Population genetic structure and plant fitness of natural and *ex situ* populations in *Silene chlorantha* (WILLD.) EHRH. and *Silene otites* (L.) WIBEL

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1 General Introduction

1.1 Effects of land-use changes on dry grasslands

Dry- and semidry grasslands of the classes *Festuco-Brometea* and *Sedo-Scleranthetea* are among the most species-rich plant habitats in Central Europe (Ellenberg 1996; Poschlod et al. 1998). In contemporary landscapes such habitats are semi-natural, which evolved by livestock grazing over the past centuries. Especially plant species of dry grasslands suffer from land-use changes as abandonment or intensification, affecting their performance and frequency (Lindborg 2007; Dostalek & Frantik 2008). Changes in traditional land-use such as a decrease of mowing and grazing by sheep and goats, eutrophication as well as intensification or abandonment of species-rich grassland, led to fragmentation and loss of plant habitats especially in north-western Europe (WallisDeVries et al. 2002). Most dry grasslands become increasingly restricted to small and isolated patches (Lauterbach 2008; Eriksson et al. 2002; Lindborg & Eriksson 2004) as formerly species-rich dry grasslands are being converted into species-poor communities dominated by grasses (e.g. Bornkamm 2006; Dostalek & Frantik 2008; Partzsch 2011; Willems 1987). In case of eutrophication and abandonment less competitive habitat specialists adapted to extensive land-use are often replaced by more competitive species such as plants forming stolons and rhizomes as well as nitrophilous and ruderal plants (Saunders et al. 1991; Thompson 1994; Fischer & Stöcklin 1997; Fischer & Matthies 1998).

Land-use often affects population structure as dispersal events such as grazing and hayseed formerly closely connected populations (Poschlod et al. 1998). Hence, land-use changes are a driving force for populations to become small and isolated. Especially for species without special dispersal vectors (autochores, barochores), seeds have difficulties to reach suitable habitats even when they are nearby (Ozinga et al. 2009). Additionally, small populations face a higher risk of extinction as they are more prone to environmental and stochastic effects (Boyce 1992).

In the investigation area of this thesis, the north-eastern part of Germany (Brandenburg), a decline in sheep and goat grazing has led to an increase in fragmentation, abandonment and succession by shrubs and trees (Kowarik 1990; Pless 1994; Ristow et al. 2011). Moreover, on fallow dry grasslands, establishment for seedlings is restricted by an accumulation of living plant biomass and litter (Lauterbach et al. 2011; 2012b).

1.2 The study species: *Silene chlorantha* (WILLD.) EHRH. and *Silene otites* (L.) WIBEL

The genus *Silene* (Caryophyllaceae) with about 600-700 species is one of the largest genera of the World's flora (Greuter 1995). The genus is widespread and most abundant in the Mediterranean and the Middle East. Based on molecular studies the genus *Silene* is included in the tribe *Sileneae* (Oxelman et al. 1997; Lidén et al. 2001). As a result of its enormous diversity of ecological and morphological characters, reproductive systems and parasite host interactions *Silene* often serves as model system for manifold scientific questions (reviewed by Bernasconi et al. 2009) e.g. the evolution of reproductive system (Delph & Meagher 1995; Desfeux & Lejeune 1996), sex chromosomes in dioecious plants (Moore et al. 2003), population structure (Tero et al. 2003), interaction with parasite fungi (Alexander & Antonovics 1988) and flower scent composition (Jürgens et al. 2002a).

The Yellowgreen Catchfly *Silene chlorantha* (WILLD.) EHRH. (Caryophyllaceae) (Fig. 1-1) is a diploid (2n = 24; Hand & Lauterbach 2012) perennial plant species of steppe-like, nutrient-poor and sandy base-rich dry grasslands. In the investigation area of Brandenburg it grows in nutrient-poor, sandy open pine forests, dry grasslands on moraine ridges, sunny hills and inland dunes. *S. chlorantha* belongs to the continental flora of xerothermic grasslands within the Festuco valesiacae-Stipetum capillatae sub-association Festuco-Koelerietum glaucae (Krausch 1961). Typical plant species associated with *S. chlorantha* in the north-eastern part of Germany are *Gypsophila fastigiata, Silene otites, Potentilla incana, Festuca psammophila, Koeleria glauca* and *Koeleria macrantha*.

In the genus *Silene*, the species *S. chlorantha* belongs to the section Sclerocalycinae (BOISS.) SCHISCHKIN which includes the section Chloranthae (ROHRB.) SCHISCHKIN (Tutin et al. 1993). It was firstly mentioned and pictured by Mentzel (1682) from a location near Fürstenwalde in the East of Germany (Brandenburg) (Krausch 2010). In the first description by Willdenow (1787) it was pictured and named as *Cucubalus chloranthus* found in Berlin: "*In editioribus gramineis prope* Spandau bei den Pulvermagazinen *passim*, hinterm Gewehrplan *frequens*" (Fig. 1-2). This population became extinct as a result of urban settlement probably in the 19th or 20th century. *S. chlorantha* is characterised by a basal leaf rosette with glabrous, lanceolate-spathulate leaves and yearly renewed flowering stems of 30-80 cm height. Flowering takes mostly place in June and often there is a second flowering peak in September. Flowers are hermaphroditic, protandric and most likely pollinated by nocturnal moths (Jürgens et al. 2002b). Selfing is possible and seems to be frequent in small populations (geitonogamy). Seeds are gravity-dispersed, with no morphological adaptation

for animal- or wind-dispersal. Germination occurs between autumn and spring, without obligatory seed dormancy. The mean seed number per plant in Brandenburg is 4450 (standard deviation (SD) 4852) with an average seed mass of 0.12 mg (SD 0.032) per seed (Lauterbach 2008).

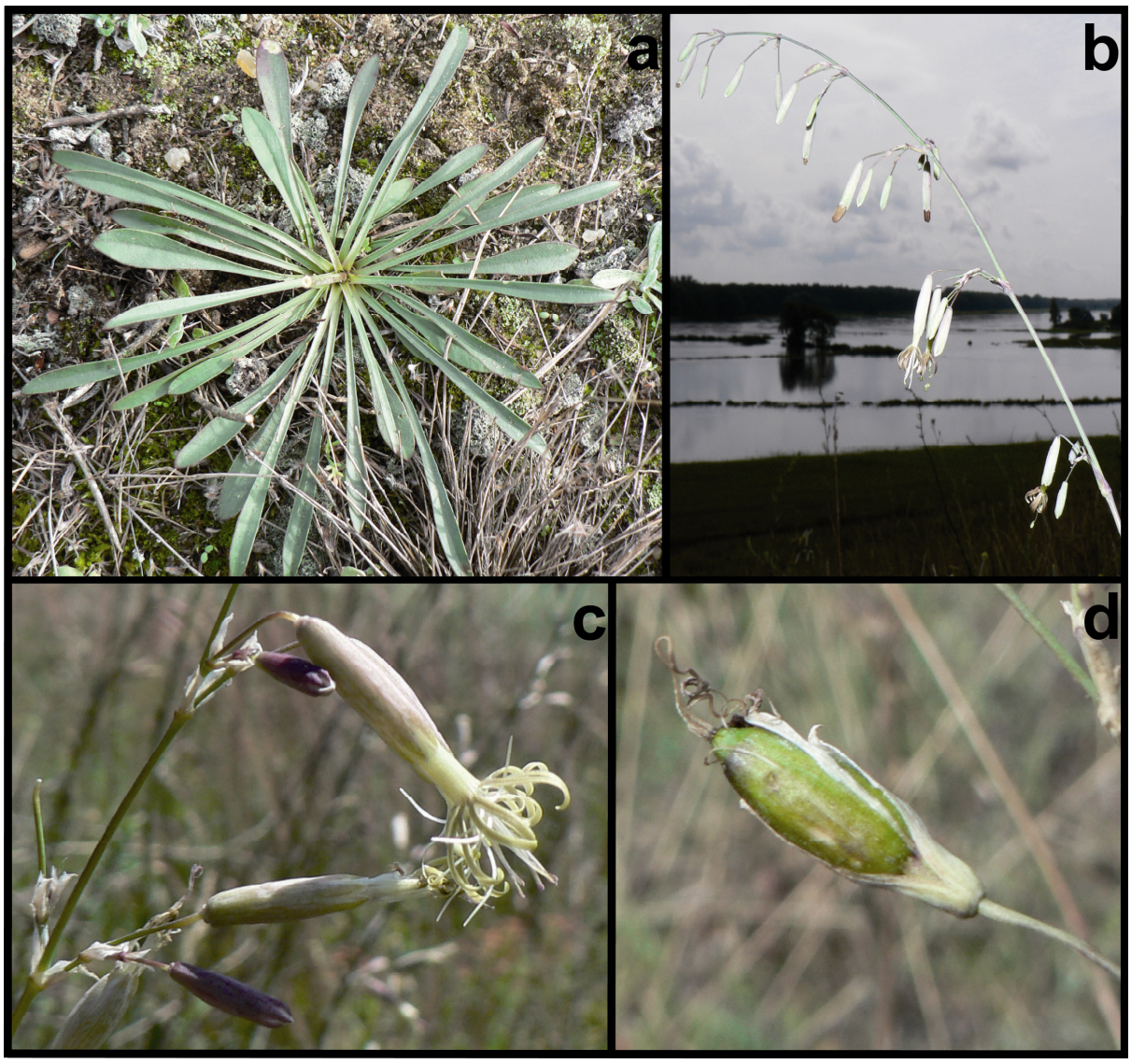

Fig. 1-1: *Silene chlorantha* north of Frankfurt/ Oder: a) leaf rosette of an adult plant, b) flowering stem, c) flower and flower buds, c) ripening seed capsule

In Europe, *S. chlorantha* can be found from sub-meridional to temperate regions but it is restricted to continental Europe and western Siberia. Its southern-most boundary is in southern Bulgaria. Its western-most boundary around 13°E in Brandenburg, north-eastern Germany, appears to be largely defined by climatic conditions, such as average temperature and the amount of rainfall (Krausch 1961; Meusel et al. 1965; Tutin et al. 1993). The

western-most boundary follows a line from Luckau in the Southeast over Glau/Trebbin, Berlin-Heiligensee, Bernau, and Greiffenberg/Angermünde to Gartz (Oder) in the North-East. The actually western-most population is located in Glau/Trebbin.

In Germany, even in historic floras this species was never reported to be common in the investigation area. In Berlin and Brandenburg, approximately 100 locations are known (Ascherson 1864; Müller-Stoll & Krausch 1960; Benkert et al. 1996) of which 80 % have become extinct over the past decades due to forestation, succession and sand mining of dry grassland habitats. At present, only 22 populations still exist in north-eastern Germany being mostly geographically isolated; only ten of them feature population sizes of more than 100 individuals (Table 1-1). *S. chlorantha* is listed as endangered in the Red Lists of Germany (Korneck et al. 1996) and Brandenburg (Ristow et al. 2006), and highly endangered in Berlin (Prasse et al. 2001).

Fig. 1-2: Probably one of the first drawings of *Silene chlorantha* in Willdenow's Florae Berolinesis (Willdenow 1787)

Table 1-1: Summary of remnant populations of *Silene chlorantha* still existing in Germany, site, coordinates, population size (number of flowering individuals), conservation status of the area, status of population in the last 10 years

Population site	Coordinates	Size	Conservation	Status
Berlin	52°35'N/13°13'E	10,000	protected	positive
Eisenhüttenstadt I	52°10'N/14°36'E	8,000	unprotected	steady
Eisenhüttenstadt II	52°11'N/14°36'E	1,000	unprotected	critical
Gabow	52°49'N/14°04'E	880	unprotected	steady
Baruth	52°02'N/13°30'E	800	unprotected	critical
Kunow	53°07'N/14°16'E	470	protected	steady
Bernau	52°41'N/13°36'E	440	unprotected	critical
Lebus I	52°23'N/14°31'E	300	unprotected	steady
Glau	52°14'N/13°09'E	300	unprotected	steady
Biesdorf	52°42'N/14°04'E	103	protected	steady
Britz	52°51'N/13°48'E	75	unprotected	critical
Eisenhüttenstadt III	52°10'N/14°36'E	60	unprotected	critical
Greiffenberg	53°05'N/13°55'E	58	unprotected	steady
Goyatz	52°00'N/14°09'E	27	unprotected	endangered
Jamikow	53°06'N/14°10'E	26	protected	endangered
Gartz I	53°13'N/14°20'E	23	protected	endangered
Podelzig	52°29'N/14°32'E	14	unprotected	endangered
Lebus II	52°24'N/14°31'E	10	protected	unknown
Gartz II	53°12'N/14°19'E	4	unprotected	endangered
Mallnow	52°27'N/14°30'E	3	protected	endangered
Groß Radden	51°52'N/13°51'E	1	unprotected	endangered
Neuhof	52°08'N/13°28'E	1	unprotected	endangered

Silene otites (L.) WIBEL (Caryophyllaceae), the Spanish Catchfly is a diploid (n = 12) dioecious perennial herb. It is also described as short-lived or biennial (Tutin et al. 1993). However, a life span up to 5 years and more in the wild could be observed (personal observations). *S. otites* colonises sandy alkaline-neutral soils of dry grasslands, railway embankments, pine forests, and sand dunes. Typical associated plant species in north-eastern Germany are *Festuca brevipila, Armeria elongata, Veronica prostrata, Dianthus carthusianorum, Scabiosa canescens, Peucedanum oreoselinum* and *Koeleria macrantha*.

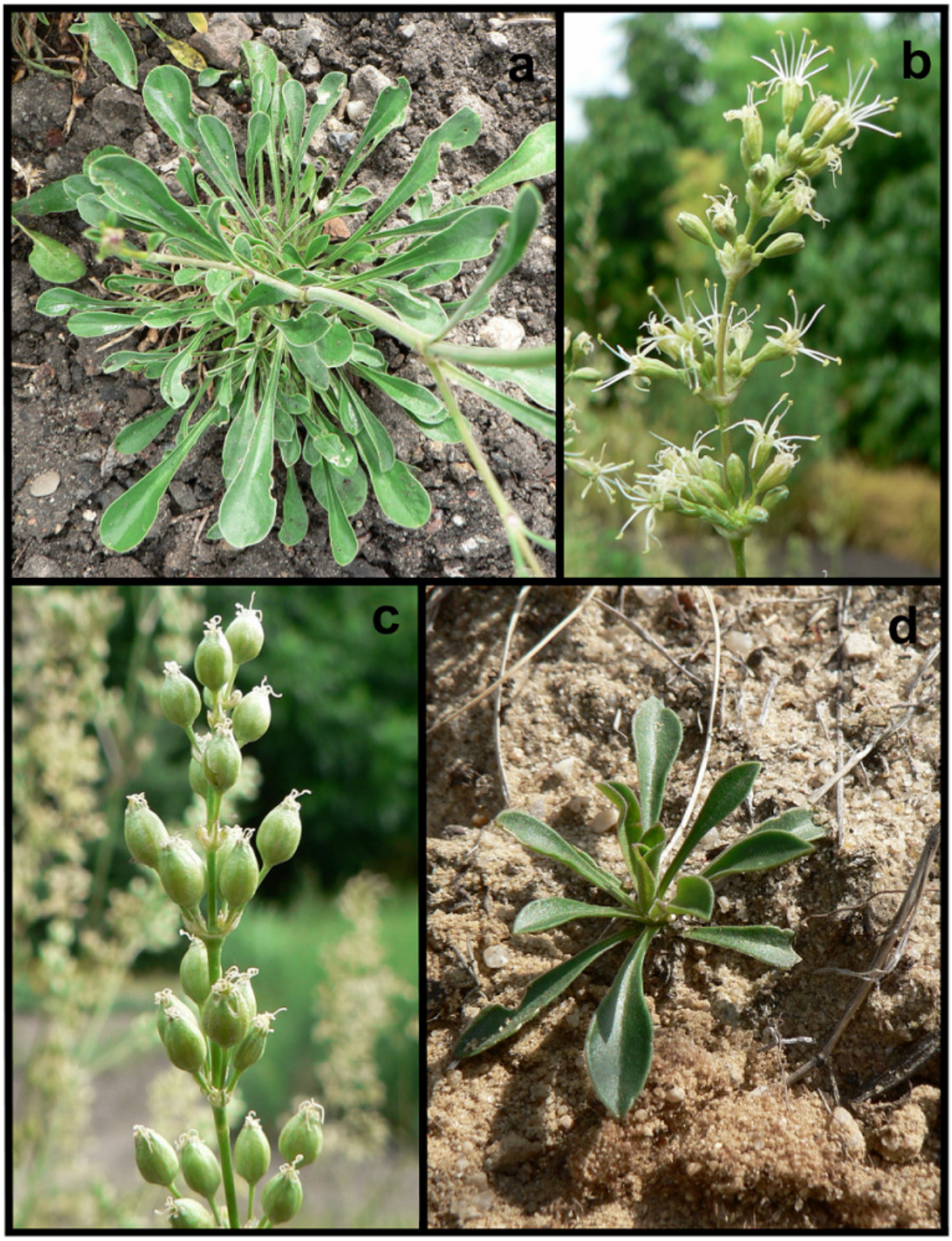

Fig. 1-3: *Silene otites* in Berlin: a) leaf rosette of an adult plant, b) male flowers, c) female seed capsules, d) juvenile plant

In the genus *Silene*, *S. otites* belongs to the section Otites OTTH (Wrigley 1986). The *S. otites* group comprises 6 species: *S. otites* (L.) WIBEL, *S. colpophylla* WRIGLEY, *S. exaltata* FRIV., *S. chersonensis* (ZAPAL.) KLEPOW, *S. donetzica* KLEPOW and *S. densiflora* D'URV which are distributed from Western Europe to Eastern Europe and Western Asia. There is a distribution overlap ("cline") among these taxa and hybrids have been reported (Wrigley 1986). The here investigated *S. otites* s. str. (Fig. 1-3) is restricted to Western Europe ranging from Central Spain to Lithuania and Bulgaria (Tutin et al. 1993). It has a stem height from 10 to 60 cm with about 20-50 flowers each. Flowering takes place between June and October. Pollinators are mostly nocturnal Lepidoptera (e.g. *Plusia gamma*) and mosquitoes (e.g. *Culex pipiens*) (Schulz 1905; Brantjes & Leemans 1976; Jhumur et al. 2007), and also ants visited flowers (personal observations). The spathulate leaves are arranged in a basal rosette. The underside of the leaves is pubescent but plants are variable in leave shape, colour and appearance. The mean seed number per plant is 5122 (SD 5195) and the average seed weight per seed is 0.16 mg (SD 0.035) (Lauterbach 2008). Soldaat et al. (2000) observed wind pollination of only a few meters. This is supported by personal investigations in a common garden experiment. Seeds are dispersed actively by sheep (Wessels-de Witt & Schwabe 2010) but more commonly in a passive way (autochor, barochor) (Watt 1981). Sometimes complete capsules are dispersed by adhering in fur or clothes with their dentate opening (personal observations). Germination occurs in autumn and spring (Soldaat et al. 2000), mean germination rate is about 80 % (Lauterbach et al. 2012a), and many seedlings die off under unfavourable weather conditions. *S. otites* is said to feature some hermaphroditic individuals (Newton 1931); which, however, was not observed during the thesis' project time. During the past decades, the number of populations distinctly declined as results of habitat loss and secondary succession of dry grassland caused by decrease of traditional sheep grazing and increased eutrophication. *S. otites* is listed as vulnerable in the Red Lists of Germany (Korneck et al. 1996), Brandenburg (Ristow et al. 2006), and highly endangered in Berlin (Prasse et al. 2001).

1.3 Population genetic structure and plant fitness

Population genetics comprise the study of allele frequency distribution under the influence of natural selection, mutation, genetic drift and gene flow. The main goal of population genetic studies is to improve the understanding of the forces that have an impact on levels of genetic variation (Hartl & Clark 1997). A population is defined as group of individuals of the same

species which are in genetic exchange with each other but separated from other groups by temporal or spatial segregation (Hartl & Clark 1997). Genetic population structure is influenced by many factors, e.g. population size, migration patterns, mutations, selection, life history traits and mating systems (Loveless & Hamrick 1984). In addition, changes in abiotic conditions, stochastic events, as well as biotic factors like competition and predator-prey interactions can influence population structure (Hutchings 1997). In summary, there is an evolutionary pressure on populations for survival where genetic diversity holds the potential of adaptation to changing environmental conditions.

The Hardy-Weinberg-Theorem assumes that a population is in equilibrium when it is infinite in size and all individuals mate at random (Futuyma 1998). In nature there is often a deviation from the equilibrium state caused by non-random mating, small population sizes and restricted gene flow. These facts decrease population genetic diversity and increase genetic differentiation among populations. Frequently, individuals of a group mate with their relatives leading to inbreeding effects. The theory of inbreeding was in detail discussed by Wright (1931; 1938). The most extreme form of inbreeding is selfing, which can often be found in hermaphroditic plant species. In contrast, some plant species avoid selfing by obligate out-crossing, e.g. as dioecious ones (Barrett & Harder 1996). Inbreeding is more likely in small and isolated populations of few individuals resulting in increased numbers of homozygotes and deceased numbers of heterozygotes. A decline in the mean phenotype of individuals, called “inbreeding depression”, is caused by an increased number of homozygotes for recessive alleles (Charlesworth & Charlesworth 1987; Futuyma 1998). Conversely, an increasing number of heterozygotes by outbreeding and so concealing more of the recessive alleles is called “heterosis” (Futuyma 1998). Inbreeding depression can cause a decline in progeny fitness characterized by lower seed set, lower germination rate and lower offspring survival (Husband & Schemske 1996). However, as expressed in the theory of “partial dominance“ (Charlesworth & Charlesworth 1987) natural selection is expected to purge deleterious recessive alleles combined with inbreeding in a population with less genetic variation (Lande & Schemske 1985).

Besides mutations, inter-population gene flow is a possibility to increase genetic diversity. Therefore, obligate out-crossing species should have greater levels of gene flow and higher intra-population genetic diversity than self-compatible ones (Glemin et al. 2006; Duminil et al. 2009). However, obligate out-crossing species are expected to show stronger effects of inbreeding depression, whereas self-compatible ones are less sensitive to such effects (Lande & Schemske 1985).

Random fluctuations in allele frequencies are often neutral to evolution and do not directly affect survival or reproductive fitness. If such fluctuations lead to a loss or fixation of an allele

it is called genetic drift (Nei et al. 1975; Barrett & Kohn 1991). Genetic drift is strongly influenced by effective population size (Nei et al. 1975). Rare alleles are predicted to disappear faster in small populations. Hence, small populations have a higher risk to loose genetic diversity from generation to generation by random genetic drift. Additionally, restricted gene flow among populations results in an increased inter-population genetic divergence. In dispersal limited species, as many insect pollinated plants without special morphological adaptation for seed dispersal, there is a higher probability of genetic exchange among neighbouring populations than long distance dispersal. Over distances, differentiation among populations increases, called “isolation by distance (IBD)” (Wright 1943). Isolation by distance is predicted to occur if genetic drift and gene flow are at regional equilibrium (Hutchison & Templeton 1999). Whereas the absence of “isolation by distance” accompanied by a high amount of genetic differentiation among populations often indicates genetic drift to be much more influential than gene flow (Futuyma 1998; Hutchison & Templeton 1999).

Population size is one of the driving forces for population genetic structure and also expected to be one of the best predictors for population viability (Schmidt & Jensen 2000). In each generation some original alleles of a population get lost. Therefore, “the smaller the population, the faster heterozygosity declines” (Futuyma 1998). The effective population size (N_e) is the number of breeding individuals in an idealised population (Wright 1931; 1938). It strongly depends on generation overlap, mating system, geographical barriers and imbalanced sex ratio as found in many dioecious plant species (Felsenstein 1971; Lauterbach et al. 2012a). Strong fluctuations in population size, more precisely periods of very small population size, lead to genetic bottlenecks and founder effects (Nei et al. 1975). In the case of genetic bottlenecks, a population undergoes a massive decline in size followed by a decreased level of genetic variation in the following generations. If afterwards the population grows rapidly, genetic variation can only slowly built up by mutations if there is absence of gene flow among populations. This was shown by Lauterbach et al. (2011) for an isolated population of *Silene chlorantha* of known population history. In this study, the observed genetic diversity was lower than expected for the population size of several thousand individuals. The founder effect is described as special case bottleneck when only a few individuals of a larger gene pool start new populations.

In fragmented landscapes, small and isolated populations are predicted to have reduced plant fitness due to genetic erosion (Ellstrand & Elam 1993; Lienert 2004; Leimu et al. 2006). Inbreeding can lead to a reduced genetic diversity and inbreeding depression, leading to a further reduction in population size. Besides the genetic influences, a reduced fitness can also be caused by inappropriate habitat conditions and missing plant pollinator interactions (Ouborg et al. 2006). Therefore, field studies in combination with common garden

experiments have the potential to investigate the effects of genetic diversity and habitat quality on plant fitness (Fischer & Matthies 1998). Husband & Schemske (1996) highlighted the importance of life cycle stage and mating system for the investigation of plant fitness. The choice of dependent fitness variables (e.g. seed set, germination, survival rate, plant size) is a key to better understand correlations between genetic diversity, habitat quality and plant performance.

In the last decades numerous different methods to investigate plant population genetic structure have been developed, each with advantages and disadvantages (e.g. Nybom 2004). The overall goal of population genetic studies is the measurement of variation of a genetic marker following the assumption that the variation of the marker is proportional to the entire variation. In this thesis I have chosen the random amplified length polymorphism (AFLP) method (Vos et al. 1999) with an optimised protocol for the study species *S. chlorantha* (Lauterbach et al. 2011) and *S. otites* (Lauterbach et al. 2012a; b). The AFLP method has been widely used for population level genetic studies (e.g. Tero et al. 2003; Bylebyl et al. 2008; Prinz et al. 2009). In comparison to other dominant markers such as random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR), AFLP has a much higher reproducibility, robustness and information content (Meudt & Clarke 2007). Codominant markers as microsatellites are individually more informative but are often restricted to a few informative loci. Additionally, for the here studied species *S. otites* and *S. chlorantha*, priori sequence information or already developed primers for microsatellite analyses did not exist so far. For other species of the genus *Silene* microsatellites loci were isolated e.g. for *S. tatarica* (Tero & Schlötterer 2005), *S. latifolia* (Teixeira & Bernasconi 2007). For *S. chlorantha* and *S. otites*, highly variable plastid markers (trnL-F, psbA-trnH; e.g. in Taberlet et al. 1991) and mitochondrial markers (cob, atpA; e.g. in Barr et al. 2007) as well as the nuclear ITS region showed no polymorphism on population level as well as among populations in the geographically small investigation area of north-eastern Germany.

1.4 *Ex situ* plant conservation

Preservation of plant biodiversity as source of food, biomass, medicine, clothing and ecosystem services and function is one of the main goals for the future (Guerrant et al. 1994). The contracting states which signed the Convention of Biological Diversity (CBD 1992) adopted the Global Strategy for Plant Conservation (GSPC) (CBD 2002) to stop the world wide loss of plant biodiversity. This strategy requests contract parties and the government to build up national and regional plans and programmes to preserve plant

species and their diversity. One aim of the GSPC is to promote research on genetic variability, taxonomy, ecology, habitat and conservation biology. Besides management and conservation in the wild (*in situ*) the GSPC also focuses on *ex situ* activities. One ambitious target (GSPC target VIII) is to safeguard 60 % of all endangered plant species in accessible *ex situ* collections preferably in the country of their origin including 10 % of them in restoration and recovery programmes. However, the national implementation is a great challenge (Burkart & von den Driesch 2006). So far, it will be mainly implemented by botanic gardens, seed banks and tissue collections. Botanic gardens cultivate endangered and rare plant species and conduct their reintroduction (Godefroid et al. 2010).

Ex situ activities comprehend different approaches as cultivation of living plants in botanic gardens and arboreta, germplasm banks and via seed storage in seed banks by cryo-conservation (Hamilton 1994; Maunder et al. 2001; Havens et al. 2006). The aim of all *ex situ* activities is to preserve samples that are representative of the extant *in situ* diversity and make the most efficient use of available resources (Falk & Holsinger 1991; Husband & Campbell 2004; Ensconet 2009). Because of financial constrains and limited space for cultivation or storage, sampling size is often restricted in living collections and seed banks. Moreover, for longer time periods in seed storage there is an absence of evolutionary processes as adaptation and selection to environmental factors. In contrast, in garden *ex situ* cultivars there is often unconscious selection and adaptation to garden conditions.

Highest conservation priority should first be given to *in situ* conservation of habitats and ecosystems since successful reintroduction and even more restoration are very costly and time-consuming efforts. Moreover, cultivation implements a change of evolutionary processes such as selective pressures and competition (Lauterbach et al. 2012a).

The differences between wild and *ex situ* demonstrate the importance of ecological and genetic studies for optimised sampling and cultivation of target species. There is a lot of theoretical knowledge about the effects of minimum population size, fragmentation, inbreeding and genetic drift on genetic structure and plant fitness from studies in the wild (e.g. Barrett & Kohn 1991; Elstrand & Elam 1992; Leimu et al. 2006). However, there is a lack of *ex situ* case studies especially for botanic garden cultivars. In garden cultivars, in the past not much attention was paid to representative sampling, unconscious selection during cultivation and minimum population size. Many *ex situ* populations are based on less than 10 source individuals (Brown & Briggs 1991; Husband & Campbell 1994). Hence, genetic bottleneck and founder effects can be expected for such small sized cultivar accessions.

Ex situ cultivation is only successful when it is possible to preserve the utility for future reintroduction (Husband & Campbell 1994). Often a species was cultivated just “somehow” in

the garden to avoid extinction (Burkart & von den Driesch 2006). This was done without paying attention to artificial and stochastic factors affecting genetic diversity and evolutionary biology of *ex situ* populations. Additionally, populations were often small, not genetically representative, poorly documented and not specifically managed for conservation purposes (Maunder et al. 2001; Maunder et al. 2004). Especially garden collections are vulnerable to hybridisation, genetic erosion, artificial selection and infection by pathogens (Maunder et al. 2004). The abiotic and biotic conditions are very different from those in the source population. Hence, in case of *ex situ* conservation purposes the application of the theoretical knowledge derived from *in situ* studies is largely restricted. The knowledge about genetic diversity, fitness, hybridisation during cultivation and suitability for reintroduction programmes from practical studies is also rather small (e.g. Enßlin et al. 2011; Rucinska & Puchalski 2011; Lauterbach et al. 2012).

1.5 Comments to the structure of the presented thesis

This is a cumulative doctoral thesis. The present thesis covers different aspects of population genetics, fitness, ecology and conservation efforts of two endangered perennial dry grassland plant species of the genus *Silene* in north-eastern Germany. Other main objectives of the presented study are investigations of genetic processes during spatial and temporal isolation of *ex situ* populations and their *in situ* source populations.

The study is divided in seven parts. Chapter 1 is a general introduction. The second part discusses the population structure and fitness of *S. chlorantha* in the investigation area of north-eastern Germany (Lauterbach et al. 2011) followed by a comparison between the Berlin Botanic Garden *ex situ* population and its *in situ* source population (Chapter 3). The fourth part comprehends experiments of artificial crossing (inbreeding and outbreeding) among different *S. chlorantha* populations to test for the effects upon genetic diversity and plant performance in the F_1 generation. It deals with a more practical approach of enhancing genetic diversity and plant performance by an intraspecific crossing experiment in the botanical garden.

In Chapter 5, an analysis of population structure and plant fitness of the dioecious *S. otites* in the area of north-eastern Germany is presented (Lauterbach et al. 2012b), followed by a comparison of population genetics between three pairs of *ex situ* and corresponding *in situ* source populations of *S. otites* (Lauterbach et al. 2012a). Thereby, the potential of integrating results from population genetics and fitness analyses into practical conservation efforts of

endangered plant species in Botanic Gardens is discussed. Chapter 7 is the comprehensive general discussion including an outlook.

Chapters 2, 5, and 6 are already published as articles in scientific journals. The other Chapters 3 and 4 follow the structure of the already published chapters and are divided into: introduction, materials and methods, results, discussion, acknowledgements and references. References for each chapter are given in particular and a reference list for the general introduction and the general discussion is given in Chapter 7.

2 Genetic population structure, fitness variation and the importance of population history in remnant populations of the endangered plant *Silene chlorantha* (Willd.) Ehrh. (Caryophyllaceae)

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2.1 Abstract

Habitat fragmentation can lead to a decline of genetic diversity, a potential risk for the survival of natural populations. Fragmented populations can become highly differentiated due to reduced gene flow and genetic drift. A decline in number of individuals can result in lower reproductive fitness due to inbreeding effects. We investigated genetic variation within and between 11 populations of the rare and endangered plant *Silene chlorantha* in northeastern Germany to support conservation strategies. Genetic diversity was evaluated using AFLP techniques and the results were correlated to fitness traits. Fitness evaluation in nature and in a common garden approach was conducted. Our analysis revealed population differentiation was high and within population genetic diversity was intermediate. A clear population structure was supported by a Bayesian approach, AMOVA and neighbour-joining analysis. No correlation between genetic and geographic distance was found. Our results indicate that patterns of population differentiation were mainly caused by temporal and/or spatial isolation and genetic drift. The fitness evaluation revealed that pollinator limitation and habitat quality seem, at present, to be more important to reproductive fitness than genetic diversity by itself. Populations of *S. chlorantha* with low genetic diversity have the potential to increase in individual number if habitat conditions improve. This was detected in a single large population in the investigation area, which was formerly affected by bottleneck effects.

Keywords: AFLP, Fitness, Population genetic structure, Population history

2.2 Introduction

Nowadays, in the industrialised parts of Europe many dry grassland plant species are suffering from habitat loss due to changing land use. In particular, the decline of traditional agriculture like sheep grazing has caused habitat changes. Additionally, secondary succession and nutrient input on the remaining habitats has led to changes in species composition, from less competitive herbs to more competitive grasses and shrubs (Bobbink & Willems 1991; Poschlod et al. 2005). In central Europe, the remaining populations of dry grassland species are often spatially isolated on small fragments of formerly bigger areas (Poschlod & WallisDeVries 2002). Even on a small geographic scale, this can lead to high genetic variability among plant populations and reduced genetic diversity of populations due to random genetic drift and loss of gene flow (Ellstrand & Elam 1993; Young et al. 1996; Booy et al. 2000; Frankham 2005).

For defining populations of major conservation importance or for replanting and linking populations, knowledge about the "genetic background" and structure of populations can support population management and conservation efforts (Stefenon et al. 2007). For effective conservation and habitat management efforts, Ellstrand & Elam (1993) and Volis & Blecher (2010) propose the preservation of representative high intra-population diversity. However, high genetic diversity does not prevent extinction per se when habitat quality is insufficient. Knowledge of habitat properties and their effects on plant fitness components, ranging from seed output to seedling survival and plant establishment, form a basis for population management.

In several studies, the effects of fragmentation and isolation on population genetics in relation to plant fitness and population size have been analysed to support conservation efforts. In most of these studies, fitness and population size were positively correlated (Oostermeijer et al. 1994; Fischer & Matthies 1998; Hensen et al. 2005; Hensen & Wesche 2006; Jacquemyn et al. 2007b). Sometimes, the results were contradictory, depending on the investigated fitness component (Schmidt & Jensen 2000; Jacquemyn et al. 2007b), life span, mating system and rarity (Leimu et al. 2006).

Schmidt & Jensen (2000) proposed population size to be one of the best predictors of population viability. Positive correlations between genetic diversity and population size were detected in several plant species (e.g. Oostermeijer et al. 1994; Gaudeul et al. 2000; Vergeer et al. 2003; Hensen & Oberprieler 2005); however, two studies of dry grassland species (Leimu & Mutikainen 2005; Jacquemyn et al. 2007b) revealed no correlations. Also, in several perennial dry grassland species, no correlation between genetic diversity and seed

set was found (Hensen et al. 2005; Leimu & Mutikainen 2005; Honnay et al. 2007). Furthermore, population history (Leimu & Mutikainen 2005) and species distribution ranges are important for the assessment of current genetic diversity and population structure, as loss of pollinators or genetic drift is sometimes stronger in marginal than in central populations of species distribution ranges (Gabrielsen et al. 1997; Schiemann et al. 2000). The species under investigation, *Silene chlorantha* (Caryophyllaceae), is regarded as highly vulnerable in Germany (Korneck et al. 1996).

The investigation area in northeast Germany comprises all German populations at the species' western-most periphery of distribution. Here, a severe population decline during the past decades could be detected. Therefore, the effects of isolation and population size on genetic variation and differentiation in 11 more-or-less isolated populations of different size were investigated. Additionally, correlations between plant reproductive fitness in different life stages and habitat characteristics, as well as population size and genetic diversity, were analysed to support conservation efforts. One of the investigated populations had a well-documented history, which gave us the opportunity to clarify whether such history leads to a genetic degradation and decline of population fitness.

2.3 Materials and Methods

Study species

Silene chlorantha (WILLD.) EHRH. (Caryophyllaceae) is a perennial plant species of steppe-like, nutrient-poor and sandy dry grasslands. It is characterised by a basal leaf rosette and yearly renewed flowering stems. Flowering takes place between June and October. Flowers are hermaphroditic, protandric and most likely pollinated by nocturnal moths. Selfing is possible among flowers of the same plant. Seeds are gravity-dispersed, with no morphological adaptation for animal- or wind-dispersal. Germination occurs between autumn and spring, without obligatory seed dormancy.

In Europe, *S. chlorantha* reaches from sub-meridional to temperate regions and is restricted to continental Europe and western Siberia. Its western-most boundary (Brandenburg in northeast Germany) appears to be largely defined by climatic conditions, such as rainfall and temperature (Meusel et al. 1965; Tutin et al. 1993). Here, even in historic floras this species was never reported as common (approximately 100 locations; Müller-Stoll & Krausch 1960) and 80 % of populations have become extinct over the past decades. At present, 20

populations still exist in northeast Germany, and are more or less geographically isolated; only eight of them feature population sizes of more than 100 individuals.

The single extant Berlin population (BB) is known from as far back as the beginning of the 19th century (Dietrich 1835), and was comprised of a large number of individuals until the 1950s (Zimmermann 1982). This population was reduced to about 20 individuals in the 1980s (Hömberg 1992) through human impact on the habitat (heavy disturbance in a military area). In 1988, seeds were taken for ex situ cultivation in the Botanic Garden Berlin-Dahlem. In 1990, the military area was closed and 279 individuals were replanted from the ex situ progeny (Hömberg 1992; T. Dürbye, personal communication). Since that time, conservation efforts have led to safeguarding of the habitat and the number of individuals has increased to approximately 10,000 today.

Sampling and AFLP analysis

For population structure and fitness evaluation, 11 populations of *S. chlorantha* were screened (Table 2-1, Fig. 2-1). Twelve individuals per population, representing all life stages, were sampled randomly during 2007 and 2008. Samples of fresh and healthy leaves were collected in the field, immediately dried in silica gel and stored at 4 °C until further processing.

In the laboratory, approximately 1 cm^2 each of the silica dried leaf material of individual plants was homogenised with a mill (Retsch MM 301; Retsch GmbH, Haan, Germany). Total genomic DNA was isolated using the NucleoSpin Plant II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's protocol, with the exception that incubation time for cell lyses was changed to 30 min, which resulted in higher DNA yields. The obtained DNA quality was checked on a 1.5 % TAE-agarose gel. DNA purity and quantity were measured for each individual via a NanoDrop1000 spectrophotometer (ThermoScientific, Wilmington, DE, USA) and diluted to 30 ng/µl. Amplified fragment length polymorphism (AFLP) was carried out after a modified protocol of Vos et al. (1995): 10 µl genomic DNA (30 ng/µl) was double-digested in a final volume of 25 µl (0.25 µl EcoRI (10 U/µl); Fermentas, St Leon Rot, Germany, 0.15 µl Tru1I (10 U/µl); Fermentas, 2.5 µl 10·buffer (Fermentas) and 12.1 µl purified H_2O at 37 °C for 3 h. The reaction was terminated by heating to 65 °C for 10 min. Ligation was carried out with 0.5 µl T4-ligase (1 Weiss units/µl); Fermentas, the double-stranded adapters 0.5 µl EcoRI (5 pmol/µl); MWG Biotech, Ebersberg, Germany and 0.5 µl True1I (50 pmol/µl); MWG Biotech, 1.2 µl ATP (10 mM; Fermentas), 0.5 µl 10 ligation buffer (Fermentas) and 1.8 µl purified H_2O at 20°C for 16 h.

Two consecutive PCR amplifications with primers containing first one (+1) then three (+3) selective nucleotides at their 3'-ends were carried out. In the first pre-selective PCR, 1.5 µl of restriction ligation product was used as a template in a total volume of 7 µl containing 0.2 µl of each primer (50 ng/µl); MWG Biotech, 0.7 µl 10 buffer (Qiagen, Hilden, Germany), 0.14 µl dNTPs (10 mm; PeqLab, Erlangen, Germany), 0.035 µl Taq polymerase (1000 U/µl); Qiagen) and 4.23 µl purified H_2O. PCR conditions for the pre-selective amplification comprised an initial step of 94 °C for 2 min followed by 30 cycles at 94 °C for 20 s, 56 °C for 30 s, 72 °C for 2 min and a final extension step at 72 °C for 30 min. The pre-selective PCR products were checked on 1.5 % TAE-agarose gels and DNA quantity was adjusted to 20–30 ng/µl. The selective PCR reaction of 4.2 µl final volume contained 3.14 µl AFLP coremix (Applied Biosystems, Darmstadt, Germany), 0.21 µl fluorescent labelled EcoRI primer (1 µM; Sigma Aldrich, Taufkirchen, Germany), 0.21 µl True primer (5 µM; MWG Biotech) and 0.65 µl adjusted pre-PCR product. PCR conditions for the selective amplification reaction started with an initial step at 94 °C for 2 min, followed by 10 cycles at 94 °C for 20 s, 66 °C for 30 s, 72 °C for 2 min, 25 cycles at 94 °C for 20 s, 56 °C for 30 s, 72 °C for 2 min, ending with a final extension step at 72 °C for 30 min.

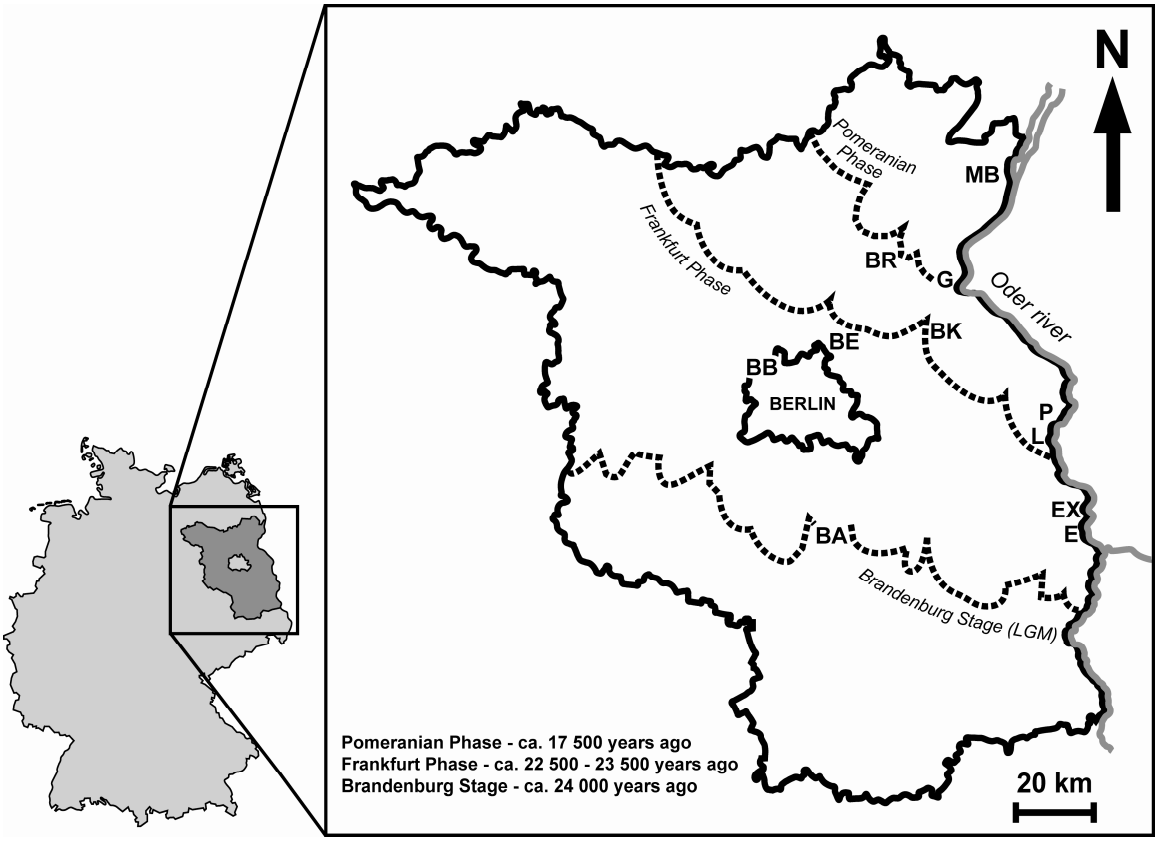

Fig. 2-1: Map of the study area in Germany and Brandenburg and Berlin, with bold one and two letter abbreviations, on the location of the 11 sampled populations of *S. chlorantha* (for further information see Table 2-1). Scattered lines depict different stages of glacier retreat after the last glacial maximum (LGM).

Table 2-1: Summary of 11 sampled *Silene chlorantha* populations and AFLP results

Pop. no	Location	Code	Coordiantes	Pop. size	Plants analy.	*P* %	H_E	Var(H_E)	DW	Priv. alleles
1	Berlin	BB	52°35'/13°13'	10,000	12	52.3	0.18084	0.000271	24.60	1
2	Baruth	BA	52°02'/13°30'	800	11	62.2	0.19037	0.000294	30.75	1
3	Bernau	BE	52°41'/13°36'	440	10	63.1	0.19561	0.000309	19.54	0
4	Britz	BR	52°51'/13°48'	75	12	51.4	0.19015	0.000287	22.69	2
5	Eisenhüttenstadt	E	52°10'/14°36'	373	10	56.8	0.20757	0.000345	15.38	0
6	Eisenhüttenstadt	EX	52°10'/14°36'	8,000	10	66.7	0.22922	0.000318	13.78	0
7	Lebus	L	52°23'/14°31'	300	11	57.7	0.20398	0.000346	45.57	3
8	Podelzig	P	52°29'/14°32'	27	11	52.3	0.17154	0.000285	17.43	2
9	Biesdorf	BK	52°42'/14°04'	103	10	52.3	0.18315	0.000342	25.39	1
10	Gabow	G	52°49'/14°04'	880	9	57.7	0.18121	0.000326	7.83	0
11	Kunow	MB	53°07'/14°16'	470	11	45.9	0.18166	0.000347	8.11	0

Pop. no = population number; Coordinates = north/east; Pop. size = population size measured as number of flowering plants; Plants analy. = Number of analysed individuals; *P* % = percentage of polymorphic loci; H_E = Nei's gene diversity; Var (H_E) = variance of H_E; DW = frequency-down-weighted-marker-value, Priv. alleles = number of private alleles per population

A preliminary primer screening of 16 individuals across four populations with 13 primer combinations was conducted to select three primer combinations providing clear reproducible bands that were sufficiently polymorphic to show variation within and between populations. Primer combinations used for the final analysis were EcoRI ACG – True CGA, EcoRI ACG – True CAA and EcoRI AAG – True C. Selective PCR products were separated on a polyacrylamide gel with an internal size standard (GenomeLab DNA Size Standard kit 400; Beckman Coulter, Krefeld, Germany) on an automated sequencer (CEQ 8000; Beckman Coulter). Bands were identified and scored semiautomatically for presence and absence using Genographer software (version 1.6.0, J.J. Benham, Montana State University, Bozeman, MT, USA). Only unambiguously scorable AFLP fragments were included in the analysis. Each AFLP fragment was scored using the "thumbnail" option, which allows comparison of signals per locus over all samples. If possible, peaks of low intensity were additionally scored by eye and included into the analysis. Standard lanes, carrying identical samples on each plate, were added as a quality check.

AFLP data analysis

The presence/absence matrix from AFLP fragments was used to calculate allele frequencies using a Bayesian method with no uniform prior distribution (Zhivotovsky 1999). From these allele frequencies, the percentage of polymorphic loci, genetic diversity (H_E) and pair-wise F_{st} values were calculated based on the method of Lynch & Milligan (1994), using the software AFLPsurv, version 1.0 (Vekemans 2002). Gene diversity, which is equivalent to expected heterozygosity (H_E) under Hardy–Weinberg conditions (Nei 1987), was used as a measure of within population genetic diversity. An additional measure of divergence, the "frequency-down-weighted-marker-value" (DW) following Schönswetter & Tribsch (2005), was applied using the program AFLPdat (Ehrich 2006). The number of private alleles per population was calculated with FAMD version 1.1 (Schlüter & Harris 2006). A neighbour-joining analysis based on *p*-distances was generated using the program PAUP* 4.0 (Swofford 2003). The tree was drawn and edited using FigTree version 1.2.1 (http://tree.bio.ed.ac.uk/software/figtree/). Analysis of molecular variance (AMOVA) was performed to evaluate the genetic variation within and among populations using the software Arlequin, version 3.1 (Excoffier & Schneider 2006) and significance tests were performed using 10,000 permutations. The assignment of individuals into genetic groups, with the program STRUCTURE version 2.2 (Pritchard et al. 2000), was examined as an alternative approach to explore genetic structure. STRUCTURE employs a model-based clustering analysis that groups individuals into genetic clusters (*K*) without a predefined delimitation of genetic

populations in the sampling area. We estimated the number of genetic clusters *K* from 20 independent runs for each *K* value (*K* = 1 - 12). The analysis was based on the non-admixture model with independent allele frequencies between populations, and was run with a burn-in period of 50,000 and 100,000 MCMC replications. Several ways of determining the optimal *K* value were explored using the method of Pritchard et al. (2000) and Evanno et al. (2005). Recently, it has been suggested that a good estimator to detect the true number of genetic groups is the modal value of *ΔK*, an ad hoc quantity that is related to the second-order rate of change of the log probability of data with respect to *K* (Evanno et al. 2005). *ΔK* values to compare the resulting assignments were calculated using STRUCTURE-sum (Ehrich 2006) in R 2.6.0 (R Development Core Team 2007). We utilised the program CLUMPP, version 1.1.1 (Jakobsson & Rosenberg 2007) with the greedy algorithm and 10^4 random input orders of 20 independent STRUCTURE runs to determine the optimal alignment of clusters across individual runs for each K. Results from CLUMPP were imported into DISTRUCT, version 1.1 (Rosenberg 2004) for viewing.

In addition, associations between the matrix of direct line geographic distance and genetic distances using pair-wise F_{st} values derived from AFLPsurv were tested using a Mantel test in R 2.6.0 (R Development Core Team 2007) with 9,999 permutations.

Fitness data sampling

Population fitness was evaluated in the field as well as in a common garden approach. In the field, in July 2007, 2008 and 2009, flowering plants of all investigated populations were counted to estimate effective population size. For each population, the density of flowering plants was counted in a plot of 10 x 10 m. In 2009, we randomly selected 30 flowering individuals along a transect spanning the largest diameter of each population. Reproductive fitness components were determined by counting the total number of capsules, the number of seeds in one randomly selected capsule, and the number of shoots for each individual. Number of seeds per plant was estimated from number of seeds per capsule multiplied by the total number of capsules per plant. In addition, for each of the 11 study sites, in 10 randomly selected plots of 0.5 x 0.5 m, vegetation characteristics (overall cover, cover of vascular plants, cover of litter and cover of cryptogams) were recorded. Additionally, the number of adults (flowering plants) and juveniles (rosette diameter under 5 cm) was counted in each plot.

In the common garden approach at the Berlin Botanic Garden, 100 seeds from 10 seed families of each population were sown in May 2008 and the germination rate recorded. For further investigations, 30 randomly selected seedlings of each population were cultivated outdoors in single pots in a randomised arrangement. In October 2009, all plants were harvested and fitness components were determined by counting the total number of capsules per plant, the number of seeds in one randomly selected capsule, and calculating the total number of seeds for each plant.

Fitness data analysis

Correlations for genetic diversity, percentage of polymorphic loci, germination rate and population size were tested using Spearman rank correlation. To correlate population size, genetic diversity and population density to plant fitness in nature and in the common garden, generalised linear models (GLM) with Poisson error distribution for count data were applied. For plant fitness, the number of capsules per plant, number of seeds per capsule and total number of seeds per plant were regarded as response variables. GLM allow for a more versatile analysis of relationships, as the error distribution of the dependent variable and the function linking predictors to it can be adjusted to the characteristics of the data. In the case of over-dispersion, a quasi-Poisson error distribution was applied (Crawley 2007). The effects of vegetation cover, number of adult plants, population size, population genetic diversity and population density on number of juvenile plants as response variable were analysed by applying a generalised linear mixed model (GLMM) with a Poisson error distribution and the lmer function in the lme4 R-package (Bates et al. 2008). Study site was regarded as a random factor; the other variables were regarded as fixed factors. Population size and population density were log-transformed to satisfy model assumptions.

After fitting the maximum models, models were reduced using step-wise backward model selection, following the recommendations of Venables & Ripley (2002). Therefore, results were compared with and without the newly removed term using the ANOVA protocol in R (Crawley 2007). In case of non-significant differences between the models (likelihood ratio tests produced $P > 0.05$), the term was removed in all further analyses to find the minimal adequate model. All analyses were calculated and plotted in R 2.6.0 (R Development Core Team 2007).

2.4 Results

AFLP pattern and polymorphisms, genetic diversity and geographic structure

One hundred and thirty-two individuals from 11 populations were analysed with three AFLP primer combinations, which revealed 111 scorable polymorphic bands with length variation from 61 to 437 bp. Some individuals failed the AFLP and were excluded from further analyses. The percentage of polymorphic bands per population (P %) varied from 45.9 % to 66.7 % (Table 2-1). Genetic diversity within populations varied from H_E = 0.172 for the location Podelzig (P), the smallest population with 27 individuals, to H_E = 0.229 for Eisenhüttenstadt (EX), one of the largest populations with 8,000 individuals (Table 2-1). The smaller population E (H_E = 0.208) had the genetic diversity of the adjacent larger population EX (H_E = 0.229). The genetic diversity of the Berlin (BB) population was lower (H_E = 0.181). The population Lebus (L) had the highest frequency-down-weighted-marker-value, with DW = 45.57, and highest number of private alleles (3). The population Gabow (G) had the lowest DW value (7.83) and no private alleles. The AMOVA results (Table 2-2) confirmed a distinct population structure between the analysed *S. chlorantha* populations. The highest molecular variance was within populations (64.15 %, $P < 0.001$), while variation among populations was 35.85 % ($P < 0.001$). The F_{st}-value was 0.36.

Table 2-2: Analysis of molecular variance (AMOVA) based on AFLP analysis of 11 *S. chlorantha* populations.

Source of variation	df	SSD	VC	%	*P*-value	F_{st}
among populations	10	412.32	3.16	35.85	$P < 0.001$	
within populations	113	638.48	5.65	64.15	$P < 0.001$	
total	123	1050.79	8.81			0.36

Statistics included degree of freedom (df), squared deviation (SSD), variance component (VC), percentage of variation (%), probability (*P*-value) and F_{st}.

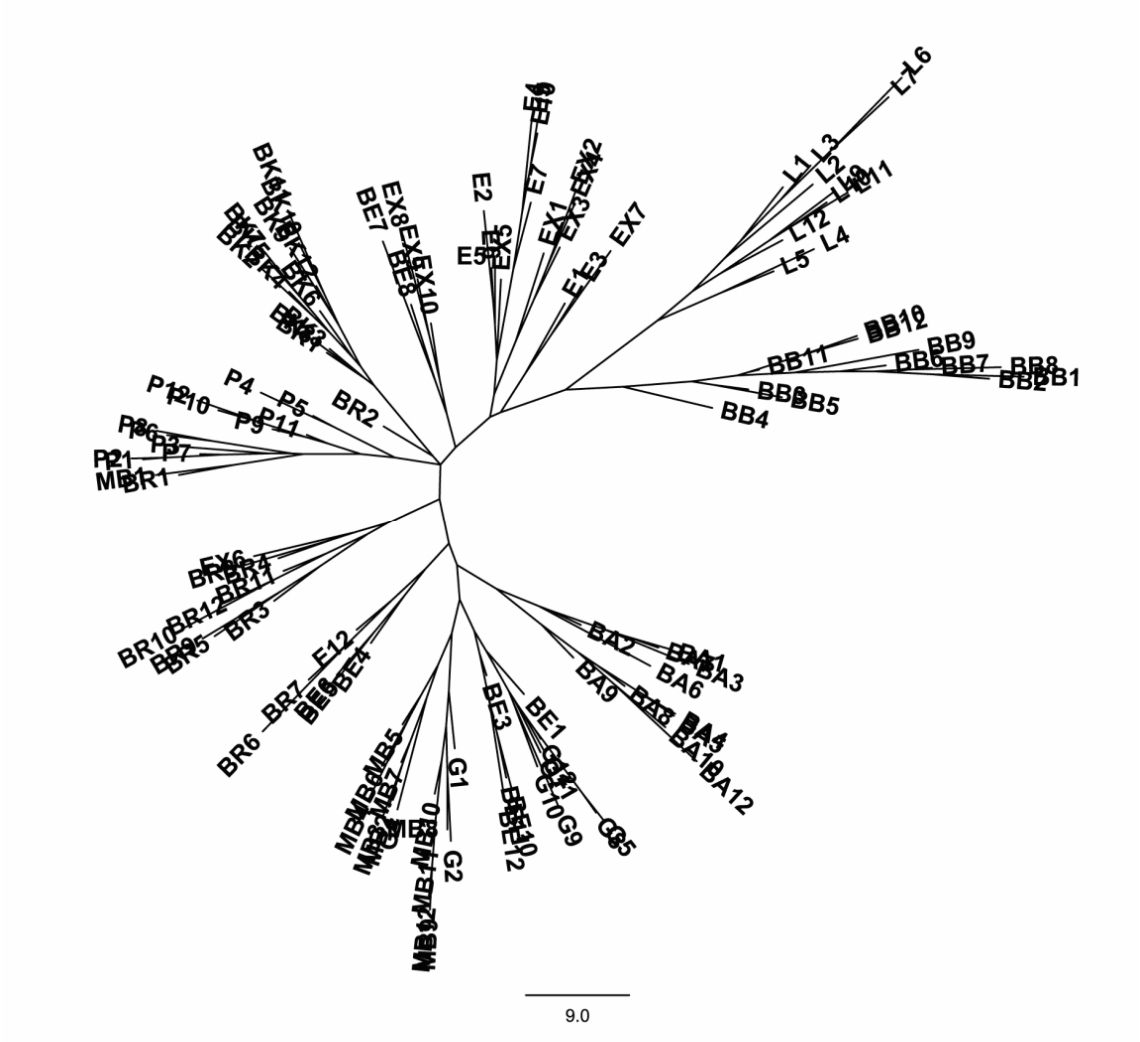

Fig. 2-2: Unrooted neighbour-joining tree (*p*-distance), depicting the genetic distance between the individuals of the 11 populations (for abbreviations see Table 2-1).

The neighbour-joining tree (Fig. 2-2) reflected a distinct population structure, but with no statistical support. Individuals mostly formed distinct groups according to their population affiliation. Individuals of the two Eisenhüttenstadt populations (E and EX) clustered together, with the exception of two outliers. Individuals of Gabow (G) clustered together with those from Kunow (MB), which is the northern-most population. Individuals of the population Bernau (BE) mixed with the populations EX, G and BR. An outlier individual of Kunow (MB) was genetically similar to individuals from Podelzig (P). Individuals of the geographically most distant southern population, Baruth (BA), formed a distinct cluster (Fig. 2-1); however, the average genetic distance was no larger than for other populations (Fig. 2-2). The average genetic distances of the Berlin (BB) and Lebus (L) populations were comparatively larger than for all other populations.

The minimum linear distance between the analysed populations was 0.25 km (E and EX), the maximum distance was 131 km (BA and MB). The Mantel test revealed no correlations between genetic and geographic distances (r_m = 0.06; *P* = 0.41, 9,999 replications, data available from the first author upon request).

STRUCTURE results showed a distinct modal maximum of *ΔK* at *K* = 2 (Evanno et al. 2005). Following Pritchard et al. (2000), the maximal mean log-likelihood value before saturation was at *K* = 9. For *K* = 2, all individuals of the populations Berlin (B) and Lebus (L) clustered together and were separated from all other populations (Fig. 2-3). For *K* = 9, the populations Berlin (BB), Baruth (BA), Lebus (L), Podelzig (P) and Biesdorf (BK) formed several distinct clusters; individuals of the populations Gabow (G) and Kunow (MB) presented one genetic uniform cluster; individuals of the populations Eisenhüttenstadt (E and EX), Britz (BR) and especially Bernau (BE) had genetic affiliation to several genetic clusters (Fig. 2-3).

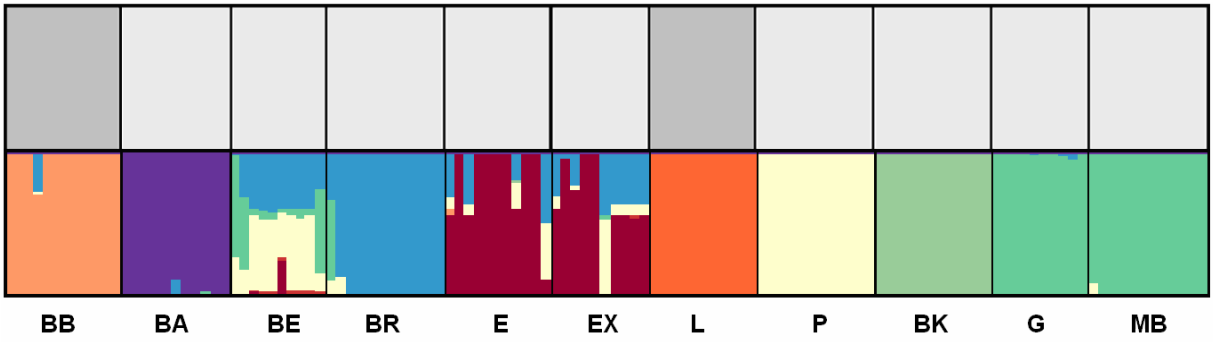

Fig. 2-3: Population structure of the analysed *S. chlorantha* populations (for abbreviations see Table 2-1) without prior population affiliation using a model-based clustering method implemented in STRUCTURE for *K* = 2 (top line) and *K* = 9 (bottom line). Each column represents the proportion in which an individual belongs to a different coloured cluster.

Fitness

No correlation could be detected between population size and genetic diversity (r_s = 0.08; *P* = 0.81) or the percentage of polymorphic loci (r_s = 0.41; *P* = 0.21) within the investigated populations. The low genetic diversity, but large population size, of the population Berlin (BB) might have influenced the overall correlation pattern. Due to the known historical bottleneck of the Berlin (BB) population, it was excluded from the analysis. This resulted in a higher, but still non-significant, relationship (population size and genetic diversity: r_s = 0.32; *P* = 0.36, and population size and percentage of polymorphic loci: r_s = 0.61; *P* = 0.06). Germination rate in the different populations differed, between 66 % for Berlin (BB) and 96 % for Bernau (BE), with an average of 82 %.

No statistically supported correlations between germination rate and genetic diversity (r_s = 0.29; *P* = 0.42) or population size (r_s = 0.13; *P* = 0.73) could be detected. In the field, the genetic diversity was slightly negatively correlated with the number of seeds ($F_{1,293}$ = 9.66; *P* < 0.01) and capsules ($F_{2,323}$ = 3.79; *P* < 0.05) per plant (Table 2-3A). Individuals of larger populations produced more seeds per capsule ($F_{1,295}$ = 55.19; *P* < 0.001), more seeds per plant ($F_{1,293}$ = 13.08; *P* < 0.001). Density of flowering plants per population was slightly positively correlated to the total number of seeds per plant ($F_{1,293}$ = 4.93; *P* < 0.05). In the common garden approach (Table 2-3B), genetic diversity and natural population size showed no correlation to measured fitness components.

Table 2-3: The minimal adequate models for I. total seeds per plant; II. seeds per capsule; and III. capsules per plant in relation to genetic diversity (H_E), population size, and population density in nature (A); and in relation to genetic diversity (H_E), population size in common garden (B). Parameter estimates as well as the results of the likelihood ratio tests for the significant terms in the minimal adequate model are presented. GLM with Quasi-Poisson error distribution.

	I Total seeds per plants			**II Seeds per capsule**			**III Capsules per plant**		
A nature	***Estimate***	***F-test***	***P***	***Estimate***	***F-test***	***P***	***Estimate***	***F-test***	***P***
H_E	-13.806	$F_{1,293}$=9.66	< 0.01	-1.497	-	-	-11.51	$F_{2,323}$=3.79	<0.05
log (pop. size)	0.179	$F_{1,293}$=13.08	<0.001	0.111	$F_{1,295}$=55.19	<0.001	0.098	$F_{2,323}$=3.17	<0.05
log (pop. density)	-0.144	$F_{1,293}$=4.93	<0.05	-0.036	-	-	-0.099	-	-
B common garden	***Estimate***	***F-test***	***p***	***Estimate***	***F-test***	***p***	***Estimate***	***F-test***	***p***
H_E	4.147	-	-	2.346	-	-	1.015	-	-
log (pop. size)	-0.0188	-	-	0.003	-	-	-0.010	-	-

The number of juvenile plants in correlation with different layers of surrounding cover, number of adult plants, population size, population density and population genetic diversity provided a negative correlation to the cover of cryptogams (χ^2 = 20.03; *P* < 0.001) and a positive correlation to population genetic diversity (χ^2 = 12.33; *P* < 0.001). High coverage of cryptogams (mosses and lichens) was correlated to lower numbers of juvenile plants (Table 2-4).

Table 2-4: The minimal adequate model for the number of juvenile plants in relation to population size, population density, population genetic diversity (H_E), overall coverage, coverage of litter, coverage of cryptogams, coverage of herbs, and number of adults plants. The table gives parameter estimates and their standard errors as well as the results of likelihood ratio tests for the significant terms in the minimal adequate model. GLMM with Poisson error distribution and random effect for population (10 plots each in 11 populations).

number of juvenile plants	**Parameter estimates**		**Likelihood ratio tests**		
	Estimate	***Std. Error***	***df***	***χ^2***	***P***
log (population size)	-0.044274	0.114231	-	-	-
log (population density)	0.235132	0.163633	-	-	-
H_E	40.97868	11.71399	1	12.33	<0.001
overall cover	0.014338	0.010620			
cover litter	-0.009479	0.010909	-	-	-
cover cryptogams	-0.020795	0.006390	1	20.03	<0.001
cover herbs	-0.005543	0.009160	-	-	-
number of adults plants	0.029468	0.053050	-	-	-

2.5 Discussion

Population genetic and geographic structure

The high genetic differentiation among populations of *S. chlorantha* at the periphery of its distribution range may be caused by restricted habitat availability and spatial isolation through human-induced landscape fragmentation. In the investigation area, the habitat requirements of open, sandy, base-rich dry grassland for *S. chlorantha* are only found in scattered locations. Hence, this species was probably never common here in the past. The high level of genetic differentiation discovered among populations (F_{st} = 0.36) is comparable to that of other *Silene* species, e.g. *S. tatarica* (F_{st} > 0.287 over a small geographic range at its edge of distribution; Tero et al. 2003), *S. rothmaleri* (RAPD variation of 25 % among populations on a small geographic range; Cotrim et al. 2003) or S. nutans (G_{st} = 0.215 allozyme variation on a broader geographic range; Van Rossum et al. 2003). Compared to other dry grassland species, the variation is intermediate, e.g. *Pulsatilla vernalis* (AFLP variation of 28.2 % among populations on a broader geographic range; Ronikier 2002), *Globularia bisnagarica* (F_{st} = 0.44 at the edge of distribution area; Honnay et al. 2007), *Anthericum liliago* (RAPD variation of 41 % between populations on a broader geographic range; Peterson et al. 2008), *Eryngium campestre* (AFLP variation of 16 % among

populations within regions in central Europe; Bylebyl et al. 2008) and *Stipa capillata* (RAPD variation of 53.1 % at the periphery of the species distribution area; Hensen et al. 2010), even though these analyses are partly based on the application of different molecular techniques.

The common garden approach revealed *S. chlorantha* to be self-compatible between different flowers of one plant. In theory, selfing supports low out-crossing rates leading to high genetic differentiation among populations and low genetic diversity within populations (Nybom & Bartish 2000; Duminil et al. 2009). No data about selfing rates in *S. chlorantha* are available, but in small populations selfing most likely occurs regularly. Pollen exchange between populations is not likely since distances of 50 km and more do not allow pollinator migration, which might occur at distances of < 1 km (Kwak et al. 1998; Peterson et al. 2008). The dispersal ability of seeds is restricted by lack of morphological adaptation for wind or animal dispersal. In summary, the high genetic population differentiation in *S. chlorantha* found here seems to be a result of limited gene flow among fragmented populations.

Within-population genetic diversities of *S. chlorantha* were similar to or slightly higher than in other perennial plant species (Gaudeul et al. 2000; Ronikier 2002; Tero et al. 2003; Honnay et al. 2007). No correlation between genetic diversity and population size could be detected, which most likely is a result of comparatively recent declines in population sizes through habitat restriction, e.g. forestation and succession. Otherwise, a recent increase of individual numbers could serve as an explanation for the absence of any correlation of population size to low population genetic diversity, as observed in the Berlin (BB) population. However, Leimu et al. (2006) already stated that in self-compatible species an association between genetic diversity and population size cannot automatically be deduced. No correlation between population size and genetic diversity was found in other dry grassland species, e.g. *Centaurea scabiosa* (Ehlers 1999), *Vincetoxicum hirundinaria* (Leimu & Mutikainen 2005), *Eryngium campestre* (Bylebyl et al. 2008) and *Stipa capillata* (Hensen et al. 2010). A missing association between population size and genetic diversity has also been confirmed in other AFLP studies, e.g. for *Pedicularis palustris* (Schmidt & Jensen 2000) and *Silene tatarica* (Tero et al. 2003). Analysis comparing population size and genetic variability in *Silene nutans* was carried out by Van Rossum & Prentice (2004) using allozyme variation, which revealed positive correlations between allelic richness and population size. In our study, effective population size, measured as number of flowering plants, was rather similar in 2007, 2008 and 2009. Thus, fluctuations in flowering as a reason for missing population size effects on genetic diversity, as mentioned by Honnay et al. (2007), can be excluded.

Leimu & Mutikainen (2005) state the importance of population history in interpreting within-population structure. This can be especially useful in the interpretation of the large Berlin

population (BB), which had a remarkably low level of genetic diversity (H_E = 0.181). Population history here is indicative of a former bottleneck effect (Nei et al. 1975) in the 1980s and subsequent increase in individual number since then. Schmidt & Jensen (2000) found a similar result of low genetic diversity and large population size in Pedicularis palustris; however, they ascribed it to founder effects and subsequent radiation. As the Berlin (BB) population of *S. chlorantha* has been known since 1835 (Dietrich 1835) and is not of recent origin, founder effects, which limit the genetic variation, can at the present stage most likely be excluded. The population genetic distance is similar to that of all other populations. Hence, population genetic diversity seems to be restricted not only by population size per se, but also by population history. Unfortunately, no information about former population dynamics of the other investigated sites was available.

As already stated, *S. chlorantha* is restricted to more-or-less fragmented populations on moraine ridges or inland dunes. Formerly, a connected distributional range in the investigation area might have occurred at the till plain edge of the Oder valley. Otherwise, we can assume that the populations without current gene flow were also historically fragmented, except for both Eisenhüttenstadt locations (E and EX), which are separated by only 0.25 km. Here, no genetic differentiation between the geographically adjacent populations could be detected, suggesting metapopulation dynamics with free gene flow across (sub)populations or more likely a former connection. As the main distributional range of *S. chlorantha* is in eastern and central Europe (Tutin et al. 1993), the population Baruth (BA) is the southwestern-most isolated outpost. Even being highly isolated, genetic diversity was still reflected by similar values to populations that are closer to the main species distributional range. This indicates no substantial loss in genetic diversity or inbreeding effects in the outpost population.

In our analysis neither an east–west relationship between the populations nor a north–south population relationship along the till plain edge of the Oder valley was detected; although the geographic distance between populations along the north–south gradient is short. Surprisingly, genetic differentiation between the geographically close populations Lebus (L) and Podelzig (P) (distance of 10 km) was high. Also, the genetic distance of the Lebus (L) population to any of the other eastern-most analysed populations was high. This finding is also unexpected, as north–south dispersal by former sheep grazing along the river valley would have been expected. Additionally, the isolation by distance relation was not statistically supported, which is in concordance to findings within *Silene tatarica* (Tero et al. 2003).

The investigation area was affected by different stages of the last glacial maximum (LGM) (Böse 2005). If species survival during glaciations outside the ice sheet is postulated (Comes & Kadereit 1998), *S. chlorantha* most likely re-colonised northeast Germany from refugial

areas from the east and southeast of Europe. This recolonising strategy has also been suggested for other European steppe species, e.g. *Clausia aprica* (Franzke et al. 2004) and *Stipa capillata* (Hensen et al. 2010). Studies on post-glacial plant recolonisation in central Europe mostly concern tree species on larger scales (Taberlet et al. 1998; Petit et al. 2002) and for herbaceous species information is scarce (e.g. *Melica nutans*, Tyler 2002; *Eryngium campestre*, Bylebyl et al. 2008; *Iris aphylla*, Wróblewska & Brzosko 2006). In the northeast German lowland region, it is difficult to correlate genetic patterns with postglacial recolonisation events, as the ice marginal position at the LGM is difficult to identify on the basis of geo-morphological features (Böse 2005). Genetically similar populations, e.g. Gabow (G) and Kunow (MB), are geographically distant (distance of 35 km). It can be assumed that these, geographically separated populations, were connected through other, now extinct populations at the till plain edge of the Oder valley, as their habitat requirements there are potentially being met. The Gabow and Kunow populations are located in the area that was still ice-covered about 17,500 years before present (Fig. 2-1), whereas the other locations had become ice-free already ca. 6,000 years before, thus probably indicating a more 'recent population establishment' which is also supported by the absence of private alleles and low detected DW values (Schönswetter & Tribsch 2005). The comparatively high number of private alleles in the populations Lebus (L) and Podelzig (P) is potentially indicative of a former stronger connection to the species main distribution area, which begins on the eastern site of the River Oder.

The Bayesian clustering approach identifies genetic clusters *K* and assigns membership of individuals to it without prior assumptions of population affiliation (Mameli et al. 2008). Almost no resolution for $K = 2$ could be detected (Evanno et al. 2005). Only individuals of Lebus (L) and Berlin (BB) clustered together, which cannot be explained by geographic proximity. Following Pritchard et al. (2000), $K = 9$ reflected a well structured genetic pattern in concordance to the results of the neighbour-joining analysis. The population Bernau (BE) was diverse, bearing genetic similarities to three other genetic clusters (BR, P, E/EX). As these populations occur along railway tracks, the pattern may be indicative for former gene flow through, e.g. seed dispersal by train or transport of construction materials like sand for the railway embankments (Tikka et al. 2001).

Fitness evaluation

Fitness was evaluated as seed output by maternal plants (reproductive fitness), germination and number of juvenile plants (recruitment). For *S. chlorantha* there was a slightly negative

correlation between genetic diversity and reproductive fitness (Table 2-3) and no correlation between germination rate and genetic diversity in nature. Lammi et al. (1999) also found no correlation between germination and genetic diversity in *Lychnis viscaria*. These results contradict the positive correlations between fitness and allozyme diversity found in e.g. *Gentianella germanica* (Fischer & Matthies 1998), *Cochlearia bavarica* (Paschke et al. 2002) and *Succisa pratensis* (Vergeer et al. 2003); however, it reflects the controversial discussion on quantitative genetics and allozyme diversity (Lammi et al. 1999). Additionally, when using a neutral molecular marker like AFLP or allozyme electrophoreses, correlations between genetic diversity and fitness may be low and no information about evolutionary potential or adaptation can be retrieved (Reed & Frankham 2001; 2003). Population size rather than genetic diversity explained differences in reproductive fitness among investigated populations.

In nature, positive correlations between population size and number of seeds per plant are in concordance to evaluations of *Gentianella germanica* (Fischer & Matthies 1998), *Cochlearia bavarica* (Paschke et al. 2002) and *Pulsatilla vulgaris* (Hensen et al. 2005). A possible explanation could be a higher pollinator attractiveness of large populations (Kunin 1997) or better habitat quality supporting pollinator abundance (Petanidou et al. 1995). In smaller populations, reduced reproductive success, due to pollination limitation, was found in Primula veris (Van Rossum & Triest 2007) and *Lythrum salicaria* (Waites & Agren 2004). The positive correlation of population density and seed set in *S. chlorantha* has also been found in *Lychnis viscaria* (Mustajärvi et al. 2001) and can also be explained by higher pollinator attraction.

The common garden approach revealed no correlation between fitness and genetic diversity or source population size (Table 2-3). In a meta-analysis, Leimu et al. (2006) discovered that the mean correlation between plant population size and fitness was generally significantly positive in self-incompatible plant species, but not significant in self-compatible plant species (see also Reed & Frankham 2003). Deduced from an equal seed set among all investigated populations, pollinator attraction was equal for all plants of different origin in the common garden approach.

Seed production is only one aspect from which to evaluate fitness, in addition to germination, recruitment and establishment. Seed germination seems not to be a limiting factor for recruitment and expansion, as revealed in the common garden approach. *S. chlorantha* produced an enormous number of seeds per single plant in one season (approximately 3,000), with high germination rates (mean = 82 %, 100 seeds from 10 plants each per population in 11 populations). Therefore, population recruitment and establishment in nature is limited by seedling survival rather than seed production. Fewer juvenile plants are present

with a higher cover of cryptogams (Table 2-4). This is most likely due to the negative effect of cryptogams or biological soil crusts upon seedling survival. Langhans et al. (2009) showed negative effects of stable biological soil crusts on plant establishment and survival of typical dry grassland species (e.g. *Silene otites*). In *Pulsatilla patens*, Röder & Kiehl (2006) also mentioned a negative effect of cover by phanerogams and litter on seedlings, even though seedling emergence and survival sometimes differs between species of the same habitat (Ryser 1993; Jakobsson & Eriksson 2000). An intermediate cover of mosses was revealed to be favourable for juveniles of *Pulsatilla patens* in the studies of Kalliovirta et al. (2006). By visiting locations where former population extinction of *S. chlorantha* has been documented, these habitats nowadays are characterised by nutrient overload and an almost complete ground cover of mosses and aboveground litter.

We found a positive correlation of population genetic diversity to number of juvenile plants in nature (Table 2-4). Hence, there must be increased offspring fitness in cases of higher genetic diversity. In selfing species, effects of inbreeding depression were more pronounced in later stages of life cycles, such as survival and growth (Husband & Schemske 1996). An effect of inbreeding upon later fitness traits, like survival rate, was also found in *Silene nutans* (Thiele et al. 2010).

Implications for conservation

In recent times, a considerable decline in populations and individual numbers for *S. chlorantha* in the area under investigation could be observed, alluding to the necessity for an active conservation management programme. Conservation strategies can be manifold. Our analysis revealed, for all investigated populations, high reproductive success in the form of good seed set with high vitality of seeds. As seed set was correlated to population size and habitat quality rather than to genetic diversity, conservation strategies focusing on reproductive fitness should aim for large populations on well-maintained habitats. In later plant life cycle stages (e.g. the establishment or survival of juvenile plants), genetic diversity was revealed to be as important as habitat quality for population sustainability. Hence, good habitats, characterised here by low cryptogam cover and high intra-population genetic diversity, are key to increasing population recruitment in the self-compatible and perennial *S. chlorantha*.

The known history of the Berlin population shows that a decline in individuals per population, leading to a loss of genetic diversity, does not inevitably result in population extinction. If habitat conditions improve again, plant populations of *S. chlorantha* have the potential to

expand. Nevertheless, no information about long-term effects of changing environmental conditions upon the genetic diversity of *S. chlorantha* is available. Marginal populations, such as those analysed in this investigation, might be especially more prone to extinction than central populations (Jacquemyn et al. 2007a; b); however, they are often important sources of genetic variation with the potential for adaptation (Hampe & Petit 2005). For the highly differentiated populations of *S. chlorantha*, the improvement of habitat conditions and protection of as many large populations as possible should therefore be prioritised.

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3 Genetic variation and fitness of the endangered *Silene chlorantha* (WILLD.) EHRH.: a comparison between a botanic garden *ex situ* population and its *in situ* source population

3.1 Abstract

Ex situ cultivation of endangered plants could be one possibility to preserve populations of rare species from extinction. Here I investigated a botanic garden *ex situ* population of the Yellowgreen Catchfly (*Silene chlorantha*) and its parental *in situ* source population after 26 years of isolation. Population genetic diversity and genetic differentiation were analysed by AFLP technique. Furthermore, a common garden approach was conducted to compare plant performance under equal environmental conditions. Genetic variability was similar in botanic garden and *in situ* source population. A low level of differentiation (F_{st} = 0.106) between parental *in situ* population and corresponding *ex situ* population could be observed. Neighbour-joining analysis and a Bayesian clustering approach showed no separation between the *in situ* and *ex situ* individuals. The negligible genetic differentiation and the maintained genetic variation during spatial and temporal isolation in the *ex situ* population can be a result of low population turnover and self-compatibility. Plant performance in terms of leaf rosette diameter and length of the longest leaf was similar in *ex situ* and *in situ* progeny. Under botanic garden conditions, the maintenance of original genetic constitution during 26 years of isolation was successful in *S. chlorantha*. However, genetic diversity of the *in situ* population was low since there was a genetic bottle neck in the 1980s and individuals of *ex situ* progeny were replanted in the wild in the early 1990s.

Keywords: Botanic garden, Conservation genetics, Fitness, Genetic bottle neck

3.2 Introduction

Conservation efforts should prior focus on natural (*in situ*) preservation of endangered species, their habitats and biodiversity (Maunder et al. 2001a). However, sometimes different approaches of *ex situ* preservation are the only way to halt the decline of biodiversity. Holding samples in living collections or seed banks lost in the wild, is one possibility preventing or delaying individuals of threatened or endangered plants from extinction (Guerrant et al. 2004; Maunder et al. 2001). This has been more or less successfully done via cultivation of living plants in botanic gardens and arboreta (Maunder et al. 2001; Havens et al. 2006; Volis & Blecher 2010; Lauterbach et al. 2012b). Living collections or seed banks, whatever, in all cases the aim is to obtain and preserve samples that are representative of the extant *in situ* diversity and make the most efficient use of available resources (Falk & Holsinger 1991; IUCN 2002; Goodall-Copestake et al. 2005; Li et al. 2002; Husband & Campbell 2004). Cultivation under artificial conditions comprises disadvantages as limited space and unconscious selection during cultivation (Falk & Holsinger 1991; Enßlin et al. 2011). The consequences of reproductive isolation and cultivation upon population genetic composition and plant performance have been rarely evaluated.

In the past, preservation mostly starts with an artificial selection of seeds from the initial pool of genotypes in the wild and genetic data was not available when *ex situ* cultivation was initiated (Helenurm & Parsons 1997; Bottin et al. 2007). During long time periods of cultivation ecological shifts due to translocation, small population size, inbreeding and gardener induced selection can lead to genetic bottlenecks and a rapid genetic decline in *ex situ* populations (Zohary 2004; Miller & Schaal 2006; Guerrant et al. 2004; Lauterbach et al. 2012). Especially small population size and unconscious selection accelerate the loss of genetic diversity and random genetic drift which is often followed by effects of inbreeding depression and reduced plant fitness (Krauss et al. 2002; Enßlin et al. 2011). This reduces the suitability of *ex situ* plant material for future reintroduction efforts (Hufford & Mazer 2003).

The genetic evaluation of old *ex situ* populations is complicated because of incomplete documentation, extinct parental *in situ* source populations and much less finding more than one *ex situ* sample of the same species (see also Lauterbach et al. 2012). Thus, so far only few studies have evaluated population structure of *ex situ* cultivars (e.g. Etisham-Ul-Haq et al. 2001; Enßlin et al. 2011; Namoff et al. 2010; Lauterbach et al. 2012b; Rucinska & Puchalski 2011).

Population structure and differentiation strongly depend on species traits as e.g. life cycle, mating system and pollination mode. In self-compatible species genetic differentiation should

be higher and intra-population diversity lower compared obligate out-crossers (Glemin et al. 2006; Duminil et al. 2009). In case of missing gene flow by habitat fragmentation self-compatible ones are expected be more stable in genetic population structure (Moyle 2006) and to be not as sensitive for inbreeding depression as out-crossers. Also a pronounced longevity, which slows down genetic decline during cultivation, counteracts a rapid population turnover as found in annuals (Husband & Campbell 2004).

In the present study I analysed corresponding *in situ* and *ex situ* populations of the endangered perennial *Silene chlorantha* in Germany. The goal of this study was to investigate the effects of reproductive isolation, small population size and unconscious selection on genetic structure and fitness of a well documented *ex situ* population and its parental *in situ* population after 26 years of isolation.

3.3 Materials and Methods

Study species

The Yellowgreen Catchfly *Silene chlorantha* (WILLD.) EHRH. (Caryophyllaceae) is regarded as highly vulnerable in Germany (Korneck et al. 1996) and highly endangered in Berlin (Prasse et al. 2001). In north-eastern Germany, a severe population decline during the past decades could be detected (Lauterbach & Gemeinholzer 2010; Lauterbach et al. 2011). *S. chlorantha* is a species of steppe-like, nutrient-poor and sandy dry grasslands. It is characterised by a basal leaf rosette and yearly renewing flowering stems. It has a lifespan up to 5 years or more (personal observation). Flowering takes place at two peaks in June and September. *S. chlorantha* is hermaphroditic, protandric and pollinators are most likely nocturnal moths. Selfing is possible among flowers of the same plant (geitogamy). Seeds dispersal is largely passive with no morphological adaptation for animal- or wind-dispersal. Germination occurs between autumn and spring without essential seed dormancy. In Europe, *S. chlorantha* reaches from sub-meridional to temperate regions and is restricted to continental Europe and western Siberia. Its westernmost boundary (Lauterbach et al. 2011) appears to be largely defined by climatic conditions, such as rainfall and temperature (Meusel et al. 1965; Tutin et al. 1993).

In the *ex situ* population at the Berlin Botanic Garden (BGBM), plants have been cultivated in a single-species bed since 1982 with a population size of about 20 individuals. There is an approximately biennial rejuvenation from the cultivated stock by the gardeners. Plants have been cultivated on a nutrient rich soil with continuous watering. The single extant Berlin *in*

situ population in Berlin Heiligensee "Baumberge" (BB) is known as far back as the beginning of the 19th century (Dietrich 1835) and was comprised of a large number of individuals until the 1950s (Zimmermann 1982). Population size was decimated to about 20 individuals in the 1980s (Hömberg 1992) by human impact on the habitat (heavy disturbance in a military area). In 1980 seeds were harvested in the wild and progeny was cultivated *ex situ* in the Berlin botanic garden since 1982. In 1988 further seeds were taken to produce progeny for resettlement of the natural population. In 1990 the military area was closed. From 1990 to 1992 in total 279 progeny plants were replanted in the "Baumberge" (Hömberg 1992; Dürbye personal comments). Since that time, conservation efforts have led to a safeguard of the habitat and the number of individuals rapidly increased to approximately 10,000 individuals today (Lauterbach et al. 2011).

Sampling

The well documented *ex situ* population and its corresponding *in situ* population were sampled (Table 3-1). Both populations are geographically separated by an air line distance of 16 km so that genetic exchange between them has been unlikely. Fresh and healthy leaf tissue samples of 12 plants per population were collected in 2008. Samples were immediately dried in silica gel and stored at 4 °C until DNA extraction.

Table 3-1: Summary of sampled *S. chlorantha* populations: code, collection site, coordinates, status, population size (Size), number of individuals analysed for AFLPs (N_{AFLPs}), method of cultivation, year of establishment (Y_{est}), number of cultivated generations (G_{cult}), percentage of polymorphic loci (P %), genetic diversity (H_E), number of private bands (pb)

Code	Collection site	Coordinates	Status	Size	N_{AFLPs}	Cultivation	Y_{est}	G_{cult}	P %	H_E	pb
BB	Berlin	52°36'N/13°13'E	*in situ*	10,000	12				52.3	0.181	8
BGBM	Botanic Garden	52°27'N/13°18'E	*ex situ*	20	12	flowerbed	1982	~13	51.4	0.186	5

DNA isolation and AFLP fingerprinting

The silica dried leaf material (1 cm²) was homogenised with a mill (Retsch MM 301, Haan, Germany). Genomic DNA was isolated using the NucleoSpin Plant II kit (Macherey-Nagel GmbH and Co.KG, Düren, Germany) according to the manufacturer's protocol with modification (incubation time for cell lyses was changed to 30 min). DNA quality and quantity were measured using a NanoDrop1000 spectrophotometer (ThermoScientific, Wilmington,

USA) and diluted to 30 ng/µl. Amplified fragment length polymorphism (AFLP) was carried out using a modified protocol of Vos et al. (1995) according to Lauterbach et al. (2011). A preliminary primer screening of 16 individuals from four populations with 13 primer combinations was conducted to select three primer combinations giving clear, reproducible, bands and appeared to be sufficiently polymorphic to show variation within and between populations. Following primer combinations were used for the final analysis: *Eco*RI ACG – *True* CGA; *Eco*RI ACG – *True* CAA and *Eco*RI AAG - *True* C. Selective PCR products were separated on a polyacrylamide gel with an internal size standard (GenomeLab DNA Size Standard Kit 400, Beckman Coulter, Krefeld, Germany) on an automated sequencer (CEQ 8000, Beckman Coulter).

Bands were identified and scored semi-automatically for presence and absence using Genographer software (version 1.6.0, J.J. Benham, Montana State University, Bozeman, USA). Each AFLP fragment was scored using the "thumbnail" option of the program, which allows for the comparison of signals per locus over all samples. Standard lanes, carrying identical samples, were run to check for reproducibility. A presence/absence matrix was exported for further calculations.

Statistical analysis

The presence/absence matrix from AFLP fragments was used to calculate allele frequencies by using a Bayesian method with no uniform prior distribution (Zhivotovsky 1999). Percentage of polymorphic loci, genetic diversity (H_E), and pairwise F_{st} were calculated based on the method of Lynch & Milligan (1994), using the software AFLPsurv, version 1.0 (Vekemans 2002). Genetic diversity, which is equivalent to expected heterozygosity (H_E) under Hardy-Weinberg conditions (Nei 1987), was used as a measure of within population genetic diversity. The number of private bands per population was calculated with FAMD version 1.1. (Schlüter & Harris 2006). Neighbour-joining analysis based on *p*-distances was generated using the program PAUP* 4.0 (Swofford 2003). The tree was drawn and edited using FigTree version 1.2.1 (http://tree.bio.ed.ac.uk/software/figtree/). Analysis of molecular variance (AMOVA) was performed using the software Arlequin, version 3.1 (Excoffier et al. 2005) (significance test 10,000 permutations). The F_{st} value was calculated after Weir and Cockerham (1984). The affilation of individuals into genetic groups was examined by using the program STRUCTURE version 2.2 (Pritchard et al. 2000). The number of genetic clusters *K* was estimated by 20 independent runs for each *K* value (*K* = 1 to *K* = 3). The analysis based on the admixture model with correlated allele frequencies to consider the common ancestry of the analysed populations and to use the most sensible and progressive model.

The model was run with a burn-in period of 50,000 and 100,000 MCMC replications. The *ΔK* values (Evanno et al. 2005) comparing the results assignments were calculated using STRUCTUE-SUM (Ehrich 2006) in R 2.7.1 (R Development Core Team 2008). We utilised the program CLUMPP (version 1.1.1) (Jakobsson & Rosenberg 2007) with the Greedy algorithm and 10,000 random input orders of the 20 independent STRUCTURE runs. Results from CLUMPP were imported into DISTRUCT (version 1.1) (Rosenberg 2004) for plotting.

Common garden experiment

In October 2009, 100 seeds from 10 seed families of each population were germinated in small pots with sand:humus soil mixture 2:1. In December 2009 the number of germinated seeds was counted. Seedlings were grown frost-free in the greenhouse of the botanical garden and separated in single pots in April 2010. 50 plants of each population were arranged in an alternating design. Since April 2010, all pots stand outside in the garden and were watered if required. In October 2010, following fitness variables were measured: diameter of leaf rosette, length of the longest leaf, number of stems, stem height, number of seed capsules, and number of seeds per capsule. Analyses of variance (ANOVA) to investigate differences in fitness variables between *in situ* and *ex situ* populations were calculated with R 2.7.1 (R Development Core Team 2008).

3.4 Results

AFLP-patterns and genetic structure

AFLP analyses with three selective primer combinations revealed polymorphic bands with length variation between 61 and 437 base pairs. In total 61 polymorphic bands were identified in the 24 analysed individuals. Genetic diversity (H_E) of *ex situ* population was at the same level as in the *in situ* population (Table 3-1). Percentage of polymorphic loci was slightly higher in the *in situ* population (Table 3-1). There were 8 private bands in the *in situ* population and 5 private band in the *ex situ* population.

The neighbour-joining analysis based on *p*-distances of *in situ* and *ex situ* individuals reflected no clear partition between *in situ* and *ex situ* origin (Fig. 3-1). Individuals of the *in situ* population were located at same branches as the *ex situ* individuals and vice versa.

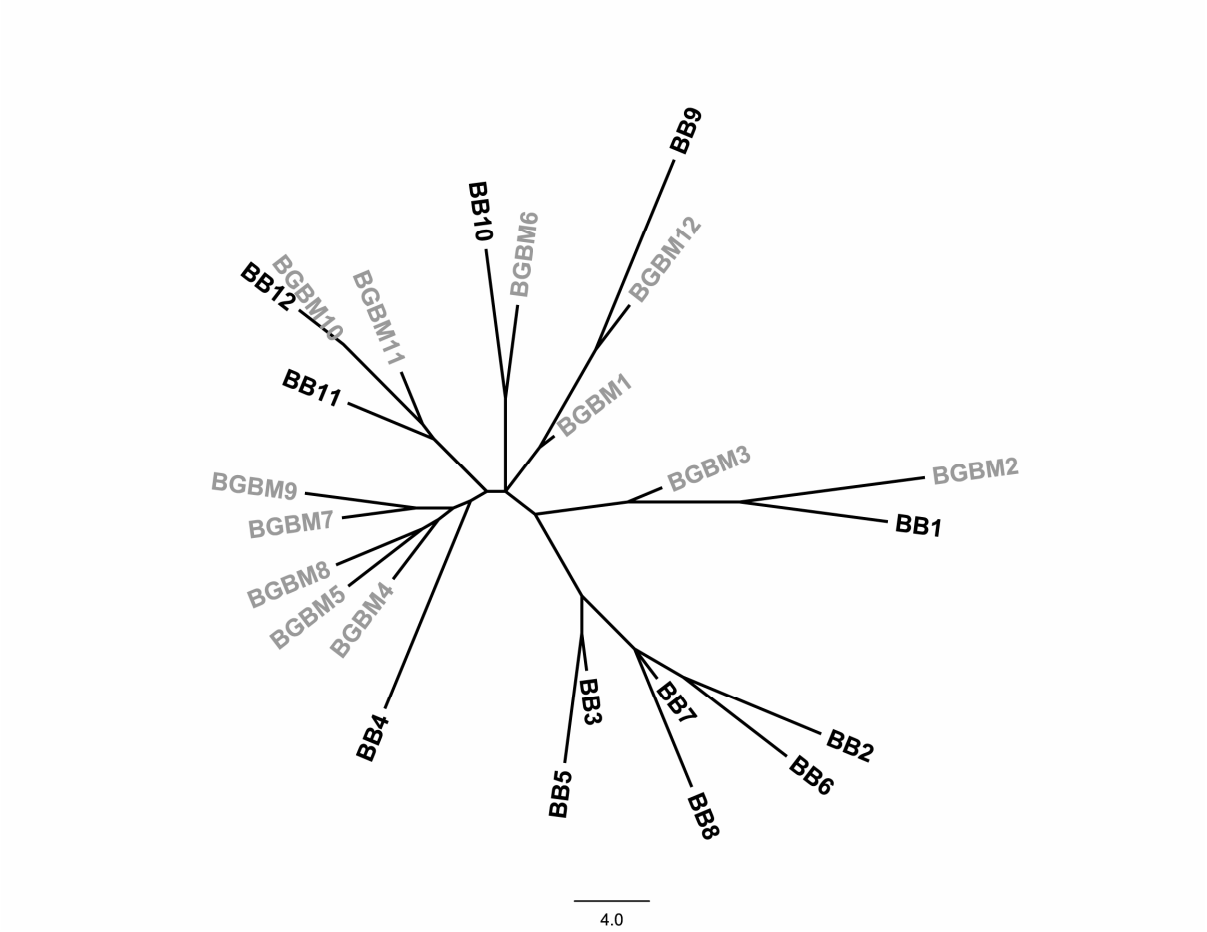

Fig. 3-1: Unrooted neighbour-joining tree based on *p*-distances obtained with the program PAUP* for *in situ* (black labelled) and *ex situ* (grey labelled) individuals of *Silene chlorantha.* Population codes correspond to those in Table 3-1. Scale bar below indicates genetic distance.

The AMOVA showed a very low level of genetic differentiation between the *in situ* and the *ex situ* population (F_{st} = 0.106, $P < 0.001$). Most of the variance (89.41 %) was found within the populations.

To estimate the most probable number of genetic units which were present in each data set a Bayesian clustering approach was performed. Following the method of Evanno et al. (2005) there was a distinct maximum of ΔK at $K = 2$. After the method of Pritchard et al. (2000) there was a maximum of the mean log-likelihood value at $K = 1$. When plotted for two genetic clusters, results not allow distinguishing between *in situ* and *ex situ* individuals (Fig. 3-2).

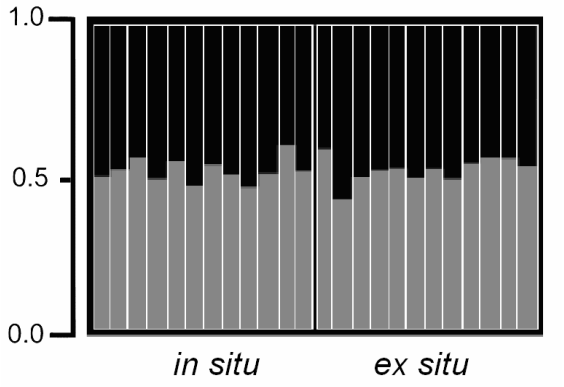

Fig. 3-2: Population structure based on AFLP data using model based clustering method implemented in STRUCTURE. Diagram showing the proportion of membership of each of the studied individuals for the *in situ* vs. *ex situ* comparison of *Silene chlorantha* to inferred Bayesian groups (*K* = 2). Vertical bars represent individuals.

Fitness

The germination rate was slightly higher in the *ex situ* seeds (90 %) compared to the *in situ* seeds (73 %). In 2010 only 38 % of the *in situ* progeny and 50 % of the *ex situ* progeny flowered. Therefore, the variable of stem height, number of capsules and seed set were excluded from the analyses. ANOVAs to test for difference between *in situ* and *ex situ* individuals showed no significant difference of rosette diameter ($F_{1,97}$ = 3.23; *P* = 0.08) and length of the longest leaf ($F_{1,97}$ = 3.71; *P* = 0.06) (Fig. 3-3).

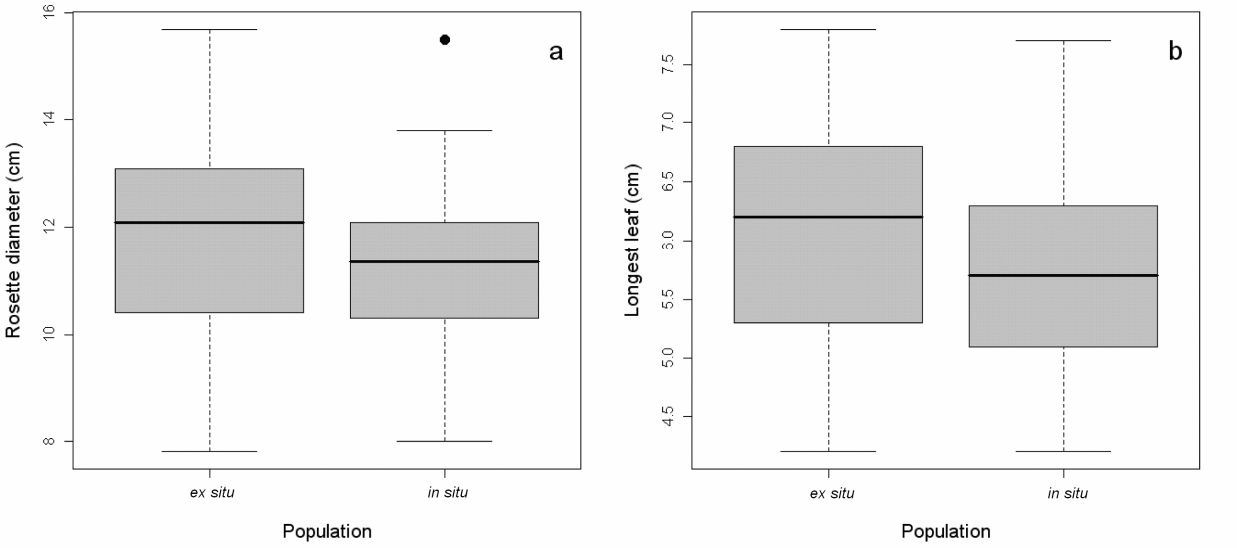

Fig. 3-3: ANOVAs for leaf rosette diameter (a) length of the longest leaf (b) investigated in 50 *S. chlorantha* individuals of each population (*ex situ* and *in situ*) in a common garden approach under same ecological conditions

3.5 Discussion

Genetic diversity

The main goal of *ex situ* conservation is restoring the genetic diversity as found in natural populations (Krauss et al. 2002). However, when establishing *ex situ* cultivars a biased or underrepresented sampling of genotypes can lead to low genetic variation and founder effects in such translocated populations (Ye et al. 2011). For *Cynoglossum officinale* (Enßlin et al. 2010) and for *Silene otites* (Lauterbach et al. 2012b), a decreased genetic diversity in botanic garden cultivars was found. This trend was not supported by my results in *S. chlorantha*. Just a lower number of private bands in the *ex situ* population indicated a genetic bottleneck which is typical for populations of more recent origin (Stehlik et al. 2001; Tribsch et al. 2002). The results revealed an almost identical level of genetic diversity in the investigated *ex situ* and *in situ* populations after 26 years of isolation. However, genetic diversity was slightly lower as compared to other natural populations of *S. chlorantha* in north-eastern Germany (Lauterbach et al. 2011); and also lower as compared to other perennial dry grassland species (Ronikier 2002; Tero et al. 2003; Bylebyl et al. 2008; Hensen et al. 2010). The low genetic diversity in the Berlin *in situ* population can be traced back to a genetic bottleneck effect (Nei et al. 1975) before seeds were sampled for *ex situ* conservation in the early 1980s. Since the plants for replanting came from a constricted gene pool of few natural plants and the *ex situ* progeny the genetic diversity have to be low. After conservation efforts as replanting and habitat improvement there was a strong increase in population size in the 1990s.

S. chlorantha is a self-compatible and long lived perennial. In theory self-compatible species are expected to be more stable with a lower population turnover (Moyle 2006). Compared to obligate out-crossers, in self-compatible species the intra-population diversity should be low and genetic differentiation among populations higher (Hamrick & Godt 1996; Lauterbach 2011a). The here investigated *S. chlorantha* population at the BGBM revealed the potential of preserving natural genetic diversity under *ex situ* conditions as there was no substantial loss in genetic diversity. This was contrarily to the results found in *Silene otites* by Lauterbach et al. (2012b). For a hermaphroditic and self-compatible species like *S. chlorantha*, a population size of 20 individuals seems to be enough to prevent genetic erosion. Nevertheless, we recommend a near natural like cultivation with evolutionary important processes as competition, generation overlap and natural selection as well as a larger *ex situ* population sizes up to 500 individuals if reintroduction programmes are intended (Godefroid et al. 2011).

Genetic differentiation

In a self-compatible species, small population sizes and loss of genetic exchange over longer time periods can result in strong genetic drift and a distinct genetic differentiation among populations (Ellstrand & Elam 1993). Linhart & Grant (1996) proposed that a rapid selective change can occur within less than 10 generations. This was also supported by the findings in *S. otites* of Lauterbach et al. (2012b). The results in *S. chlorantha* showed a surprisingly low degree of genetic differentiation between corresponding *in situ* and *ex situ* populations during about 13 generations of isolation. This could mainly be explained by restricted gene pool of the wild population in the 1980s when the *ex situ* cultivar was established. Additionally the replanting of many progeny plants from the *ex situ* cultivar in the wild enhanced the genetic harmonisation. *S. chlorantha* is a more long lived perennial species (life-span more than 5 years pers. observ.) probaly having a low extant of genetic turnover of individuals in a population. In contrast, short lived plant species as annuals, are expected to be most strongly affected by genetic erosion (Aguilar et al. 2008).

Fitness

The results revealed no difference in plant performance between *in situ* and *ex situ* individuals when cultivated under same ecological conditions. Positive correlations between fitness and genetic diversity or population size were found in a number of species (e.g. Fischer & Matthies 1998; Leimu et al. 2006; Hensen et al. 2004) whereas not for *S. chlorantha* (Lauterbach et al. 2011a). Nevertheless, the expression of inbreeding effects depend on the environmental conditions (Armbruster & Reed 2005) and the conditions in the common garden approach were not nutrient or water limited as in the field. Alternatively, effective purging of deleterious alleles through the strong genetic bottleneck effect can be an explanation of non-restricted fitness (Crnokrak & Barrett 2002).

In *Cynoglossum officinale,* reduced seed dormancy was observed in garden collections (Enßlin et al. 2011). The loss of dormancy under domestication is a typical evolutionary change in Botanic Gardens (Hilu & de Wet 1980), which is mainly induced by unconscious selection of early germinants (Havens et al. 2004). According to that, my results also indicated a higher level of germination of *ex situ* seeds. However, we have no information about seed dormancy or infertility of non germinated seeds as the experiment was stopped when seedlings were transplanted in single pots.

Implications for conservation

We found no reduction of genetic variation and plant fitness in the *ex situ* plants compared to the parental *in situ* ones. Hence, in the self-compatible and long lived *S. chlorantha* a proposed minimum population size of 20 individuals (Gale & Lawrence 1984) was enough to preserve genetic diversity during cultivation of 26 years. However, especially in short lived, annual or dioecious species such a minimum population size should be handled with care (Husband & Campbell 2004). A higher population turnover and imbalanced sex ratios could accelerate genetic erosion under human induced selection (Duminil et al. 2009; Lauterbach et al. 2012b). Cultivation inevitably implies some kind of selection different from natural conditions. Here, it should be aimed for larger population sizes, repeated seed collection throughout seed ripening, and reduced seedling selection during cultivation. If later relocation or reintroduction programs are planned, time of cultivation should be kept to a minimum. I recommend a careful selection of founder individuals to maintain the original genetic diversity of wild populations. Based on the presented results with only one example of a well documented *ex situ* population, no general recommendations for cultivation of other plant species can be given so far. This highlight the importance of further studies of documented *ex situ* collections in botanic gardens, especially if corresponding wild populations are still accessible.

3.6 Acknowledgements

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3.7 References

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4 How to prevent genetic erosion? Effects of inbreeding and outbreeding in fragmented populations of the endangered *Silene chlorantha* (Caryophyllaceae)

4.1 Abstract

Many species of fragmented and isolated populations suffer from genetic erosion. Here I investigated the effects of artificial intra-population crosses as possibility for nature conservation management strategies to re-introduce genetic variability in depleted populations. F_1 progeny can feature enhanced genetic diversity leading to a better plant performance (heterosis). However, in case of adaptation to local environments inter-population crosses can also have negative effects on plant performance (outbreeding depression). A cross-pollination experiment was conducted among 5 different sized, geographically isolated and genetically variable populations in the self-compatible endangered dry grassland perennial *Silene chlorantha*. Genetic diversity (AFLP) and plant performance in a common garden experiment was analysed of the F_1 progeny. By crossing individuals of depleted populations, the genetic diversity could not be increased. Genetic diversity could only be enhanced if one partner originated from a genetically diverse population. Progeny of outbreeding from genetically diverse populations featured significantly higher genetic diversities than progeny of self-pollinations. Significant inbreeding depression was found in the offspring of self-pollinations in genetically depleted populations. F_1 progeny of inter-population crosses between genetically diverse and large populations featured an increased plant performance. Plant performance was increased at crosses between geographically distant populations. The results of this study are discussed in the context of local adaptation, reinforcements and promotion of gene flow among small and isolated populations for optimized conservation strategies.

Keywords: AFLP, Inbreeding, Outbreeding, Plant performance, Pollination

4.2 Introduction

Genetic diversity of populations is a main factor for population viability and individual plant fitness (Leimu et al. 2006). Rare and endangered plant species often feature geographically distant populations with no or reduced gene flow inbetween. Especially small and isolated populations are more prone to stochastic effects, loss of genetic diversity and genetic drift leading to a reduced plant performance (Lande 1988; Leimu et al. 2006). Gene flow among geographically fragmented populations is often restricted by missing long distance seed dispersal and limited pollen transfer (Lauterbach et al. 2011). Hence, limited gene flow among populations increases the level of genetic differentiation and promotes the mating between relatives within populations (Ellstrand & Elam 1992; Young et al. 1996). An increased level of homozygosity may result in inbreeding depression, e.g. reduced offspring fitness and plant performance (Charlesworth & Charlesworth 1987; Barret & Kohn 1991). Especially in self-compatible species, selfing is enhanced is small isolated populations and inbreeding may occur when pollen is transferred within an individual or when mating occurs between close relatives (Collin et al. 2009). Again, selfing populations also tend to show reduced inbreeding depression for early fitness traits as would be expected if selection preferentially purges lethal mutations in early life stages (Husband & Schemske 1996). So far, effects of inbreeding depression and purging of genetic loads are inconsistent and difficult to detect depending on experimental design, life history traits, life cycle stage and genetic basis (Byers & Waller 1999).

In contrast, offspring from crossings between distant populations may suffer from outbreeding depression in case of localized adaptation (Waser & Price 1993). Therefore, in the field of restoration ecology there is an ongoing discussion concerning the translocation of organisms for stabilizing populations of rare and endangered plant species.

To develop strategies to prevent the loss of biodiversity on population level is complicated by the interaction of stochastic and deterministic factors (Schemske et al. 1994). When reintroductions or reinforcements of plant populations are planned, attention has to been paid for genetic composition of source material and the effects of inbreeding and outbreeding (Hufford & Mazer 2003). Artificial genetic exchange between populations by pollen or seeds can counteract reduction in genetic diversity and heterozygosity leads to increased plant fitness (Luijten et al. 2002). Hence, is it possible to increase genetic diversity and fitness of populations by singular gene flow? Some studies also showed dependence of genetic and geographic distance affecting offspring performance by inbreeding or outbreeding depression e.g. in *Delphinium nelsonii* (Waser & Price 1994), in *Lotus scoparius* (Montalvo & Ellstrand

2001) and *Grevillea mucronulata* (Forrest et al. 2011). Outbreeding depression leads to a maladaptive disruption of co-adapted beneficial gene complexes by crossing of dissimilar individuals of the same species (Price & Waser 1979; Lynch 1991; Waser & Price 1994). In case of strong inbreeding and co-adapted gene complexes irrespective of the environment, outcrossing can disrupt such modification and enhance plant fitness (Luijten et al. 2002). Hence, gene flow between depleted populations may be favourable by masking deleterious alleles and a reintroduction of new alleles (Ingvarsson 2000; Ingvarsson & Whitlock 2000). Therefore, enhancing genetic diversity by artificial gene flow as transplantations can be a suitable way to prevent genetic erosion (Willi et al. 2007).

However, there is a controversial discussion about artificial mixture between remnant populations to increase genetic diversity (Tallmon et al. 2005; Edmands 2007). An obvious strategy of stocking small populations is to use individuals or seed material of as possible near located populations. Gene flow can quickly attenuate inbreeding depression due to heterosis (Westemeier et al. 1998; Richards 2000). The often observed heterosis effect is maximised in the first generation (Lynch 1991). Hence a monitoring of population fitness in the subsequent populations is important to evaluate the outcome of crossing experiments (Edmands 2007). However, especially in perennial plant species long term evaluations are difficult and studies are often restricted to measurements of the first generation.

In related studies of inbreeding and outbreeding effects in plants, measurements of only quantitative genetics without parallel molecular analyses have been frequent (e.g. Fenster & Galloway 2000; Bailey & McCauley 2006; Collin et al. 2009). In the here presented study, I combined analyses of molecular genetics and measurements of plant fitness to investigate the effects of inbreeding and outbreeding. Additionally, considering population size, genetic distance and geographic distance is very important for evaluations of inbreeding and outbreeding effects (Willi et al. 2007). In a previous study (Lauterbach et al. 2011), small populations with a low genetic diversity suffered from a reduced seed set and lower offspring fitness in the rare *Silene chlorantha*. The here investigated populations of *Silene chlorantha* were genetically differentiated and there was no support of isolation by distance (Lauterbach et al. 2011).

In the here present study, I crossed five populations of *Silene chlorantha* located in the region of north-eastern Germany. I chose different crossing designs as: inbreeding, outbreeding between depleted populations and outbreeding between depleted and genetically diverse populations. Furthermore I chose populations of different geographic distances (no distance, < 10 km, > 20 km) to each other to test for geographic effects. The five investigated parental populations also featured different population sizes. In a common garden approach, the effects of (i) parental population genetic diversity, (ii) parental

population size, and (iii) geographic distance between parental populations upon plant performance variables in the F_1 progeny of cross pollinations in *Silene chlorantha* were analysed. Genetic diversity of F_1 progeny was analysed by AFLP to investigate the results of different crossing designs in addition to fitness evaluation.

4.3 Materials and Methods

Study species and plant material

S. chlorantha (WILLD.) EHRH. is a self-compatible endangered perennial dry grassland species. It has protandric flowers but selfing seems to be very common between flowers of different developmental stages at the same flower stem. Remnant populations in north-eastern Germany (Brandenburg) are genetically strongly differentiated and variable in genetic diversity (Lauterbach et al. 2011). Based on these investigations, five different populations (Fig. 4-1) were selected for a cross-pollination experiment.

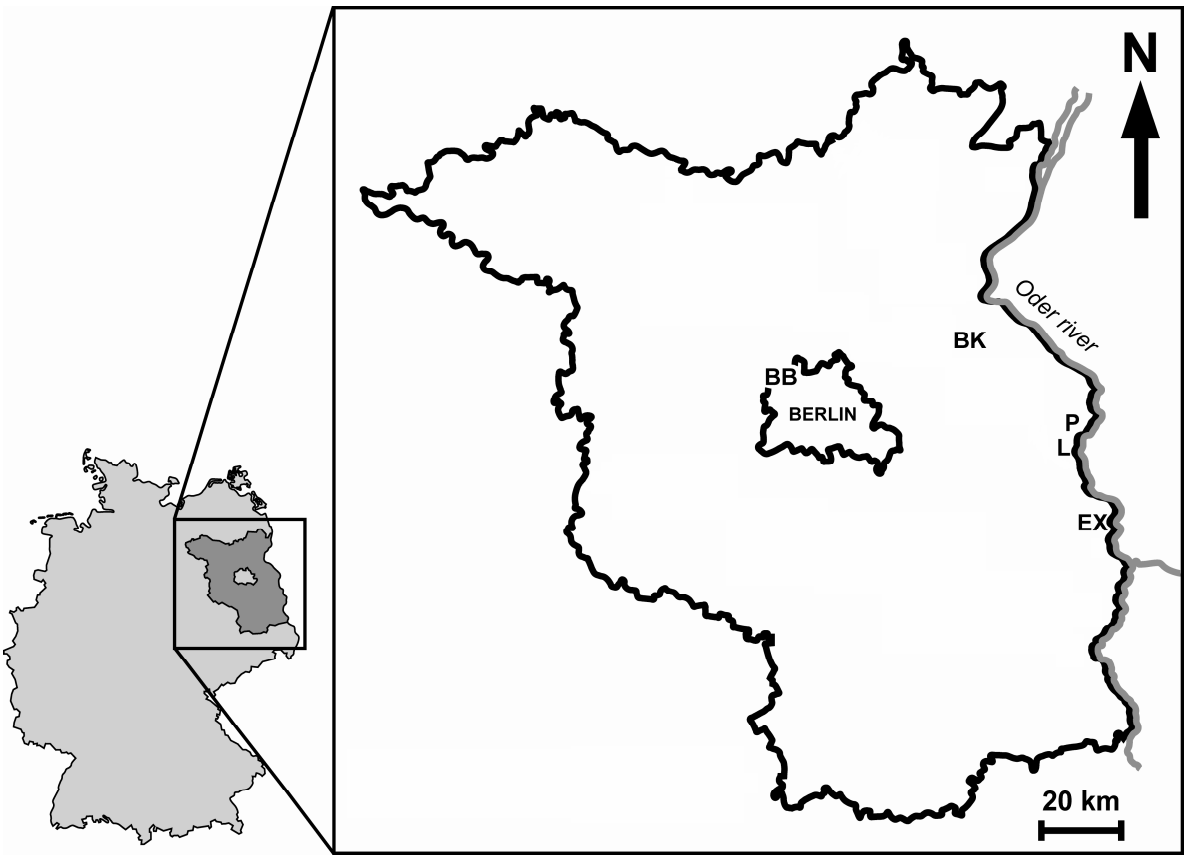

Fig. 4-1: Distribution map of parental populations used for crossing experiments of *Silene chlorantha*, population codes are as in Table 4-1

A common garden experiment was conducted in botanical garden of Berlin (BGBM). In April 2008, I sowed 100 seeds from 10 seed families of each population (sampled in 2007, see Lauterbach et al. 2011) and 35 seedlings were randomly selected as “parental plants” for further investigations and crossing experiments. Each plant was planted individually in a clay pot and placed into a sand bed of the common garden.

Crossing design

To produce F_1 progeny, cross pollination of 13 individuals among 5 different populations were conducted (Tab. 4-1). Following treatments were conducted: self-pollination (inbred) and across population pollination - hereby, crosses between plants of genetically diverse populations, genetically depleted populations, small populations, large populations, geographically distant populations, geographically near populations were conducted (Table 4-2).

From June to September 2009 intraspecific pollination crosses were performed in a common garden experiment. For this, anthers of receptor plant flowers (female) were removed by hand in the bud stage and inflorescences were bagged in a frame of fine gauze. Anthers from the donor plant flowers were clipped by tweezers and brushed against the stigmas of the flowers of the female plant. Each female flower received pollen from one selected donor two times in a time span of two days to ensure pollination. Best time of female receptivity was in the afternoon on sunny days when the stigmas were light pinkish coloured. The donor plants were also bagged to avoid pollen transfer from other plants. The donor plants simultaneously act as in-breeders and flowers in different developmental stages were cross pollinated inside each baggage. Seeds were harvested 4 to 6 weeks after crossing and pooled of a single maternal plant.

Table 4-1: Crossing-treatment scheme of parental populations and number of replicates (colour-coded) among 5 populations of *Silene chlorantha*

Parental populations					**Genetic diversity**			**Population size**			**Geographic region**		
Population	Code	Size	Genetic diversity	Region	Inbred	vs. high	vs. low	Inbred	vs. small	vs. large	no	vs. high	vs. low
Berlin	BB	10 000 (large)	0.181 (low)	central	-	2	2	-	3	1	-	4	-
Eisenhüttenstadt	EX	8 000 (large)	0.230 (high)	south	1	-	1	1	-	1	1	1	-
Lebus	LL	300 (large)	0.204 (high)	east	1	-	2	1	1	1	1	1	1
Podelzig	P	27 (small)	0.172 (low)	east	3	1	4	3	3	2	3	2	2
Biesdorf	BK	103 (small)	0.183 (low)	north	3	-	2	3	2	-	3	-	1

Table 4-2: Summary of pairwise genetic (upper triangle, F_{st}) and geographic distances (lower triangle, km) among parental populations of *Silene chlorantha* based on the results of Lauterbach et al. (2011)

Code	**BB**	**EX**	**LL**	**P**	**BK**
BB	-	0.184	0.278	0.301	0.305
EX	105	-	0.221	0.120	0.175
LL	91	25	-	0.335	0.331
P	90	35	10	-	0.266
BK	59	71	47	41	-

Common garden experiment and plant performance

From each crossing treatment, if available 100 seeds per cross treatment were harvested in October 2009 and immediately sawn in pots filled with sand in the glasshouse. In some cases the number of fertile seeds per treatment was lower, mainly caused by suboptimal conditions e.g. rainy days at pollination time. In April 2010, 30 randomly chosen seedlings of each crossing treatment (Table 4-1) were planted in single clay pots each, containing a 2:1 mixture of sand:humus. After two weeks in the glasshouse, pots were transferred to the outside. Pots were randomised dug in grid to avoid dehydration with a barrier of gauze to the top soil. Randomization was rearranged again 4 weeks later. The plants were watered if necessary. In October 2010 following fitness variables were measured for each plant: leaf rosette diameter and length of the longest leaf. In the first year 2010 less than one third of the plants flowered. Therefore, variables as plant height, number of stems, number of flowers, and number of seeds per capsule were excluded from the here presented analysis.

AFLP procedure

Samples of fresh leaf material were dried and stored in silica gel until DNA extraction. From 12 individuals per pollination treatment DNA was extracted using the NucleoSpin Plant II kit (Macherey-Nagel GmbH and Co.KG, Düren, Germany) according to the manufacturer's protocol with modification (incubation time for cell lyses was changed to 30 min). Amplified fragment length polymorphism (AFLP) was carried out after a modified protocol of Vos et al. (1995) following Lauterbach et al. (2011): 10 µl Genomic DNA (30 ng/µl DNA) was double-digested in a final volume of 25 µl (0.25 µl fast digest *Eco*RI (10 U/µl; Fermentas, St. Leon Rot, Germany), 0.25 µl fast digest *Mse*I (10 U/µl; Fermentas), 2.5 µl 10x fast digest buffer (Fermentas) and 12 µl purified H_2O at 37 °C for 10 min. Reaction was terminated by 80 °C for 5 min. Ligation was carried out with total volume of restriction products and: 0.5 µl T4-ligase (1 Weiss units/µl; Fermentas), double stranded adapters 0.5 µl *Eco*RI (5 pmol/µl; MWG Biotech, Ebersberg, Germany), 0.5 µl *Mse*I (50 pmol/µl; MWG Biotech), 1.5 µl ATP (10 mM; Fermentas), 0.5 µl 10x fast digest buffer (Fermentas) and 1.5 µl purified H_2O at 20 °C for 16 h. Selective amplifications were performed by using four primer combination *Eco*RI ACG – *Mse*I CAA; *Eco*RI ACG - *Mse*I CGA; *Eco*RI AAG - *Mse*I CAC; *Eco*RI ACC - *Mse*I CTC. Fragments were separated on a polyacrylamide gel with an internal size standard (GenomeLab DNA Size Standard Kit 400, Beckman Coulter, Krefeld, Germany) on an automated sequencer (CEQ 8000, Beckman Coulter).

Statistical analyses

The presence/absence matrix from AFLP fragments was generated to calculate allele frequencies by using a Bayesian method with non uniform prior distribution (Zhivotovsky 1999). From these allele frequencies, the percentage of polymorphic loci and genetic diversity (H_E) were calculated based on the method of Lynch & Milligan (1994) assuming Hardy-Weinberg conditions, using the software AFLPsurv, version 1.0 (Vekemans 2002). Genetic diversity, which is equivalent to expected heterozygosity (H_E) under Hardy-Weinberg conditions (Nei 1987), was used as a measure of within population genetic diversity.

I used ANOVA models and Tukey's post hoc test to test for the effects of parental population size, parental genetic diversity and parental population distance upon plant performance. All calculations were performed with R 2.7.1 (R Development Core Team 2008).

4.4 Results

Genetic diversity

The four primer combinations provided 110 polymorphic loci. Inbreeding treatment of populations with a low genetic diversity resulted in the lowest values for F_1 genetic diversity (Table 4-3). ANOVA resulted in a significant difference of genetic diversity between the different cross-treatments ($P < 0.001$). Highest genetic diversity was found in crossings between genetically diverse and poor populations (Fig. 4-2). Progeny plants from self-pollinations of low-diversity populations and progeny from outcross between low-diversity populations featured lower genetic diversities than progeny from high-diversity outcrosses (Fig. 4-2). Progeny plants from inbred of high-diversity populations showed no reduced genetic diversity (Fig. 4-2).

Table 4-3: Results for genetic diversity of cross pollinations between 5 populations of *Silene chlorantha*. Code of F_1 crossings (F_1), Parental plants, number of analysed individuals by AFLP (n_{AFLP}), crossing scheme of parental genetic diversity (genetics), geographic distance between parental populations (Dist.), parental population sizes (Size), percentage of polymorphic loci (PL), genetic diversity (H_E), number of measured plants for fitness calculations (n_{Fit})

F_1	Parents (f x m)	n_{AFLP}	Genetics	Size	Dist.	PL	H_E	n_{Fit}
PW	PW1 x PW1	12	inbred low	inbred small	no	33.3	0.14083	23
PP	PW2 x PW2	12	inbred low	inbred small	no	38.5	0.15179	28
PS	PW3 x PW3	11	inbred low	inbred small	no	45.8	0.14123	30
BK	BK1 x BK1	11	inbred low	inbred small	no	35.4	0.14331	30
BB	BK2 x BK2	12	inbred low	inbred small	no	31.3	0.14603	28
LL	LL1 x LL1	11	inbred high	inbred large	no	52.7	0.17550	28
EE	EE1 x EE1	12	inbred high	inbred large	no	43.8	0.17541	29
BW	BB2 x PW2	12	outcross low low	small large	high	36.5	0.14984	15
BP	BB2 x PW3	12	outcross low low	small large	high	37.5	0.15918	25
PY	PW3 x BK1	11	outcross low low	small small	low	40.6	0.15638	24
PV	PW1 x BK2	3	outcross low low	small small	low	31.3	0.14468	4
LP	LL1 x PW4	12	outcross high low	large small	low	52.7	0.18175	30
LB	LL1 x BB3	6	outcross high low	large large	high	61.8	0.23642	30
EB	EE1 x BB1	11	outcross high low	large large	high	64.6	0.20542	30

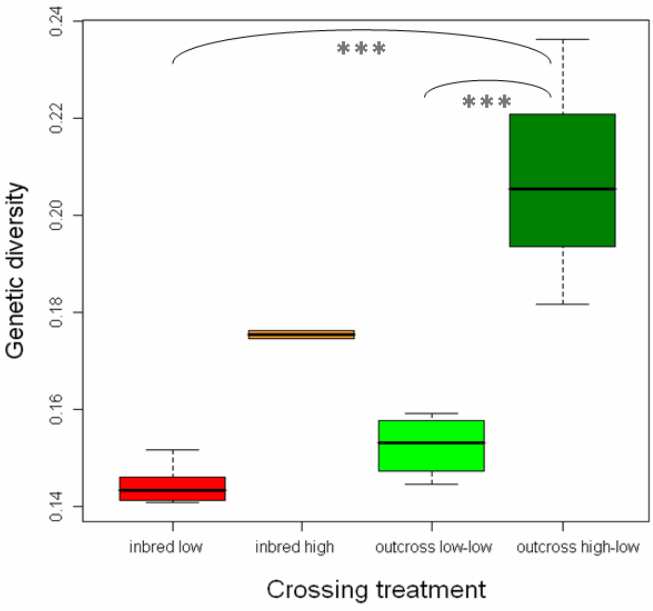

Fig. 4-2: Results for genetic diversity (H_E) of F_1 progeny plants of different crossing-treatments; *** $P < 0.001$ for multiple comparisons (Tukey's test)

Plant performance

The rosette diameter of the F_1 generation differed significantly between the different cross-treatments for parental genetics, parental population size, and parental population distances (Table 4-4). A significant difference between the treatments was also found for the length of the longest leaf. Selfing in populations with low genetic diversity (inbred low) resulted in significant smaller rosette diameters and leaf length than any other cross-treatment (Fig. 4-3). Highest plant performance was found in the treatment "outcross low-low" (Fig. 4-3a). There was no difference in plant performance between the two outcrossing treatments. Selfing of genetically diverse populations (inbred high) resulted in a significant smaller plant height as compared to all other treatments (Table 4-5a; Fig. 4-3a).

F_1 plants from crossings of large with small populations showed significantly smaller diameters and longer leaves as F_1 plants from crossings of small with small populations, and crossings of large with large populations (Table 4-5b; Fig. 4-3b).

Crossing of plants between geographically distant populations resulted in significantly larger rosette diameter as F_1 plants from crossings of populations with a low geographic distance (Fig. 4-3c).

Table 4-4: ANOVAs testing the effect of cross-treatment, parental population size and parental population distance on rosette diameter, length of the longest leaf, and plant height in *Silene chlorantha*.

		Rosette diameter			**Longest leaf**		
source of variation	df	MS	F	*P*	MS	F	*P*
genetics	3	28.51	6.94	< 0.001	10.15	9.39	< 0.001
size	2	75.09	18.29	< 0.001	12.84	11.89	< 0.001
distance	1	94.25	22.96	< 0.001	9.20	8.52	0.004
residuals	347	4.11			1.08		

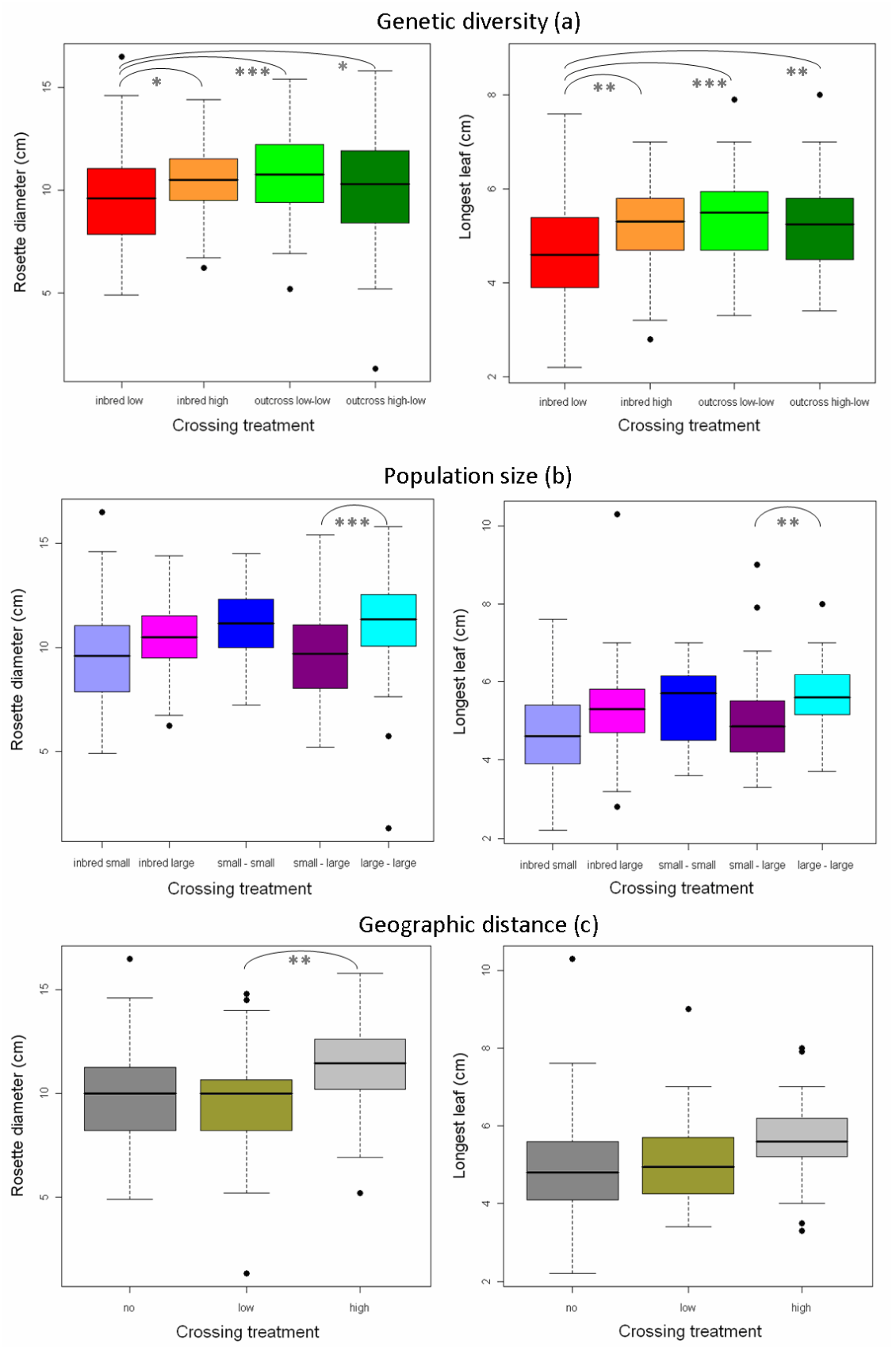

Fig. 4-3: Plant performance of different cross-treatments of the F_1 generation of cross pollinated *Silene chlorantha* plants: a) parental genetic diversity, b) parental populations size and c) parental population distance; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ for multiple comparisons (Tukey's test)

Table 4-5: Multiple comparisons of plant performance of the F_1 cross pollinated *Silene chlorantha* populations, ANOVA post hoc tested by Tukey's test for the different treatment levels, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

crossing treatment	plant performance	
a) Genetic diversity	***rosette diameter***	***longest leaf***
inbred low vs. inbred high	0.029 *	0.003 **
high low vs. inbred high	0.954	0.999
low low vs. inbred high	0.730	0.946
high low vs. inbred low	0.049 *	0.001 **
low low vs. inbred low	<0.001 ***	<0.001 ***
low low vs. high low	0.322	0.888
b) Population size		
small small vs. large large	0.618	0.609
small large vs. large large	<0.001 ***	0.001**
small large vs. small small	0.098	0.489
large large vs. inbred large	0.114	0.287
small small vs. inbred large	0.984	1.000
small large vs. inbred large	0.113	0.361
inbred small vs. inbred large	1.000	1.000
large large vs. inbred small	0.033	0.132
small small vs. inbred small	0.976	1.000
small large vs. inbred small	0.028	0.175
c) Geographic distance		
low vs. high	0.009 **	0.168
no vs. high	0.139	0.478
no vs. low	0.229	0.577

4.5 Discussion

Since genetic diversity is often positively correlated with plant fitness (Leimu et al. 2006), an increase of intra-population genetic diversity is aimed by artificial gene flow among populations to avoid genetic erosion of small and isolated populations. Genetically depleted populations featuring inbreeding effects can be rescued by the introduction of only few immigrants (Tallmon et al. 2005; Edmands 2007). However, in the presented study outcrossing between two genetically depleted populations resulted not in an increased

genetical diversity of F_1 progeny. Increased genetic diversity in the F_1 progeny of *Silene chlorantha* could only be detected if one partner derived from a genetically diverse population. This highlights the importance of maintaining genetically diverse populations as reservoir of intra-specific diversity. Populations should retain enough genetic variability to withstand stochastic effects, changes in environmental conditions and evolutionary processes (Hamrick et al. 1991). Since the highest genetic diversity was found in the "high-low" crossing scheme, I recommend crossings with at least one genetically diverse population to increase genetic diversity of depleted populations.

Molecular markers are indicative for the analysis of population genetic diversity, whereas quantitative measurements in plant performance are directly acted on by natural selection (Vitt & Havens 2004). As expected, the outcross progeny showed a better plant performance whereas progeny from the selfing treatment of genetically depleted populations showed effects of inbreeding depression e.g. in form of smaller leaf rosette diameters. Reduced plant fitness from inbred populations is well known (reviewed by Husband & Schemske 1996). Nevertheless, in self-compatible species the influence of inbreeding upon fitness is sometimes cryptic (Byers & Waller 1999). Inbreeding depression could decline with increased inbreeding by purging deleterious alleles (Husband & Schemske 1996). However, the effects of inbreeding depression are also life cycle dependent. Hauser & Siegismund (2000) found maternal discrimination against selfing in a study of pollen fitness and zygote survival in *Silene nutans*. In general, life cycle dependent inbreeding depression was observed either early, during seed maturation, or in later life cycle stages, during growth, but rarely during germination or juvenile survival (Husband & Schemske 1996). A slight heterosis effect by crossing genetically depleted populations of *S. chlorantha* could be observed (Fig. 4-3a). Here the F_1 progeny of the treatment "outcross low-low" featured lower genetic diversity but an increased plant performance. Heterosis effects were also found in of other studies e.g. in *Silene douglasii* (Kephart 2004), *Silene vulgaris* (Bailey & McCauley 2006), *Arnica montana* (Luijten et al. 2002), *Chamaecrista fasciculata* (Fenster & Galloway 2000) and *Ranunculus reptans* (Willi et al. 2007).

In the F_1 generation of *S. chlorantha*, no clear evidence for outbreeding depression was found. However, outbreeding depression can also be expressed in later generations (Fenster & Galloway 2000; Keller et al. 2000). Outbreeding by intra-specific hybridisation is a scarce phenomenon (Edmands 2007). It has been found e.g. in *Gentianella germanica* (Fischer & Matthies 1997) and *Calylophus serrutalus* (Heiser & Shaw 2006). Additionally, in case of very small population sizes and high selfing rates, positive effects of inter-population crosses should outweigh the risk of outbreeding depression. Nevertheless, at the current stage of the study we only observed short term genetic effects. There is a need to evaluate the plant

performance of further generations (Willi et al. 2007) since the effects of inbreeding and outbreeding would be more apparent if examined over long time periods or in different life cycle stages (Armbruster & Reed 2005). However, long time evaluations are difficult especially under financial constraints and limited conditions for long term common garden experiments. Furthermore, experimental garden conditions are different from those in nature. Hence, local adaptation to environmental conditions can be masked. Based on the results found here, a transplantation experiment in nature would be interesting to test for effects of local adaptation. However, such transplantation experiments bear the risk of irreversible changes in genetic composition and outbreeding depression by mixing different genotypes (Schaal & Leverich 2004). This should be considered when working experimentally with rare species in ecological restoration (Hufford & Mazer 2003).

Parental population size did not influence the fitness of the progeny with the exception of a difference between crosses of “large with large” populations and “small with large” populations. Small populations did not benefit from outcrossing with large sized populations, which was also found in *Ranunculus reptans* by Willi et al. (2007). The smaller plant size in case of “small-large” crossings lacks explanation and should be followed up in further generations.

Simulated long distance pollination resulted in an increased rosette diameter of the progeny. Forrest et al. (2011) found an intermediate distance (adjacent cluster) to produce the best responses in plant fitness of the first generation for the outcrossing shrub *Grevillea mucronulata*. There, genetic provenance does effect optimized population management. In *S. chlorantha*, progeny fitness of self-pollinations was not reduced compared to near and distant population crosses. A possible explanation can be the clear genetic differentiation among parental populations as e.g. the nearby located populations Podelzig and Lebus (distance 10 km) showed the highest pairwise genetic differentiation. So far, it would be hard to predict the outcome of translocation experiments as parallel population genetic studies additional to quantitative measurements are not always feasible.

Implications for conservation

In case of strong genetic population differentiation and absence of isolation by distance support, rather ecological distance than geographic distance should be considered in case of artificial inter-population crosses. Nevertheless, introduction of novel genotypes should be handled with care because of local adaptation to environmental conditions (Vander Mijnsbrugge et al. 2010). In each case, actors have to decide to do nothing or introduce

novel genotypes. An increase of genetic diversity by introduction of novel genotypes should be one of the last activities. Priority should be given first on habitat improvement. Only if there is a strong evidence of inbreeding depression and populations fail to become self-sustaining without reinforcement (Kephart 2004) we should think about artificial gene flow, wherever applicable of surrounding populations. This must be weighted against potential consequences of outbreeding (Forrest et al. 2011). In *S. chlorantha* population genetic differentiation in the investigation area was high (Lauterbach et al. 2011). However, genetic differentiation does not have to be adaptive when neutral forces dominate selective ones (Linhart and Grant 1996). In the F_1 fintess, the study revealed positive effects of inter-population crossings; however, there is a need of further investigations for the effects of inbreeding and outbreeding in the next generations and also in other species with different mating- and pollination systems (Duminil et al. 2009).

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5 Population genetics and fitness in fragmented populations of the dioecious and endangered *Silene otites* (Caryophyllaceae)

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5.1 Abstract

Population fragmentation is often correlated with loss of genetic diversity and reduced fitness. Obligate out-crossing (dioecy) is expected to enhance genetic diversity, reduce genetic differentiation, and avoid inbreeding depression through frequent gene flow. However, in highly fragmented populations dioecy has only diminishing effects upon genetic structure as pollination limitations (e.g. flight distance of pollinators) most often restrict inter-population gene flow in insect pollinated species. In fragmented dry grasslands in north-eastern Germany, we analysed genetic structure, fitness, and habitat quality of the endangered dioecious *Silene otites* (Caryophyllaceae). Using AFLP markers, a high level of differentiation among ten populations was found (F_{st} = 0.36), while the intra-population genetic diversities (H_E = 0.165 - 0.240) were similar as compared to hermaphroditic species. There was neither a correlation between geographic and genetic distance nor between genetic diversity and population size, which indicates reduced gene flow among populations and random genetic drift. Plant size was positively correlated with genetic diversity. Seed set and number of juveniles were positively related to population size. Higher total coverage resulted in reduced plant fitness, and the number of juveniles was negatively correlated to cryptogam cover. Additionally, we found a sex ratio bias towards more male plants in larger populations. Overall, our results indicate that on a regional geographic scale dioecy does not necessarily prevent genetic erosion in the case of habitat fragmentation, especially in the absence of long distance seed and pollen dispersal capacity.

Keywords: AFLP, Isolation by distance, Mating system, Population size, Sex ratio

5.2 Introduction

In Europe, the ongoing human-mediated fragmentation and habitat loss by changes of land use during the last century have led to a decline in plant species diversity (Young et al. 1996; Booy et al. 2000; Maurer et al. 2006). In addition, factors such as a decreased plant-pollinator interaction across populations (Kwak et al. 1998; Steffan-Dewenter & Tscharntke 1999) and missing seed dispersal (Poschlod & WallisDeVries 2002; Poschlod et al. 2005) affect plant population structure. As a consequence, small populations in fragmented remnant habitats exhibit reduced gene flow, random genetic drift and inbreeding. This may result in reduced genetic diversity and reduced plant performance (Ellstrand & Elam 1993). Additionally, small populations are more prone to stochastic effects and have a higher risk of extinction (Reed & Frankham 2003; Leimu et al. 2006; Honnay & Jacquemyn 2007). So far, population size seems to be one of the best predictors for population fitness (e.g. Fischer & Matthies 1998; Schmidt & Jensen 2000; Hensen et al. 2005; Lauterbach et al. 2011). Nevertheless, there is an ongoing controversial discussion about the importance of genetic diversity versus habitat conditions upon plant fitness. The effects of low genetic diversity upon fitness are sometimes cryptic or affect different life cycle stages (Husband & Schemske 1996; Lauterbach et al. 2011). Results vary depending on the investigated fitness component and life history traits such as life span and mating system (Leimu et al. 2006). Therefore, good knowledge about genetic diversity and habitat conditions in interaction with plant performance in different life cycle stages are necessary for adequate species conservation (Duffy et al. 2009).

Mating systems and pollination modes seriously affect genetic population structure (Hamrick & Godt 1996). Obligate out-crossing (e.g. dioecy, self-incompatibility) is said to maintain genetic diversity, reduce genetic differentiation and avoid inbreeding depression through frequent gene flow, whereas selfing decreases genetic variation, enhances differentiation and can generate fitness loss (Loveless & Hamrick 1984; Ellstrand & Elam 1993; Hamrick & Godt 1996; Linhart & Grant 1996; Guibert et al. 2009). Hence, dioecy can be seen as one strategy for forcing out-crossing and avoiding self pollination. It is frequently associated with mobility traits as an increased extant of pollen dispersal giving an evolutionary advantage in comparison to hermaphroditism (Wilson & Harder 2003). However, dioecy is with only 6 % relatively infrequent in angiosperms (Renner & Ricklefs 1995), and population genetics of dioecious plant species have rarely been studied (e.g. in woody species: Hilfiker et al. 2004; Ueno et al. 2007; Zhou & Chen 2010). Especially studies of herbaceous dioecious species are scarce (e.g. Richards et al. 2003; Vandepitte et al. 2010). Additionally theory predicts an even sex ratio for balancing selection in dioecious plant populations. However, Barrett et al.

(2010) in an analysis of 126 dioecious plant species found that only one-third exhibit a close to even sex ratio, while the others showed a strong bias either to the male or the female side.

The dioecious Spanish Catchfly, *Silene otites* (L.) WIBEL (Caryophyllaceae) occurs throughout central and western Europe (Tutin et al. 1993). In the investigation area of north-eastern Germany, most populations are found on scattered remnants of dry grassland. During the past decades the population number has declined through habitat loss and secondary succession of dry grasslands (Lauterbach et al. 2012). This is due to the loss of traditional sheep grazing and increased eutrophication (Pless 1994). *Silene otites* is listed as vulnerable in Germany (Korneck et al. 1996) and Brandenburg (Ristow et al. 2006), and highly endangered in Berlin (Prasse et al. 2001). *Silene otites* is a perennial herb, growing on alkaline-neutral dry grasslands and sand dunes. The flowering period is from June to September. Putative pollinators are nocturnal moths and mosquitoes (Schulz 1905; Brantjes & Leemans 1976). Soldaat et al. (2000) observed wind pollination in *S. otites* of only a few meters. In contrast to theory (Barrett 2010), *S. otites* features no general dioecious traits such as pronounced longevity, clonal growth, woody structures and wind pollination. Seeds can be dispersed by sheep (Wessels-de Wit & Schwabe 2010) or largely passive (Watt 1981). Sometimes complete capsules are dispersed by adhering to fur or clothes with their dentate opening (personal observation). Germination occurs in autumn and spring (Soldaat et al. 2000); many seedlings die off under unfavourable weather conditions.

Focussing on fragmented populations of north-eastern Germany, we analysed genetic variation and plant fitness within and among different sized populations of the dioecious, insect-pollinated, herbaceous *Silene otites*. The aims of this study are to investigate (1) the genetic population structure, (2) the effect of population size on sex ratio, and (3) plant fitness in relation to population genetic diversity, habitat conditions and population size.

5.3 Materials and Methods

Sampling and AFLP analysis

Ten differently sized populations were investigated featuring two geographic regions with a distance of 143 km (Fig. 5-1; Table 5-1): along the Havel river system in Brandenburg (KM, MM, SW, KS, BL, BG, BS) and along the Oder river system in the eastern part of Brandenburg (PW, PP, LE). Along the Oder river system, *S. otites* is more abundant because of more favourable habitat conditions as dry grasslands are more widespread. According to sample size evaluations by Campbell et al. (2003) and Singh et al. (2006), we collected leaf

material of fresh and healthy leaves from 12 flowering plants per population in summer 2008. All samples were immediately dried in silica gel and stored at 4°C until DNA extraction. Total DNA was extracted from 1 cm homogenised (Retsch MM 301, Haan, Germany) leaf material using NucleoSpin Plant II kit (Macherey-Nagel GmbH and Co. KG, Düren, Germany) according to the manufacturer's protocol (incubation time for cell lyses was changed to 30 min). DNA quality and quantity were measured via NanoDrop1000 spectrophotometer (ThermoScientific, Wilmimgton, DE, USA) and diluted to 30 ng/µl. AFLP analysis was performed according to the protocol of Vos et al. (1995) with modifications (Lauterbach et al. 2011). DNA was restricted with endonucleases EcoRI and True, and ligated to double stranded adaptors. After a preliminary primer screening of 16 individuals from 4 populations with 18 primer combinations, 3 different selective primer combinations were chosen for the analysis: EcoRI ACG - True CTG, EcoRI ACA - True CTG, and EcoRI AGA - True CTC. Selective PCR products were separated on an automated sequencer (CEQ 8000, Beckman Coulter, Krefeld, Germany) with an internal size standard (GenomeLab DNA Size Standard Kit 400, Beckman Coulter).

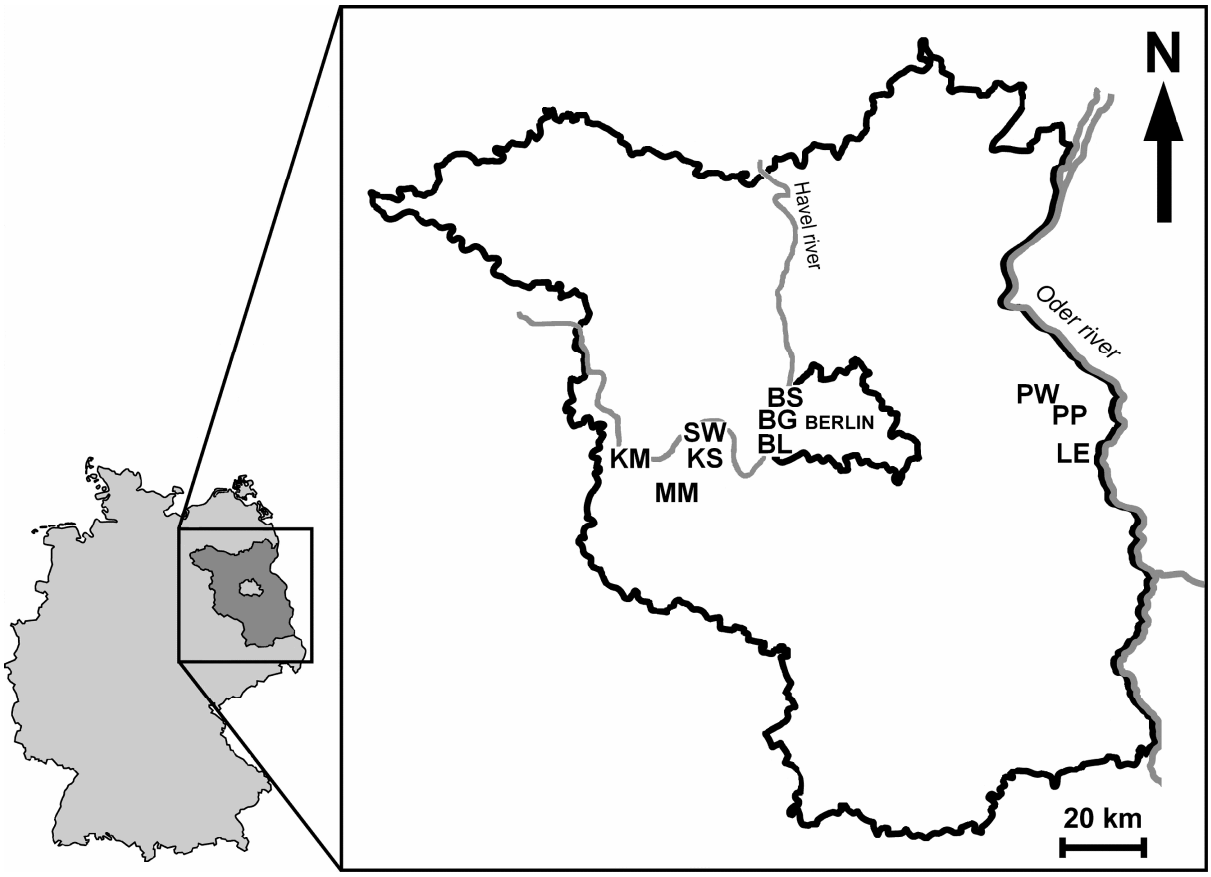

Fig. 5-1: Map of sampled *Silene otites* populations in the area of Berlin and Brandenburg in north-eastern Germany. Abbreviations are as in Table 5-1

Bands were identified and scored semi-automatically using Genographer software (version 1.6.0, JJ Benham, Montana State University, Bozeman, MT, USA). Only AFLP fragments with a sufficient intensity, being unambiguously scorable, were included in the analysis. For 22 samples, the AFLP analysis for each primer combination was repeated to calculate the phenotypic error rate as number of phenotypic differences related to the total number of phenotypic comparisons. The value was subsequently averaged over the three AFLP combinations (Pompanon et al. 2005). A presence/absence matrix was exported for further calculations.

Table 5-1: Summary of sampled *Silene otites* populations: code, geographical group, coordinates, population size, plants analysed by AFLP (n), sex ratio (females/(females + males), number of polymorphic loci (PL), genetic diversity (H_E), and germination rate (GR)

Population	Code	Group	Coordinates	Size	*n*	Sex ratio	PL	H_E	GR
Berlin Lieper Bucht	BL	Havel	52.47°N/ 13.19°E	15	12	0.67	51.7	0.180	0.98
Berlin Gatow	BG	Havel	52.48°N/ 13.17°E	42	12	0.38	51.2	0.165	0.78
Berlin Spandau	BS	Havel	52.52°N/ 13.13°E	2730	12	0.35	56.7	0.214	0.92
Kirchmöser	KM	Havel	52.38°N/ 12.44°E	113	12	0.70	50.2	0.202	0.61
Michelsdorf	MM	Havel	52.31°N/ 12.70°E	775	12	0.40	45.6	0.167	0.54
Krielow	KS	Havel	52.41°N/ 12.82°E	890	12	0.47	64.0	0.232	0.85
Schmergow	SW	Havel	52.46°N/ 12.79°E	2760	12	0.38	51.7	0.206	1.00
Lebus	LE	Oder	52.39°N/ 14.42°E	500	12	0.33	66.0	0.240	0.78
Podelzig I	PP	Oder	52.48°N/ 14.54°E	850	12	0.51	61.1	0.218	0.72
Podelzig II	PW	Oder	52.48°N/ 14.53°E	970	12	0.45	58.1	0.197	0.79
Wriezen	WR	-	52.41°N/ 14.08°E	134	-	0.54	-	-	-
Götz	GO	-	52.25°N/ 12.44°E	38	-	0.42	-	-	-
Marzahne	MZ	-	52.30°N/ 12.32°E	24	-	0.70	-	-	-
Jahnberge	JB	-	52.42°N/ 12.42°E	23	-	0.50	-	-	-

Plant performance and fitness

For each population the number and density of flowering *S. otites* individuals was determined. In large populations (n > 500 plants) flowering plants were recorded in an area of 100 m² and extrapolated according to the expansion area of the population. To asses the populations' sex ratio [number of females/ (females + males)], all individuals in the area of

100 m² were considered. To enlarge sample size, sex ratios were determined on four additional populations (Table 5-1). Our study was conducted shortly after the flowering maxima, so sex could doubtlessly be determined.

In late July 2008 at 30 randomly chosen plants per location along a transect following fitness variables were measured: number of stems, stem height, rosette diameter, number of capsules, seeds per capsule (counting the seed set in one randomly chosen capsule per plant), and seeds per plant by extrapolating the number of seeds per number of capsules. For each of the ten study sites, in ten randomly selected plots of 0.5 x 0.5 m arranged over a transect spanning the population's largest diameter, the total coverage, cover of cryptogams, cover of litter, cover of herbal layer, and the number of juvenile plants (rosette diameter less than 3 cm) were estimated using the percentage scale. In a greenhouse experiment, seed germination was carried out in small pots with a 2:1 sand:humus mixture. Therefore, 100 seeds from 10 seed families (offspring of the plants used for genetic analyses) for each population were examined. The number of seedlings was counted after 3 months.

Statistical analyses

The presence/absence matrix from AFLP fragments was used to calculate allele frequencies, using a Bayesian method with non-uniform prior distribution, assuming Hardy-Weinberg genotypic proportions (Zhivotovsky 1999). The percentage of polymorphic loci and gene diversity (H_E) were calculated based on the method of Lynch & Milligan (1994) with the software AFLPsurv, version 1.0 (Vekemans 2002). Gene diversity (H_E), which is equivalent to expected heterozygosity under Hardy-Weinberg conditions (Nei 1987), was used as a measure of within population genetic diversity.

Genetic differentiation was tested by analysis of molecular variance (AMOVA) using the software Arlequin version 3.1 (Excoffier & Schneider 2006). F_{st} values were calculated after Weir & Cockerham (1984). To test possible correlations between pairwise genetic and geographic distances, a Mantel test was carried out using R 2.7.1 (R Development Core Team 2008). A neighbour-joining (NJ) tree based on *p*-distances was produced using the program PAUP* version 4.0b10 (Swofford 2003). The tree was drawn and edited using FigTree version 1.2.1 (Rambaut 2008). In addition, a Bayesian clustering method implemented in STRUCTURE version 2.2 (Pritchard et al. 2000) was carried out to assign individuals to genetic groups. For each cluster ranging from 1 to 10, we ran 20 independent replicate chains. The analysis was based on the admixture model with correlated allele frequencies. The model was run with a burn-in period of 50,000 and 100,000 MCMC

replications. To determine the most likely number of clusters, the maximum log-likelihood value (Pritchard et al. 2000) and the maximum value of ΔK (Evanno et al. 2005) were calculated using STRUCTURE-SUM (Ehrich 2006) in R 2.7.1 (R Development Core Team 2008). We utilised the program CLUMPP, version 1.1.1 (Jakobsson & Rosenberg 2007), with the Greedy algorithm and 10,000 random input orders of the 20 independent STRUCTURE runs to determine the optimal alignment of clusters across individual runs for K. Results from CLUMPP were imported into DISTRUCT, version 1.1 (Rosenberg 2004), for viewing the individuals' assignment probabilities. Relationships between genetic diversity, germination rate, and population size were tested by Spearman rank correlation. Values of population size were log transformed. Relationship between sex ratio and population size was analysed by linear regression. Relationships between plant fitness traits and explanatory variables such as population gene diversity (H_E), population size, population density, and cover (total, cryptogams, litter, herbal layer) were tested using generalised linear mixed effect models (GLMM) with a Poisson error distribution and population as random factor in the lme4 R-package (Bates et al. 2008). Population size was log-transformed to satisfy model assumptions. After fitting the maximum model, step-wise backward model selection was applied to obtain the minimum adequate model (Venables & Ripley 2002). If likelihood ratio tests produced $P > 0.05$, the term was removed from the model. The simplified model was tested against the more complex model by ANOVA (Crawley 2007). All calculations were performed with R 2.7.1 (R Development Core Team 2008).

5.4 Results

Population genetic structure

The AFLP analysis of 120 analysed individuals provided 203 polymorphic bands ranging from 59 to 479 base pairs. The mean phenotypic error rate (Pompanon et al. 2005) among replicated samples (22 samples x 89 loci) amounted to 4.3 %, which is similar to previously published reports below 5 % in plants (Bonin et al. 2004). Gene diversity (H_E) ranged from 0.165 to 0.240 (Table 5-1). There was no correlation between population genetic diversity and population size ($r_s = 0.41$; $P = 0.24$) or germination rate ($r_s = 0.16$; $P = 0.66$).

The AMOVA (Table 5-2) revealed significant genetic differences among populations (F_{st} = 0.36; $P < 0.001$) with 64.40 % of the variance at the within-population level. According to the two sampled geographic regions (Havel group, Oder group), the level of variation among the groups was very low (1.20 %). Mantel test showed no significant correlations between pairwise geographic and genetic distances: (r_m = -0.12, P = 0.66). Neighbour-joining analysis depicted a clear differentiation of populations (Fig. 5-2). Individuals clustered together in groups according to their population origin. Individuals of the populations PP and PW clustered at the same branch with some genetic admixture. The nearby located populations BL and BG also clustered. The STRUCTURE analysis resulted in a distinct modal maximum of ΔK and a beginning saturation of mean log-likelihood at K = 6. Here, the populations KM, MM, and BG/BL formed three distinct clusters. There was genetic similarity among populations KS, SW, and BS. The three eastern populations PP, PW, and LE consisted of two genetic clusters with more similarity between LE and PP (Fig. 5-3).

Table 5-2: Analysis of molecular variance (AMOVA) of AFLP data for ten *Silene otites* populations with two geographic groupings: Havel (KM, MM, KS, SW, BG, BL, BS); Oder (PP, PW, LX)

source of variation	df	Sum of Squares	VC	% Variation	F_{st}
among groups	1	76.09	0.17	1.20	
among populations	8	573,50	4.88	34.40	
within populations	109	996.21	9.14	64.40	
total	118	1645.80	14.19		0.36 ***

Degrees of freedom (df), variance components (VC) *** $P < 0.001$

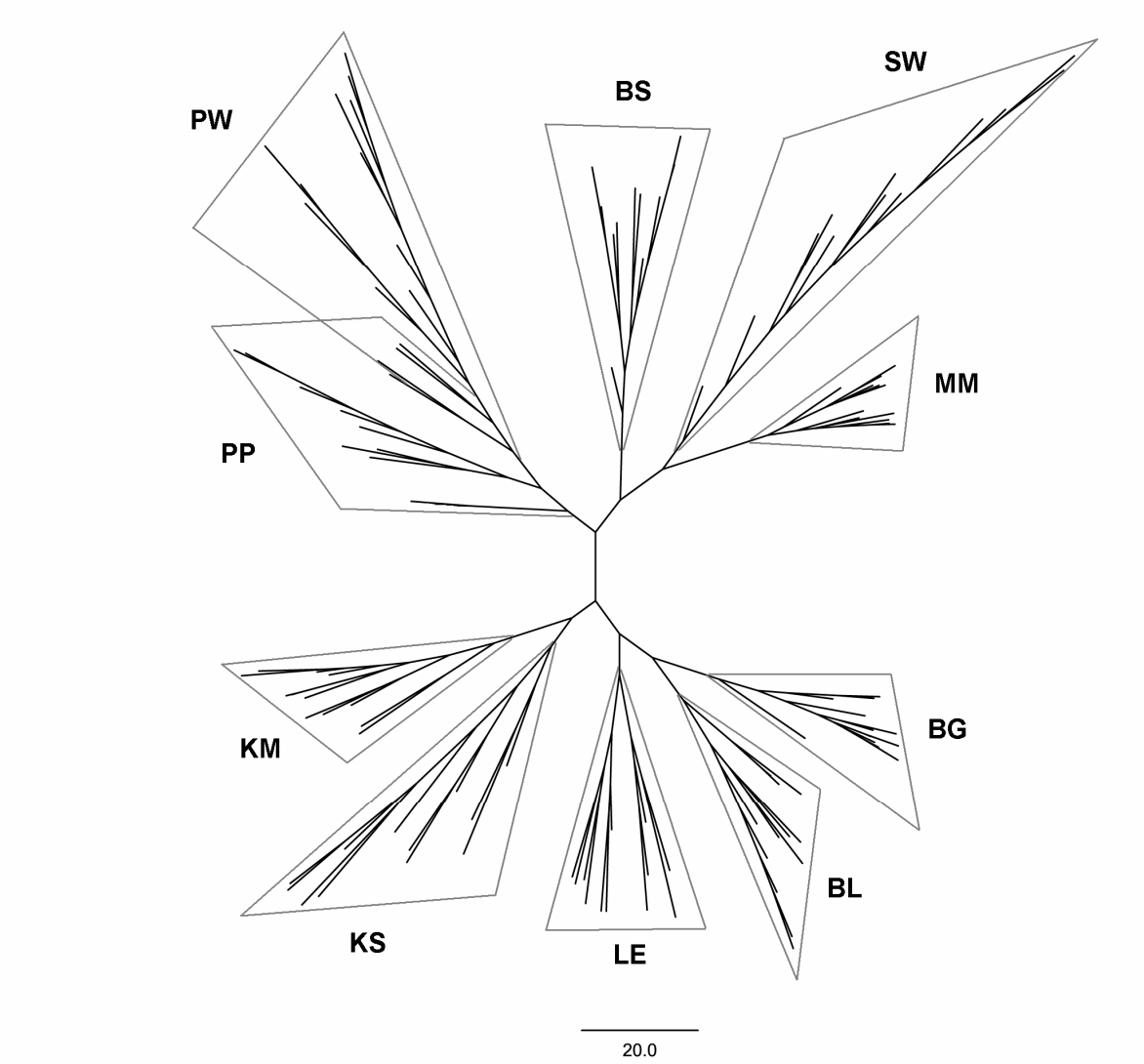

Fig. 5-2: Unrooted neighbour-joining tree (PAUP) of the ten *Silene otites* populations based on *p*-distances. Population abbreviations are as in Table 5-1.

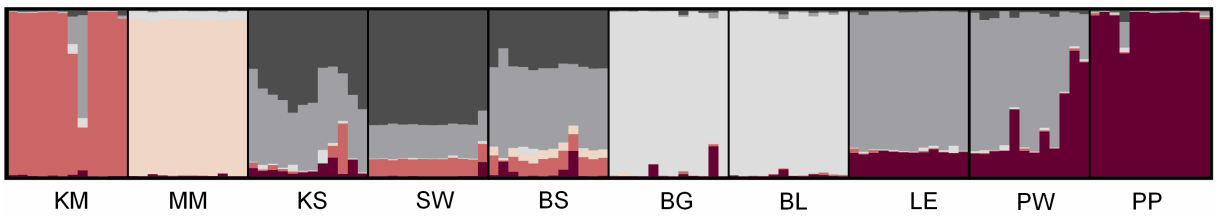

Fig. 5-3: Population structure of ten *Silene otites* populations using the model-based Bayesian algorithm implemented in the program STRUCTURE for K = 6. Each individual is represented by a vertical bar, and fractional membership in each of the clusters is indicated by colour. The ten populations are separated by vertical black bars. Population abbreviations are as in Table 5-1.

Sex ratio, plant performance, and fitness

The overall sex ratio was 0.49. There was a negative relationship between population size and sex ratio (R^2 = - 0.34; $P < 0.05$; Fig. 5-4). The overall mean germination rate was 80 %. Highest germination rates were found in the largest but also in the smallest populations (Table 5-1). In nature we found a mean number of 0.78 juveniles at 0.25 m². In populations with higher genetic diversity, individuals had more capsules, larger rosettes, and more stems (Table 5-3), but the number of juveniles was lower.

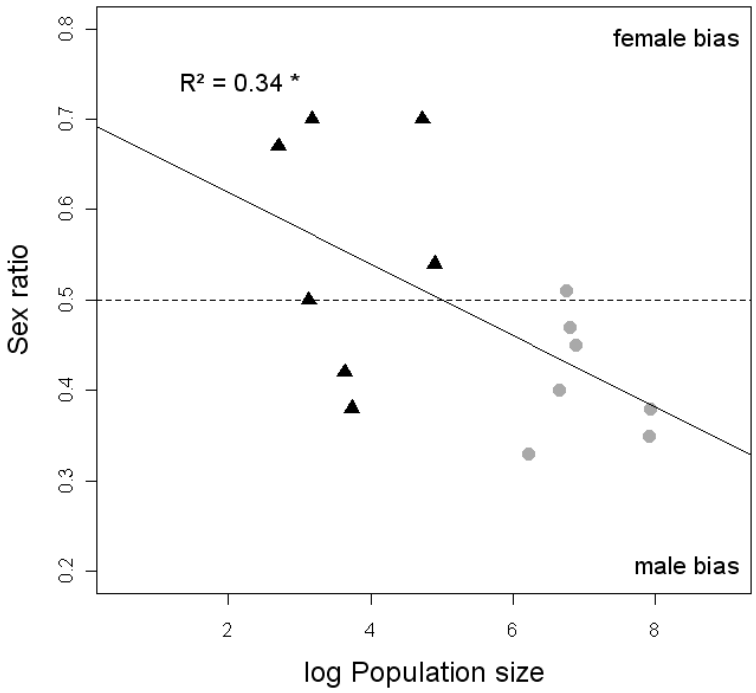

Fig. 5-4: Linear regression between sex ratio and population size of 14 *Silene otites* populations. Black triangles are small populations (< 150 individuals) and grey dots are large populations (> 500 individuals); dashed line indicates 1:1 sex ratio, * $P < 0.05$

The number of seeds per plant and juveniles per plot increased with larger population size. A higher degree of total coverage had a negative effect upon number of seeds per plant, number of capsules, rosette diameter and stem height. Moreover, a higher coverage of cryptogams was negatively associated with the number of juveniles and seeds per capsule. In addition, a higher coverage of herbal layer had a positive effect upon the number of seeds per plants and stem height (Table 5-3). In the GLMM analysis, no relationship between sex ratio and the explanatory variables could be observed.

Table 5-3: Minimal adequate models for plant fitness variables of *Silene otites* calculated by generalised linear mixed effect modelling (GLMM) with population location as random factor, parameter estimates (est) and results of likelihood ratio tests (χ^2) for significant terms after stepwise backward model selection

	Seeds plant		**Seeds capsule**		**Capsules**		**Juveniles**		**Diameter**		**Stems**		**Stem height**	
	est	χ^2	est	χ^2	est	χ^2	est	χ^2	est	χ^2	est	χ^2	est	χ^2
H_E					+	7.55 **	-	5.23*	+	8.20 **	+	9.41 **	+	5.60 *
Log population size	+	12.67 ***					+	15.66 ***					+	6.26 *
Population density	-	10.61**							-	7.56 **			-	6.89 *
Total cover %	-	13.79 ***			-	22.77 ***			-	23.60 ***			-	16.12 ***
Cover litter %			-	6.58*										
Cover cryptogams %			-	34.28 ***			-	4.80 *						
Cover herbal layer %	+	4.98 **											+	6.39 *

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

5.5 Discussion

Population genetic structure

One of the best predictors of population structure is expected to be the mating system (Loveless & Hamrick 1984; Duminil et al. 2009). Theory predicts that high outcrossing rates as found in dioecious plant species reduce inter-population differentiation measured by F_{st} (Giles & Goudet 1997; Hamrick & Godt 1996). Therefore, in obligate out-crossers genetic differentiation should be lower and intra-population diversity higher as compared to self-compatible ones (Linhart & Grant 1996; Glemin et al. 2006; Duminil et al. 2009). However, this was not supported by our results in our highly fragmented populations of *S. otites*. The values of genetic differentiation obtained in this study were similar or slightly higher to those in other *Silene* studies (Moyle 2006). Genetic diversity values within populations of *S. otites* were similar as compared to hermaphroditic rare species like *Silene tatarica* (Tero et al. 2003), *Silene chlorantha* (Lauterbach et al. 2011), *Pulsatilla vernalis* (Ronikier 2002) and *Globularia bisnagarica* (Honnay et al. 2007), but lower as in dioecious common species like the insect-pollinated herbaceous *Silene latifolia* (Richards et al. 2003), the wind-pollinated woody *Taxus baccata* (Hilfiker et al. 2004), or the wind-pollinated herbaceous *Mercurialis perennis* (Vandepitte et al. 2010). The comparatively lower diversities in the predominantly insect-pollinated *S. otites* may be due to genetic drift and restricted gene flow among the investigated populations due to restricted pollination capacities.

Brantjes & Leemans (1976) stated *S. otites* are wind and insect pollinated. Soldaat et al. (2000) observed wind pollination in climate chambers only over very short distances. But the main pollinators are nocturnal Lepidoptera (e.g. *Plusia gamma*) and mosquitoes (e.g. *Culex pipiens*) (Schulz 1905; Brantjes & Leemans 1976; Jhumur et al. 2008). Our own observations in the common garden and nature showed mainly hoverflies, mosquitoes and nocturnal moths visiting the flowers. So probably pollen dispersal is mainly limited by the foraging radius of pollinators. Tsuda et al. (2008) found mean flight distances of *Culex pipiens pallens* in an urban area of Japan of about 400 m up to a maximum of about 1,200 m. In *Plusia gamma* long-distance migration and wind-induced drift are well known (Cardé 2008). Nevertheless, studies on pollinator migration revealed distances of more than 1 km to be already exceptional in fragmented habitats (Kwak et al. 1998; Peterson et al. 2008). The AFLP results with respect to the geographically close populations BL and BG (distance 2 km, partitioned by a river) and PP and PW (distance 0.5 km, partitioned by a forest) depict some extent of admixture. Wind pollination in *S. otites* over such distances is most likely inefficient and negligible, especially as forests and settlements are in between, which is confirmed by

the high genetic differentiation among populations and low level of intra-population genetic diversity. The detected genetic admixture between populations PP and PW seems to be rather a relict of historic gene flow. For *S. otites*, Wessels-de Wit & Schwabe (2010) showed the importance of seed dispersal by sheep. Till the early twentieth century, the populations PP and PW were connected via sheep grazing on more expanded dry grasslands (Pless 1994). Some kind of genetic admixture between both populations most likely reflects former connectivity between both sites. This highlights the importance of integrating population and habitat history when discussing current population structure (Leimu & Mutikainen 2005; Lauterbach et al. 2011). Population structure of *S. otites* was similar to the situation of the hermaphroditic and self-compatible *Silene chlorantha* (Lauterbach et al. 2011) in the same region. Thus, in *S. otites* dioecy per se did not prevent from genetic erosion and differentiation among populations. Here, the influences of ongoing habitat fragmentation seem to be stronger than the expected increased amount of genetic variation and exchange among populations by obligate out-crossing (Loveless & Hamrick 1984). For the wind-pollinated und mating system variable *Mercurialis annua*, Obbard et al. (2006) observed a lower genetic differentiation of dioecious populations than in monoecious populations.

In *S. otites* no correlation between genetic and geographic distance could be observed, although some populations are geographically quite close to each other. Within the genus *Silene*, Moyle (2006) found isolation by distance not to be significant in more than one-third of the examined studies. However, a missing isolation by distance correlation was found more frequently in clearly obligate out-crossing species as they may experience higher population turnovers in contrast to genetically more stable populations of self compatible ones (Moyle 2006).

Also no correlation between population size and genetic diversity was found in other plant species of fragmented habitats, e.g. *Silene tatarica* (Tero et al. 2003), *Silene chlorantha* (Lauterbach et al. 2011), *Vincetoxicum hirundinaria* (Leimu & Mutikainen 2005) and *Stipa capillata* (Hensen et al. 2010). In our investigation area, there has been an intense habitat decline during the last 50 years (e.g. KM: this population was much larger until the 1960s when there was habitat destruction by removing a hill, undocumented comment by W. Lauterbach). In such an evolutionary short context, some remnant populations possibly retained genetic diversity even when population size decreased. Thus, fluctuations in effective population size do not immediately result in a loss of genetic diversity (Ellstrand & Elam 1993).

Besides population size, in dioecious species mainly sex ratio influences genetic structure (Barrett et al. 2010). Due to stochastic effects, there is an increased variation of sex ratio in small populations (Soldaat et al. 2000; Nilsson & Agren 2006). Some studies in *Silene otites*

revealed a bias in sex ratio caused by environmental factors and habitat conditions (Soldaat et al. 1997; 2000). In agreement with an expected sex ratio of 1:1 (Barrett et al. 2010), our results showed an overall even sex ratio, but there was a bias in relationship to population size (Fig. 5-4). Deviation from a balanced sex ratio depending on population size was also found in other plant species such as the gynodioecious *Plantago maritima* (Nilsson & Agren 2006), *Lobelia syphilitica* (Caruso & Case 2007), and the dioecious *Taxus baccata* (Hilfiker et al. 2004). Under unfavourable environmental conditions, female frequencies increase (Case & Ashman 2007). Based on field studies of *S. otites* populations on porphyritic soils, higher vegetation coverage was associated with an increased number of females (Soldaat et al. 1997), which was not supported by our findings. In contrast to our studied populations on sandy soils, the effects may be more visible under stressful conditions of resource limitation on porphyritic outcrops, which are strongly variable in soil layer. In other species there was also a variable mortality by different resource utilisation of the sexes (Freeman et al. 1976). Additionally, a low overall vegetation cover can also be an indicator for drought stress, which induced higher male mortality in *S. otites* (Soldaat et al. 2000).

Population fitness

Especially in perennial species, fitness and inbreeding depression are closely linked to the reproductive system and life cycle stage (Husband & Schemske 1996; Richards et al. 2003). Self-incompatible species are more sensitive to inbreeding depression and its negative effects upon plant fitness than self-compatible ones (Leimu et al. 2006). In self-compatible species deleterious recessive mutations should be effectively purged from highly inbred populations (Charlesworth & Charlesworth 1990). In our investigated populations higher genetic diversity was correlated with higher individual plant fitness in terms of taller plants and more capsules per plant. This is in accordance with the findings of Husband & Schemske (1996) that out-crossing species express inbreeding depression either early or late in the life cycle. Positive correlations between genetic diversity and fitness traits were also found in other studies of perennials, e.g. in the self-incompatible *Cochlearia bavarica* (Paschke et al. 2002), the protogynous predominantly self-incompatible *Pulsatilla vulgaris* (Hensen et al. 2005), the mainly out-breeding but self-compatible *Dictamnus albus* (Hensen & Oberprieler 2005), and the self-compatible but predominantly out-crossing *Anthericum liliago* (Peterson et al. 2008). The negative correlation between population genetic diversity and number of juveniles in the dioecious self-incompatible S. otites is in contrast to the results of the same habitat preferring but hermaphroditic and self-compatible *Silene chlorantha* (Lauterbach et al. 2011).

In *S. otites* we found a positive correlation between seed set and population size, which has also been documented for several other herbaceous plant species (e.g. Fischer & Matthies 1998; Paschke et al. 2002; Hensen et al. 2005). Small and isolated populations often suffer from pollinator limitation, which strongly influences self-incompatible and obligate out-crossing species (Jennersten & Nilsson 1993; Paschke et al. 2002). Thus, our results support population size to be one of the best predictors for population fitness (Schmidt & Jensen 2000; Lauterbach et al. 2011).

Next to population size and genetic structure, we also examined the correlation between habitat quality and plant fitness. For typical dry grassland species higher degrees of total coverage, including herbal and cryptogam layer indicate an increased competition of resources (Leimu 2010). In *S. otites*, total coverage had a negative impact on plant performance caused by competition for light and resources. The germination rate of *S. otites* was high under greenhouse conditions without inter-specific competition, which is also supported by the findings of Soldaat et al. (2000). However, under natural conditions only a low number of juveniles could be detected. Insufficient recruitment in nature can either be due to competition, phytochemical interactions, or high mortality of juveniles due to unfavourable weather conditions. The negative relationship between cryptogams and biological soil crusts to number of juveniles was also detected in other studies, e.g. in *S. otites* (Langhans et al. 2009), *S. chlorantha* (Lauterbach et al. 2011) and *Pulsatilla patens* (Kalliovirta et al. 2006).

Conclusions

In *Silene otites* dioecy did not prevent loss of genetic diversity, random genetic drift and genetic differentiation among fragmented populations. Even on a comparatively regional geographic scale (~150 km), population structure seems to be more strongly affected by habitat fragmentation than by the effects of a dioecious mating system. In terms of ongoing fragmentation and isolation, genetic exchange among populations becomes less likely, since among-population structure depends more on pollinator flight distance and dispersal capacities instead of mating system. Thus, we presume that especially short-lived insect pollinated out-crossing species are more vulnerable to genetic erosion and a decreased fitness than wind pollinated and self-compatible ones. Our results also indicate that interactions among habitat quality, population size and population history seem to be of the same importance for population structure and plant fitness in dioecious plants as in other mating systems too. In accordance with Wilson & Harder (2003), dioecy and self-incompatibility are advantageous to prevent inbreeding depression within populations but

does not enhance inter-population gene flow per se when there is limitation or absence of long-distance pollen dispersal.

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6 Rapid genetic differentiation between *ex situ* and their *in situ* source populations: an example of the endangered *Silene otites* (Caryophyllaceae)

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6.1 Abstract

Ex situ cultivation in botanic gardens could be one possibility to preserve plant species diversity and genetic variation. However, old *ex situ* populations are often sparsely documented. We were able to retrieve three different *ex situ* populations and their source *in situ* populations of the endangered plant species *Silene otites* after 20 - 36 years of isolation. Furthermore, three additional wild populations were included in the analysis. Population genetic diversity and differentiation were analysed using AFLP markers. Genetic variation in the *ex situ* populations was lower than the variation found in the *in situ* populations. Strong differentiation (F_{st} = 0.21 - 0.36) between corresponding *in situ* and *ex situ* populations was observed. Bayesian clustering approach also showed a distinct genetic separation between *in situ* and *ex situ* populations. The high genetic differentiation and loss of genetic diversity during spatial and temporal isolation in the *ex situ* populations can be attributable to small population sizes and unconscious selection during cultivation. Therefore, adequate sampling prior to *ex situ* cultivation and large effective population sizes are important to preserve genetic diversity. Near-natural cultivation allowing for generation overlap and interspecific competition without artificial selection is recommended as being best for the maintenance of the genetic constitution.

Keywords: AFLP, Botanical garden, Conservation genetics, Founder effect, Population size

6.2 Introduction

Safeguarding at least 60 % of threatened plant species in *ex situ* collections is one of the goals of the *Global Strategy for Plant Conservation* (CBD Secretariat 2000). Different approaches of *ex situ* preservation in plants exist, e.g. via seed storage in seed banks, germplasm banks and cultivation of living plants in botanic gardens and arboreta (Hamilton 1994; Maunder et al. 2001a; Havens et al. 2006; Oldfield 2009). For such approaches advantages and disadvantages have been widely discussed elsewhere (e.g. Falk & Holsinger 1991; Volis & Blecher 2010). Especially if *in situ* conservation cannot be guaranteed, as a result of habitat destruction or changes in land use, *ex situ* collections are one possibility for safeguarding species diversity and genetic variation that can be used as source for reintroduction programmes in case of extinction. The aim of *ex situ* activities is to preserve samples that are representative of the extant *in situ* diversity and make the most efficient use of available resources (Falk & Holsinger 1991; IUCN 2002; Goodall-Copestake et al. 2005; Li et al. 2002; Husband & Campbell 2004; Ensconet 2009; Fay 2010). However, whether cultivated *ex situ* collections in botanic gardens still genetically correspond to the once sampled *in situ* source populations and if the genetic constitution was maintained after defined time spans, has rarely been evaluated (e.g. Enßlin et al. 2011; Rucinska & Puchalski 2011).

Cultivation is expensive and in general there is a resource limitation for the cultivation of living collections (Namoff et al. 2010). For this, *ex situ* cultivation must be as efficient as possible. Preservation mostly starts with an artificial selection of seeds from the initial pool of genotypes in the wild, and *ex situ* collections are often initiated without available genetic information (Helenurm & Parsons 1997; Bottin et al. 2007). Furthermore, ecological shifts, small population size, genetic drift, inbreeding and gardener-induced selection may negatively affect population structure after several generations of *ex situ* cultivation (Krauss et al. 2002; Enßlin et al. 2011). Techniques used (e.g. micropropagation) can also cause deleterious side effects (Ye et al. 2011). In the worst case, all these factors result in a loss of genetic diversity, causing genetic bottlenecks and/or founder effects (Zohary 2004; Miller & Schaal 2006; Guerrant et al. 2004). A decreased genetic diversity is expected to reduce the suitability of *ex situ* cultivars for future reintroduction efforts by effects of inbreeding depression and reduced plant fitness (Ellstrand & Elam 1993; Hufford & Mazer 2003; Leimu et al. 2006).

Evaluating the long-term efficiency of *ex situ* conservation is important but complicated (Li et al. 2002), due to the difficulty of finding more than one sample of a documented (origin and

cultivation) *ex situ* population and its corresponding still existing *in situ* source population. Most studies of *ex situ* genetics in plants concentrate on tree or shrub species (Etisham-Ul-Haq et al. 2001; Miller & Schaal 2006; Krauss et al. 2002; Goodall-Copestake et al. 2005; Li et al. 2005), ornamental plants and their wild relatives (Chwedorzewska et al. 2008; Honjo et al. 2008; Maunder et al. 2001b) or medicinal plants and crop species (Anthony et al. 2002; Shi et al. 2008; He et al. 2009). Investigations in endangered herbaceous plant species are scarce (Enßlin et al. 2011; Rucinska & Puchalski 2011).

Here, we analysed several corresponding *in situ* and *ex situ* populations of the endangered dioecious plant species *Silene otites* (L.) WIBEL (Caryophyllaceae) in Germany. To the best of our knowledge, only one comparable study, with only one population comparison of the endangered hermaphroditic *Cochlearia polonica* FROHL. (Brassicaceae), has been published so far (Rucinska & Puchalski 2011). The present study is the first one comparing population genetics between three samples of botanic garden *ex situ* populations and their respective *in situ* source populations. As the botanic gardens followed different cultivation strategies, recommendations for the optimisation of *ex situ* conservation management in botanic gardens are given. *Silene otites* is a dry grassland perennial. It is listed as vulnerable in the Red List of Germany (Korneck et al. 1996). The species distribution range covers Western to Eastern Europe (Tutin et al. 1993). Flowering takes place between June and October. Pollinators are nocturnal Lepidoptera and mosquitoes (Brantjes & Leemans 1976). Seed dispersal is largely passive (Watt 1981), and germination occurs in autumn and spring (Kupferschmid et al. 2000; Soldaat et al. 2000). In the investigation area the number of populations declined in recent decades due to habitat loss and secondary succession of dry grassland caused by the decline of sheep grazing and eutrophication (Lauterbach et al. 2012).

In three German botanic gardens, independent *ex situ* populations of *S. otites* were established in the late 1970s and 1980s. These *ex situ* populations and their corresponding *in situ* source populations still exist today. Hence, three independent pairs of corresponding *ex situ* and *in situ* populations of *S. otites* were available for comparable analyses of population genetic diversity and between population differentiations. During cultivation there were conditions of temporal and spatial isolation, small population size and ecological changes. In addition, three more wild populations were included into the analysis to better represent the species genetic diversity in the investigation area and to evaluate population size effects upon genetic diversity.

We here used AFLP (amplified fragment length polymorphism) markers to investigate population structure. The anonymous AFLP marker technique (Vos et al. 1995) has been routinely applied to assess the genetic diversity at population level (e.g. Mariette et al. 2002;

Nybom 2004; Woodhead et al. 2005; Chwedorzewska et al. 2008), even though the method has disadvantages as little is known about mutation processes and fragment homoplasy (Meudt & Clarke 2007; Anderson et al. 2010). However, for species where no established other marker techniques are available, AFLP provides numerous, highly variable, genome wide dominant loci without *a priori* sequence information.

6.3 Materials and Methods

Sampling

Three pairs of well documented German *ex situ* botanic garden populations and corresponding *in situ* populations were sampled (Table 6-1). At the Berlin Botanic Garden (*ex situ A*) and the Marburg Botanic Garden (*ex situ B*), plants have been cultivated in a single-species bed without interspecific competition and with biennial rejuvenation from the cultivated stock by the gardeners. At Mainz Botanic Garden (*ex situ C*) plants have been cultivated in near natural dry grassland on sand with spontaneous rejuvenation, interspecific competition and overlapping generations of different ages. Therefore, no detailed information about cultivated generations since establishment is available. In all three cases, e*x situ* populations and *in situ* source populations are geographically separated by > 3 km so that genetic exchanges between *in* and *ex situ* populations have been unlikely. Additionally, three differently sized wild German populations without corresponding *ex situ* populations (*in situ W*) from western Brandenburg (W1), northern Brandenburg (W2), and Lower Saxony (W3) were included to represent the genetic diversity in the region. Fresh, healthy leaf material was sampled in 2008 or 2009 and immediately dried in silica gel and stored at 4 °C until DNA extraction. According to evaluations by Campbell et al. (2003) and Singh et al. (2006), because of limited time and financial constraints, a sample size of 12 individuals per population was used to analyse genetic structure. The sample size was rather small; however, for five of the nine populations analysed it covers > 50 % of the population size. Additionally, for each population the number of flowering plants was counted to estimate population size.

Table 6-1: Summary of sampled *Silene otites* populations: code, collection site, coordinates, status, population size (Size), number of individuals analysed for AFLPs (N_{AFLPs}), method of cultivation, year of establishment (Y_{est}), number of cultivated generations (G_{cult}), percentage of polymorphic loci (*P %*), genetic diversity (H_E), number of private bands (pb).

Code	Collection site	Coordinates	Status	Size	N_{AFLPs}	Cultivation	Y_{est}	G_{cult}	*P %*	H_E	pb
BE	Berlin	52°29'N/ 13°10'E	*in situ A*	42	12				38.5	0.142	27
BBG	Berlin Botanic Garden	52°27'N/ 13°18'E	*ex situ A*	20	12	flowerbed	1988	~10	36.7	0.132	6
EF	Erfurt	51°01'N/ 10°57'E	*in situ B*	~1,000	12				40.7	0.165	34
MAG	Marburg Botanic Garden	50°48'N/ 08°46'E	*ex situ B*	26	12	flowerbed	1973	~20	38.1	0.125	2
MZ	Mainz	50°00'N/ 08°12'E	*in situ C*	~150	12				34.1	0.134	27
MBG	Mainz Botanic Garden	49°59'N/ 08°14'E	*ex situ C*	40	12	dry grassland	1982	unknown	41.6	0.127	10
W1	Michelsdorf	52°18'N/ 12°42'E	*in situ W*	800	12				39.8	0.156	11
W2	Frauenhagen	53°05'N/ 14°01'E	*in situ W*	2500	12				44.7	0.183	24
W3	Brünkendorf	53°03'N/ 11°26'E	*in situ W*	13	12				36.3	0.142	12

DNA extraction and AFLP procedure

The silica-dried leaf material (1 cm²) was homogenised with a mill (Retsch MM 301, Haan, Germany). Genomic DNA was isolated from leaf material using the NucleoSpin Plant II kit (Macherey-Nagel GmbH and Co. KG, Düren, Germany) according to the manufacturer's protocol with modification (incubation time for cell lyses was changed to 30 min). The obtained DNA quality was checked on 1.5 % TAE-agarose gel. DNA quality and quantity were measured for each individual using a NanoDrop1000 spectrophotometer (ThermoScientific, Wilmimgton, USA) and diluted to 30 ng/µl. AFLP was carried out using a protocol modified from Vos et al. (1995): 10 µl genomic DNA (30 ng DNA /µl) was double-digested in a final volume of 25 µl (0.25 µl *Eco*RI (10 U/µl; Fermentas, St. Leon Rot, Germany), 0.15 µl *Tru*1I (10 U/µl; Fermentas), 2.5 µl 10x buffer (Fermentas) and 12.1 µl purified H_2O at 37 °C for 3 h. The reaction was terminated by 65 °C for 10 min. Ligation was carried out with 0.5 µl T4-ligase (1 Weiss units/µl; Fermentas), double stranded adapters 0.5 µl *Eco*RI (5 pmol/µl; MWG Biotech, Ebersberg, Germany), 0.5 µl *Tru*1I (50 pmol/µl; MWG Biotech), 1.2 µl ATP (10 mM; Fermentas), 0.5 µl 10x ligation buffer (Fermentas) at 20 °C for 16 h. Two consecutive PCR amplifications with primers that contained first one (+1) then three (+3) selective nucleotides at their 3'ends were carried out. In the first pre-selective PCR 1.5 µl of restriction ligation product was used as template in a total volume of 7 µl containing 0.2 µl primers (50 ng/µl; MWG Biotech), 0.7 µl 10x buffer (Quiagen, Hilden, Germany), 0.14 µl dNTPs (10 mM; PeqLab, Erlangen, Germany), 0.035 µl Taq polymerase (1000 U/µl; Quiagen) and 4.2 µl purified H_2O. The PCR conditions for the pre-selective amplification comprised an initial step of 94 °C for 2 min followed by 30 cycles at 94 °C for 20 s, at 56 °C for 30 s, at 72 °C for 2 min, and a final extension step at 72 °C for 30 min. The pre-selective PCR products were checked on 1.5 % TAE-agarose gels and DNA quantity was adjusted to 20-30 ng/µl for the selective PCR using a NanoDrop1000 spectrophotometer. The selective PCR reaction of 4.2 µl final volume contained 3.14 µl AFLP coremix (Applied Biosystems, Darmstadt, Germany), 0.21 µl fluorescent labelled *Eco*RI primer (1 µM; Sigma Aldrich, Taufkirchen, Germany), 0.21 µl *Tru* primer (5 µM; MWG Biotech) and 0.65 µl adjusted pre-PCR product. The PCR conditions for the selective amplification reaction started with an initial step at 94 °C for 2 min followed by 10 cycles at 94 °C for 20 s, 1 at 66 °C for 30 s, at 72 °C for 2 min, 25 cycles at 94 °C for 20 s, at 56 °C for 30 s, at 72 °C for 2 min, ending with a final extension step at 72 °C for 30 min. A preliminary primer screening of 16 individuals from four populations with 18 primer combinations was conducted to select three primer combinations giving clear, reproducible bands and appeared to be sufficiently polymorphic to show variation within and among populations. For the final analysis following three primer

combinations were chosen: *Eco*RI ACG - *Tru* CTG, *Eco*RI ACA - *Tru* CTG, *Eco*RI AGA - *Tru* CTC. Fragments were separated on a polyacrylamide gel with an internal size standard (GenomeLab DNA Size Standard Kit 400, Beckman Coulter, Krefeld, Germany) on an automated sequencer (CEQ 8000, Beckman Coulter).

Bands were identified and scored semi-automatically for presence and absence using Genographer software (version 1.6.0, J. Benham, Montana State University, Bozeman, USA). Only AFLP fragments with a sufficient intensity, being unambiguously scorable, were included in the analysis. Each AFLP fragment was scored using the "thumbnail" option of the program, which allows for the comparison of signals per locus over all samples. If possible, peaks of low intensity were additionally scored by eye and included into the analysis. Standard lanes, carrying identical samples, were run on each plate to compare data sets from two or more runs. For 22 samples, the AFLP analysis for each primer combination was repeated and the phenotypic error rate was calculated as number of phenotypic differences related to the total number of phenotypic comparisons, and subsequently averaged over the three combinations (Pompanon et al. 2005). A presence/absence matrix was exported for further calculations.

Statistical analysis

The AFLP data set was first analysed for all data to evaluate genetic diversity. Then, the analysis was split into three data sets for pairwise *in* and *ex situ* comparison. For each of the three data sets A, B, C (*ex situ* population and *in situ* population) a single analysis was performed to guarantee data independence. The presence/absence matrix from AFLP fragments was used to calculate allele frequencies by using a Bayesian method with no uniform prior distribution, assuming Hardy-Weinberg genotypic proportions (Zhivotovsky 1999). From these allele frequencies, the percentage of polymorphic loci and genetic diversity (H_E) were calculated based on the method of Lynch & Milligan (1994), using the software AFLPsurv, version 1.0 (Vekemans 2002). Genetic diversity, which is equivalent to expected heterozygosity (H_E) under Hardy-Weinberg conditions (Nei 1987), was used as a measure of within-population genetic diversity. The number of private bands per population was calculated with FAMD version 1.1 (Schlüter & Harris 2006). Relationships between genetic diversity and population size, pairwise F_{st} values and age of *ex situ* population were tested by Spearman rank correlation. Values of population size were log transformed. Differences of genetic diversity, percentage of polymorphic loci and number of private bands

between *in situ* and *ex situ* populations were tested using a Mann-Whitney test. All calculations were performed with R 2.7.1 (R Development Core Team 2008).

Analyses of molecular variance (AMOVAs) and calculation of pairwise F_{st} values were performed using the software Arlequin, version 3.1 (Excoffier et al. 2005) and significance tests were run using 10,000 permutations. The AMOVA was calculated for the six *in situ* populations with two groups (group 1: W1, W2, W3, BE; group 2: MZ, EF). F_{st} values were calculated after Weir & Cockerham (1984). A neighbor-joining (NJ) analysis based on *p*-distances was generated using the program PAUP* version 4.0b10 (Swofford 2003) to analyse genetic structuring of populations. The tree was drawn and edited using FigTree version 1.2.1 (Rambaut 2008). In a more detailed analysis, the three independent *in* and *ex situ* comparisons were analysed to examine the assignment of individuals into genetic clusters by using the program STRUCTURE version 2.2 (Pritchard et al. 2000). Calculations were conducted separately for each population pair. STRUCTURE employs a model-based clustering analysis that groups individuals into genetic clusters (*K*) without a pre-defined delimitation of genetic populations in the sampling area. We estimated the number of genetic clusters *K* by 20 independent runs for each *K* value (*K* = 1 to *K* = 3). The analysis was based on the admixture model with correlated allele frequencies to consider the common ancestry of the analysed population pairs and use the most sensible and progressive model. The model was run with a burn-in period of 50,000 and 100,000 Markov chain Monte Carlo (MCMC) replications. The optimal value of *K* was explored using the method of Evanno et al. (2005). Recently it has been suggested that a good estimator to detect the true number of genetic groups is the modal value of *ΔK*, an *ad hoc* quantity related to the second order rate of change of the log probability of data with respect to *K* (Evanno et al. 2005). The *ΔK* values comparing the results assignments were calculated using R-script STRUCTUE-SUM (Ehrich 2006) in R 2.7.1 (R Development Core Team 2008). We utilised the program CLUMPP version 1.1.1 (Jakobsson & Rosenberg 2007) to determine the optimal alignment of clusters across individual runs for each *K*. Results from CLUMPP were imported into DISTRUCT version 1.1 (Rosenberg 2004) for viewing.

6.4 Results

AFLP patterns and genetic diversity

AFLP analyses with three selective primer combinations revealed polymorphic bands with length variation between 55 and 444 base pairs. For the total dataset 226 scorable polymorphic bands were identified. For the three independent *in situ* vs. *ex situ* datasets (A, B, C) we found 110 polymorphic bands in the *A* data set, 110 polymorphic bands in the *B* data set, and 128 polymorphic bands in the *C* data set. The mean phenotypic error rate (Pompanon et al. 2005) among replicated samples (22 samples x̣ 89 loci) amounted to 4.3 %, which is similar to previously published reports below 5 % in plants (Bonin et al. 2004).

The genetic diversity (H_E) of the *in situ W* populations ranged from 0.142 (W3) to 0.183 (W2) (Table 6-1). Genetic diversity of *ex situ* populations ranged from 0.125 (B) to 0.132 (A) (Table 6-1). Overall, there was a positive relationship between genetic diversity and population size (r_s = 0.75, *P* = 0.026, n = 9), (Fig. 6-1). Furthermore, we detected a negative but not significant trend between the age of *ex situ* populations and their population genetic diversity (r_s = -1, *P* = 0.33, n = 3), (Fig. 6-2).

The three comparisons between *ex situ* and *in situ* populations revealed the percentage of polymorphic loci to be higher in the *in situ* than in the *ex situ* populations with the exception of data set *C* (Table 6-1). The genetic diversity was higher in all *in situ* populations than in the related *ex situ* populations (Table 6-1). The number of private bands (pb) was much higher in the *in situ* populations than in the *ex situ* populations (Table 6-1). Considering all populations (n = 9), the Mann-Whitney test revealed a significant difference between *in situ* and *ex situ* populations for genetic diversity (*P* = 0.028) and number of private bands (*P* = 0.028) but not for percentage of polymorphic loci (*P* = 1).

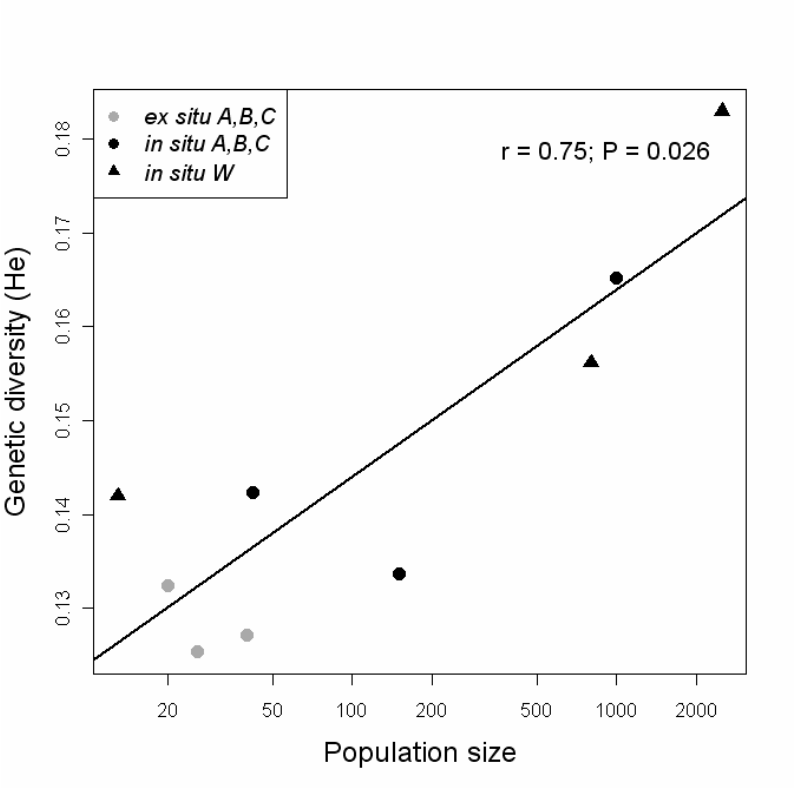

Fig. 6-1: Relationship between genetic diversity (H_E) and population 1 size of nine populations in *Silene otites*: *in situ W* (black labelled triangles), *in situ A, B, C* (black labelled dots), and *ex situ A, B, C* (grey labelled dots)

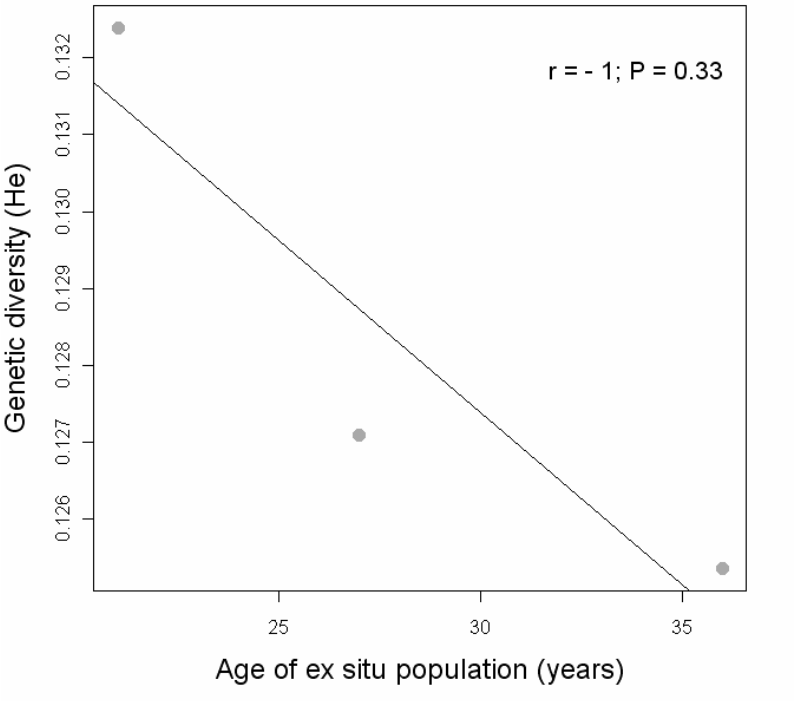

Fig. 6-2: Relationship between genetic diversity (H_E) and age of three *ex situ* populations in *Silene otites*

Population structure and genetic differentiation

All six *in situ* populations were genetically strongly differentiated (F_{ct} = - 0.033, *P* = 0.80; F_{sc} = 0.36, *P* < 0.001; F_{st} = 0.34, *P* < 0.001). The pairwise F_{st} values for the three independent data sets ranged from 0.212, *P* < 0.001 (C) to 0.363, *P* < 0.001 (B) and confirmed a clear differentiation between corresponding *in situ* and *ex situ* populations (Table 6-2). There was no correlation between pairwise F_{st} and age of *ex situ* populations (r_s = 0.5, *P* = 1, n = 3).

Table 6-2: Matrix of pairwise F_{st} values between three *in situ* and corresponding *ex situ* populations of *Silene otites*.

	***in situ* A**	***in situ* B**	***in situ* C**
***ex situ* A**	0.279, *P* < 0.001	-	-
***ex situ* B**	-	0.363, *P* < 0.001	
***ex situ* C**	-	-	0.212, *P* < 0.001

The neighbor joining analysis revealed clear population structuring and also reflected clear-cut partitioning between corresponding *in situ* and *ex situ* individuals (Fig. 6-3). In all three data sets, the STRUCTURE analyses resulted in a most probable number of *K* = 2, where the distinct modal maximum of *ΔK* was achieved. Results allowed for clear distinguishable genetic clusters between *in situ* and *ex situ* individuals in each of the three data sets (Fig. 6-4A-C). In data set C, four *in situ* individuals showed high proportions of affiliation to the *ex situ* cluster (Fig. 6-4C).

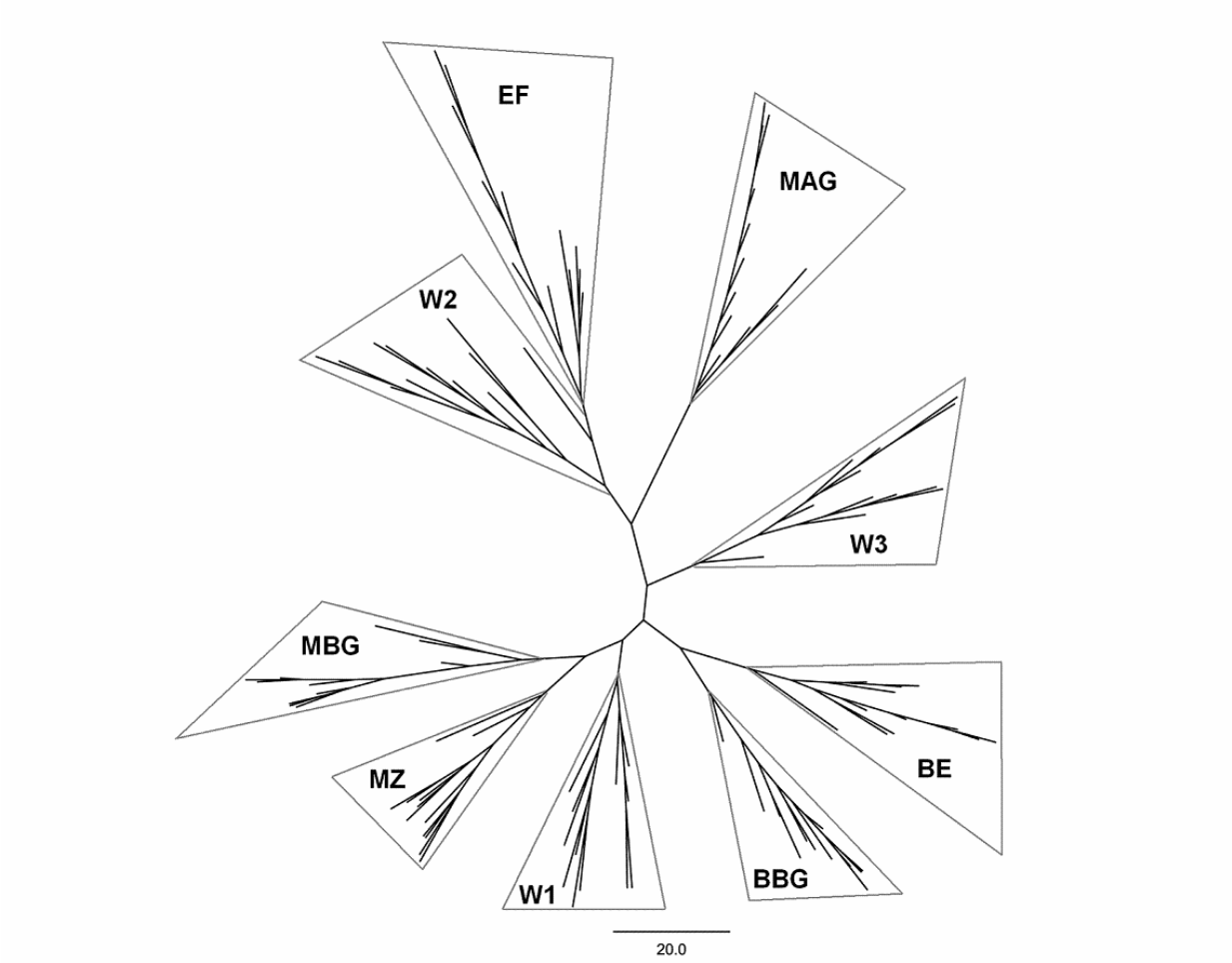

Fig. 6-3: Unrooted neighbour-joining tree based on *p*-distances obtained with the program PAUP* for nine populations of *Silene otites*. Population codes correspond to those in Table 6-1. Scale bar below indicates genetic distance.

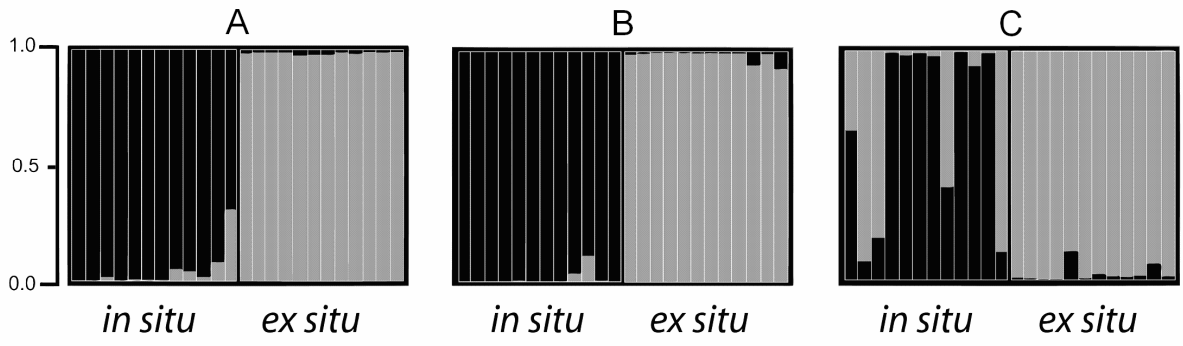

Fig. 6-4: Population structure based on AFLPdata using model based clustering method implemented in STRUCTURE. Diagram showing the proportion of membership of each of the studied individuals for all three data set comparisons (*in situ* and *ex situ* populations A, B, C) of *Silene otites* to inferred Bayesian groups (*K* = 2). Vertical bars represent individuals.

6.5 Discussion

Genetic diversity

Due to limited cultivation space in botanic gardens, *ex situ* collections can be recognized as small and isolated populations. After several generations of isolation, small populations often feature reduced genetic variability due to inbreeding caused by reduced interpopulation gene flow, random genetic drift and founder effects (Young et al. 1996; Leimu et al. 2006). A reduction in genetic diversity can result in a reduced plant fitness through increased homozygosity and inbreeding depression (Ellstrand & Elam 1993; Reed & Frankham 2003). However, contrasting relationships between genetic diversity, population size and fitness have also been reported (Reed & Frankham 2003; Vitt & Havens 2004; Lauterbach et al. 2011; 2012).

Similar to other studies of rare, perennial plant species (e.g. Vergeer et al. 2003; Hensen & Oberprieler 2005; Leimu et al. 2006) we detected a positive relationship between population size and genetic diversity in our analysis (Fig. 6-1). Genetic diversity of the smallest *in situ* population (W3, 13 individuals) was still higher than in any *ex situ* population (≥ 20 individuals), which can probably be explained by a recent decline in population size of former larger and genetically diverse wild populations. Not only the genetic diversity but also the number of private bands was lower in the *ex situ* populations confirming the loss of genetic variability during cultivation. A lower number of private bands indicates a genetic bottleneck or a founder effect typical for populations of more recent origin (Stehlik et al. 2001; Tribsch et al. 2002). Over all, the statistical support for lower genetic variability in *ex situ* populations was weak, most likely attributable to the problem of small sample size. However, this is hard to overcome, as additional conspecific long-term *ex situ* populations with corresponding *in situ* source populations were not available.

Only a few other studies investigated genetic variation in botanic garden populations; however, they also revealed reduced *ex situ* genetic variability (Krauss et al. 2002; He et al. 2009; Asmussen-Lange et al. 2011; Rucinska & Puchalski 2011). In the endangered *Primula sieboldii* E.MORREN, Honjo et al (2008) found higher plastid haplotype diversity in cultivated garden stocks as compared to the overall genetic diversity the wild. Accordingly, they assumed an *ex situ* preservation of genetic diversity that has been lost in the wild. However, since hybridization with related species of the same genus in botanic gardens is frequent (Maunder et al. 2001a; Maunder et al. 2004), especially in the investigated *Primula* section *Cortusoides* (Köhlein 1984), spontaneous interspecific *ex situ* hybridisation could also

explain such a pattern. For *Cynoglossum officinale* L., Enßlin et al. (2011) found a decreased genetic diversity with increased duration of garden cultivation. This finding is in concordance to our analyses in *S. otites*. The oldest *ex situ* population (MAG) also featured the lowest genetic diversity, even although *in situ* genetic diversity and population size of the source population (EF) was rather large (Table 6-1).

In *S. otites*, the Mainz data set (C) revealed the best potential to preserve *in situ* genetic diversity under *ex situ* conditions, as here the loss of genetic diversity was comparatively low. This might be a result of the near-natural *ex situ* reproduction instead of periodical offspring cultivation as in the other gardens. In the Mainz *ex situ* collection, overlapping generations of different life cycle stages and lack of artificial selection of seeds and juveniles as in bed cultivation seem to support the preservation of genetic diversity. The *ex situ* population size, in Mainz (40) was higher than in Marburg (26) and Berlin (20), which might also have positive influences upon the preservation of genetic diversity. The Marburg and Berlin *ex situ* populations might have been affected by unconscious selection through manual seed harvesting and selection of juveniles for biennial rejuvenation. Several decades ago when the garden populations were established less attention was paid to guidelines for material collection. Instead, old *ex situ* populations often originate from few collected individuals rather than from representative sampling of the whole gene pool (collecting guidelines see: Ensconet 2009), resulting in bottleneck events and founder effects right from the beginning of *ex situ* cultivation (Parzies et al. 2000; Rice & Emery 2003; Miller & Schaal 2006). As founder effects are characterized by the loss of genetic variability when a new population of only a few individuals is being established from a larger population (Provine 2004), the lower genetic *ex situ* diversity can possibly be traced back to the time of *ex situ* population establishment. Unfortunately, as is typical for older *ex situ* collections, no information is available concerning the genetic diversity and method of sample selection at time of collection for *ex situ* establishment.

Genetic differentiation

Small effective population sizes, lack of genetic exchange over long time periods and genetic drift can result in a distinct genetic differentiation between populations (Ellstrand & Elam 1993). In *S. otites* we observed a high degree of genetic differentiation between populations of same ancestry (Table 6-2), indicating selective evolutionary forces during *ex situ* cultivation. A similar value of genetic differentiation between an 18-year-old *ex situ* population and its source population of *Cochlearia polonica* was found by Rucinska &

Puchalski (2011). In our study, the highest differentiation was found between the oldest *ex situ* population (36-year-old) and its source population over about 20 documented generations. Differentiation was somewhat lower between the 27-year-old *ex situ* population and its respective *in situ* population; however, here the number of generations is unknown. The findings highlight the impact of cultivated generations in addition to isolation time upon genetic differentiation. Linhart & Grant (1996) proposed that rapid selective changes in plant populations can even occur within < 10 generations. In *S. otites*, this was supported by the rapid observed genetic differentiation between populations of the same ancestry.

The values of genetic differentiation between *in situ* and *ex situ* populations of *S. otites* were similar to those between the investigated six *in situ* populations and similar to the values in investigations of population structure in the rare *Silene rothmaleri* P. SILVA (Cotrim et al. 2003), the endangered *Silene tatarica* PERS. (Tero et al. 2003), and the endangered *Silene chlorantha* (WILLD.) EHRH. (Lauterbach et al. 2011). Moyle (2006) postulated a more rapid population turnover for dioecious *Silene* spp., in contrast to genetically more stable populations of hermaphroditic or self-compatible species. For outbreeding species such as the dioecious *S. otites*, most likely only large effective population sizes can prevent genetic decline caused by inbreeding effects (Soulé 1980; Ellstrand & Elam 1993; Krauss et al. 2002). Unfortunately, no information about former sex ratios in the *ex situ* populations was available. However, imbalanced sex ratios or imbalanced pollinations accompanied by small numbers of individuals can accelerate genetic differentiation by isolation. *Silene otites* is a long-lived perennial species (life-span up to five years and more, personal observation DL). Under natural conditions with nutrient poor soils and drought stress, plants outlive those that are cultivated under garden conditions in humus rich soils with fertilizer (*S. otites* is sensitive to stagnant moisture). Under *ex situ* conditions, some individuals died after pronounced, strenuous flowering at the end of the second year (personal observation DL). The biennial *ex situ* rejuvenation accompanied by no or reduced generation overlap, unconscious selection and a small initial gene pool could have accelerated genetic differentiation especially in the flower bed cultures of Marburg and Berlin. By contrast, the lowest F_{st} value was found in the Mainz data set. Here the number of cultivated generations is unknown, as juvenile and adult plants grow together and may hybridize across generations instead of a reproductive scheme involving strictly separate cohorts without generation overlap.

Implications for conservation

The main goal of *ex situ* conservation is the preservation of endangered populations from extinction and safeguarding representative material for reintroduction and reinforcement programmes. The *ex situ* populations of *S. otites* investigated here failed the conservation requirements in a strict sense as they diverged genetically from their source populations and featured only a reduced genetic diversity after several generations of cultivation. Even though the genetic diversities of the populations could not be preserved; the species itself was successfully propagated *ex situ* over the last few decades. For highly endangered plant species such *ex situ* preservation is better than none, as offspring might still be used for *in situ* reinforcement of endangered populations with low recruitment (but see, e.g. Ye et al. 2011). However; nowadays, stronger conservation requirements need to be met. For this, our analysis provides information about the potential of *ex situ* optimization in botanic gardens.

Gale & Lawrence (1984) proposed the cultivation of 20 individuals for crop species to preserve genetic diversity. However, crop plants are selected for genetic and phenotypic uniformity so the initial genetic variability is presumably lower than in wild plants (e.g. Pickersgill 2009). To preserve wild plants, the effective population size in botanic gardens must be raised. To prevent genetic decline in out-crossing wild species, Krauss et al. (2002) deduced a theoretical minimum effective population size of 50 individuals. For reintroduction of wild plants a minimum viable population size of 500 individuals or more was suggested (Godefroid et al. 2011), but from an economic point of view, botanic gardens can only provide these high population sizes for selected taxa over short time spans. Additional recommendations for *ex situ* handling are repeated seed collection for rejuvenation throughout the period of seed ripening, reduced selection of early germinants (Enßlin et al. 2011) and reduced selection of the tallest juveniles during cultivation. We also recommend *ex situ* cultivation in a near-natural environment allowing for generation overlap, competition and seed dormancy (see also Volis & Blecher 2010). In addition, special emphasis should be given to the maintenance of balanced sex ratios in dioecious species (Givnish 2010). Cultivation inevitably implies some kind of selection pressure different from natural conditions (e.g. soil type, water and nutrient supply, competition, pollination) (Zohary 2004). Therefore, the time of isolation should be kept to a minimum, if relocation or reintroduction programmes are planned. For future establishment of *ex situ* collections in botanic gardens, a careful selection of founder individuals is recommended to maintain the original genetic diversity of wild populations (Rice & Emery 2003; Cieslak et al. 2007; Wang et al. 2008; Ensconet 2009; Namoff et al. 2010). Also successive transfer of *in situ* material into *ex situ* populations could

help to improve genetic diversity in botanic gardens. However, as the effects of genetic drift and loss of genetic diversity depend on breeding system and species traits such as lifespan (Duminil et al. 2009), no general recommendations for *ex situ* cultivation of other plant species can be given so far. In accordance with the view of Schwartz et al. (2007), our results highlight the importance of further genetic monitoring in *ex situ* collections, especially if corresponding source populations are still accessible.

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7 General Discussion and Outlook

In Europe, dry grassland plant communities are one of the most vulnerable plant communities and they feature the largest numbers of endangered plant species (Dostalek & Frantik 2008). Many dry grassland species are restricted to this habitat and local extinction is more likely for species with high habitat specificity (Fischer & Stöcklin 1997). Ongoing land use changes as intensification of agriculture or abandonment of formerly used dry grassland in terms of the collapse of traditional sheep herding (Lindborg 2007; Dostalek & Frantik 2008) lead to an accelerated decrease in plant population size and increase of genetic drift followed by a loss of within-population genetic diversity and reduced fitness (Leimu et al. 2006).

The distinct population structure, typical for fragmented populations in remnant habitats, found in both investigated *Silene* species can either be explained by reproductive isolation of populations due to land use changes in the last centuries or as consequence of historical events such as recolonization after the last glaciation. However, in *S. chlorantha* so far no correlation between the ice marginal position at the last glacial maximum (LGM) in Brandenburg (Böse 2005) and the genetic divergence of populations could be detected (Chapter 2). Due to the facts that the investigation area is rather small and that during the last two centuries the extinction of many populations within this area was recorded (Chapter 1), too few populations are left for drawing detailed conclusions. Hence, strong genetic differentiation as result of post glacial recolonization needs to be investigated either on a more widely distributed plant species with similar ecological and plant functional traits or on a broader geographic scale with an enlarged data set comprising populations from the edge of distribution in north-eastern Germany to the main distribution area more eastwards (see also for *Stipa capillata* in Hensen et al. 2010). In *S. otites*, the population structure investigated on an east-west gradient was not representative for the ice marginal position at the LGM (Chapter 5).

By visits to all known historical localities of *S. chlorantha* in Brandenburg (Chapter 1), dry grassland habitats were often overgrown by perennial tall grasses and/or woody species, forested by pine or used as sand pits during the last 50 years. Hence, abrupt land use changes resulting in habitat loss probably led to the extinction of some and the reproductive isolation of the remaining populations. In the past, local nomadic sheepherds and transhumance were more common (Poschlod et al. 1998) and dispersal events, connecting populations, were more likely than today. Furthermore, in the investigation area, seed dispersal via sheep and goat herding, typical on dry grasslands at moraine outcrops and at the slopes of the Oder valley till the 20th century (Ristow et al. 2011), promoted gene flow

among populations. The here found strong genetic differentiation and absence of isolation by distance among populations in *S. chlorantha* characterize restricted gene flow among the investigated populations (Chapter 2). The same effect is true for the investigated *S. otites* populations (Chapter 5). A high level of genetic differentiation was also found in other endangered and rare dry grassland species in Central Europe (e.g. *Pulsatilla vernalis*: Ronikier 2002; *Anthericum liliago*: Peterson et al. 2008; *Stipa capillata*: Hensen et al. 2010). Hence, at present it is more feasible to interpret the detected population structures by habitat loss and fragmentation leading to reproductive isolation, with the absence of seed dispersal vectors and extinction of connecting populations, than by post glacial recolonization.

The “habitat quality” paradigm and the “conservation genetics” paradigm have been used sometimes controversial to describe species threats (Ouborg et al. 2006). The “habitat quality” paradigm means that loss of populations is a result of habitat quality changes. Here habitat management strategies are the solution for population problems. According to the “conservation genetics” paradigm, loss of genetic diversity may cause loss of fitness (inbreeding depression) leading to an increased probability of population extinction. However, genetic effects resulting in inbreeding depression would be a meaningless concept without considering environmental/ecological conditions (e.g. habitat quality, dispersal rate) and species interactions (Ouborg et al. 2006). The simultaneous study of both effects in the here presented thesis demonstrate the two paradigms are not mutually exclusive. Rather, the reconciliation of both concepts may be the key to understand the complexity of population fitness. In this thesis, fitness variables and habitat quality of populations were scored in the field and in an experimental common garden study, while genetic differentiation and diversity of the same populations were analysed in the laboratory. In both *Silene* species, the relationship between genetic diversity and fitness was inexplicitly depending on investigated fitness variables and life cycle stages. This was also found in other studies (reviewed by Leimu et al. 2006). In the common garden approach of *S. chlorantha*, none of the explaining variables correlated with plant performance could be found. Whereas in the field, a positive effect of genetic diversity upon number of juveniles was found in *S. chlorantha* (Chapter 2). In the field study of *S. otites*, genetic diversity was positively correlated to plant size (Chapter 5).

According to Husband & Schemske (1996), fitness depends on life cycle stage and mating system. In self-compatible species as *S. chlorantha*, genetic diversity positively affects earlier life cycle stages (Chapter 2), whereas in obligate out-crossers as *S. otites* positive effects of genetic diversity are typical for later life cycle stages (Chapter 5) (Husband & Schemske 1996). Overall, population size is expected to be one of the best predictors for population

fitness (Schmidt & Jensen 2000; Hensen et al. 2005; Leimu et al. 2006). This was confirmed by the results found in both investigated *Silene* species.

In summary, the results of the fitness investigations in *S. chlorantha* and *S. otites* presented here highlight the importance of habitat quality as well as genetic diversity for threatened plant species. This was also shown in other studies of endangered plant species (e.g. *Pulsatilla patens*: Röder & Kiehl 2006). Genetic diversity per se did not prevent population extinction (Chapter 2). Rather, habitat conditions, namely low competition of dominating higher plant species and cryptogams are the key for a better seedling recruitment and plant fitness (Chapter 2 & 5; Kalliovirta et al. 2006; Langhans et al. 2009).

Besides environmental conditions as habitat fragmentation, species specific traits as mating system, life cycle and pollination modes affect genetic population structure (Hamrick & Godt 1996). In both investigated *Silene* species, life cycle (perennial) and pollination mode (insect pollinated) are similar but there is a difference in mating system (hermaphroditic vs. dioecious). In contrast to theory of higher gene flow and reduced inter-population differentiation in obligate out-crossers (Giles & Goudet 1997; Hamrick & Godt 1996), population structure of the dioecious *S. otites* was similar to high genetic differentiation in the hermaphroditic and self-compatible *Silene chlorantha* (Chapter 2 & 5). In self-compatible species, small seed size is expected to be more important for gene flow than pollen dispersal (Ouborg et al. 1999). However, seed dispersal is also restricted under current habitat conditions in both species. In summary, on a comparatively regional geographic scale (~150 km), population structure seems to be more strongly affected by habitat fragmentation than by the mating system.

The distinct effects of reproductive isolation upon population structure are also reflected by the fast population genetic differentiation and loss of genetic diversity observed between the spatial and temporal isolated *ex situ* cultivars and corresponding *in situ* populations of *S. otites* (Chapter 6). Concerning the *in situ* versus *ex situ* evaluation in this study, both *Silene* species produce different pictures (Chapter 3 & 6). However, for interpreting the results in *S. chlorantha* the population history (Chapter 2 & 3) must be taken into account. Due to the first 2 years of population management in the 1990s that treated the genetic bottleneck of the *in situ* population with the replanting of *ex situ* individuals from a constricted gene pool, no distinct genetic differentiation was found between *in situ* and *ex situ* populations of *S. chlorantha*. Therefore, the results of *S. otites* are more adequate to interpret the effects of *ex situ* cultivation. The investigated *ex situ* populations of *S. otites* failed the general goal to preserve samples representing the extant *in situ* diversity (Falk & Holsinger 1991; Husband & Campbell 2004). The low genetic diversity found in the investigated *ex situ* cultivars is congruent with the expected positive correlation between genetic diversity and population

size (Elstrand & Elam 1992; Fischer & Matthies 1998; Leimu et al. 2006). Population sizes in *ex situ* cultivars were below the theoretical minimum of effective population size of 50 -500 individuals (Krauss et al. 2002; Godefroid et al. 2011), probably leading to a decline in genetic diversity. In summary, for the establishment of *ex situ* cultures, representative field sampling of parental material and large *ex situ* population sizes are important to preserve genetic diversity. Furthermore, cultivation time for *ex situ* cultures should be minimized and human induced artificial selection during cultivation should be reduced to a minimum by near-natural cultivation allowing for generation overlap, competition and seed dormancy (Chapter 6).

In case of strong inbreeding depression and small population size, conservation management as habitat networking or artificial transplantations of plants can result in an introgression of new alleles with unknown effects upon genetic diversity and plant fitness. The crossing experiments in *S. chlorantha* (Chapter 4) resulted in positive effects of inter-population crossings whereas no outbreeding effects were detected. Selfing resulted in inbreeding depression. However, at present only results of the F_1 generation are available. As alternative to negative effects of inbreeding in plant fitness, deleterious alleles can also be purged quickly by selection (Lande & Schemske 1985). Hence, in comparison to populations that declined rapidly in size the magnitude of inbreeding depression could be lower in populations that gradually declined in size or featured a genetic bottle neck e.g. as in the well documented population of *S. chlorantha* (Chaper 2; Leimu & Mutikainen 2005).

In general, inter-population crossings in nature should be handled with care (Vander Mijnsbrugge et al. 2010). In case of strong inbreeding depression, detected by population genetic studies and studies of population fitness, crossings or transplantations among populations of the same geographic region “Naturraum” may be a useful tool for species conservation. Alternatively, among population crossings can be recommend e.g. for the establishment of new populations especially in compensation measures or at revegetation areas with no indigenous populations in close vicinity to avoid introgression of new alleles in indigenous populations. For successful local adaptation in new habitats, high levels of genetic diversity should be aimed for to provide the evolutionary potential for adaptation (see also Hampe & Petit 2005).

For further studies concerning *ex situ* conservation, additional perennial species with similar or different mating systems need to be investigated to allow for general recommendations about cultivation strategies and changes of genetic composition during cultivation. Furthermore, long time experiments with different cultivation approaches and more replicates are needed to propose recommendation for *ex situ* conservation in botanic gardens.

An extended common garden experiment could be useful to test for the effect of different habitat management approaches and for the development of better management recommendations. Also landscape modelling of gene flow (Holderegger & Wagner 2008; Dyer et al. 2010) could allow for the correlation between population structure and contemporary ecological influences. Detailed knowledge about spatial and temporal isolation upon genetic changes in populations can also be useful for plant population modelling (Jeltsch et al. 2008).

Furthermore, also other genomic tools as studies in non-neutral genetic variation, the screening of sequence variation in functional genes and gene expression would be interesting to study changes in selectively important variation in model species (Van Tienderen et al. 2002; Ouborg et al. 2006). The fast genetic differentiation in *S. otites ex situ* populations also raises the question how to interpret temporal scale and population structure revealed by anonymous fingerprint techniques as AFLP (amplified fragment length polymorphism)? AFLP is an adequate way to investigate population structure (Nybom 2004; Meudt & Clarke 2007) but are we able e.g. to reconstruct post glacial recolonization e.g. by an AFLP clock approach (Kropf et al. 2009)? Ehrich et al. (2009) mentioned dating of shallow-time evolutionary history based on AFLP data is too good to be true. Further investigations in biogeography may comprise a larger set of populations, especially from the eastern parts of the main distribution area. Additionally, other molecular approaches (nuclear and chloroplast DNA sequence data) could better elucidate the post-glacial recolonization routes and biogeographic history of the species under investigation.

7.1 References General Introduction and General Discussion

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8 Summary

Within the presented thesis „Population genetic structure and plant fitness of natural and *ex situ* populations in *Silene chlorantha* (WILLD.) EHRH. and *Silene otites* (L.) WIBEL“ the effects of temporal and spatial isolation on population genetic structure and plant fitness were analysed. The hermaphroditic *Silene chlorantha* (Caryophyllaceae) and the dioecious *S. otites* in north-eastern Germany served as model species. Effects of inbreeding and outbreeding on genetic diversity and plant performance were investigated in a pollination experiment. Additionally, population structures between botanic garden *ex situ* populations and corresponding *in situ* source populations were compared.

Genetic population structure was analysed using the DNA fingerprinting technique AFLP. Fitness evaluations were conducted in the field and in common garden approaches and data were analysed by statistical modelling.

In both *Silene* species, population genetic studies, which used a Bayesian approach, AMOVA and neighbour-joining analyses, revealed a strong genetic differentiation among populations. Additionally, in both species no correlation between geographic and genetic distance could be found. The results indicate that patterns of population differentiation are mainly due to reproductive isolation and genetic drift. In *S. chlorantha*, fitness evaluation revealed pollinator limitation and habitat quality to be more important for reproductive fitness than genetic diversity by itself. Genetic diversity was similar in the botanic garden *ex situ* and the *in situ* source population. A very low level of differentiation between both populations could be observed. In *S. chlorantha*, plant performance was similar in *ex situ* and *in situ* progeny. F_1 progeny of cross-pollinations in *S. chlorantha* featured heterosis and inbreeding depression whereas no outbreeding depression could be observed. Genetic diversity could only be enhanced, if one partner originated from a genetically diverse population.

A high level of genetic differentiation among populations of *S. otites* in north-eastern Germany was found. Neither a correlation between geographic and genetic distance nor between genetic diversity and population size could be detected, which indicates reduced gene flow among populations and random genetic drift. Plant performance was positively related to population size and genetic diversity. Higher total vegetation coverage resulted in reduced plant fitness. Additionally, in *S. otites* a sex ratio bias towards more male plants in larger populations could be observed. The results indicate that dioecy does not necessarily prevent from genetic erosion in case of habitat fragmentation. In *S. otites*, genetic variation in the *ex situ* populations was lower than the variation found in corresponding *in situ* populations. Strong differentiation between corresponding *in situ* and *ex situ* populations was

observed. This could be the result of small population sizes and unconscious selection during cultivation. Therefore, adequate sampling prior to *ex situ* cultivation and large effective *ex situ* population sizes are important to preserve genetic diversity.

9 Zusammenfassung

Die vorliegende Arbeit mit dem Titel „Population genetic structure and plant fitness of natural and *ex situ* populations in *Silene chlorantha* (WILLD.) EHRH. and *Silene otites* (L.) WIBEL“ analysiert die Effekte zeitlicher und räumlicher Isolation auf die genetische Populationsstruktur und Fitness der zwittrigen Pflanzenart *Silene chlorantha* (Caryophyllaceae) und der zweihäusigen Pflanzenart *Silene otites* (Caryophyllaceae) in Nordostdeutschland. Die Auswirkungen von Inzucht und Auszucht auf die genetische Diversität und Fitness wurden mittels eines Bestäubungsexperiments untersucht. Des Weiteren wurde die genetische Populationsstruktur zwischen *ex situ* Kulturen in Botanischen Gärten und deren dazugehörigen Freilandpopulationen verglichen.

Die genetische Populationsstruktur wurde mit Hilfe der DNA-fingerprint Technik AFLP untersucht. Die Datenerhebungen zur Fitness wurden an den Wildstandorten und in experimentellen Ansätzen im Versuchsgarten durchgeführt. Analysiert wurden die Daten mit Hilfe verschiedener statistischer Modelle.

Bei beiden *Silene*-Arten waren die Populationen genetisch stark differenziert, dies wurde durch Bayesische Ansätze, AMOVA und neighbour-joining Analysen gestützt. Es konnte keine Korrelation zwischen genetischer und geographischer Distanz nachgewiesen werden. Die Ergebnisse lassen vermuten, dass die Populationsstruktur maßgeblich durch reproduktive Isolation zwischen den Populationen und durch genetische Drift beeinflusst wird. Die Analysen zur Fitness bei *S. chlorantha* ergaben einen positiven Einfluss der Populationsgröße und einen deutlichen Effekt der Habitatqualität. Die genetische Diversität hatte im Gegensatz zu den Habitatfaktoren einen geringeren Effekt auf die Fitness. Die genetische Diversität der *in-* und *ex situ* Population war bei *S. chlorantha* gleich. Die genetische Differenzierung zwischen beiden war sehr niedrig und es konnte kein Unterschied in der Pflanzengröße nachgewiesen werden. Die F_1 Generation der Kreuzbestäubungen zeigte Effekte von Inzucht und Heterosis, Auszuchtdepression konnte zum gegenwärtigen Zeitpunkt nicht nachgewiesen werden. Die genetische Diversität von Populationen konnte nur dann erhöht werden, wenn mindestens ein Kreuzungspartner aus einer genetisch diversen Population kam.

Auch bei *S. otites* wurde eine deutliche Differenzierung der untersuchten Wildpopulationen in Nordostdeutschland gefunden. Weder eine Korrelation zwischen geographischer und genetischer Distanz, noch zwischen genetischer Diversität und Populationsgröße konnte nachgewiesen werden. Die Ergebnisse lassen auf einen reduzierten Genfluss zwischen den Populationen und genetische Drift schließen. Die Fitness korrelierte positiv mit der

Populationsgröße und der genetischen Diversität. Bei den Freilandstudien führte eine höhere Vegetationsdeckung zu einer verringerten Fitness von *S. otites*. Außerdem konnte eine Verschiebung des Geschlechterverhältnisses in Richtung Männchenüberschuss in großen Populationen nachgewiesen werden.

Die Untersuchung an *S. otites* ergab eine deutliche Differenzierung zwischen den *in*- und den *ex situ* Populationen. Die genetische Diversität der *ex situ* Populationen war niedriger als die der dazugehörigen Wildpopulationen. Mögliche Ursachen sind kleine Populationsgrößen in den *ex situ* Kulturen sowie eine unbewusste Selektion während der Kultivierung. Daher werden eine optimale Besammlung des Ausgangsmaterials und größere Populationsgrößen während *ex situ* Kutlivierung empfohlen, um die genetische Diversität längerfristig erhalten zu können.

10 Appendix

10.1 List of figures

10.2 List of tables

10.3 Curriculum vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

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