fruit production (F 5.943, p<0.05), as indicated by the different slopes from non-infected and infected individuals (Figure 2.3). Infected trees tended to produce more fruits when larger, while non-infected individuals showed the opposite trend. In addition, the interaction between population and fungal infection also had a significant effect on fruit set (F 4.336, p<0.05) (Figure 2.4), because infected trees from BP have more fruits that non-infected ones; contrary to BH and GB where infected trees show a reduced reproductive output.

Table 2. 5. ANCOVA of the relationship between fragmentation (Pop), fungal infection (Fungus) and *PC1* with fruit set.

Effect	DF	SS	MS	F
Pop	2	0.895	0.448	15.801***
Fungus	1	0.083	0.083	2.956
PC1	1	0.089	0.089	3.149
Pop x Fungus	2	0.245	0.122	4.336*
***PC1* x Fungus**	1	0.168	0.168	5.943*
Residuals	48	1.359	0.028	
Multiple r^2	0.522			

* $p<0.05$; *** $p< 0.0001$

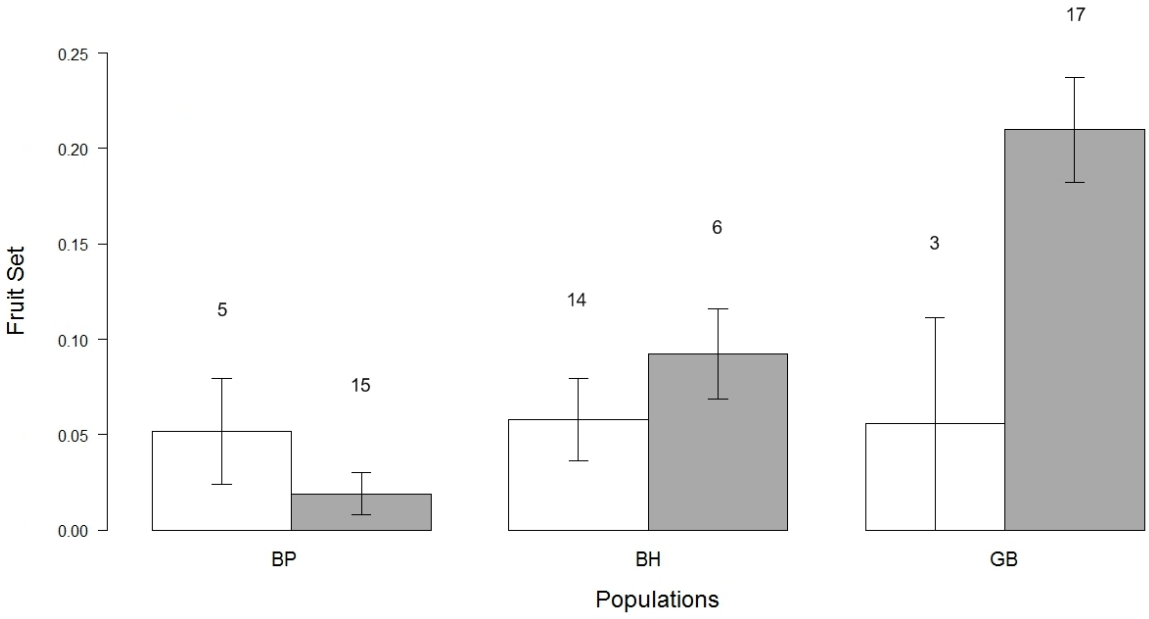

Figure 2. 3. Relationship between population and fungal infection, and their effect on fruit set. Fruit set from non-infected (grey bars) and infected (white bars) individuals is displayed in each population. The numbers of infected and non-infected individuals in each population is indicated at the top of each bar.

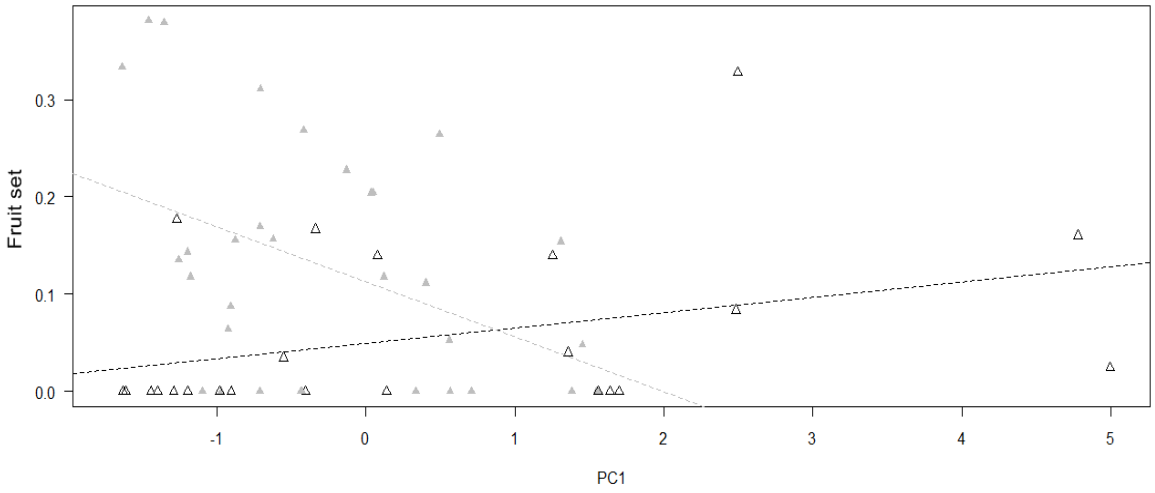

Figure 2. 4. Relationship between *PC1* and presence of fungus, and its effect on open fruit set. This is a significant relationship due to the different slopes from non-infected (grey slope and grey triangles) and infected trees (black slope and open triangles).

POLLEN LIMITATION: HAND-CROSSES AND NEAREST NEIGHBOURS

Cross-pollinations revealed the presence of pollen limitation in both fragmented populations. In BP and BH, the mean number of fruits set following hand cross-pollination was consistently higher than that in the open controls; however the difference was only significant in BP in 2010 (Figure 2.5). In GB, on the other hand, both treatments resulted in very similar fruit set. Hand cross-pollination also revealed a reduction in the fruit set in BP when pollen is supplemented, compared to BH and GB (Figure 2.5), suggesting that factors others than pollen may exacerbate the reduction of fruit production in BP.

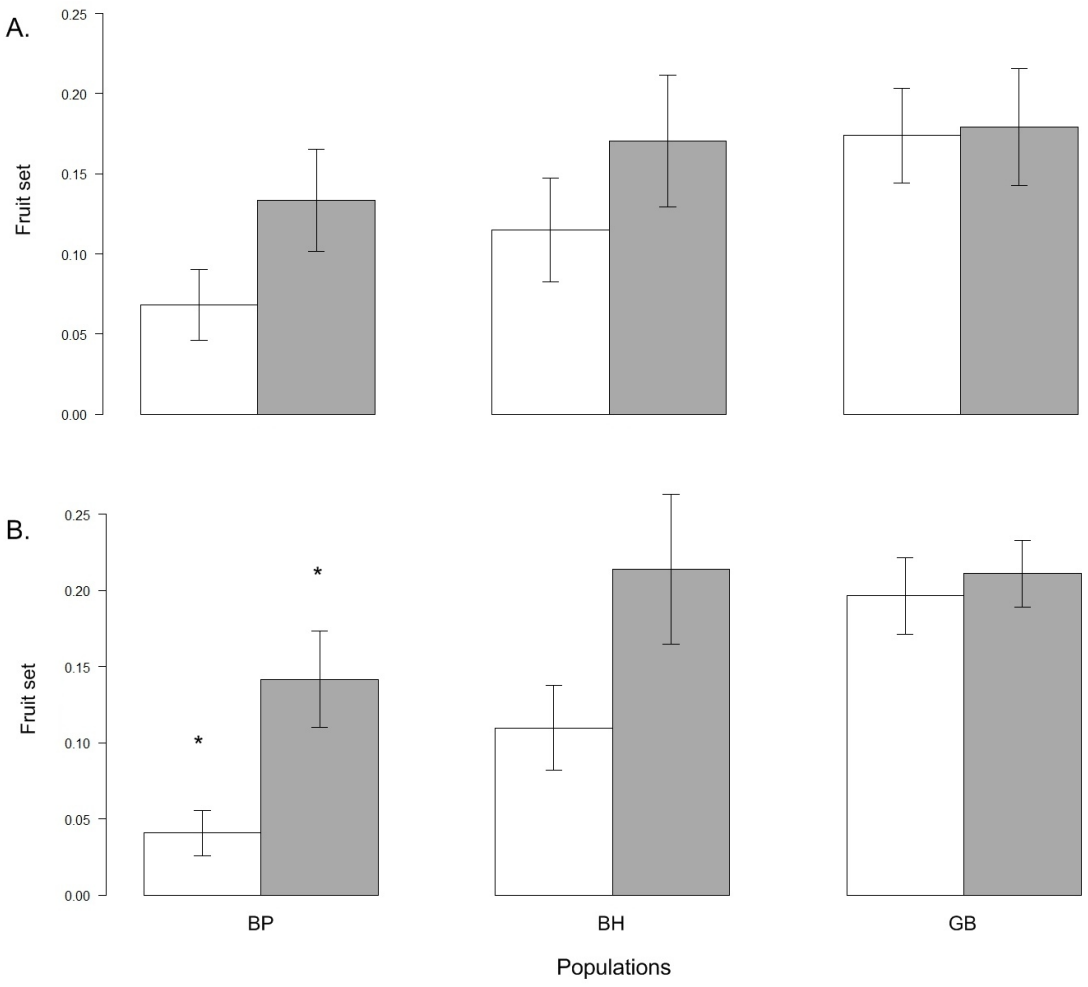

Figure 2. 5. . Mean fruit set for three populations (BP, BH, and GB) of chokecherry trees following open controls (white bars) and cross-pollinations (grey bars); from 2009 (A) and 2010 (B). Asterisks (*) represent significant difference between the two treatments within a population.

The estimates of the pollen limitation index (*L*) for both seasons are shown in Table 2.6. Results from the one way ANOVAs using *L* as the response variable, indicated that there was a significant difference in pollen limitation among populations in both years (Table 2.7). In 2009, both fragments had similar levels of pollen limitation, and in

both populations, pollen limitation was significantly greater than in GB (BP - GB: Tukey's test $p<0.05$; BH - GB: Tukey's test $p<0.005$). In 2010, BP showed not only higher pollen limitation than in 2009 but also higher limitation than BH. However, the difference between *L* in the fragments was not significant. Once more, when pollen limitation from each fragment was compared to GB, significant differences were found (BP - GB: Tukey's test $p<0.001$; BH - GB: Tukey's test $p<0.05$).

Table 2. 6. Pollen limitation (*L*) in two fragments of forest and one continuous population of chokecherry, in two consecutive years. *L* was defined as $1-P_o/P_c$, where Po is the fruit set from open controls, and Pc fruit set from hand-cross pollinations. Standard deviations are in parentheses.

	BP	**BH**	**GB**
2009	0.555 (SD 0.407)	0.598 (SD 0.381)	0.172 (SD 0.295)
2010	0.701 (SD 0.414)	0.477 (SD 0.347)	0.155 (SD 0.236)

Table 2. 7. Analysis of variance of pollen limitation (*L*) among two fragments of forests (BP and BH) and one continuous population (GB) of chokecherry, over two consecutive years.

	2009				**2010**			
Effect	DF	SS	MS	F	DF	SS	MS	F
Populations	2	3.436	1.718	6.339**	2	4.366	2.182	9.039***
Residuals	38	10.292	0.271		34	8.219	0.241	

**$p<0.005$; *** $p< 0.0001$

Estimation of the local population density showed that there was considerable variation in density of individuals among and within populations. Excluding the patches in which I did not find chokecherry trees, the density in BP ranged from 1.445 to 0.025 trees/ m^2, in BH ranged from 0.795 to 0.013 trees/ m^2 and in GB from 0.451 to 0.013 trees/ m^2. Due to the variation among patch density within populations, the average density in the populations, including patches with no trees, did not show significant differences (Figure 2.6); however, general patterns were identified. The highest density patches were found in BP but there were also patches with no trees. In BH, the density of individuals among patches was intermediate (compared to BP and GB), and all selected patches had at least one tree. On the other hand, densities were, on average, lower in GB and there was less variation in density among patches.

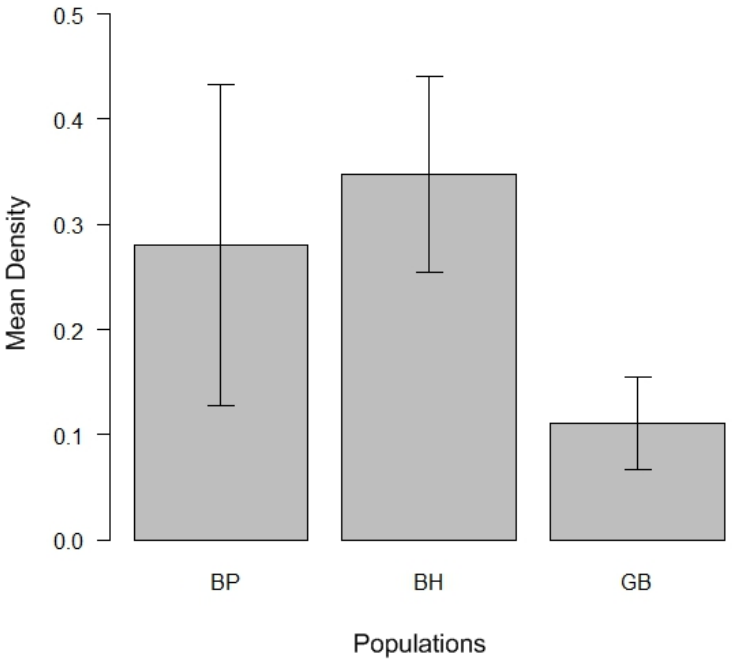

Figure 2. 6. Mean density per population, estimated as the mean of the total number of individuals recorded in 10 randomly chosen patches of 100m^2 in each population.

The average distance to the four nearest neighbours within a 15m radius circle, was similar for all individuals in the three populations, and was not more than 5m (averages in meters: BP 3.852 SD 4.707; BH 4.577 SD 4.548; GB 2.576 SD 4.369). Only 7 of the 63 individuals sampled in the three populations had nearest neighbours beyond 5m. Beyond the 15m circle, most of the individuals had the four neighbours within 25m, and only 4 out of the 63 individuals had neighbours beyond that distance (averages in meters: BP= 23.201 SD 11.451; BH=20.658 SD 3.994; GB=19.852 SD 4.032). I did not find any relationship between open fruit set and the average distance to the neighbours within and outside a 15m radius circle, based on regression analyses (data not shown).

SELF INCOMPATIBILITY

Although chokecherry has a SI system, production of fruits after self-pollination (assisted) and closed controls (unassisted) was observed. The average *SC* index was slightly different among population (mean ± standard error: 0.076 ± 0.067, 0.183 ± 0.093 and 0.184 ± 0.120 for BP, BH and GB respectively).

The frequency distribution of trees according to the four categories of the *SC* index, and the population in which they occur, is shown in Figure 2.7. Individuals from GB and BH were found to have individuals from all four SC categories, and the former had the highest proportion of self-compatible individuals of all three populations. BP on the other hand, did not have any self-compatible individuals, and most trees were strongly self-incompatible. A chi-square test of independence to examine the frequency distribution of SC category among populations, did not show significant differences among populations ($\chi^2_{df=6}$ 5.188, p= 0.527).

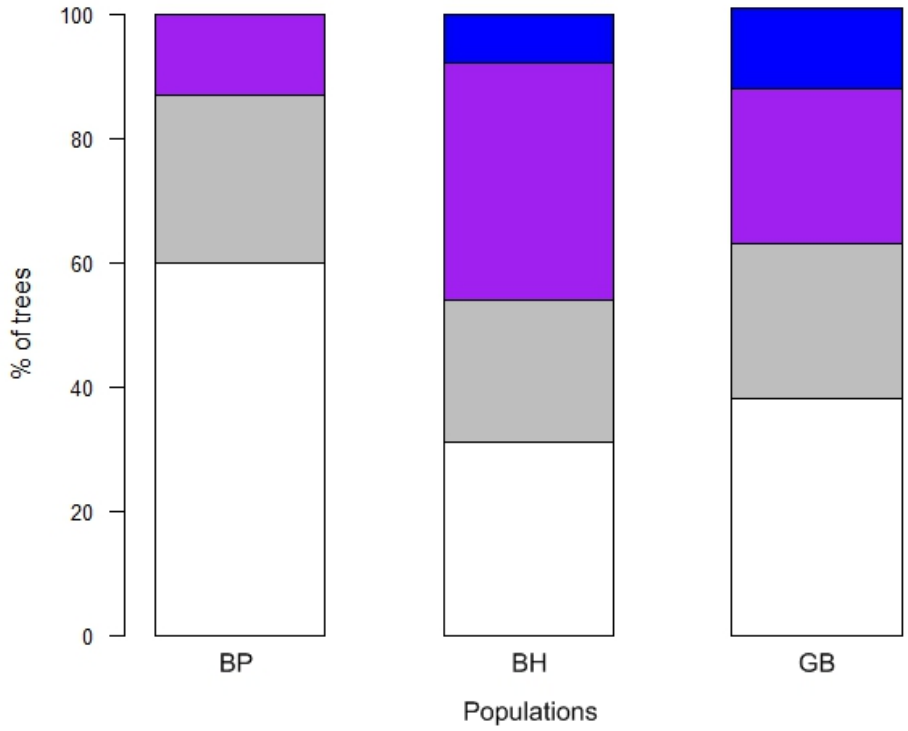

Figure 2. 7. Frequency distribution of chokecherry trees in two fragments of forest (BP and BH) and a continuous population (GB), according to SI categories. SI categories were defined as follow: (i) strongly self-incompatible (*SC* index = 0) (white bars); (ii) self-incompatible (0 < *SC* index < 0.149) (grey bars); (iii) partially self-incompatible (0.15 < *SC* index < 0.49) (purple bars); self-compatible (*SC* index > 0.5) (blue bars). See methods for definition of *SC* index.

The aggregation of plants within and outside a 15m radius circle did not have an effect on *SC* index, as revealed by the linear regression analyses (data not shown). Similarly, the ANCOVA showed no significant contribution of *SC* to fruit set, or interaction in the slopes of *SC* among populations (Table 2.8).

Table 2. 8. ANCOVA of the relationship between populations and *SC* index, as predictor variables of the fruit set in chokecherry trees.

Effects	DF	SS	MS	F
Populations	2	0.271	0.134	18.487***
SC	1	0.006	0.006	0.760
Populations x *SC*	2	0.013	0.007	0.932
Residuals	35	0.256	0.007	

*** $p < 0.0001$

The Autogamy index, *A*, was slightly higher for GB (0.120 ± 0.087) compared to BP (0.110 ± 0.093), and was lowest in BH (0.069 ± 0.061), however the differences were not significant. Similarly, the correlations between *A* and the distance to nearest neighbours, and between *A* and fruit set in the open controls, were not significant (data not shown), indicating effectively no effect of *A* on the production of fruits.

Examination of styles following self- and cross-pollination revealed conspicuous differences in pollen tube growth in strongly self-incompatible individuals (Figure 2.8). After self-pollination, pollen tube growth was typically arrested in the first half of the style (Figure 2.8A), whereas after cross-pollination, pollen tubes grew to the ovary (Figure 2.8B). In individuals that were able to set fruits after self-pollination, self pollen tubes were observed to grow further, although somewhat more slowly than cross pollen tubes, and may not have reached the ovary (Figure 2.8C).

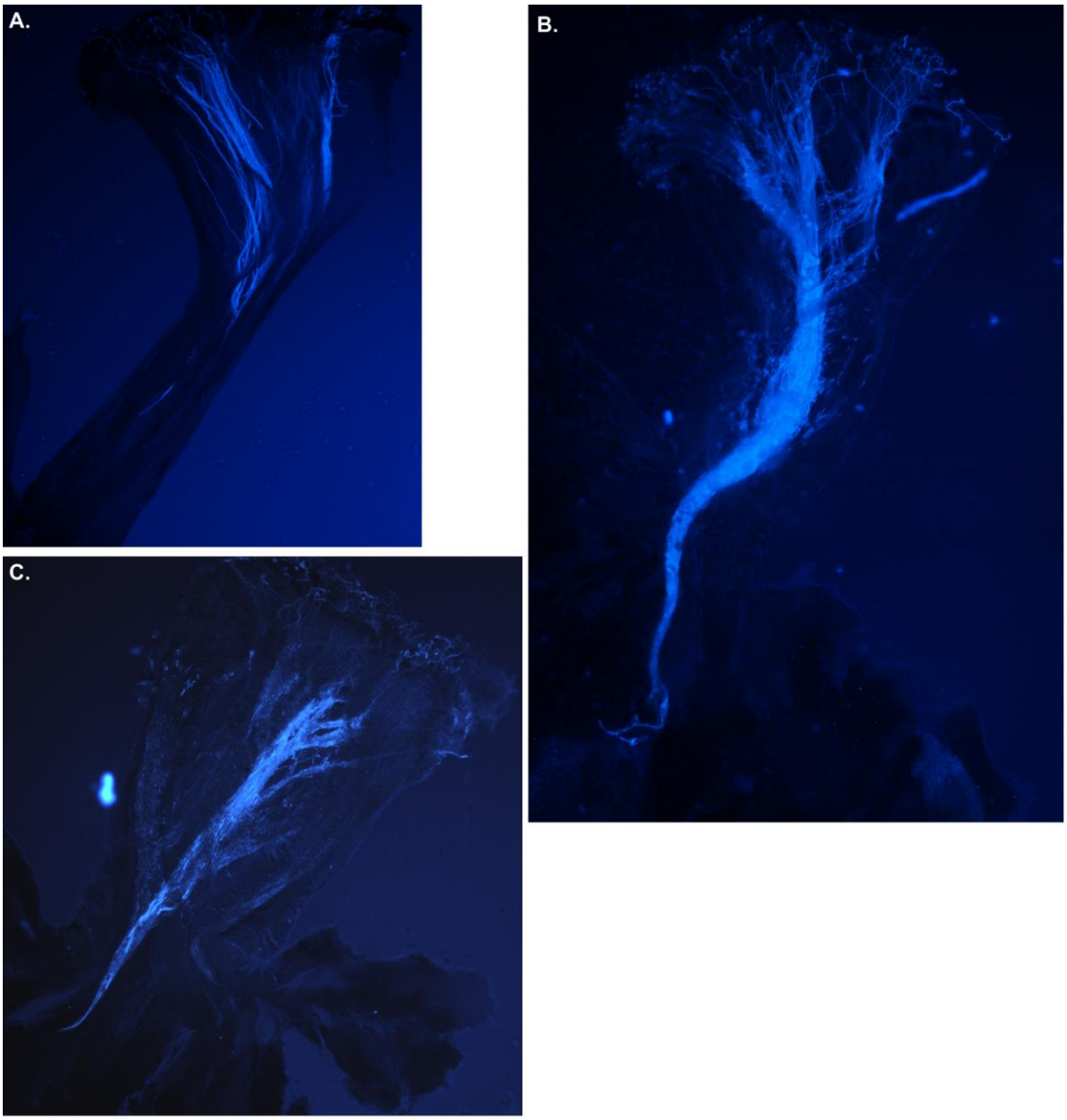

Figure 2. 8. Growth of pollen tubes in the style of chokecherry flowers 42 h following hand self- (A) and cross-pollination (B) in a strongly self- incompatible individual; and after self- pollination in a partially self-incompatible individual (C) 100X.

BIPARENTAL INBREEDING DEPRESSION IN CROSS POLLINATIONS

According to results from the genetic analyses, most of the crosses in BP were between non-related pairs; however three trees received pollen from half-sibs (Figure 2.9A). These crosses did exhibit a reduction in fruit set compared to that set for non-related pairs. However, most of the mating pairs, even those with no evidence of relatedness, showed low fruit set. In GB almost all pairs were not related except for two full-sibs pairs. As expected, the fruit set from these pollinations was highly reduced (Figure 2.9B), supporting the hypothesis that after crosses between close relatives there would be a reduction in reproductive output.

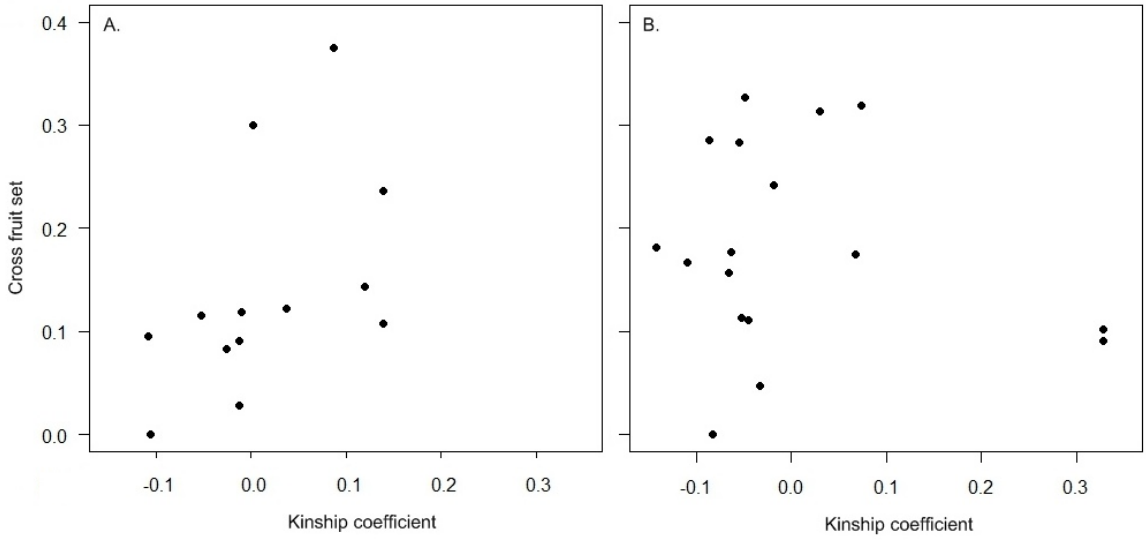

Figure 2.9. Relationships between kinship coefficient and fruit set after cross-pollination in BP, a fragment of forest (A), and a continuous population, GB (B), of chokecherry trees.

2.4. DISCUSSION

The results obtained in this research clearly indicate a reduction in reproductive output (namely fruit set) in the fragmented populations compared to the continuous population. After exploring possible causes for this outcome, my results highlight pollen availability as one of the most important limiting factors for fruit production; a finding that is in agreement with other studies in which fragmentation has been observed to cause an increase in pollen limitation (Wagenius *et al.* 2007). One possible cause of pollen limitation in the fragments, could be variation in the density of individuals in continuous compared to fragmented sites (Ghazoul 2005). I estimated the distance to nearest neighbours and evaluated its contribution to pollen limitation in the fragments, but failed to detect a significant effect of neighbourhood size on pollen limitation. However, the density of individuals was greater in the fragmented sites, and this may favour shorter inter-mate distances and thereby increase the relatedness of male-female pairs. Because these chokecherry populations harbour a SI system, if the relatedness of male-female pairs increases, there could be an increase in the number of incompatible mates, which would reduce the "quality" of pollen.

In addition to the effect of pollen limitation, I also explored the effect of maternal resources (evaluated using the first principal component, *PC1*, from the analyses of size and number of inflorescences variables) and disease (fungal infection), as proximate factors controlling fruit production and maturation. Although neither *PC1* nor fungus showed a direct effect on fruit set, their interactions was found to have a significant impact on fruit production; smaller infected trees tend to produce less fruit set compared to bigger infected individuals. This and the interaction between fungus x population,

suggest that the low incidence of fungal infection in GB may contribute to the higher levels of fruit set in this population; however, it does not explain the reduction in reproductive output in BP.

Finally, I found that there is little variation in the SI response among populations, indicating that pollen limitation does not contribute to the variation in the strength of SI. In addition, I did not find a relationship between fruit set and the strength of SI, suggesting that self-fertilization does not occur in open-pollinated flowers. This result is supported by genetic analyses (chapter 2) that show that virtually all seeds from open-pollinated arrays were the result of outcrossing. Factors such as pollen competition may be accounted for the predominance of outcrossing in these populations (Vogler & Stephenson 2001).

FRUIT SET FROM OPEN POLLINATIONS

The finding that the open-pollinated fruit set was higher in the continuous than in the fragmented populations in both years, suggests that either environmental/ecological conditions were similar between years, or that they did not influence seed set. Among the many environmental factors known to affect fruit production, temperature has been shown to be the most important, in particular the temperature during the bloom period (Lewis 1942; Sanzol & Herrero 2001). Temperature did not vary among chokecherry populations within years, but it did between years. In 2009, the mean and maximum temperature in bloom was 3°C and 6°C higher respectively, compared to 2010. This suggests that this range of temperature variation does not affect the ratio of flowers that develop into fruit in these chokecherry trees. This coincides with reports in apricot

(*Prunus armeniaca*), that show that temperature during bloom did not influence the potential of flowers to become fruits (Alburquerque *et al.* 2004). However, bloom temperature has been shown to influence fruit set in cherry (*Prunus avium*), and peach (*Prunus persica*), such that small increases in temperature can drastically reduce fruit set (Kozai *et al.* 2004; Hedhly *et al.* 2005). In general, empirical evidence shows that temperature affects the pollination process, but the range of temperature under which there is significant variation in fruit set varies greatly among species, and via interactions with other environmental conditions, such as rainfall. The results from my study indicate that the range of temperatures observed in 2009 and 2010 did not influence fruit set in these chokecherry populations.

The mean fruit set in each of the three populations studied is slightly lower (0.04-0.20) than that reported in cultivated chokecherry trees, where mean fruit sets were found to range from 0.16 to 0.37 in two consecutive years (Shiell *et al.* 2002). However, the levels of fruit set observed in this study are similar to those reported in natural populations of wild black cherry (*Prunus serotina*), a close relative to chokecherry (Pairon *et al.* 2006). In any case, the mean levels of fruit production observed in this study, and observed in other both cultivated and natural populations of *Prunus* species, indicate that the genus has a low fruit/flower ratio (Guitian *et al.* 1993; Jordano & Schupp 2000; Kollmann & Pflugshaupt 2001).

Different hypotheses have been put forward to explain the excess of flower production and/or low fruit set ratios observed in many species. These include the resource availability hypothesis, the attraction hypothesis, and the selective abortion hypothesis (Holland *et al.* 2004). The former predicts that under occasional favourable

conditions, an excess of flowers allows plants to take advantage of the additional resources and produce more fruits (Stephenson 1981; Holland *et al.* 2004). This situation is more likely to occur in species that are strongly limited by resource availability, which does not seem the case for chokecherry. The availability of maternal resources, estimated as both tree size and the number of inflorescences, was not significantly correlated with fruit set, and fruit set did not vary among years, suggesting that resource availability is not a likely explanation of the low fruit-flower ratio.

The attraction hypothesis on the other hand, postulates that in animal-pollinated species, an excess production of flowers will increase the likelihood of pollinator visits, consequently improving fertilization rates (Schaffer & Schaffer 1979; Holland *et al.* 2004). This is especially true when plants are pollen limited, and an increase in pollen transfer rates leads to an increase in fruit set (Knight *et al.* 2005). Given that I found higher fruit set following pollen supplementation in the fragmented populations, this suggests that the large floral display in chokecherry may partially serve to attract pollinators. Finally, the selective abortion hypothesis postulates that an excess of flowers allows for selection of "high quality" fruits (e.g., those produced from unrelated pollen), and abortion of "low quality fruits" (Stephenson 1981; Hirao 2010). Results from hand cross-pollinations following genetic determination of kinship between the maternal plant and pollen donor chosen for cross-pollination, indicate that fruit set is reduced after pollination with closely related pollen, in particular between full-sibs. This supports the hypothesis that biparental inbreeding depression occurs in chokecherry, and can lead to the early abortion of inbred progeny (Charlesworth & Willis 2009; Hirao 2010). This suggests that selective fruit abortion may also partially explain the low fruit-to-flower

ratio. In summary, it appears that the large number of flowers observed in chokecherry relative to the fruit set may be attributed to both pollinator attraction and selective abortion of fruits.

THE EFFECTS OF FRAGMENTATION ON REPRODUCTIVE SUCCESS AND POLLEN LIMITATION

I found significantly lower reproductive success in the fragments of forests compared to the continuous population during both years. As other empirical evidence has shown, pollen limitation appears to be the most proximate factor causing a reduction in reproductive success (Wagenius *et al.* 2007). This was demonstrated by hand cross-pollinations with supplemented pollen, because they produced higher fruit set than open controls in the fragments but not in the continuous population. Similarly, levels of pollen limitation estimated as *L* also showed significantly higher values in the fragments compared to the continuous population. This suggests that habitat fragmentation may be an important factor affecting pollen limitation, as has been found for other plant populations (Quesada *et al.* 2003; Aguilar *et al.* 2006).

In animal-pollinated species, ecological factors affecting different aspects of the ecology of pollinators may influence pollen limitation. After fragmentation, the spatial distribution of plants may change and affect pollinator assemblages and/or pollinator behaviour, including alterations in flight pattern, visitation rates and handling time, leading to alterations in the quality and quantity of pollen arriving to the stigma (Collevatti *et al.* 2010; Trant *et al.* 2010). I observed differences in the density of individuals in the fragments compared to the continuous population; the fragments were

observed to have denser patches of trees while the continuous population had a lower stand density. Although previous studies indicate that higher plant density typically increases pollinator attraction and visitation frequency (Ghazoul 2005), if denser patches consist of extensive clonal genets or are caused by restricted seed dispersal, higher density would increase the probability of pollination by related males. The clumped arrangement of trees in the fragments may favour shorter inter-mate distances, resulting in more mating among neighbouring related trees, while in GB the lower tree density within patches might increase pollinator flight distances, resulting in the sampling of a wider array of pollen donors. This interpretation is also supported by genetic analysis on mating dynamics in the populations (chapter two), in which I found that in BP, maternal trees received pollen from fewer close neighbouring pollen donors, which tend to be more closely related compared to GB. These results suggest that the higher density in the fragments is increasing the frequency of short distance flights in pollinators, and increasing mating events among related pairs, resulting in reduced fruit set.

If trees in the fragmented populations are receiving more related pollen because of a higher density of trees in patches, biparental inbreeding may be an important factor causing the reduction of fruit set in the fragmented populations. Traditionally, studies on natural populations have reported reductions in fruit set when neighbouring plants are intercrossed (Byers 1998; Robertson & Ulappa 2004); and recently, a study using kinship coefficients found that biparental inbreeding depression lead to a reduction in seed set (Hirao 2010). Hirao (2010) also found a negative correlation between kinship values of maternal-paternal pairs and their resulting seed set, suggesting that higher levels of consanguinity led to lower reproductive success. This is in line with my results, because I

found a considerable reduction in fruit set after cross-pollination between full sibs. Overall, my results suggest that the increase of mating between near neighbours, which are likely to be related, is causing a reduction in the reproductive success due to a reduction in "quality" of pollen.

In species with SI systems, such as chokecherry, the increase in biparental inbreeding may also result in the reduction of fruit set when related parents share *S*-alleles. The probability of having mating events between individuals that share *S*-alleles increases in very small and isolated populations, in which random genetic drift must be strong enough to overcome the strong selective forces operating on *S*-allele diversity (Glemin *et al.* 2005). If fragmentation causes a reduction in genetic diversity, and an increase in genetic differentiation among chokecherry populations, *S*-allele diversity could theoretically limit fruit set. However, these conditions do not easily arise, except in highly endangered species (Demauro 1993), or in populations that have gone through long, protracted bottlenecks (Busch & Schoen 2008; Young & Pickup 2010). If population bottlenecks are not severe enough to affect *S*-allele diversity, studies indicate that decreases in *S*-allele diversity do not mirror those at neutral loci (Busch *et al.* 2010). Not surprisingly, given the widespread occurrence of this species, analyses using genetic markers show that these chokecherry populations are highly variable, and maintain high levels of gene flow among populations (chapter two). This suggests that the fragments may not be limited by compatible mates, but rather by the type of pollen brought by pollinators, which appears to be biparentally inbred.

THE EFFECTS OF FUNGAL INFECTION AND MATERNAL RESOURCES ON REPRODUCTIVE SUCCESS

The amount of maternal resources available for fruit development can be reduced after fragmentation if there are changes in biotic interactions such as parasitism (Burdon 1987; Benítez-Malvido & Arroyo-Rodríguez 2008). After fragmentation, the level of genetic diversity in plant populations may decrease, followed by genetic erosion through increases in random genetic drift, leading to high levels of inbreeding depression and increased susceptibility to diseases and pests (Young *et al.* 1996; Ouborg *et al.* 2000). In addition, fragmentation may also cause changes in the incidence of pathogens and increase the rate of infection in plant populations. Chokecherry is highly susceptible to attack by the fungus *Apiosporina morbosa* (Zhang *et al. 2005)* which causes knotlike cankers to develop on the stems. These swellings are likely to reduce fitness and alter the allocation of nutrients with a shift in resources towards defense and away from fruit production, as has been previously reported for *Prunus cerasus,* L. (McFadden-Smith *et al.* 2000). As expected, the occurrence of the fungus was significantly lower in the continuous population compared to the fragments, in particular to BH. The lower incidence of the fungus in GB may be explained by the spatial distribution of individuals, because in this population, the chokecherry trees are surrounded by many non-susceptible species which may help minimise the spread of fungal spores (Carlsson *et al.* 1990; Lienert 2004). On the other hand, one of the fragmented populations, BP, had an intermediate frequency of fungal infection while the other, BH, had a high frequency. In BP, more than half of the trees sampled were healthy, and the expected number of infected trees did not deviate from that observed. In BP, the spatial distribution of trees is

different from that in GB, but it may also act as a barrier to fungal infection, since there are dense clusters of trees but also large patches without chokecherry trees. The spatial distribution of individuals at BH, where there is a more uniform density of individuals, seems to be optimum for the proliferation of the disease, as indicated by the significantly higher proportion of infected individuals compared to healthy one. These results suggest that fragmentation is affecting the incidence of the fungus in chokecherry, and that the spatial distribution of the individuals is a critical factor controlling the spread of the fungal spores.

The ANCOVA analysis of the effect of fragmentation, fungal infection and *PC1* on fruit set, showed that neither maternal resources nor fungal infection are main factors limiting reproductive output. However, the observation of a significant interaction between fungal infection and *PC1* revealed that infected trees tended to have higher fruit set when larger; while non-infected trees showed the opposite trend. A significant interaction between population and fungal infection was also observed, and indicated that fungal infection generally had a negative effect on fruit set as expected, except in BP, where infected trees produced slightly more fruits that non-infected ones. This last interaction is probably due to the presence of larger trees in BP that, even when infected, set the highest levels of fruit set in this fragment. These larger trees may be able to allocate more resources to reproduction, even while defending themselves, because they invest less in vegetative growth compared to smaller infected trees (Obeso 2002).

A number of studies have found a positive correlation between plant size and fruit set (Dudash 1991; Deckers *et al.* 2005), which is typically attributed to an increase in

pollen deposition on stigmas of large plants compared to less attractive smaller individuals (Dudash 1991). However, when both infected and non-infected chokecherry trees were included, I found a negative correlation between *PC1* and fruit set, that only became significant when infected trees were removed from the analysis. This trend was also observed when hand-pollinated fruit set was related to plant size (data not shown), suggesting that the variation in fruit production according to size is not determined by the delivery of pollen by pollinators. Instead, my results suggest that the allocation of resources may change according to plant size, causing variation in the reproductive output of small and large trees (Andersson 1988). It is possible that in chokecherry there is a different energetic trade-off according to tree size, and in small trees the allocation of resources to individual flowers is higher than in large plants.

In summary, the analyses of fruit set following open pollination showed a significantly higher reproductive output in the continuous population compared to both fragments of forests, which indicates an effect of population on the reproductive output of chokecherry trees. This was mainly explained by the occurrence of pollen limitation in the fragments. However, GB also showed a lower incidence of fungus infection, which seems to have a slight negative effect on fruit set. This suggests that the higher frequency of healthy individuals in GB may also contribute to the higher reproductive success in this large continuous population of chokecherry. Nevertheless, it does not explain why I observed a reduction in fruit set in BP, which did not show a significantly higher number of infected individuals, and further support the hypothesis that pollen limitation is a major cause for the reduction in reproductive success in the fragments.

BP in particular, showed to be the fragment with the highest pollen limitation

during both years, and revealed the lowest fruit set not only after open pollinations but also when pollen was supplemented. Kinship analysis showed that most of the hand-crosses were between unrelated pairs, ruling out the possibility that the low fruit set from these pollinations was due to mating among relatives. My results suggest that additional factors others than pollen and fungal infection are exacerbating the reduction of fruit production in this fragment of forest. Possible candidate factors such as other diseases, fruit predation and herbivory may be causing the lower fruit set in BP, even when pollen is supplemented (Stephenson 1981; Niesenbaum 1996; Vallius & Salonen 2006).

SELF INCOMPATIBILITY

The results of the hand-pollinations suggest that chokecherry is predominantly self-incompatible, although many individuals were capable of limited self-fertilization. A number of studies have shown that the expression of genetic modifiers influenced by external environmental conditions such as temperature; or internal stylar conditions such as the age of the flower, may cause variation in the strength of SI (Stephenson *et al.* 2000; Good-Avila *et al.* 2008). My results suggest that in chokecherry, rates of self-fertilization are not enhanced by these condition, since the temperature during the bloom period was similar among locations, and the ability to self-fertilize, i.e., the *SC* index, was not positively correlated with floral age at the time of pollination, i.e., the production of fruits did not increase with floral age at pollination time (data not shown).

Other important factors that may cause the invasion of modifiers increasing self-fertility in species with SI systems are population bottlenecks and/or the failure of pollinators. After habitat fragmentation, populations may become limited by outcross

pollen which eventually will lead to conditions favouring modifiers that increase rates of self-fertilization (Byers & Meagher 1992; Willi 2009). In fact, individuals with mutations that weaken the SI response have been shown to be more effective colonizers in conditions of pollen limitation (Vallejo-Marin & Uyenoyama 2004). My results detected significantly higher levels of pollen limitation in the fragments than in the continuous population, and I also observed the occurrence of PSC individuals in all three populations. However, contrary to what was expected, there is not an increase of self-fertilization in BP or BH, and in fact, the number of individuals able to self is somewhat higher in GB. This suggests that the variation in the strength of the SI occurred in these populations prior to fragmentation, and the presence of partially self-compatible individuals is not driven by the current levels of pollen limitation.

This result is further supported by the observation that even though some individuals are capable of self-fertilization as revealed by hand self-pollinations, no seeds appear to be set via self-fertilization in open-pollinated flowers. Results from the genetic analyses (chapter two) show that virtually all seeds from open-pollination were the result of outcrossing; suggesting that outcross pollen may experience a siring advantage over self pollen when both are present in the stigma. This competition by outcross pollen has been reported several times in other species (Vogler & Stephenson 2001), including *Prunus* species (Alonso 2005), where PSC individuals coexist with self-incompatible ones. However, if self-fertilization occurs, it may be followed by selective embryo abortion caused by maternal control, embryo competition (Korbecka *et al.* 2002) or inbreeding depression (Husband & Schemske 1996).

The different number of seeds produced after self-pollinations among populations, suggests that the degree of inbreeding depression may also differ. Individuals with partial SI were more frequent in GB, and the population also harboured the greatest number of individuals that were categorized as self-compatible, suggesting that levels of inbreeding depression may be lower in GB compared to the fragmented populations. This is consistent with previous studies in *Campanula rapunculoides,* L., a PSC species that show lower levels of inbreeding depression in families with weak SI compared to families with strong SI (Vogler *et al.* 1999; Good-Avila *et al.* 2003). In addition, inbreeding depression has also shown to be higher in more stressful environments (Hedrick & Kalinowski 2000), and variation is expected to occur according to the fitness effects of the deleterious alleles involved (Charlesworth & Charlesworth 1987). Stressors such as pollen limitation and fungal infection in the fragmented populations may increase the levels of inbreeding depression, explaining the lower number of PSC individuals in these populations, and the potentially more negative consequences of biparental inbreeding.

CONCLUSIONS

Overall, my results suggest that fragmentation is causing a reduction in the reproductive output of chokecherry. In fragmented populations (BP and BH), natural pollinations revealed lower fruit set compared to hand cross-pollinations, suggesting that pollen is an important factor limiting fruit production. A possible explanation for this outcome is the higher density in both fragments of forest, which may favour shorter inter-mate distances and thereby increase the relatedness of male-female pairs (i.e., biparental

inbreeding). The increase in biparental inbreeding may result in more frequent pollination events between incompatible mates (i.e., pairs sharing *S*-alleles) or in biparental inbreeding depression (i.e., pairs sharing deleterious alleles), having negative consequences on reproductive success (Nason & Ellstrand 1995; Hirao 2010).

In addition, I also explored the impact of fungal infection and maternal resources (evaluated using *PC1*, from the analyses of size and number of inflorescences) on reproductive success. The interactions fungus x *PC1* (smaller infected trees tend to produce less fruit set than bigger infected ones, and smaller non-infected trees tend to produce more fruit set than bigger non-infected ones) and fungus x population (reduced fruit set in infected individuals only in BH and GB), revealed significant indirect effects on fruit production. The significantly lower incidence of the fungus in the continuous population may contribute to the higher reproductive success compared to the fragmented populations. Nevertheless, it does not explain the reduction in fruit set in plants from BP, where infected trees did not show reduced fruit set and are not majority. This further supports the hypotheses that; (i) pollen limitation is a major cause for the reduction in reproductive success in the fragments, and that; (ii) fragmentation may affect the incidence of the fungus. However, the latter is also determined by intrinsic characteristics of the fragments, such as density, which is crucial for the spread of the fungal spores.

Finally, the results from the self-pollinations suggest that chokecherry is predominantly self-incompatible, although many individuals were capable of limited self-fertilization. I did not find a relationship between fruit set and the strength of SI, suggesting that self-fertilization does not occur in open-pollinated flowers. This result is

supported by genetic analyses (chapter two) that show complete outcrossing in open-pollinated arrays.

3.0. CHAPTER TWO: Genetic diversity, mating system patterns and pollen gene flow of chokecherry trees in two fragments of forest and one continuous population

3.1. INTRODUCTION

Forest fragmentation via anthropogenic disturbance, is a process in which large areas of forest are cleared for agriculture and urban sprawl, resulting in a mosaic of small, separated remnants (Fahrig 2003). The two major impacts of habitat fragmentation on natural populations stem from the reduction in size and the increase of isolation of habitats. Under population genetics theory, populations in fragmented habitats are expected to experience a loss of genetic diversity (because of the reduction in population size), and a disruption of gene flow (because of the increase in isolation). The combined effects of these processes will exacerbate the effect of random genetic drift, and increase the genetic differentiation among populations (Levins 1969; Young *et al.* 1996; Lowe *et al.* 2005), which will limit the ability of the population to adapt to environmental changes, and therefore increase the probability of local extinction (Isagi *et al.* 2007). Nevertheless, empirical evidence has provided mixed results, and implies that forest fragmentation may not always result in genetic erosion of plant populations (Lowe *et al.* 2005; Aguilar *et al.* 2008).

Variation in the response of plant populations to habitat fragmentation will depend on the different ecological and genetic factors influencing a species or population, including its mating system, the methods of pollen and seed dispersal, the effective population size and standing levels of genetic diversity in the populations prior to fragmentation (Hamrick 2004; Aguilar *et al.* 2008). For instance, recent studies have

shown that factors such as long distance pollen flow will maintain population connectivity and may buffer the adverse effects of habitat fragmentation (Craft & Ashley 2010; Wang *et al.* 2010). For this reason, studying the effect of fragmentation is challenging, and requires the evaluation of multiple factors influencing populations both prior to and post fragmentation where possible.

The mating system is an important factor shaping the response of plant populations to habitat fragmentation because it partially determines the structure of genetic variation within and among plant populations (Hamrick & Godt 1996). After a sudden reduction in population size, predominantly outcrossing species are expected to exhibit a greater loss of genetic diversity than selfing species, since in the former most of the genetic variability is found within populations. This has been demonstrated by studies in natural populations, that have found a greater loss of alleles and polymorphic loci in mainly outcrossing compared to predominantly selfing species or species with mixed mating system (Aguilar *et al.* 2008).

Of more serious concern is the possibility that fragmentation induces a change in the mating system of a species, which may negatively impact its future survival. An abrupt reduction in habitat may lead to heterogeneity in outcrossing rates among populations, due to the increase of non-random mating. In self-incompatible or partially-self-incompatible species, fragmentation may cause variation in consanguineous mating and/or correlation of paternity, affecting the levels of inbreeding, and causing biparental inbreeding depression, which is the reduction in the fitness of the inbreed offspring (Uyenoyama 1986). An increase in mating among related individuals after fragmentation can result from assortative movements by pollinators, decreased pollen flow and/or

spatial structuring of the genotypes in a population, when related individuals are likely to be neighbours and to cross among them (Uyenoyama 1986; Zhao *et al.* 2009). However, these factors are not mutually exclusive, and their interaction may cause important changes in mating dynamics and aggravate the levels of biparental inbreeding depression. An increase on correlated paternity, i.e., the probability that two siblings are outcrossed full-sibs (Ritland 1989), may also exacerbate this condition (Hardy *et al.* 2004), and may arise when a pollinator deposits pollen from the same donor on several flowers in a maternal tree, or when different pollinators carry pollen from the same donor (Hardy *et al.* 2004). This last situation may be potentially important in plant species with restricted pollen dispersal and vegetative reproduction, since clones are typically close to each other decreasing paternal diversity (Llaurens *et al.* 2008). Overall, the effect of these changes will be mainly determined by features of the reproductive system, such as the presence of self-incompatibility (SI); the selective abortion of seeds by maternal regulation of seed quality or by sibling rivalry; and changes in the density of individuals within populations, which may affect pollinator behaviour and, ultimately, outcrossing rate (Fernandez-Manjarres *et al.* 2006; Jacquemyn & Honnay 2008).

Therefore, to understand how fragmentation affects mating dynamics, it is crucial to evaluate different features of the mating system within populations. The pattern of genetic transmission from parents to progeny shown by genetic markers has been widely used to estimate plant mating system parameters, usually following the 'mixed mating model', where mating events are of two types: random outcrossing and self-fertilization (Ritland 2002). Outcrossing is often estimated using multilocus procedures since they have lower statistical variance and higher robustness against the violation of model

assumptions, compared with single-locus methods (Ritland & Jain 1981). However, comparison of multilocus and single locus estimates of outcrossing provides an estimate of the levels of biparental inbreeding (Shaw *et al.* 1981).

Ultimately, changes in the outcrossing-selfing rate *per se* are determined by the impact of habitat fragmentation on gene dispersal. Since plants are stationary as adults, dispersal of gametes and offspring occurs via pollen and seeds (Adams 1992), and after colonization by seed, pollen-mediated gene movement becomes a major factor shaping the distribution of genetic diversity in plant populations (Vekemans & Hardy 2004). Pollination patterns will influence levels of spatial genetic structure and connectivity of populations, and determine different mating system parameters such as reproductive neighbourhood size and levels of biparental inbreeding (Coates *et al.* 2007; Yates *et al.* 2007).

The patterns and extent of pollen dispersal within and among populations have been estimated using different methods, including those that use highly polymorphic genetic markers (e.g., microsatellites). Traditional indirect methods to infer levels of gene flow, use estimates of the spatial distribution of genetic diversity (such as F_{ST} statistics) for a set of populations, assuming equilibrium between migration and genetic drift; however these methods cannot estimate the level of contemporary gene flow (e.g., after anthropogenic landscape alteration) (Smouse & Sork 2004). Direct parentage analysis, in contrast, estimates contemporary gene flow directly from the progeny of a population, but is usually limited to single populations because it requires considerable field and lab work, since all the individuals from the populations have to be sampled (Dunphy & Hamrick 2007). Smouse *et al.* (2001) proposed an indirect method of estimating

contemporary pollen dispersal called TwoGener, a hybrid of traditional genetic structure and parentage analyses, where the basic idea is to compare the gametic pollen profiles identified from different maternal parents, to determine if there is heterogeneity in the pollen donor pools sampled. This method, however, requires independent (or joint) estimation of the effective density of pollen donors (*de*) (Austerlitz & Smouse 2002), and failure to accurately estimate this demographic parameter may affect the accuracy of the dispersal function parameters. Additionally, TwoGener assumes equal male fecundity (flowering intensity) and synchronous flowering, which may overestimate the effective density of pollen donors (Kang *et al.* 2003). To overcome these biases, Robledo *et al.* (2007) released KinDist, a procedure that is independent of the unknown effective density of males, making it more robust for estimation of the dispersal kernel parameters. KinDist estimates the structure of the pollen pool as $\Psi_{(z)}$, a measure of correlated paternity between maternal sibship pairs separated by the distance z, relative to the population average, and calculates the parameters of the pollen dispersal curve.

Numerous studies on the pollen dispersal curve have shown that the pattern of pollen flow tends to be leptokurtic, with large amounts of near-neighbour mating events (Austerlitz *et al.* 2004). However, the characterization of the tail of the curve can be problematic; in particular when the scope is restricted to relatively small areas (Garcia *et al.* 2007; Larsen & Kjær 2009). In some cases, this will limit the detection of long distance pollen movements, which are crucial in various ecological contexts. For instance, the occurrence of large distance pollen flow after habitat fragmentation will maintain population connectivity, and help to counteract the effects of random genetic drift (Sork & Smouse 2006; Kamm *et al.* 2009). For this reason, obtaining reliable

measures of long-distance gene flow is important to understand the response of plant populations to habitat fragmentation.

To assess the genetic consequences of forest fragmentation, I compared plants from 2 forest fragments with those from a continuous forest remnant of chokecherry (*Prunus virginiana*, L.), a widely distributed species from the Rosaceae family. I used microsatellite markers to explore levels of genetic variability within and among populations, and tested if there is a loss of allelic diversity, or an increase in genetic structure in fragmented populations. I also estimated different aspects of the mating system using progeny arrays from one of the fragments (BP) and from the continuous population, to detect changes in mating dynamics after fragmentation. Finally, I evaluated the pollen dispersal curve with a recently developed indirect approach, to estimate contemporary pattern of pollen dispersal.

3.2. METHODS

MICROSATELLITE GENOTYPING

To assess the consequences of forest fragmentation on the genetic diversity of chokecherry, I compared plants from 2 fragments of forest (BP and BH), with those from a continuous forest remnant (GB) in Southern Manitoba (see chapter one for details). A total of 22, 31 and 49 individuals were sampled from BP, BH and GB respectively. Initially, 13 microsatellite primers designed for peach (*Prunus persica*) that were found to be polymorphic and transferable to other species of *Prunus* (Cipriani *et al.* 1999; Testolin *et al.* 2000; Dirlewanger *et al.* 2002), were tested in a representative sample of

each population. DNA was extracted with a modified version of the CTAB method (Cheng *et al.* 1997), using no more than 30mg of frozen young leaf tissue. PCR reactions were carried out in a total volume of 25 ul containing 2X of GoTaq® (Promega), 0.2 uM of each primer and 40 ng of extracted DNA. Amplifications were performed on a PTC-100TM thermal cycler (MJ Research) using the following protocol: an initial denaturation step at 94°C for 2 min; 35 cycles of 94°C for 45 s, annealing at 53–48 °C for 45 s and extension at 72°C for 1 m; and a final extension at 72°C for 5 min. PCR products were loaded onto 1.5% agarose gels, run at constant voltage (45 V) for 1.5 h and detected by staining with ethidium bromide. To assess genetic variation within and among the three populations, four of the 13 loci were selected for further analyses (Table 3.1). PCR of these four loci was carried out using fluorescent primers and the reactions contained 2X of GoTaq® (Promega), 0.2uM of reverse primer, 0.12 uM of forward primer, 0.08 uM of fluorescent forward primer and 60 ng of extracted DNA. For detection and scoring, the MJ basestation genotyper with its licensed genotyping software, Cartographer, were used respectively.

GENETIC DIVERSITY AND GENETIC STRUCTURE

To estimate the genetic diversity and differentiation among chokecherry populations, observed heterozygosity (H_o), expected heterozygosity (H_e), and the Weir & Cockerham estimator of F_{ST} were calculated using the software FSTAT 2.9.3 (Goudet 2001). For the evaluation of the effect of forest fragmentation on genetic diversity, two estimations were calculated independently for each population using the software FSTAT: (i) an unbiased estimator of genetic diversity (Nei 1987):

$$H_{sk} = \frac{n_k}{n_k - 1} (1-\Sigma p^2_{ik} - H_{ok} / 2n_k)$$

where n_k is the size of population k, p_{ik} the frequency of allele A_i in population k and H_{ok} the observed proportion of heterozygotes; and (ii) allelic richness, where the number of alleles is measured independently from sample size (Petit *et al.* 1998). FSTAT was also used to detect significant differences between the fragments and the continuous population in mean within-group observed and expected heterozygosity, allelic richness and genetic diversity. To test for significance, a two-tailed test with 1000 random permutations was used, where variation among populations within categories was compared to that detected between categories. Two categories were defined; one as fragmented and one as continuous. In the former, BP and BH were included. In the latter, GB was divided in two groups, one with samples from the east and the other with samples from the west of this population. The samples from GB were divided in two groups only to be able to perform this test since it requires at least 2 populations per group.

Additionally, to explore the effect of forest fragmentation on allele frequencies, gene frequencies were estimated and the number of rare alleles ($p \leq 0.05$) present in each population was counted. Also, the number of private alleles (those occurring in only one population) for each population was identified. To test for significant differences in the number of rare and private alleles among populations, chi-square contingency table tests were performed. The allelic frequencies were estimated using the software FSTAT 2.9.3 (Goudet 2001).

To detect if there has been a significant recent reduction in effective population size for each population, the program BOTTLENECK 1.2.02 (Cornuet & Luikart 1996) was used. Populations that have experienced a recent bottleneck may exhibit a reduction in allele number and heterozygosity at polymorphic loci, although the former is expected to decline faster than observed heterozygosity because rare alleles are the first ones to be lost and, consequently, an excess of heterozygosity can be observed in the generations proceeding a bottleneck. BOTTLENECK tests for the occurrence of significantly larger observed heterozygosity compared to expected equilibrium heterozygosity (H_{eq}) from the observed allele number in which loci are assumed to be in mutation-drift equilibrium (Cornuet & Luikart 1996). Ten thousand simulations were performed for each population based on a two-phase model (TPM), consisting of 90% single-step mutations and 10% multistep changes, as suggested by Luikart & Cornuet (1998). The TPM of evolution has been found to fit microsatellite loci better than either the pure stepwise or infinite allele models (Luikart & Cornuet 1998). The Wilcoxon sign-rank test was used across loci to determine whether a population exhibited a significant number of loci with heterozygosity excess, compared with gene diversity predicted at equilibrium on the basis of the observed number of alleles.

SAMPLING FOR MATING SYSTEM, SPATIAL GENETIC STRUCTURE AND POLLEN DISPERSAL ANALYSES

For subsequent analyses of the genetic diversity of adults vs. offspring generations, the spatial genetic structure, the pollen dispersal curve and the mating system, only samples from BP (one of the fragments) and GB (continuous population)

were analyzed, and the number of microsatellite loci scored was increased to eight (Table 3.1). BP was selected between the two fragments based on results obtained in chapter one, in which it was found that there were stronger negative ecological consequences of fragmentation (a reduction in fruit set and an increase in pollen limitation) in BP compared to BH. Additionally, BP is further geographically from GB, and therefore a higher degree of genetic differentiation could be expected under the isolation by distance model (Wright 1943). A total of 24 and 36 adult individuals were sampled from BP and GB respectively.

To estimate the mating system and pollen dispersal curves within the populations, 15 and 20 maternal parents were selected and 20 embryos per mother were sampled, for a total of 150 and 200 progeny arrays from BP and GB respectively. The embryos were obtained from seeds after removing the endocarp, testa and endosperm. DNA was extracted using ID labs extraction kits, since DNA extracted with CTAB did not amplified the microsatellites in the embryos.

The software MICROCHECKER 2.2.3 (Cock *et al.* 2004) was used to detect null alleles in the eight microsatellite loci, and to test for errors in scoring due to stuttering and allelic dropout. FSTAT 2.9.3.2 was used to asses Hardy-Weinberg equilibrium (HWE) at each locus, and to test for linkage disequilibrium (LD) between each pair of microsatellite loci.

Table 3. 1. Microsatellite primer names, annealing temperatures, sequences, repeat motifs and size ranges (base pairs) used in the analyses of genetic diversity and mating system in the chokecherry populations. Asterisks (*) represent the four microsatellites used in the initial screening analysis in BP, BH and GB.

Name	Temperature	Primer sequence (5′→ 3′)	Repeat motif	Range size
UDP96-005	53	GTAACGCTCGCTACCACAAA CCTGCATATCACCACCCAG	(AC)16TG(CT)2CA(CT)11	100-174
*UDP96-018	53	TTCTAATCTGGGCTATGGCG GAAGTTCACATTTACGACAGGG	(AC)21	243-269
*UDP97-402	48	TCCCATAACCAAAAAAAACACC TGGAGAAGGGTGGGTACTTG	(AG)17	114-174
BPPCT007	53	TCATTGCTCGTCATCAGC CAGATTTCTGAAGTTAGCGGTA	(AG)22 (CG)2 (AG)4	108-158
*ps08e08	48	CCCAATGAACAACTGCAT CATATCAATCACTGGGATG	(CAA)7	138-171
ps12a12	53	GCCACCAATGGTTCTTCC AGCACCAGATGCACCTGA	(GA)22	140-176
*M4c	48	GAATTTGTTCTCTCTCTCTC GGAAGCGTTCGTCTGCAAAT	(TC)17	76-108
Ma006b	48	ACAACTTACCATTTGAGGCT CAATCATTCAAGCTCTCTCC	(AG)18	238-276

SPATIAL AUTOCORRELATION ANALYSIS

The decay in genetic similarity associated with increasing distance between individuals, and the spatial genetic autocorrelation coefficients (*r*), were estimated and plotted at six distance intervals. The distance classes were chosen so that the number of observations in each class was equal. The autocorrelation coefficient, similar to Moran's-*I,* is a measure of the genetic similarity between pairs of individuals whose geographic separation falls within the specified distance class. The coefficient is bounded by –1, +1 and has a mean of '0' when there is no correlation (Smouse & Peakall 1999). For this analysis, 999 permutations were used to estimate the two-tailed 95% confidence intervals around the null hypothesis of no autocorrelation (r =0). Presence of a significant autocorrelation was declared in distances where the estimate of r is greater than the 95% confidence interval about zero. This spatial autocorrelation analysis was performed using the software GENEALEX 6.2 (Peakall & Smouse 2006).

POLLEN DISPERSAL CURVE

The average probability of exclusion (E_L) when only one parent is known was calculated for individual loci and across all loci following Jamieson and Taylor (1997) using GENEALEX 6.2.

Current pollen dispersal was estimated using KinDist (Robledo-Arnuncio *et al.* 2007), an indirect method based on the genetic structure of the pollen cloud as implemented in POLDISP 1.0c (Robledo-Arnuncio *et al.* 2007). KinDist estimates the allele frequencies of the pollen pool by inferring the male gametic contribution to each diploid seedling. The structure of the pollen pool is then estimated as $\Psi_{(z)}$, a measure of

correlated paternity between maternal sibship pairs separated by the distance *z*, relative to the populations average. $\Psi_{(z)}$ is defined as the ratio $Q_{(z)}/Q_0$, where Q_0 is the probability that a female mates twice with the same male, and $Q_{(z)}$ the probability that two females at a distance *z* apart will mate with the same male. Finally, a dispersal function with parameter set θ is chosen and a set of expected $\Psi_{(z,\theta)}$ values are estimated for each pair of maternal trees. The expected $\Psi_{(z,\theta)}$ values along with the observed pairwise $\Psi_{(z)}$ values are used in a least-square regression estimation of the dispersal parameters.

To estimate the parameters of the pollen dispersal curve, the correlated paternity among maternal trees ($\Psi_{(z)}$) was calculated for each population, and a Spearman's rank correlation test was then used to detect for a significant decay in correlated paternity with increasing spatial distance between female pairs. An approximated threshold distance, a value beyond which correlated paternity among mothers stabilized, was estimated to calibrate the kinship coefficients. Subsequently, a one-parameter (exponential) and two two-parameter (exponential power and gamma) dispersal distributions were tested to determine which was a better fit to the observed pollen dispersal parameters, based on the least-square residuals (LSR) values. The estimated parameters of the pollen dispersal kernels included the scale (*a*), shape (*b*) (only in two parameter models), and average effective pollen dispersal (δ). The parameters for the best fitting model were used to estimate the moments of the distribution, and construct the pollen dispersal curve.

MATING SYSTEM ANALYSIS

The multilocus mating system program MLTR V.3.2 (Ritland 2002) was used for mating system analyses on progeny arrays with known maternal genotypes. Since the

populations did not show genetic differentiation based on F_{ST} analysis, gene frequencies were assumed to be homogenous in both populations, providing more statistical power to the analysis. The estimated parameters included the multilocus outcrossing rate (t_m), single-locus outcrossing rate (t_s), and multilocus paternity correlation (r_p). The t_m and t_s values were compared in order to assess the degree of biparental inbreeding. In the absence of biparental inbreeding, the values will be the same, whereas in the presence of biparental inbreeding, t_s will be lower than t_m because outcrossing events that are not detected at a single locus have a higher probability of being detected as more loci are examined (Ritland 1990). MLTR was run using initial default values of outcrossing rate $t_m = 0.9$, and paternity correlation $r_p = 0.1$. Standard errors (SE) were obtained by 1000 bootstraps using families as the resampling unit, and used to calculate the 95% confidence intervals (CI) of each estimate. Significant differences between two parameter estimates were declared if their 95% CI did not overlap. In addition, to determine whether the values were significantly lower than 1 (t_m) or greater than zero ($t_m - t_s$), mean ± 1.96 x SE was used. The number of pollen donors contributing to a maternal tree (i.e neighbourhood size), was estimated as $1/rp$ (Ritland 1989).

In addition, I used the TwoGener model as a second approach to estimate the paternity of correlation and the mean number of pollen donors per maternal plant. TwoGener was used to estimate the pairwise differentiation between pollen clouds of maternal trees (ϕ_{FT}), a measure of pollen pool structure which is fundamentally based on the pattern of correlated paternity (Austerlitz & Smouse 2001a). To detect a significant difference of ϕ_{FT} between the fragment and continuous populations, 95% confidence intervals around ϕ_{FT} were calculated based on the estimated standard deviation ($s\phi$)

assuming a normal distribution. The standard deviation was calculated from the variance, $s^2\phi$, following Smouse *et al.* (2001). The number of effective pollen donors (N_{ep}) was then estimated under TwoGener approach based on the relationship $N_{ep} = 1/(2\ \phi_{FT})$ (Smouse *et al.* 2001). This TwoGener-based analysis was performed in POLDISP 1.0c.

Other important parameters required to estimate mating dynamics are the pollen and ovule frequencies. Since chokecherry is widely distributed, it is expected that a large number of potential pollen donors were not included in the sampling. For this reason, pollen and ovule allele frequencies were not constrained to be equal, and were estimated for each locus using the expectation-maximization method (EM) (Ritland 2002). Differences between these frequencies per locus were tested by the chi-square test of heterogeneity: $\chi^2 = 2N\ F_{ST}\ (n - 1)$, df = $(n - 1)$, where N is the number of seeds examined, n is the number of alleles at the locus, and F_{ST} is a measure of the genetic diversity between the pollen and ovule pools (Hall *et al.* 1994). F_{ST} was calculated using ARLEQUIN V. 3.1 (Excoffier *et al.* 2005), based on the allelic frequencies estimated with the EM method on MLTR.

GENETIC DIVERSITY OF ADULTS VS. OFFSPRING

To compare levels of genetic diversity in the adult and offspring populations, the number of alleles (N_a), the effective number of alleles (N_e), the observed heterozygosity (H_o), the expected heterozygosity (H_e) and the fixation index (F) were estimated using GENEALEX 6.2 (Peakall & Smouse 2006). For the adult populations, the mean and 95% confidence intervals of each genetic diversity estimate were calculated using bootstrapping with 100 bootstrapped replicates. For the progeny, one offspring per

maternal adult was randomly chosen 100 times to create data sets consisting of 15 and 20 offspring in BP and GB respectively, avoiding the effect of family structure on measurements of genetic diversity for the offspring population. Bootstrapping with replacement for the adult population estimates and the random sampling from each maternal plant were performed in R V. 2.10.0 (R Development Core Team, http://www.r-project.org). The mean and its 95% confidence intervals were calculated for each genetic diversity parameter and significant differences between parameter estimates between populations, as well as between adults and offspring, were considered if their 95% CI did not overlap.

S-ALLELE

Previous screening for *S*-linked *RN*ase activity in pistils suggested the presence of the *S*-allele in these chokecherry populations (data not shown). To explore the genetic diversity of this gene within and among populations, cDNA from different combinations of primers were initially used (Raspe & Kohn 2002; Sutherland *et al.* 2004; Sutherland *et al.* 2008). Flowers were sampled and RNA was extracted using PureLink™ RNA Mini Kit (Invitrogen). First-strand cDNA was synthesized with SuperScript III RNase (Invitrogen), and an aliquot of this reaction was used directly for 3'RACE (Frohman *et al.* 1988). Samples obtained with degenerate primers designed from the conserved regions 1 and 2 from the *S*-allele (Raspe & Kohn 2002) and the cDNA synthesis tag (GACTCGAGTCGACATCGA) were sent for sequencing. However, I was not able to obtain *S*-alleles following this protocol. Genomic DNA was then used with combinations of consensus degenerate primers flanking the second intron of known *S*-alleles in the

Rosaceae (Sutherland *et al.* 2004). Different combination were tested including primers from C1 and C2 (EM-PC1consFD and EM-PC2consFD respectively) as forward primers, and the regions upstream to C3 and C5 (EM-PC3consRD, EM-PC5consRD respectively) as reverse. PCR products were cloned with pGEM® (Promega), and after detection by PCR plasmid, were extracted using QIAprep® Spin Miniprep Kit (QIAGEN). Sequences were analyzed using Blast (http://ncbi.nlm.nih.gov/BLAST) to detect similarities with *S*-alleles from other species of *Prunus.*

3.3. RESULTS

HIGH GENETIC DIVERSITY AND LACK OF GENETIC STRUCTURE

The four microsatellite loci used to explore levels of genetic diversity in chokecherry (Table 3.1) were polymorphic with a total of 52 alleles. The mean observed heterozygosity per individual was 0.724 and the mean expected heterozygosity within populations was 0.741.

Analysis on the observed heterozygosity and expected heterozygosity at equilibrium, revealed a recent reduction on the effective population size in the two fragments (BP, $p<0.05$; BH, $p<0.05$; Wilcoxon sign-rank test) but not in the continuous population (GB, $p=0.061$). This confirmed the expected hypothesis that the forest fragments have passed through a recent bottleneck, as opposed to being historically small populations. Nevertheless, this reduction did not affect the levels of genetic diversity, as revealed by Nei's unbiased estimator which displayed similar values in the three populations (Table 3.2). There was a slight reduction of allelic richness and the

percentage of rare and private alleles, especially in BP (Table 3.2), but none of these values were significantly different in the fragments compared to the continuous population. In addition, the analysis of genetic structure indicated very low levels, effectively no, genetic differentiation among populations (F_{ST}=0.004).

Table 3. 2. Estimates of genetic diversity in two fragments of forest and one continuous population of chokecherry using four microsatellite loci. Gene diversity was calculated using Nei's unbiased estimator (Nei 1987). Allelic richness, a measure of the number of alleles independent of sample size, was estimated using the approach from Petit *et al.* (1998). The percentage of rare and private alleles was calculated in each population.

	BP	BH	GB
Gene diversity	0.752 (0.13)	0.704 (0.21)	0.766 (0.13)
Allelic Richness	8.253 (6.18)	9.943 (6.93)	10.627 (6.83)
Total of alleles	34	41	48
% of alleles			
rare p≤0.05	44%	61%	63%
Privates	3%	5%	17%

HWE AND LINKAGE EQUILIBRIUM FOR THE EIGHT SSR LOCI

There was no indication of a deviation from HWE for any of the loci or any evidence of LD between loci. MICROCHECKER detected the presence of null alleles at two loci (BPPCT007 and UDP96-18) but there was no indication of large allele dropouts

at any locus. When loci with null alleles were removed from the analyses of genetic diversity and pollen dispersal, the estimations did not differ considerably, thus the results shown here are from calculations using the eight loci. However, this was not the case for the mating system analysis, where BPPCT007 and UDP96-18 were excluded, since the inclusion of null alleles may reduce the power to estimate mating system parameters (Ritland 2002).

SPATIAL AUTOCORRELATION ANALYSIS

The results from the fine genetic structure analyses showed that the values of *r* (autocorrelation coefficient) from BP and GB at the first distance class (32m), fall outside the confidence belt were no spatial structure is assumed (Figure 3.1). The fact that the *r* value was considerably higher in BP (0.306) compared to GB (0.085), suggests that a pair of individuals between 0 and 32m apart, have greater probability of being related if they belong to BP compared to GB. For a more conservative interpretation one might use additional statistical tests to declare presence of spatial genetic structure. Peakall *et al*. (2003) developed a bootstrap method as an additional statistical test, however, it is less powerful than permutation tests because the number of samples per distance class is much smaller than the comparisons used during permutation. Using this type of test in small sample size, such as the one used in this research, would favour the acceptance of the null hypothesis and might unable the detection spatial autocorrelation.

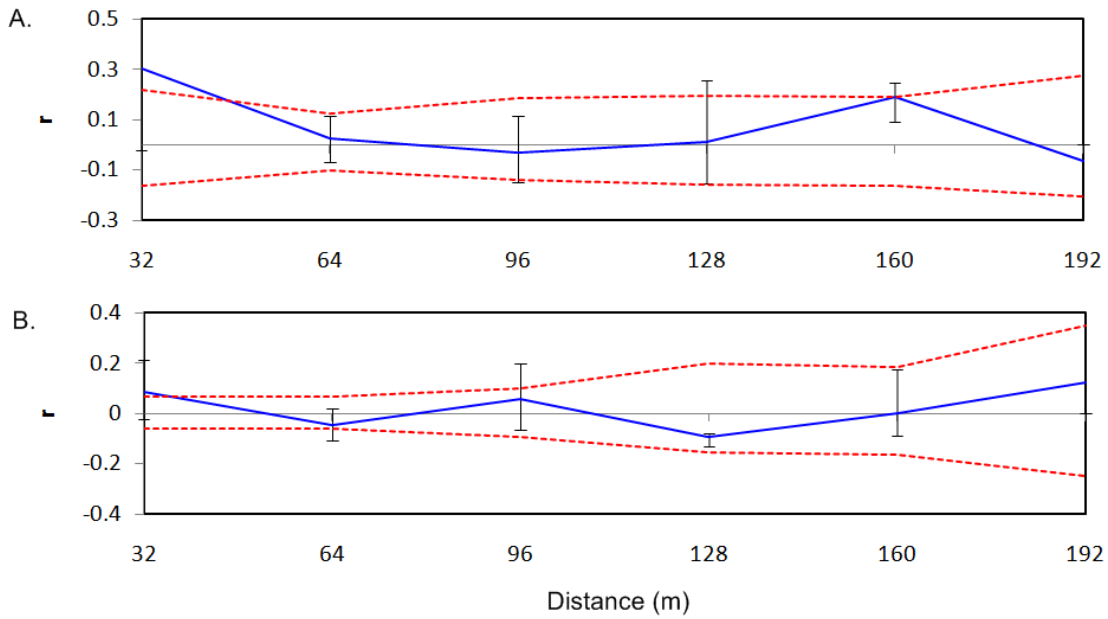

Figure 3. 1. Correlograms showing the spatial genetic structure for BP (A), a fragment of forest and GB (B), a continuous population, of chokecherry. The autocorrelation coefficient (*r*), represented as the blue line, is a measure of the genetic similarity between pairs of individuals whose geographic separation falls within the specified distance class. The red dashed lines represent the upper and lower 95% confidence intervals around 0 (no autocorrelation).

POLLEN DISPERSAL CURVE

The probability of exclusion after combining the information from the eight SSR loci was high in both populations (E_L=0.961 for BP and E_L=0.978 for GB). According to Smouse *et al*. (2001), E_L should be higher than 0.7 for reliable estimates of ϕft, suggesting that the eight markers used here will provide sufficient excluding power to infer the male gametic contribution to each diploid seedling.

Analysis of the pollen cloud sampled by each maternal tree, revealed a significant negative correlation between the correlated paternity and the distance, only in the continuous population (GB, Spearman's rank=-0.225, $p<0.005$; BP, Spearman's rank= 0.004 $p=0.967$) (Figure 3.2), which indicates that KinDist can only be used to model pollination distances in GB. The threshold value was set at 20 km based on Figure 3.2B. Different distances were tested around this value and similar results were obtained, indicating that at this approximate distance the correlated paternity rates stabilize.

The threshold value was then used to estimate the parameters of the pollen dispersal curve. Under the exponential-power (two-parameter) distribution, the average pollen dispersal distance was considerably higher (δ = 3.9 km) and better fit (LSR=12.5) than the exponential (one-parameter) distribution (δ = 28.6 m; LSR=13.2) (Table 3.3). The exponential power distribution indicates leptokurtic pollen dispersal with large amounts of near-neighbour mating events together with long-distance pollen flow. The evidence for the latter is strongly supported by the small value of the shape parameter (b=0.146), which indicates that the dispersal curve in GB is fat-tailed (Figure 3.3). Although the occurrence of long distance pollen dispersal is consistent with other results discuss here, 3.9 km appears to be an exceptionally high value, suggesting some overestimation caused by the small value of *b.* The value of the median, which is more stable to small changes in *b* (Robledo-Arnuncio, *personal communication*), was smaller (928.5 m), and may reflect a better estimation of the real effective pollen dispersal distance.

The gamma distribution did not converge, and resulted in infinite estimates of average effective pollen dispersal, which is unrealistic and will not be discussed further.

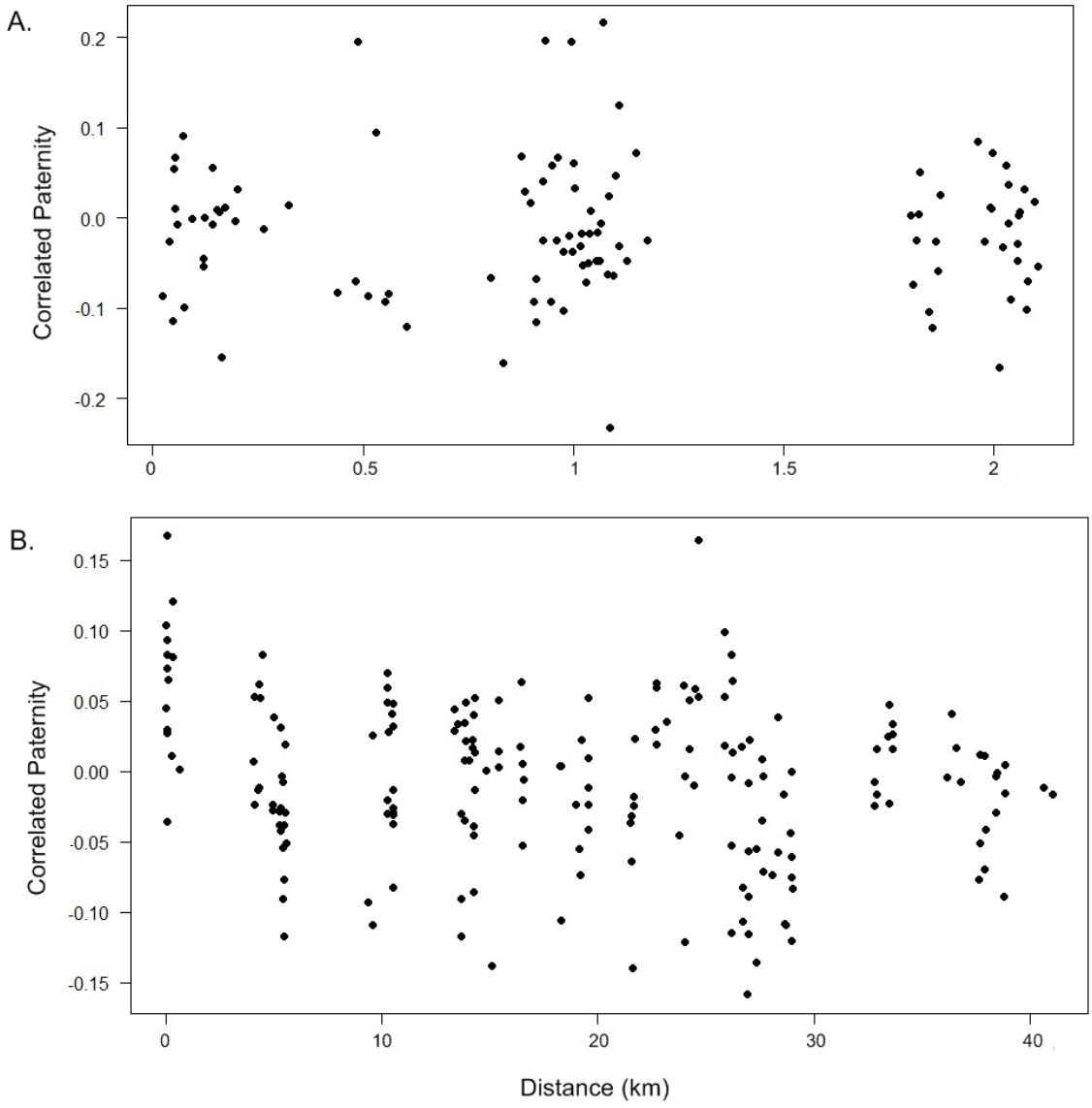

Figure 3. 2. Relationship between pairwise correlated paternity, $\Psi_{(z)}$, and geographical distance among maternal trees, in BP (A) and GB (B). Only for GB there was a significantly negative correlation.

Table 3. 3. Estimates of the effective pollen dispersal kernels from a continuous population (GB) of chokecherry using one- and two-parameter models under KinDist approach. δ = Average dispersal distance, *a* = scale and *b* = shape.

Model	δ	a	b	LSR
Exponential	28.551	14.275	-	13.169
Exponential Power	3,861.110	1.8x10E-04	0.146	12.510
Gamma	infinite	10.350	2.000	12.769

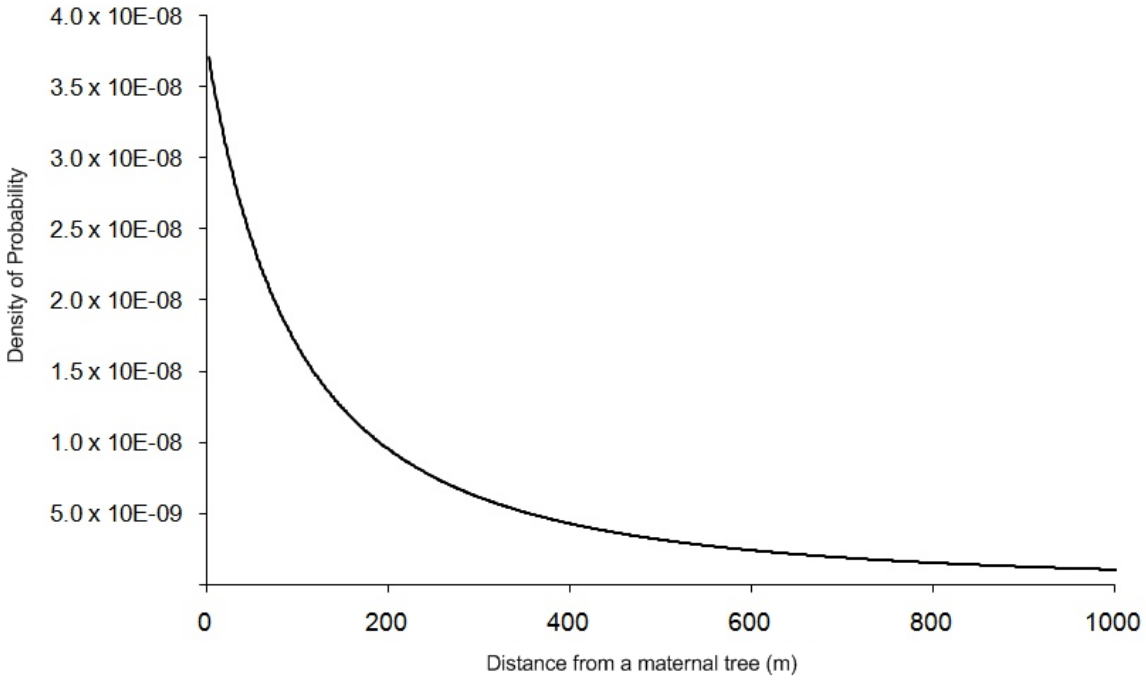

Figure 3. 3. Effective pollen dispersal curve from a continuous population of chokecherry from Southern Manitoba (GB). The curve is derived from the exponential power distribution model, with shape *b*=0.146, and scale *a*= 1.8x10^{-04}.

MATING SYSTEM ANALYSIS

The estimates of the mating system parameters from the fragment (BP) and continuous population (GB) are shown in Table 3.4. The outcrossing rates were not significantly lower than 1 (t_m + 1.96 x $SE \geq 1$) in both populations, which suggests an absence of self-fertilization. Although, GB displayed a lower outcrossing rate (0.992) than BP (1.116), this is just an artifact of the parameter bounds since both populations showed complete outcrossing. Levels of biparental inbreeding, defined as the difference between t_m–t_s, was low but significantly greater than 0 (t_m - 1.96 x $SE > 0$) in both populations, which suggests the presence of mating among relatives. Biparental inbreeding and correlated paternity were significantly higher in BP (t_m–t_s 0.175, r_p 0.437), compared to GB (t_m–t_s 0.072, r_p 0.249). Similar results were obtained with TwoGener method (Table 3.4), which analyzes the genetic structure of pollen pools sampled by individual trees relative to the global pollen pool. Using either method, there were fewer pollen donors in BP than in GB (Table 3.4).

Finally, significant differences in pollen and ovule allele frequencies were detected in 5 of the 6 loci used for the mating system analysis (Table 3.5). Rare pollen alleles not present in ovules were found in four of the five loci with heterogeneity of allelic frequencies, suggesting that pollen may have migrated from outside the area sampled. Although this indicates a violation of one of the assumptions of the mixed mating model, these deviations do not compromise the estimates of the outcrossing rate (Ritland & El-Kassaby 1985).

Table 3. 4. Results from the mixed mating system and pollen structure model analyses using MLTR and TwoGener respectively, in one fragment (BP) and one continuous population (GB) of chokecherry. The parameters estimated included the multilocus outcrossing rate (t_m), mean single-locus outcrossing rate (t_s), biparental inbreeding rate (t_m-t_s), correlation of paternity (r_p), paternity differentiation between families (ϕ_{FT}) and neighborhood size as (1/ r_p) and N_{ep}. Standard errors from the mixed mating model are shown in parentheses next to the estimates. 95% Confidence intervals (CI) are shown for t_m , t_m - t_s, r_p and ϕ_{FT}.

Model		**BP**	**GB**
Mixed mating	t_m	1.166 (0.074)	0.992 (0.024)
(MLTR)		(1.129-1.203)	(0.981-1.003)
	t_s	0.990 (0.032)	0.919 (0.030)
	$t_m - t_s$	0.175 (0.079)	0.072 (0.035)
		(0.135-0.215)	(0.057-0.083)
	r_p	0.437 (0.066)	0.249 (0.069)
		(0.404-0.470)	(0.219-0.279)
	1/ r_p	2.3	4
Pollen pool structure	ϕ_{FT}	0.160	0.120
(TwoGener)		(0.140-0.180)	(0.107-0.133)
	N_{ep}	3.1	4.2

Table 3. 5. χ^2 test results and F_{ST} for the differences between the genetic frequencies of pollen and ovule. *n* is the number of alleles at the locus. (†) indicates loci with rare pollen alleles not present in the ovules, asterisk (*) indicates when $p < 0.001$.

Loci	*n*	F_{ST}	χ^2
ps08	3	0.31106	435.484*
M4c	11	0.00054	-3.78
ps12a12 †	17	0.00553	61.936*
97-402 †	25	0.00335	56.28*
Ma006b †	13	0.00858	72.072*
96-005 †	17	0.00508	56.896*

GENETIC DIVERSITY OF ADULTS VS. OFFSPRING

Comparison of genetic diversity parameters, and the fixation index between the adults and offspring, revealed a reduction of all genetic diversity estimates, and an increase in the fixation index in the offspring compared to the adults in both populations; however, none of the differences were statistically significant (Table 3.6). The sample size for the adult populations is considerably lower compared to the offspring generation, which will partially account for the high error values in the former, and might hinder the detection of statistically significant differences between adults and offspring. In the adult populations, GB also showed higher values of the genetic diversity estimates than BP but again, none of the differences were statistically significant. On the other hand, when the offspring of both populations are compared, all the genetic diversity estimates showed significantly higher values in GB compared to BP. Finally, the fixation index presented similar values in the offspring and adults of both populations revealing no presence of inbreeding.

Table 3. 6. Genetic diversity parameters and fixation index, and their 95% CI for adult and offspring populations from one fragment of forest (BP) and one continuous population (GB) of chokecherry, using eight microsatellites loci.

	BP		GB	
	Adults	**Offspring**	**Adults**	**Offspring**
Total number of samples	24	150	36	200
Number of alleles, N_a	8.125	7.350	10.25	9.250
	6.046 - 10.203	7.265 - 7.435	8.171 - 12.328	9.138 - 9.362
Effective number of alleles, N_e	4.4485	4.300	5.1936	4.977
	2.369 - 6.527	4.241 - 4.359	3.114 - 7.272	4.865 - 5.09
Observed heterozygosity, H_o	0.8120	0.781	0.8276	0.804
	0.728 - 0.890	0.773 - 0.789	0.740 - 0.918	0.798 - 0.81
Expected heterozygosity, H_e	0.7260	0.706	0.7518	0.735
	0.651 - 0.797	0.704-0.709	0.675 - 0.828	0.732 - 0.738
Fixation index, F	-0.123	-0.070	-0.120	-0.067
	-0.316 - 0.045	-0.08 - -0.061	-0.397 - 0.070	-0.073 - -0.061

S-ALLELE

So far, I have identified 11 *S*-alleles from 15 sampled individuals from GB (Table 3.7). The *S*-alleles ranged in size from 509 bp to 1.2 kb, with introns that ranged from 77 bp to 805 bp. The dramatic difference in intron size among different alleles will allow subsequent detection using electrophoresis techniques, such as single-strand conformation polymorphism (SSCP). By separating the PCR amplicons by SSCP, unique alleles can be identified and sent for DNA sequencing. This is an ongoing research project that unfortunately will not be included in this thesis.

Table 3. 7. Summary of *S*-alleles identified. Size of the gene from the second to the fifth conserve region, and size of the intron, is given in base pairs.

Sample	Gen size	Intron size
BP4	509	77
BP26	512	71
BP1	550	130
BP8A	550	130
BP7	613	190
BP26	613	190
BP17	614	200
BP2	680	221
BP6	754	337
BP7	754	337
BP8A	754	337
BP23	804	381
GB7	855	441
BP18	956	506
BP3	1228	805

3.4. DISCUSSION

Habitat fragmentation brings about major changes in the environment of plants which, in turn, affects pollination process, the genetic structure of populations and their mating systems. The two major impacts of habitat fragmentation on natural populations, arise from the reduction of size of populations and the increase of isolation. In this regard, it is expected that after fragmentation, new small populations will experience an increase in random genetic drift, inbreeding and disruptions of pollen and seed dispersal, which may lead to higher inter-population differentiation (Young *et al.* 1996; Lowe *et al.* 2005). Contrary to these expectations, I did not find evidence of either a reduction in genetic diversity or the presence of inbreeding after fragmentation, nor did I find evidence of genetic structure among the adult populations of chokecherry. However, when other aspects were explored, particularly the mating system and genetic diversity estimates of the offspring, some impacts of habitat disturbance were detected, supporting the findings from chapter one, where I found negative consequences of fragmentation on some ecological processes.

NEUTRAL EFFECTS OF FRAGMENTATION ON GENETIC DIVERSITY AND STRUCTURE

My results demonstrated relatively high levels of heterozygosity in chokecherry, compared to those found in other *Prunus* species. A study using 25 microsatellite markers on 11 species from the subgenus *Cerasus*, a close group from the subgenus *Padus* where chokecherry is classified, reported an average observed heterozygosity of 0.48 (Ohta *et al.* 2005), which is considerably lower than the levels found in these populations of

chokecherry (H_o= 0.724, H_e=0.741). However, the levels of expected heterozygosity were similar to those found in wild populations of *Prunus avium* (another *Prunus* from the subgenus *Cerasus*), where eight microsatellite markers were implemented (Stoeckel *et al.* 2006). In any case, the high levels of heterozygosity suggest extensive genetic variability in these chokecherry populations in both the fragmented and continuous populations.

Overall, analyses of the genetic diversity in populations showed little genetic differentiation, but some differences in allelic diversity among sites. Analyses of the observed and expected heterozygosity levels within populations revealed a significant loss of allelic diversity in the fragments, suggesting that they were historically larger populations that have suffered a recent reduction of their effective population size (Cornuet & Luikart 1996). According to theoretical predictions, these new smaller populations will suffer a reduction in genetic diversity and an increase in the inbreeding coefficient if they are isolated, consequently leading to an increase in the genetic differentiation among populations (Young *et al.* 1996). However, analysis from four microsatellite loci revealed similar levels of genetic diversity in the three adult populations and, although I detected a reduction on the allelic richness in the fragments compared to the continuous population, the differences were not statistically significant. These analyses were consistent with the results obtained with four additional loci for the comparison of one of the fragments (BP) to the continuous population, in which a non-statistically significant reduction in genetic diversity was also detected in the fragment. Lastly, while there were some effects of fragmentation on allelic diversity, I found no evidence of genetic differentiation among populations. Overall, these results appear to disagree with both theoretical models and empirical studies that have shown that

fragmentation can contribute to a considerable erosion of genetic variability (Young *et al.* 1996; Lowe *et al.* 2005). However, they seem to be in line with other studies that find similar levels of genetic variability in fragmented compared to continuous populations and high levels of genetic connectivity at the landscape scale after fragmentation (Craft & Ashley 2007; Andrianoelina *et al.* 2009)

The effect of fragmentation on genetic diversity is mainly determined by internal factors arising from the life histories and ecological traits of the plant populations studied. Mating systems, in particular, are critical and help to determine the susceptibility of species to fragmentation. Hand-pollination experiments (chapter one) and amplification of *S*-alleles, indicate that chokecherry has the SI typical of the Rosaceae family, the so called gametophytic self-incompatibility. SI systems prevent fertilization by self-pollen and promote outcrossing, maintaining low among population differentiation and high levels of within genetic diversity (Hamrick & Godt 1996; Vekemans & Hardy 2004); similar to those found in the chokecherry populations. This suggests that the mating system is an important factor preserving the high levels of genetic variability within these populations, together with other ecological characteristic of chokecherry that may have buffered the adverse effects of habitat fragmentation (Hamrick 2004). For instance, the long distance pollen dispersal detected in GB may be an important factor maintaining the connectivity among the populations, even when there is considerable geographic distance, such as the case of BP and GB, which are separated by more than 100 km of fragmented habitat. In addition, gene flow through seeds may also maintain high connectivity among the populations, as has been found in other plant populations (Bacles *et al.* 2006; Figueroa-Esquivel *et al.* 2009). In chokecherry, seeds are dispersed by

mammals and more commonly by birds (Webb & Willson 1985; Parciak 2002), which can travel long distances effectively spreading the seeds far from the maternal source and achieving long distance gene flow.

In addition to high levels of gene flow and genetic diversity maintained by mating dynamics, external factors determining the extent of habitat reduction/disturbance, such as the age of fragmentation, are decisive in explaining variability in response to habitat reduction. Simulations have shown that after a reduction in population size, levels of heterozygosity will take several generations to reach equilibrium (Varvio *et al.* 1986). In addition, a review on the effects of fragmentation on more than 100 plant populations, demonstrated that the larger the number of generations under fragmentation conditions, the larger the negative effect on heterozygosity (Aguilar *et al.* 2008). Since the generation time of chokecherry trees ranges from 30 to 40 years (Leigh 1999), I suggest that a relatively recent time has elapsed under fragmentation (3-4 generations), since the massive conversion of land to crop in Southern Manitoba occurred approximately 120 years ago (Ramankutty & Foley 1999). The fact that fragmentation was relatively recent, and that populations bear high levels of genetic diversity; partially explain the similarity of heterozygosity levels among fragmented and continuous populations. Analyses of allelic richness, on the other hand, detected a slight reduction in the fragments and a depletion of rare alleles, in particular for BP. This is due to the higher sensitivity, and faster reduction, of allelic diversity in response to a decrease in population size (Cornuet & Luikart 1996). Similar results have been found in other studies examining recent fragmentation events, in which levels of gene diversity did not differ between fragmented

and continuous forest, but significant reductions in allelic richness and rare alleles were detected (Jump & Peñuelas 2006; Bittencourt & Sebbenn 2009).

Even though there was no evidence of genetic divergence of populations, the finding that the population fragments had a loss of rare alleles may have important impacts on the populations, especially if this loss of diversity also occurs for rare alleles at the *S*-locus. If there is a concomitant loss of *S*-allele diversity, this can impact the mating system by limiting the number of genetically compatible mates (mate limitation) (Young & Pickup 2010), which can be a powerful force in populations causing lower seed set and reproductive performance, and may eventually represent a risk for population persistence (Young & Pickup 2010). Analyses on the reproductive output of populations, found a significant reduction in the fragments compared to the continuous populations mainly caused by pollen limitation (see chapter one for details), probably due to a reduction in the "quality" of pollen. This may occur when pollinators deposit self- or incompatible pollen on stigmas, situation that is likely to occur after a reduction in population size, if *S*-alleles are lost via random genetic drift (Busch & Schoen 2008). Thus, the occurrence of pollen limitation in the fragments may suggest that a reduction in compatible mates is causing mate limitation. However, previous reports on natural populations supported by theoretical models, have shown that small populations will loss considerably *S*-allele diversity only when the effective population size is very small, genetic drift is strong and gene flow is highly restricted, because *S*-alleles are subject to negative frequency-dependent selection which favour males carrying rare *S*-alleles (Glemin *et al.* 2005; Holderegger *et al.* 2008; Busch *et al.* 2010). According to my results, the fragments maintain high levels of within genetic variability and the

populations are interconnected by high levels of gene flow, ruling out mate limitation as the main force increasing the levels of pollen limitation and consequent reduction of reproductive success in the fragments. Instead, factors involve in mating and ecological dynamics may better explain the impact of fragmentation on the reproduction in the fragments.

POLLEN DISPERSAL CURVE

Analysis of the pollen dispersal curve in GB indicated that the exponential power curve model was the distribution that best describes the data. The fitted expo-power distribution had a very fat-tail ($b = 0.146$), and suggests very long distance pollen dispersal ($\delta = 3.9$ km). The exponential curve on the other hand, predicted a higher proportion of short dispersal distances and a considerably lower average pollen dispersal distance ($\delta = 29$ m) compared to the exponential power curve. Such underestimation of the average dispersal distance might occur when it is assumed a function that is significantly less leptokurtic (exponential) than the actual dispersal pattern (exponential power) (Robledo-Arnuncio, *personal communication*). This was revealed by the lower LSR and supported by the low value of *b* (0.146), which determines the tail-fatness, obtained under the exponential power model compared to the exponential model estimations. If these results are extrapolated to the landscape level, they would suggest the occurrence of large amounts of near-neighbour mating events in addition to high amounts of long-distance pollen flow, which is consistent not only with the results from pollen and ovule allelic frequencies that showed heterogeneity due to presence of rare

alleles, but also with the lack of genetic structure among the populations despite the considerable geographic distance.

These results are also in line with many other reports that collectively suggest that the fat-tailed pollen dispersal curve might be a common trend among plant species, at least in the several species studied so far (Austerlitz *et al.* 2004; Wang *et al.* 2010). However, the average pollen dispersal found here is even larger than that reported for other animal-pollinated and self-incompatible species (Smouse & Sork 2004; Garcia *et al.* 2007), and trees from the Rosaceae family (Oddou-Muratorio *et al.* 2005; Kamm *et al.* 2009). Studies in the Rosaceae have reported mean average distances of 1.1 km-750 m and 1.2 km for *Sorbus torminalis* and *Sorbus domestica* respectively, which are almost 2 km lower than the average found in the continuous population of chokecherry. It is possible that this unusually high value has been the product of an overestimation caused by the small value of *b.* In this case, the value of the median, which is more stable to small changes in *b,* might reflect a better estimation of the real effective pollen dispersal distance. The median for GB, based on the parameters of the exponential power model, was 928.5 m, which is more similar to reports of long pollen dispersal on other self-incompatible Rosaceae trees.

The large pollen distance dispersal in the chokecherry populations may be accounted for by the ability of pollinators to travel across large fragmented landscapes (Ghazoul 2005). Previous research on the foraging behaviour of pollinators reports early-flying bees, especially in the genera *Andrena* and *Bombus*, as important pollinators for chokecherry (Vicens & Bosch 2000). Studies using marked-recaptured methods and pollen analyses have shown that these bees can reach flight distance up to 1150 m

(Walther-Hellwig & Frankl 2000) and 663 m (Beil *et al.* 2008), for *Andrena* and *Bombus* species respectively. This long foraging distances jointed with the wide spatial distribution of chokecherry, may allow the formation of corridors where high levels of gene flow among the populations is maintained, supporting the results of the pollen dispersal parameters obtained for GB.

Unfortunately, I was not able to estimate the parameters of the pollen dispersal curve in BP, since I did not detect a significant correlation between correlated paternity and distance between maternal trees. It is possible that the decay of correlated paternity with distance might have not been captured due to the different sampling scheme used and the occurrence of large pollen flow distances in the fragmented population. Robledo *et al.* 2006 suggested that to capture this decay, KinDist method requires a maximum among-maternal tree distance of at least 2-5 times the mean dispersal distance. The maximum distance among maternal trees in GB was 40 km, which was sufficiently efficient for detecting the correlation between correlated paternity and the distance with the average pollen dispersal distance found in this population. However, in BP the maximum distance was only 2 km, which may not have been enough to detect the decay, since in this population it is also likely the occurrence of long distance pollen dispersal.

Studies on the effects of fragmentation on the pattern of pollen dispersal in plant populations have revealed that habitat modification may increase (Dick *et al.* 2003) or decrease (Hoebee *et al.* 2007; Mimura *et al.* 2009) pollen flow distance, situations that have been explained by changes in pollinator behaviour and assemblies (Dick *et al.* 2003; Gonzales *et al.* 2006; Dunphy & Hamrick 2007). Results on the mating system show higher levels of biparental inbreeding and paternity of correlation in BP compared to GB,

implying that in the former, pollinators sample pollen from fewer donors which are more likely to be near neighbours. It is possible that, in spite of the occurrence of long distance gene flow, the pollen dispersal curve in this fragmented population is less fat-tailed than that found in GB, due to a variation in pollinator behaviour that increases short pollen movements. In addition, variation in gene flow by pollen may also result from changes on pollinator diversity, which has been demonstrated by numerous reports on fragmented landscapes (Wilcock & Neiland 2002; González-Varo *et al.* 2009). For instance, Dick *et al.* (2003) explained the variation of the pollen dispersal among disturbed and undisturbed forest, by the predominant presence of African honeybees (*Apis mellifera*) in highly disturbed habitats. Thus, understanding the effects of habitat fragmentation on this aspect of pollinator ecology may bring valuable insights to this type of analysis. For chokecherry, it has been described a wide range of non-specialist pollinators (Vicens & Bosch 2000; Geyer *et al.* 2008; Miliczky 2008), which may indicate less susceptibility to changes on pollinator diversity, compared with tree species with more specialist pollinator relationships. If this is the case, the effects of fragmentation in mating dynamics of chokecherry may be mainly due to changes in pollinator flight patterns rather than effects pollinator assembly. However, without an exhaustive study on pollinator ecology in the populations, it is difficult to draws precise conclusions about the response of the pollinators to fragmentation.

MATING SYSTEM AND FRAGMENTATION

Analysis of the mating system in chokecherry showed that although outcrossing was absolute (t_m=1), some mating events occur among relatives (t_m- t_s > 0). Biparental inbreeding was significantly higher in BP (18%) compared to GB (7%); a finding that agrees with several studies that have found higher levels of biparental inbreeding in fragmented compared to continuous populations (Fernandez-Manjarres & Sork 2005; Mimura *et al.* 2009). Results from the correlated paternity (r_p) estimates using either MLTR or TwoGener, also displayed a significantly higher value in BP compared to GB. In BP, approximately 50% (t_m x r_p) of the offspring were produced by correlated outcrossing, while in GB only 27% were full sibs, suggesting that in BP more flowers are pollinated by the same pollen donor. Two main factors may be accounted for the increase of biparental inbreeding and paternity of correlation: adult inbreeding and spatial population structure (Austerlitz & Smouse 2001b). I did not detect inbreeding in any of the populations, but I did find modest levels of spatial genetic structure at short distances (32m), in particular for BP. Thus, the spatial distribution of the genotypes may be an important factor not only determining the correlation of paternity but also affecting the levels of biparental inbreeding in these chokecherry populations, especially for BP.

In addition to the fine genetic structure within populations, variation in density due to fragmentation may also determine mating dynamics when it affects the activity of pollinators (Garcia *et al.* 2005; Knight *et al.* 2005). Estimates of the local population density showed differences among populations, due to the occurrence of more dense patches in BP than GB (see chapter one for details). This variation in the spatial distribution of individuals, may reflect an increase in the clonal propagation in the

fragments (Ortego *et al.* 2010), explaining both the high density of patches and the increase in correlation of paternity, since pollinators may fertilize many flowers with pollen from different individuals that may belong to the same ramet (Llaurens *et al.* 2008). The clumped arrangement of trees in BP may also favour shorter inter-mate distances, resulting in more mating among neighbouring trees, which are more likely to be related. In GB, on the other hand, the lower tree density within patches might increase pollinator flight distances resulting in the sampling of a wider array of pollen donors, which are less likely to have similar genealogies.

According to theoretical predictions and some empirical data, an increase in the paternity of correlation and biparental inbreeding, are likely to increase levels of inbreeding (Lienert 2004; Coates *et al.* 2007). Although, the observed heterozygosity in the offspring from BP is significantly lower than that in GB, levels of inbreeding were not significantly different in the offspring or adult populations, suggesting that the biparentally inbred progeny do not survive to adulthood. This may be explained by the exposure of the considerable genetic load that is usually maintained in outcrossing species. Mating among related individuals increases the probability that deleterious recessive loci will be exposed in homozygous form (Charlesworth & Charlesworth 1987), and cause a decline in the mean phenotype fitness, i.e., inbreeding depression (Lynch & Walsh 1998). Thus, inbreeding depression seems to maintain high levels of genetic diversity in chokecherry, avoiding inbred seeds to develop in the adult population, even when mating dynamics is affected.

Another interesting result obtained from the examination of the mating system in these populations, was the heterogeneity of pollen allele frequencies found among

maternal trees, which was detected in 5 out 6 loci. Several factors could account for the observed discrepancies in allele frequencies between the ovule and outcrossing pollen, including nonrandom mating of genotypes during outcrossing events and pollen migrating from outside the population (Doligez & Joly 1997; Lee *et al.* 2000). My results suggest that these two processes might both be important in determining the heterogeneity of pollen and ovule allele frequencies in chokecherry. The significant level of biparental inbreeding and the high paternity of correlation indicate the occurrence of nonrandom matings, in particular for BP. However, they do not explain the presence of rare alleles found in the pollen but not present in the ovules. This observation could be explain by migration of pollen from outside the area sampled, which is consistent with results from estimates of pollen dispersal, where I found long distance pollen movements.

CONCLUSIONS

Forest fragmentation is a complex process that involves several factors simultaneously, thus in order to better understand its impacts on genetic processes, it is crucial to explore different genetic parameters. The two major impacts of habitat fragmentation, the reduction of size and the increase of isolation, revealed neutral effects on these chokecherry populations. I did not find evidence of a reduction in genetic diversity, inbreeding, or significant genetic structure among the adult populations. Factors such as long distance pollen dispersal, high levels of gene flow, a predominantly outcrossing mating system and a widespread distribution may have buffered the effects of fragmentation, and help to maintain the high levels of within genetic diversity and connectivity of populations.

However, when the mating system and genetic diversity parameters of the offspring were estimated, I found evidence of the impacts of habitat disturbance. These analyses showed an increase in biparental inbreeding and paternity of correlation in one of the fragmented populations (BP) and a reduction in the genetic diversity in the offspring from BP compared to GB. The higher spatial genetic structure found in the former may partially explain these results, since pollinators are more likely to sample pollen from related near-neighbouring trees. This increase in biparental inbreeding may result in negative consequences for plant reproduction, as shown by the analyses on ecological processes (chapter one), in which I found significant negatives effects of forest fragmentation on reproductive success and pollen availability.

4.0. GENERAL CONCLUSIONS AND FUTURE RESEARCH

This study has illustrated the importance of exploring different genetic and ecological processes to elucidate the impact of habitat fragmentation on plant populations. The observation of similar levels of genetic diversity within populations and the absence of genetic structure and inbreeding, suggest that the two major impacts of fragmentation, reduction in size and increase in isolation, had neutral effects. This is consistent with the life-history traits and ecological characteristics of these chokecherry populations, such as the maintenance of high levels of gene flow, a predominantly outcrossing mating system in which the presence of SI helps to maintain connectivity of populations, a widespread distribution and the relatively recent occurrence of fragmentation. However, when I examined the mating system parameters and the genetic diversity estimates of the offspring in one of the fragments (BP), and compared them with those in the continuous population, I found evidence of negative impacts of habitat disturbance. These analyses showed higher biparental inbreeding and paternity of correlation in the fragmented population, and a reduction in the genetic diversity in the offspring from BP compared to GB. These results support the analyses on ecological processes, which also showed significant negatives effects of forest fragmentation, in particular on reproductive success and pollen availability.

Hand-pollinations with supplemented cross pollen showed that the reduction in reproductive success in the fragmented chokecherry populations is mainly due to pollen limitation. However, the higher fruit set in GB may also be partially explained by the low

incidence of the fungus *Apiosporina morbosa,* since infection seems to have a negative effect on the reproductive output in this population. In contrast, infected trees from BP show the opposite response, and seem to have higher reproductive success than non-infected individuals. This is because the infected trees from this fragment are larger (bigger and with more inflorescences), and may be able to allocate more resources to reproduction than to vegetative growth, compared to the smaller infected trees in GB. This suggests that both the presence of fungal infection and the availability of maternal resources, as estimated by tree size and the number of inflorescences, have a minor but significant effect on the production of fruits, and further support the hypothesis that pollen limitation is the main cause for the reduction in reproductive success in the fragments.

Generally, pollen limitation is a consequence of a reduction in the quantity and/or quality of pollen. In self-incompatible species, the latter is usually referred to as the lack of availability of compatible mates, i.e., mate limitation, due to the paucity of individuals carrying different *S*-alleles from the maternal tree. Population genetic theory predicts that after a reduction in effective population size there will be a loss of genetic diversity, which may translate in loss of compatible mates when the number of *S*-alleles is also reduced (Busch & Schoen 2008). Although my results support the assumption that the fragments were historically larger populations and then suffered an abrupt reduction of population size, there was no evidence of a reduction in the levels of genetic diversity or significant loss of the allelic richness at eight microsatellite markers, which is consistent with the panmictic mating system and the long distance gene flow found in chokecherry. This suggests that mate limitation is not a main factor contributing to the higher levels of

pollen limitation in the fragmented populations, and the result from the *S*-alleles sequences, which is a work in progress, will help to support this hypothesis.

My results also suggest that although there are probably sufficient *S*-alleles (i.e., compatible mates), fragmented populations are still pollen limited due to the type of pollen that arrives to the receptive stigma. The higher levels of biparental inbreeding and paternity of correlation in BP, suggest that the flowers from this fragmented population are more likely to be pollinated by the same pollen donor which is also more likely to be a related tree. These results are explained by the spatial distribution of the genotypes in the populations, which affect that type of pollen that is sampled by the pollinators (Austerlitz & Smouse 2001b); and by the variation of density among populations, which may affect the activity of the pollinators (Garcia *et al.* 2005; Knight *et al.* 2005). Estimates of plant density in each population, suggest that the clumped arrangement of trees in the fragmented populations is favouring shorter inter-mate distances, and enhancing pollen transfer among nearby trees. According to the results from the fine spatial genetic structure analysis in BP, it is likely that mating between neighbouring individuals results in biparental inbreeding. In GB, on the other hand, the lower tree density within patches might increase pollinator flight distances resulting in the sampling of a wider array of pollen donors, which are less likely to have similar genealogies. Since chokecherry is able to reproduce vegetatively, an analysis of the clonal structure in these populations may help explain the variation in the spatial distribution of individuals, and further support the higher levels of correlation of paternity found in BP.

Overall, these results suggest that the higher levels of biparental inbreeding and the reduction in the number of pollen donors, are major factors determining the reduction

on the reproductive success in the fragmented populations. The increase in consanguineous mating will increase the probability of encountering related pairs of individuals that may share *S*-alleles or bear highly detrimental receive mutations (Hirao 2010). If the former occurs, fertilization will be prevented but if fertilization does ensue, the resulting inbred zygotes may be aborted due to maternal choice, embryo competition (Korbecka *et al.* 2002) or inbreeding depression (Husband & Schemske 1996). Examination of the rate of germination and seedling survival from seeds collected during the second year will be useful to look for differences in levels of inbreeding depression among populations at different life stages, and to help further elucidate the impact of the higher levels of biparental inbreeding in the fragmented populations.

Since the increase in levels of biparental inbreeding in the fragmented populations may be mostly caused by the flight patterns and foraging behaviour of pollinators, research on the pollinator ecology and pollinator diversity in these populations would be useful to support my results on the mating system dynamics and also to: (i) provide further information about the "quantity" of pollen arriving to the stigma, and determine if it is also a limiting factor for fruit production; (ii) explore the effects of fragmentation on the extent of the pollen dispersal, and (iii) support the results obtained on the pollen dispersal curve in GB. Estimates of contemporary pollen dispersal in GB revealed a fat-tailed curve, suggesting that large amounts of near-neighbour mating events occur in addition to some very long-distance pollen flow. Although I was not able to estimate the pollen dispersal curve in BP, the increased biparental inbreeding observed in this population suggests that the mating events in the population occur more frequently between nearer neighbour pairs compared to GB. An analysis of the pollinator ecology

will provide data to test the hypothesis that the increased in mating events between nearer neighbour pairs in BP compared to GB, will result in a less fat-tailed curve in the former. In GB, on the other hand, I found an extraordinary high value for the mean pollen dispersal distance (3.9 km) that may be a statistical artefact (due to the small estimated value of *b*); but it is not an unrealistic value since it is not higher than that observed in a few other reports on other insect pollinated species. For instance, the mean pollen dispersal distance of 4.5 km reported for *Swietenia humilis* (White *et al.* 2002) or the remarkable mean of 88.6 km in *Ficus sycomorus* (Ahmed *et al.* 2009), are both higher than that observed here. White *et al.* (2002) attributed their result to the capability of *S. humilis* insect pollinators to move long distances, and the possibility of ‘‘carry-over’’ due to the extensive metapopulation system in this species. Ahmed *et al*. (2009), on the other hand, explained their results by the wind-borne dispersal of fig wasps, the main pollinator for *F. sycomorus* in the area sampled**.** Thus, information about the pollinator assembly in the chokecherry populations will be useful to both determine how precise the estimation of the mean pollen dispersal is, and explore the effects of fragmentation on the pollen dispersal distance.

In general, my results show that some ecological consequences of fragmentation (e.g., pollen limitation) are, to some extent, being buffered by the genetic characteristic of these chokecherry populations (high gene flow – high genetic diversity) (Aguilar *et al.* 2008; Kramer *et al.* 2008). However, the major ecological factor altered by fragmentation was reproductive success, which may increase the probability of local extinction if it results in demographic consequences for the plant populations (Knight *et al.* 2005). If the reduction in reproductive output is not demographically critical, it may still interfere with

broader community dynamics, in particular for common and widespread species such as chokecherry (Ashman *et al.* 2004; Broadhurst *et al.* 2008). The reduction in seed production in the fragments of forest might alter the association with other plant species, lead to lower resource availability for seed predators, and reduce habitat for wildlife.

My research provides the first report of the effects of fragmentation on ecological and genetic process of a plant species in Manitoba. To reach the goals of the provincial government for a sustainable development of Manitoba, where conservation of *critical* and *significant habitat* is one of the strategic priorities (Manitoba 2005), more research is needed. Studies on other chokecherry populations with different degrees of fragmentation but located in similar landscape conditions, would provide further information about pollinator behaviour and disturbance. In addition, future research in other plant species with different life-history and ecological characteristics is crucial to further understand the effects of the fragmentation in this region and to develop proper conservation management. My research revealed the importance of studying the effects of fragmentation not only at a landscape scale, but also at a local scale, where the variation in ecological and genetic processes may have important consequences, in particular for widely distributed plant species. These species may have intrinsic characteristic that will buffer some of the impacts of habitat disturbance at a broader scale, but changes within populations may have important effects on plant persistence and consequently interfere with broader community dynamics.

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