Plant conservation - genetic variation, phylogeography and seed banking

Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.) der Fakultät für Biologie und Vorklinische Medizin der Universität Regensburg

vorgelegt von

MARTIN LEIPOLD

aus Schwandorf
im Jahr 2018

Das Promotionsgesuch wurde eingereicht am:	26.10.2018
Die Arbeit wurde angeleitet von:	Prof. Dr. Peter Poschlod

Regensburg, den 26.10.2018

Martin Leipold

Table of contents

DECLARATION OF MANUSCRIPTS

Chapter 2 was published with the thesis' author as main author:

Leipold, M., Tausch, S., Hirtreiter, M., Poschlod, P., & Reisch, C. (2018). Sampling for conservation genetics: how many loci and individuals are needed to determine the genetic diversity of plant populations using AFLP? Conservation Genetics Resources: 1-10.

Chapter 3 and 4 were published with the thesis' author as main author:

Leipold, M., Tausch, S., Poschlod, P., & Reisch, C. (2017). Species distribution modeling and molecular markers suggest longitudinal range shifts and cryptic northern refugia of the typical calcareous grassland species *Hippocrepis comosa* (horseshoe vetch). Ecology and Evolution, 7(6), 1919-1935.

Chapter 5 was published with the thesis' author as co-author:

Leipold, M., Tausch, S., Reisch, C., & Poschlod, P. (2019). Genbank für Wildpflanzen-Saatgut - Bayern Arche zum Erhalt der floristischen Artenvielfalt. UmweltSpezial, Bayerisches Landesamt für Umwelt, Augsburg, 66 S.

General introduction

VEGETATION, SPECIES RANGES AND SPECIES DIVERSITY

Global vegetation, habitat as well as species abundances and distributions have consistently changed during the course of Earth's history (Schroeder, 1998). Today's natural plant distribution in Central Europe is the result of range shifts after the last glaciation and synchronous human impact (Lang, 1994; Taberlet et al., 1998; Poschlod, 2017). However, since the beginning of the modern age the rate and extent of environmental deterioration continuously increased due to human activities (Poschlod & WallisDeVries, 2002; Poschlod, 2017).

The distribution of plant species would -without human interaction- mostly depend on climate, weather, soil and interspecific competition (Woodward, 1987). Despite other factors such as constrained dispersal (Normand et al 2011), climate is the most influential natural broad-scale parameter for species' ranges (Willis & Whittaker, 2002; Pearson & Dawson, 2003). Without a suitable climate, a plant can't germinate, repopulate, grow or reproduce in a habitat. Hence, for the prediction of future or the reconstruction of past plant species' ranges, climate data can be used in species distribution models (SDM) (Elith & Leathwick, 2009). While such models point out the potential distribution of habitats of species, the analysis of genetic diversity and relatedness in phylogeographic studies can help to identify colonisation routes or glacial refugia (Taberlet et al., 1998; Hewitt, 1999, 2004).

The human impact on ecosystems, vegetation composition and species distribution is significant: climate change by rising atmospheric concentrations of carbon dioxide and other greenhouse gases, anthropogenic nitrogen deposition, changing land use and altered disturbance regimes, deforestation and habitat destruction (Vitousek et al., 1997; Scheffer et al., 2001; Tilman et al., 2001).

Recent industrialisation accompanied with growth of human population have caused unprecedented severe loss of the three levels of plant biological diversity: habitats and ecosystems, species and communities as well as plant genetic diversity. The European Red List of Habitats (Janssen et al., 2016) considers 36 % of the 233 habitats assessed (31 % for EU28+) as threatened: < 2 % as critically endangered, 11 % as endangered and 24 % as vulnerable. On the species level the red list of endangered plant species in Germany (Ludwig & Schnittler, 1996) states that 40 % of all native plants are at least very rare. On population level a number of factors like fragmentation and size of populations, genetic drift and bottlenecks strongly influence the genetic diversity and must therefore be considered when depicting the diversity of plants and the value and threats of single populations (Reisch & Poschlod, 2003; Reisch et al., 2003; Reisch et al., 2005; Reisch & Kellermeier, 2007).

ACTIONS FOR PRESERVATION OF DIVERSITY

As a counterpart to previously ratified conservation strategies concerning animals and its habitats, the Global Strategy of Plant Conservation (GSPC) from 2002 for the first time focused on the prevention of further losses of plant biological diversity (CBD, 2010). The GSPC contained 16 targets, which should stop or at least slow down the loss of phytodiversity by the year 2010. In 2010, when the failure was inevitable, the targets were updated at COP10 (Nagoya) and are expected to be implemented until 2020. The first two objectives ("Plant diversity is well understood, documented and recognized" and "Plant diversity is urgently and effectively conserved") encompass "Information, research and associated outputs, and methods necessary to implement the Strategy developed and shared" (target 3) and "At least 75 per cent of threatened plant species in *ex situ* collections, preferably in the country of origin, and at least 20 per cent available for recovery and restoration programmes" (target 8).

THESIS OUTLINE

The thesis at hand was prepared within the framework of the foundation of the Genbank Bayern Arche, a Bavarian seed bank for rare and threatened plant

species. Its single chapters are supposed to help towards achieving the targets of the GSPC.

In order to provide assistance for the collection of plant material for genetic studies based on AFLP markers (objective I) or for seed collections for *ex situ* conservation (objective II), Chapter 2 intends to identify patterns in the assessment of genetic diversity of 16 different plant species of the habitat calcareous grasslands. The number of plant individuals that must be collected to reflect the genetic diversity of a single population can be important to increase efficiency and productivity through reduced sample sizes. The study also investigates whether and to what extent plant functional traits have an influence on the sample size.

Using climate modelling in Chapter 3 as well as phylogeographic analysis in Chapter 4, this thesis provides further knowledge about the origin of calcareous grasslands and thereby a better understanding of plant diversity (objective I). Numerous studies have examined calcareous grasslands from different perspectives (Cornish, 1954; Gibson & Brown, 1991; Dutoit & Alard, 1995; Poschlod & Bonn, 1998; Poschlod et al., 1998; Poschlod & WallisDeVries, 2002; Kahmen & Poschlod, 2004; Karlik & Poschlod, 2009; Römermann et al., 2009). But still, the origin of its plant inventory and the potential migration routes after the last glaciation are widely unknown. It is generally assumed that plants from Central Europe outlasted the ice age in Mediterranean refugia. Recent research also point to additional refugia north of the Alps (Provan & Bennett, 2008; Svenning et al., 2008; Tzedakis et al., 2013). We therefore conducted an AFLP analysis of the horseshoe vetch (Hippocrepis comosa) across the whole species distribution range to provide insights into the postglacial migration routes to Central Europe.

Finally, in Chapter 5, we gather information about the operation of a small-scale seed bank for rare and threatened plant species, which was set up to collect and store seeds listed on the Bavarian Priority List for Botanic Species Conservation (Woschée, 2009) and alpine rarities. The documentation provides time spans for every process step within the seed bank and can therefore serve as a basis for calculation of expenses for seed banks.

QUESTIONS AND AIMS

The key elements that are addressed in this thesis are:

1. Sampling for conservation genetics: how many loci and individuals are needed to determine the genetic diversity of plant populations using AFLP markers? (Chapter 2)
2. Species distribution model reveals suitable climates for oceanic species during the Last Glacial Maximum. (Chapter 3)
3. Insights into the European postglacial colonization of the horseshoe vetch (*Hippocrepis comosa*). (Chapter 4)
4. Short communications of a seed bank for threatened plant species on the example of the Genbank Bayern Arche. (Chapter 5)

CHAPTER 2

Sampling for conservation genetics: how many loci and individuals are needed to determine the genetic diversity of plant populations using AFLP?

ABSTRACT

Molecular markers such as AFLPs are frequently applied in molecular ecology and conservation genetics to determine the genetic diversity of plant populations. However, despite the extensive utilization there is little consensus about the number of loci and individuals which should be used to estimate genetic diversity. As a consequence, these two parameters strongly vary among investigations.

In our study we analysed the impact of loci and individual number on the determined level of genetic diversity of 15 calcareous grassland species using AFLP. We investigated curve progressions of genetic diversity with an increasing number of individuals and computed the appropriate number of loci and plant individuals to reach 90 % and 95 % of a population's genetic diversity. According to our results approximately 120 loci are sufficient for a stable estimation of genetic diversity. Regarding the number of analysed individuals on average about 14 samples are needed to cover 90 % and about 23 samples are needed to cover 95 % of the genetic variation estimated from the total population sample. Wind-pollinated species require, however, larger sample sizes than insect-pollinated species.

Since funding is often limited, especially in conservation, our results may help to avoid time-consuming and costly surveys and may contribute to a more efficient use of the financial resources available for the determination of genetic variation.

KEYWORDS

AFLP, genetic variability, sample size, pollination type

2.1 INTRODUCTION

Estimates of genetic diversity is a core question in molecular ecology and conservation genetics (Nybom & Bartish, 2000) and the aspect of genetic diversity is included in a wide range of conservation decisions. Genetic diversity is for example taken into account when high priority populations have to be identified (Kaulfuß & Reisch, 2017) or when populations have to be selected for the collection of seeds to produce regional seed mixtures (Durka et al., 2017). Recently, molecular analyses have also been applied to identify the number of populations necessary to preserve 70 % of the total *in situ* genetic variation (Whitlock et al., 2016) or to determine the number of individuals which have to be sampled to conserve 90 % of

the genetic variation via seeds for *ex situ* seed banks (McGlaughlin et al., 2015).

The methodical range is large and very different molecular markers, either dominant or codominant, can be used to determine genetic diversity. Among many other techniques, microsatellites and AFLP belong to the most often applied methods. Microsatellites (Litt and Luty 1989) are still very popular (Zane et al. 2002), as they are extremely variable on locus level. In former times the design of appropriate primers needed time-consuming pre-screenings (Gaudeul et al. 2004), but the application of next generation sequencing methods makes the development of microsatellites much easier than in the past (Taheri et al. 2018; Zalapa et al. 2012). Although cutting edge technologies such as single nucleotide polymorphisms are meanwhile frequently applied in molecular ecology (Morin et al 2004), AFLP markers are still important in conservation genetics, since they do not require sequence data and are very cost-effective. Using sufficiently high numbers of loci, AFLPs markers (Vos et al. 1995) can broadly cover the genome and are therefore more suitable for estimating genetic variation than microsatellites (Eidesen et al. 2007; Gaudeul et al. 2004; Mariette et al. 2002) although it should not be concealed that their dominant character and the reproducibility represent a certain disadvantage.

Methods and samples must be adequate to addressing the biological question at hand. There are, however, numerous publications comparing the pros and cons of different methods, informing about this decision (Jarne & Lagoda, 1996; Sunnucks, 2000; Schlötterer, 2004; Semagn et al., 2006). Far more unclear is, however, how many loci and individuals need to be used for the determination of genetic variation. Previous reviews revealed a wide range among studies concerning these two parameters (Nybom, 2004; Reisch & Bernhardt-Römermann, 2014). Despite the awareness, that the number of analysed loci and individuals are of great significance (Bonin et al., 2007) there is no scientific consensus concerning this question. Whereas Bonin et al. (2007) showed that the number of analysed loci had no strong effect on the resulting estimates, Hollingsworth and Ennos (2004) stated that the number of loci should be high enough (250 loci) for an accurate result in joining trees of poorly differentiated populations. Other authors reported increasing levels of polymorphisms with increasing sample size (Sinclair & Hobbs, 2009). Regardless, there is by trend a striking increase of used individuals and loci for genetic studies in all markers, dominant and codominant. However, funding is often limited, especially in conservation, and financial resources for molecular analyses should therefore be used efficiently. This means that the number of loci and individuals should be limited to the minimum necessary to draw conclusions for conservation.

In this study we analysed therefore, how many loci are needed to get a stable determination of genetic diversity and how many individuals have to be analysed to cover respectively 90 % and 95 % of the total genetic diversity. We assume that the measured genetic diversity of a population increases by the number of investigated individuals until a plateau is reached. This curve progression of genetic diversity should either follow a steep increase within few individuals resulting rapidly in a high genetic variation or a very steadily increase might be possible; with a genetic diversity remaining at low levels, reaching high genetic diversity not until a high number individuals was added. Such curve progressions most probably influence the number of individuals (minimum sample size = MSS) that needs to be collected in order to detect a sufficient genetic variation e.g. 95 % of the genetic variation estimated from the total population sample following Sedcole (1977). A similar approach was recently applied to determine the number of populations necessary to conserve the genetic variation of a species within a specific geographic region (Whitlock et al., 2016). Here, we investigated 15 different plant species in order to describe the curve progressions, which define the relation of genetic variation and the increasing number of individuals. More specifically, we answered the following two questions:

1. How many loci are needed to receive a stable and resilient determination of the genetic diversity of a plant population?
2. How many individuals have to be analysed to cover 90 % and 95 % of a population's genetic diversity?

2.2 MATERIALS AND METHODS

SAMPLING OF PLANT MATERIAL

Our approach to measure a minimum sample size for genetic studies implied a satisfactory number of repetitions in the sense of plant species and leave samples. Furthermore, with regard to a plant functional trait analysis we conducted our plant sampling at one site situated north of Regensburg, Bavaria, to guarantee homogenous abiotic factors within one habitat. With a total area of approximatively 24 ha the site was large enough for an intensive sampling. At this loca-

TABLE 2.1 Investigated plant species. Names of the study species, used primer pairs, ind = number of analysed individuals, frag = number of detected fragments. *B. pinnatum* and *T. chamaedrys* marked with "#" are repetitions from a second calcareous grassland site

Species	D2	D3	D4	ind	frag
Anthyllis vulneraria L. s.l.	CAA-AAC	CAG-AAG	CAG-ACA	29	310
Brachypodium pinnatum (L.) P. Beauv.	CTC-ACC	CAA-AAG	CTG-ACA	37	168
Brachypodium pinnatum # (L.) P. Beauv.	CTC-ACC	CAA-AAG	CTG-ACA	40	187
Centaurea stoebe L. s.l.	CTT-AAC	CAT-AAG	CTT-ACA	47	197
Cerastium arvense L.	CTA-AAC	CAT-AGG	CTA-ACA	36	249
Chamaecytisus ratisbonensis (Schaeff.) Rothm.	CTA-AAC	CTA-AGG	CTG-ACA	38	244
Dianthus carthusianorum L.	CTT-AGC	CAA-AAG	CAA-ACT	47	233
Galium verum L. s.str.	CAA-AAC	CAC-AAG	CAC-ACA	43	227
Helictotrichon pratense (L.) Besser	CTG-AGC	CAT-AAG	CTT-ACT	43	219
Koeleria pyramidata (Lam.) P. Beauv.	CTG-AGC	CAT-AAG	CAG-ACT	43	212
Medicago falcata L. s.str.	CAC-ACC	CTA-ACG	CTT-ACA	37	274
Phleum phleoides (L.) H. Karst.	CAA-AAC	CAC-AAG	CTT-ACT	35	167
Sanguisorba minor Scop. s.l.	CAC-ACC	CAA-AGG	CTA-ACA	40	178
Teucrium chamaedrys L.	CAC-AGC	CAC-AAG	CTC-ACA	45	229
Teucrium chamaedrys # L.	CAC-AGC	CAC-AAG	CTC-ACA	44	226
Teucrium montanum L.	CAC-ACC	CAA-AGG	CAA-ACA	39	197
Thymus pulegioides L. s.l.	CTA-AAC	CAA-ACG	CTG-ACT	46	133

tion 15 representative plant species including grasses and herbs of calcareous grasslands (listed in Table 2.1) were collected. We projected a grid with an edge length of 5 x 5 m on the area and randomly chose 50 squares where we collected green leaf material of each occurring species. That way the total number of individuals for each species also represents its distribution and abundance at the site. Consequently the samples sample sizes varied from 29 to 47 individuals per species across the whole study site. The sample sizes differed due to the abundance of the species on the grassland. The fresh plant material was placed in plastic bags, stored in cooling containers during collection and subsequently at -20 °C until further processing. In total 689 samples were analysed.

MOLECULAR ANALYSIS

Following the CTAB protocol from Rogers and Bendich (1994) adapted by Reisch (2007) the cellular DNA extraction was carried out with leaf samples of 20 mg. The DNA contents were determined with a photometric analysis and afterwards diluted to the same level of 7.8 ng DNA per µl H_2O. For the analysis of genetic variation within populations, we chose the analysis of amplified fragment length polymorphism (AFLP, Zabeau & Vos, 1993). As standardized in several previous studies the AFLP-protocol from Beckmann Coulter (Brea, USA) was used. DNA adapters were built from initially single strand DNA adapters EcoRI and MseI (MWG Biotech, Ebersberg, Germany) by heating equal volumes to 95 °C for 5 min and following a 10 min cooling period at 20 °C.

For DNA restriction and adaptor ligation the adjusted DNA solution (6.4 µl) was blended with 3.6 µl of a restriction ligation mixture containing 2.5 U EcoRI (MBI Fermentas, St. Leon-Rot, Germany), 2.5 U MseI (MWG Biotech), 0.1 µM EcoRI and 1 µM MseI adapter pair, 0.5 U T4 Ligase with its corresponding buffer (MBI Fermentas), 0.05 M NaCl and 0.5 µg BSA (New England BioLabs, Ipswich, USA). After two hours of incubation at 37 °C and a final enzyme termination step at 70 °C for 15 min the resulting products were diluted 10-fold with 1x TE buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0). For the pre-selective amplification 1 µl of the diluted restriction-ligation product was added with a mixture of pre-selective 0.25 U EcoRI and MseI primers (MWG Biotech), H_2O, 1x Buffer S, 0.2 mM dNTPs and 0.25 U Taq-Polymerase (PeqLab, Erlangen, Germany). The total volume of 5 µl per sample was processed in a thermocycler under following PCR conditions: initial 2 min at 94 °C, 30 cycles each with denaturation at 94 °C for 0.3 min, annealing at 56 °C for 0.5 min, elongation at 72 °C for 2 min and another 2 min at 72 °C after finishing the cycles. 30 min at 60 °C and a cool down to 4 °C complete the procedure. Afterwards the resulting products were dilut-

ed 20-fold with 1x TE buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0). For each tested plant species three primer pairs were selected after executing a primer screening. The chosen primers are shown in Table 2.1. A separate PCR per primer pair was carried out containing 0.75 µl of the diluted pre-selective amplification product, and in total 4.25 µl of H_2O, 0.25 µM selective MseI (MWG Biotech) primer, 0.05 µM selective EcoRI primer (Proligo, Paris, France), 10x Buffer S, 0.2 mM dNTP and 0.25 U Taq-Polymerase (PeqLab, Erlangen, Germany). To enable a simultaneous detection of resulting fragments the EcoRI primers were labelled, each pair with a different fluorescent dye (Beckmann Coulter, D2, D3, D4). The PCR run included consecutive steps: initial 2 min at 94 °C, 10 cycles each with denaturation at 94 °C for 0.3 min, annealing starting at 66 °C for 0.5 min and elongation at 72 °C for 2 min. With every cycle passed through the annealing temperature was lowered by 1 °C. Once the 10th cycle was finished 25 more iteration with following run parameters were carried out: denaturation at 94 °C for 0.3 min, annealing at 56 °C for 0.5 min and elongation at 72 °C for 2 min. The PCR ended with 30 min at 60 °C and a cool down to 4 °C. For achieving optimal results, the resulting amplified products were diluted differently with 1x TE buffer. 5 µl of each primer pair were mixed and 5 µl stop buffer (Na acetate (3 M, pH 5.2), Na_2EDTA (100 mM, pH 8) and glycogen (20 mgml-1 Roche, Manheim, Germany) at a ratio of 2:2:1) added. 60 µl of 96 % EtOH (4 °C) in combination with shaking causes the precipitation of the DNA and 20 min of centrifugation at 14k rpm its aggregation. The supernatant was removed from the DNA pellet, washed with 200 µl of 76 % ethanol (4 °C) and centrifuged (20 min, 14k rpm) again. After discarding the supernatant, the pellet was dried in a concentrator (Eppendorf concentrator 5301; Eppendorf, Hamburg, Germany). Prior to the sequencer run, the DNA pellets were dissolved for 30 min in a mixture of 24.8 µl Sample Loading Solution (Beckmann Coulter, USA) and 0.2 µl CEQ Size Standard 400 (Beckmann Coulter, USA). The sequencer used for fragment detection was a CEQ 8000 (Beckmann Coulter, USA). For every investigated individual we exported the received data into three curve-files, each representing one primer pair. Those virtual gels were analysed manually for the occurrence of strong, well defined fragments (bands) using Bionumerics 6.6 (Applied Maths, Kortrijk, Belgium). The presence or absence of bands for every particular fragment size and individual was transformed into a binary (1-0) matrix, which served as basis for all further analysis. Individuals showing no clear banding signals due to unsuccessful AFLPs were repeated or ultimately excluded.

STATISTICAL ANALYSIS

In total, the AFLP analysis delivered 17 binary matrices with a widespread range of detected bands from 133 to 319. For all subsequent calculations of genetic diversities, Nei's Gene Diversity index (Nei, 1972) was used:

$$\mathrm{He} = 1 - \sum (p_i)^2$$

All analyses were conducted either in R 2.15 or when multiple iteration computations were necessary, by a self-developed web-based client/server application, based on Google Web Toolkit (GWT) (Kastlwerk, 2018).

GENE DIVERSITY AND NUMBER OF ANALYSED FRAGMENTS

In a first step, a possible correlation between the number of detected fragments and Nei's Gene Diversity (He) was investigated. As the result indicated a strong correlation, secondly, in order to analyse all data uniformly, we searched for a threshold beyond which an increasing number of loci would not affect the calculated He of a species. This was done by calculating He for each plant species using all available individuals and consecutively changing the number of loci as basis of the computation. The tested loci levels were 20, 40, 60, 70, 80, 90, 100, 110, 120, 130, 140, 160, 180, 200, 220 and 240. The loci were randomly selected without allowing duplicates; each step was repeated 10 times and here from one variance-value was calculated. This procedure was rerun 10 times to finally obtain 10 variance-values per fragment level and plant species. All resulting variances of all species and fragment levels were evaluated with a generalized linear model (McCullagh & Nelder 1989).

GENE DIVERSITY AND NUMBER OF ANALYSED INDIVIDUALS

Based on the foregoing findings the number of randomly selected fragments (m) was set to 120. With this new data set, we repeatedly calculated the Nei's Gene Diversity (He) for k individuals, with k ranging from two to the maximal number of sampled individuals (Table 2.1). The number of repeats (n) was set to 50,000. All 50,000 He values for each k were summarised in one mean value:

$$\overline{\mathrm{He}} = \frac{\sum_{i=1}^{n} \mathrm{He_i}}{n}$$

After that the procedure was rerun 9 times beginning with drawing 120 random fragments. The resulting 10 He values for each number of tested individual and

TABLE 2.2 Results of the MSS_{90} and MSS_{95} analyses. Investigated plant species (# = species collected on another sample site), calculated factors (a, b, c) for describing a sigmoid equation, Nei's Gene Diversity calculated with the maximum number of investigated species (=He_{max}), the predicted Nei's Gene Diversity for 100 individuals (=He_{100}), minimum sample size for 90 % (MSS_{90}) respectively 95 % (MSS_{90}) of the predicted Nei's Gene Diversity.

Species	a	b	c	He_{max}	He_{100}	MSS_{90}	MSS_{95}
Anthyllis vulneraria L. s.l.	0.264	3.060	-1.303	0.25649	0.2618	12.046	20.285
Brachypodium pinnatum (L.) P. Beauv.	0.236	3.805	-1.268	0.22936	0.2339	14.942	24.992
Brachypodium pinnatum # (L.) P. Beauv.	0.262	3.615	-1.243	0.23189	0.2339	15.055	25.333
Centaurea stoebe L. s.l.	0.283	3.709	-1.287	0.27803	0.2806	14.179	23.695
Cerastium arvense L.	0.303	3.526	-1.343	0.29730	0.3012	12.462	20.704
Chamaecytisus ratisbonensis (Schaeff.) Rothm.	0.279	3.028	-1.432	0.27642	0.2777	9.772	16.023
Dianthus carthusianorum L.	0.306	3.197	-1.338	0.30257	0.3040	11.734	19.594
Galium verum L. s.str.	0.262	3.257	-1.223	0.25578	0.2592	14.473	24.583
Helictotrichon pratense (L.) Besser	0.273	4.543	-1.162	0.25997	0.2670	20.610	34.068
Koeleria pyramidata (Lam.) P. Beauv.	0.289	4.003	-1.203	0.28021	0.2848	17.424	29.168
Medicago falcata L. s.str.	0.317	3.543	-1.435	0.30866	0.3112	10.804	17.628
Phleum phleoides (L.) H. Karst.	0.254	3.164	-1.228	0.24633	0.2510	14.036	23.872
Sanguisorba minor Scop. s.l.	0.225	4.366	-1.163	0.21339	0.2203	19.996	33.194
Teucrium chamaedrys L.	0.293	3.259	-1.300	0.28706	0.2903	12.664	21.274
Teucrium chamaedrys # L.	0.286	3.715	-1.368	0.28249	0.2838	12.400	20.465
Teucrium montanum L.	0.271	3.739	-1.357	0.26571	0.2689	12.686	20.971
Thymus pulegioides L. s.l.	0.296	3.432	-1.307	0.29131	0.2939	12.974	21.711

each plant species were again summarised in one mean value.

These values were firstly plotted and secondly the best equation describing the obtained non-linear saturation curves for every plant species was fitted in R using nls function within the 'stats' package (R Core Team, 2013). Therefore, we tested following function types:

root function:

$$y = ax^b$$

monod hyperbola:

$$y = a \times \frac{x}{b + x}$$

exponential saturation function:

$$y = a\left(1 - e^{bx}\right)$$

sigmoid function:

$$y = \frac{a}{1 + bx^c}$$

and chose the model with the smallest Standard Error of the Regression (S) for further analysis. As the sigmoid function was revealed as best fitting model the function parameters a, b, c were calculated for the existing data (k = 1 to k_{max} = maximal individuals). All calculated parameters are shown in Table 2.2. Moreover, the values from k_{100} were predicted using the resulting equation. He at a predicted population size of 100 individuals served as default value. Based on this value a 90 % and 95 % He threshold following Sedcole (1977) was calculated and the associated number of individuals was determined, which therefore represents a minimum sample size (MSS_{90}, MSS_{95}). Both collection numbers were tested against each other for significant differentiation via a paired t-test. For a graphical display the saturation curves for every species were plotted using the calculated factors a, b and c. In addition, the confidence intervals are shown.

IMPACT OF LIFE HISTORY TRAITS

In order to investigate the influence of life history traits on the resulting MSS we used Pearson's product moment correlations for following continuous/metric variables 'canopy height', 'release height of seeds', 'seed mass', 'longevity index', 'number of dispersal vectors' and 'length of flower season'. One-way ANO-

VAs were used for the examination of the correlations of 'pollination vector' (wind-pollinated, insect-pollinated) and the 'ability to grow clonal' (yes, no) with MSS. As prerequisite, Nei's Genetic Diversity (He) values were tested for a correlation with the life history traits to exclude any dependencies. As there could be another outcome with MSS_{90} we repeated the tests with those integers and as the results did not differ we excluded them from further analysis (= not shown in this work). The data for all life history traits were taken from two databases, BioPop (Poschlod et al., 2003) and Leda (Kleyer et al., 2008) (Table 2.5).

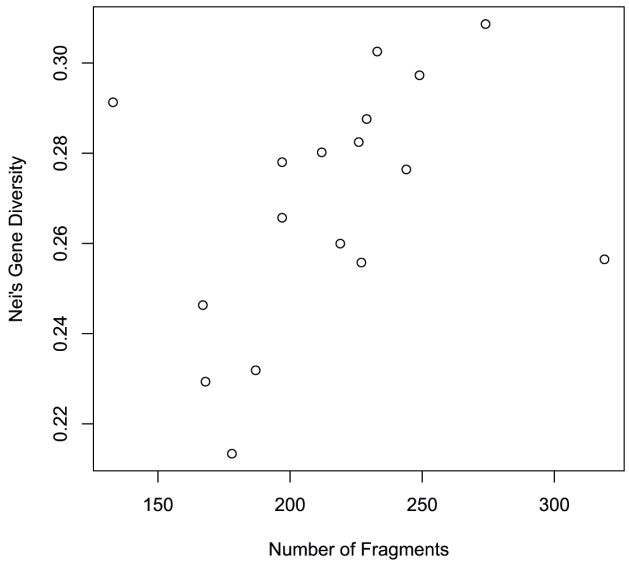


FIGURE 2.1 Relationships between the number of fragments and the estimated genetic diversity for studied plant species (N = 17). Pearson correlation: r = 0.372, t-value = 1.552, df = 15, p-value = 0.142

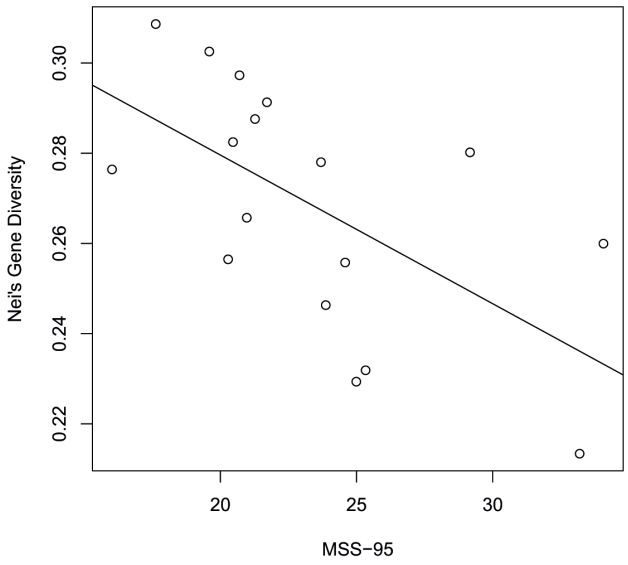
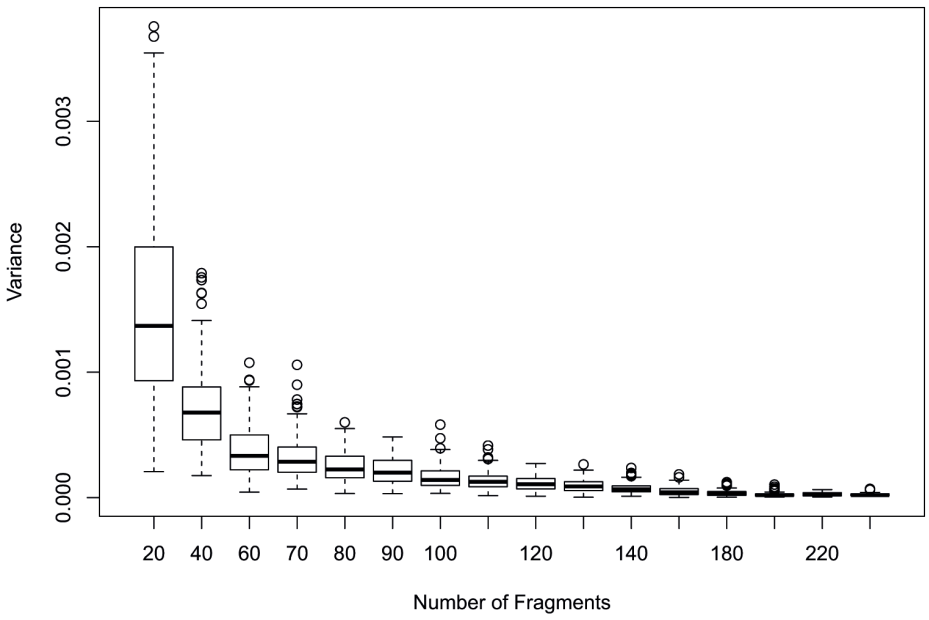


FIGURE 2.2 Relationships between the MSS_{95} the estimated genetic diversity for studied plant species (N = 17). Pearson correlation: r = -0.602, t value = -2.929, df = 15, p-value = 0.011 *

FIGURE 2.3 Number of fragments plotted against the variance of all investigated species (N = 17).



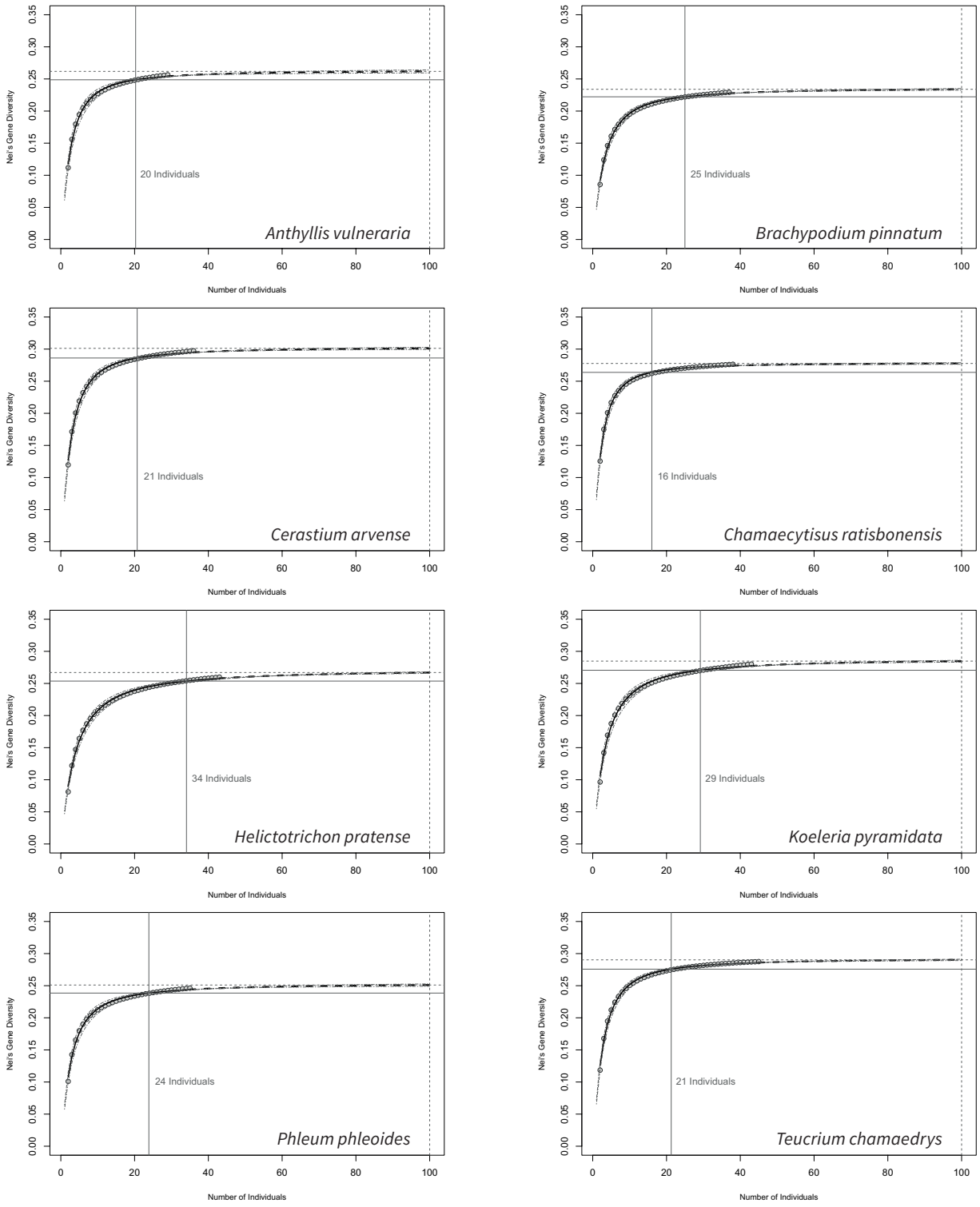
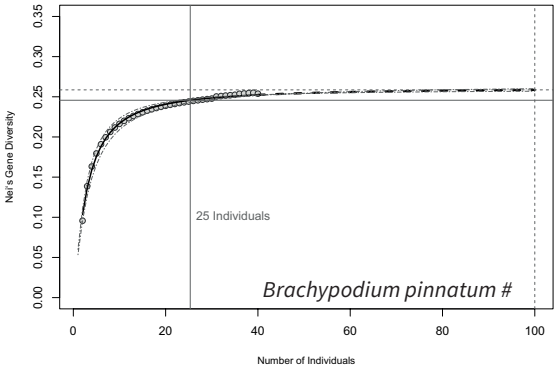
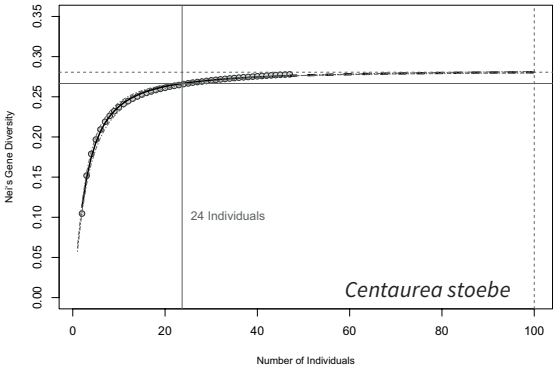
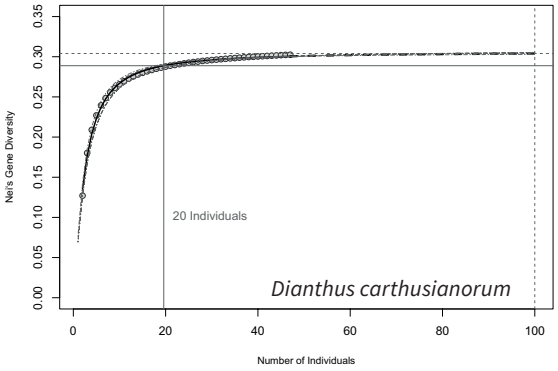
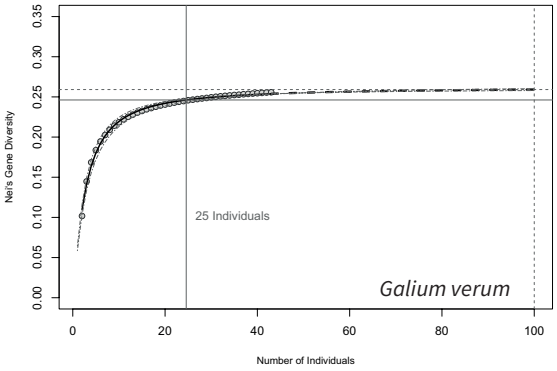


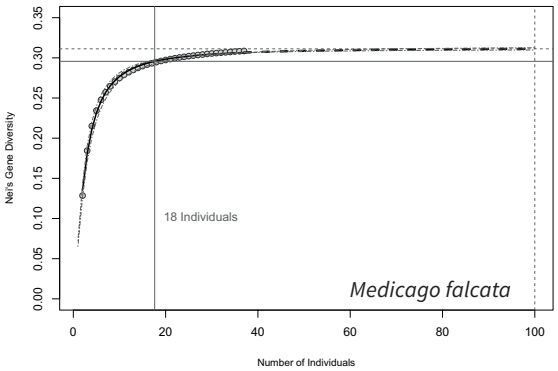
FIGURE 2.4 Display of all investigated plant species. Number of species is drawn against Nei's Gene Diversity. Measured data of the calculated curves are pictured with a solid black line; predicted data is shown in dashed black. Confidence intervals are given as dashed grey lines. The horizontal dashed grey line gives information about the maximum predicted Nei's Gene Diversity of 100 individuals (He_{100}) and serves as base for the solid grey line that displays the Nei's Gene Diversity at a 95 percent level (MSS_{95}). The associated vertical solid grey line marks the intersections with the curve and x-axis and presents the minimum sample size (MSS_{95}).

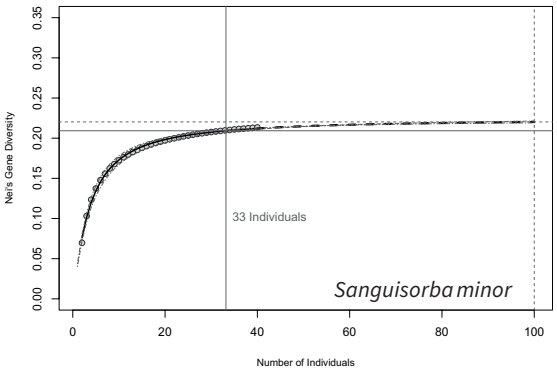

Nei's Gene Diversity
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35
0 20 40 60 80 100
Number of Individuals
25 Individuals
Brachypodium pinnatum #

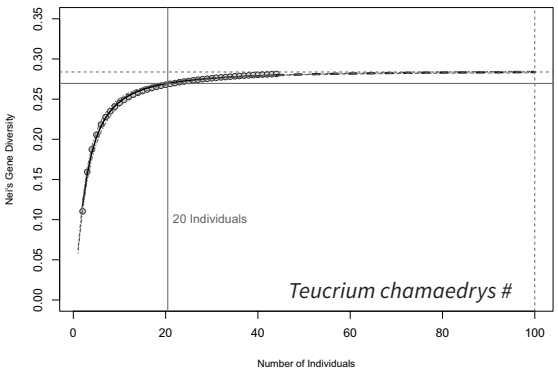

Nei's Gene Diversity
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35
0 20 40 60 80 100
Number of Individuals
24 Individuals
Centaurea stoebe

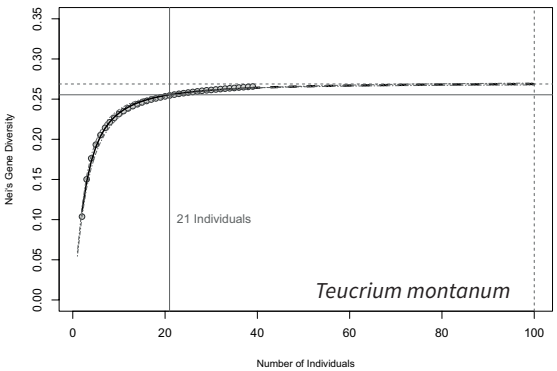

Nei's Gene Diversity
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35
0 20 40 60 80 100
Number of Individuals
20 Individuals
Dianthus carthusianorum


Nei's Gene Diversity
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35
0 20 40 60 80 100
Number of Individuals
25 Individuals
Galium verum


Nei's Gene Diversity
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35
0 20 40 60 80 100
Number of Individuals
18 Individuals
Medicago falcata


Nei's Gene Diversity
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35
0 20 40 60 80 100
Number of Individuals
33 Individuals
Sanguisorba minor


Nei's Gene Diversity
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35
0 20 40 60 80 100
Number of Individuals
20 Individuals
Teucrium chamaedrys #


Nei's Gene Diversity
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35
0 20 40 60 80 100
Number of Individuals
21 Individuals
Teucrium montanum

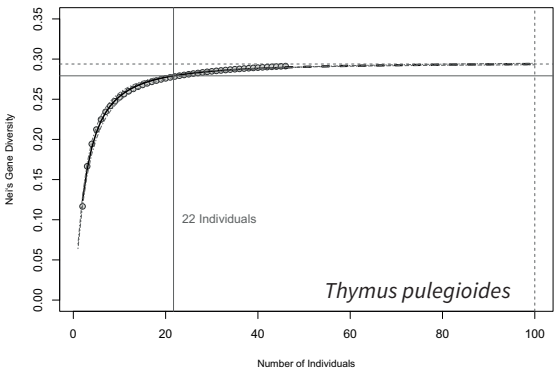

Nei's Gene Diversity
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35
0 20 40 60 80 100
Number of Individuals
22 Individuals
Thymus pulegioides

TABLE 2.3 Output of a generalized linear model. Results of a GLM in which the differentiation of the variance of the maximum number of detected fragments (240) was tested against different fragment-levels (20 - 220). Up to 100 fragments there was a significant difference in the variance noticeable and also 110 fragments displayed a tendency. Using 120 fragments or more showed no significant difference between the maximum fragment variance and the tested fragment-level.

	Estimate	Std. Error	t-value	Pr(>|t|)	
(Intercept)	2.59E-05	5.31E-05	0.488	0.6255	
Frag_240 vs. 20	1.48E-03	5.59E-05	26.406	<2e-16	***
Frag_240 vs. 40	6.83E-04	5.59E-05	12.210	<2e-16	***
Frag_240 vs. 60	3.47E-04	5.59E-05	6.211	6.20E-10	***
Frag_240 vs. 70	2.95E-04	5.59E-05	5.278	1.42E-07	***
Frag_240 vs. 80	2.19E-04	5.59E-05	3.914	9.33E-05	***
Frag_240 vs. 90	1.88E-04	5.59E-05	3.357	0.000801	***
Frag_240 vs.100	1.38E-04	5.59E-05	2.472	0.013498	*
Frag_240 vs. 110	1.08E-04	5.59E-05	1.936	0.052982	.
Frag_240 vs. 120	8.96E-05	5.59E-05	1.603	0.109140	
Frag_240 vs. 130	6.80E-05	5.59E-05	1.216	0.224038	
Frag_240 vs. 140	5.00E-05	5.61E-05	0.891	0.372925	
Frag_240 vs. 160	2.37E-05	5.61E-05	0.422	0.673291	
Frag_240 vs. 180	1.11E-05	5.70E-05	0.194	0.846090	
Frag_240 vs. 200	-1.83E-06	5.81E-05	-0.032	0.974839	
Frag_240 vs. 220	1.45E-06	6.85E-05	0.021	0.983161	
Residual std. error: 0.0002373 on 2404 degrees of freedom					
Multiple R-squared:0.724					

2.3 RESULTS

GENE DIVERSITY AND NUMBER OF ANALYSED FRAGMENTS

The number of detected fragments ranged from 133 for *Thymus pulegioides* to 319 for *Anthyllis vuleraria*. Although Figure 2.1 appears to show a correlation between the number of detected loci and the height of Nei's Gene Diversity (He), this result was not statistically significant ($p = 0.142$, $t = 1.552$, $r = 0.372$).

As Figure 2.2 shows, MSS_{95} was significantly correlated with He ($p = 0.011$, $t = -2.929$, $r = -0.602$). The same applied for MSS_{90} ($p = 0.013$, $t = -2.815$, $r = -0.588$).

The analysis of the different variances for the tested numbers of loci showed that the minimum number of fragments to estimate genetic diversity was 120. Below this threshold, the fluctuation of the calculated data had a significant influence on the variance of the data and therefore on He. Above this value, there was no significant difference to the maximum He_{max} (Figure 2.3, Table 2.3).

GENE DIVERSITY AND NUMBER OF ANALYSED INDIVIDUALS

The run of the sigmoid saturation curves showed that the different number of sampled individuals had a clear impact on the maximum measured Nei's Gene Diversity (He_{max}). For most of the tested species like *Chamaecytisus ratisbonensis* or *Anthyllis vulneraria*, the curves showed a steep ascending slope for a sample size between 2 and 15. On the other hand we also detected flat inclinations for species like *Helictotrichon pratense* or *Koeleria pyramidata*. In the subsequent development, the curves plateaued. The sigmoid curve consisted of three factors a, b, c. While a describes the maximum possible genetic variation of the tested species, both remaining factors b and c together define the steepness of the curves progression. The smaller b and c, the steeper the slope, i.e. the faster MSS_{95} is reached (Figure 2.4).

The values for MSS_{95} were significantly higher than for MSS_{90} ($p < 0.0001$, $t = -19.323$). The calculated values ranged from a minimum of $MSS_{90} = 9.77$ respectively $MSS_{95} = 16.02$ for *Chamaecytisus ratisbonensis* to a maximum of $MSS_{90} = 20.61$ respectively $MSS_{95} = 34.07$ for *Helictotrichon pratense*. The mean value for a minimum sample size including all species is 14.0 for a 90 % coverage of the population's estimated total Gene Diversity ($= MSS_{90}$) and 23.4 for a 95 % coverage ($= MSS_{95}$), respectively. The highest measured Nei's Gene Diversity (He) with 48 investigated individuals had *Medicago falcata* with 0.309, the lowest value had *Sanguisorba minor* (40 tested individuals) with 0.213. The mean calculated He for all species was 0.268. It is worth mentioning that the repeated collection of the two species *Brachypodium pinnatum* and *Teucrium chamaedrys* at different field sites did not influence the MSS_{90}, which is 14.94 vs. 15.06 for *Brachypodium pinnatum* e.g. 12.66 vs. 12.40 for *Teucrium chamaedrys*. Figure 2.4 shows He values which were plotted against the gradually increasing number individuals and the fitted curves for each species. The related factors a, b, c and the expected maximum Nei's Gene Diversity (He_{100} = calculated Nei's Gene Diversity for 100 individuals) compared to the He_{max} (calculated value with the all sampled individuals per species) are shown in Table 2.2. Both values, He_{max} ($p = 0.011$, $t = -2.919$, $r = -0.602$) and He_{100} ($p = 0.014$, $t = -2.781$, $r = -0.583$) correlated significantly with MSS_{95}, showing a negative dependency between the minimum sample size and the height of He. On the other hand, MSS_{95} did not depend on the total number of tested individuals (p-value = 0.498, $t = 0.694$, $r = 0.176$).

TABLE 2.4 Analyses of variance conducted with different life history traits as responsive variables. Number of studies in each group (n), mean values, standard derivation (SD), t-values, residual standard errors, degrees of freedom, p-values, Fstat and adjusted r are given.

	n	mean	SD	t-value	Residual SD error	degrees of freedom	p-value	Fstat	r
MSS_{90}									
Pollination vector					1,949	15	0.0003	21,91	0.75
Insect	11	12,381	0,590	21,074					
Wind	6	17,011	0,990	4,681					
MSS_{95}									
Pollination vector					3.219	15	0.0002	22.84	0.76
Insect	11	20.631	0.947	21.258					
Wind	6	28.438	1.634	4.779					
Clonality					5.070	15	0.6230	0.25	0.13
yes	8	24.041	1.793	13.412					
no	9	22.804	2.464	-0.502					
Canopy height	17	0.497		1.373		15	0.1900		0.33
Release height	17	0.559		1.622		15	0.1256		0.39
Seedmass	17	2.009		0.965		15	0.3501		0.24
Longevity index	17	0.093		-0.522		10	0.6133		-0.16
Dispersal vectors	17	2.846		0.899		11	0.3880		0.26
Flower duration	17	3.000		0.273		15	0.7890		0.07

PLANT FUNCTIONAL TRAITS CORRELATE WITH MSS BUT NOT WITH NEI'S GENE DIVERSITY

The investigation of a possible relation between the minimum sample size (MSS) and life history traits revealed only one trait with a significant correlation. The pollination type correlated significantly with MSS_{90} ($r = 0.75$, $p = 0.0003$, Table 2.4) and MSS_{95} ($r = 0.76$, $p = 0.0002$, Table 2.4). In both cases insect-pollinated species had a significantly lower MSS (MSS_{90} with 12.381 ± 0.40 SE, MSS_{95} with 20.630 ± 0.73 SE) than wind-pollinated plants where 17.011 ± 1.14 SE (MSS_{90}) and 28.438 ± 1.80 SE (MSS_{95}) individuals must be collected to reach 95 % of the total Nei's Gene Diversity. Insect-pollinated species had higher measurements of Nei's Gene Diversities (0.28 ± 0.01 SE) than wind-pollinated species (0.24 ± 0.02 SE), but like all other tested life history traits, this difference was not significant (Table 2.4).

2.4 DISCUSSION

In this study we analysed how many loci and individuals are needed for a stable determination of 90 % and 95 % of the total genetic diversity of a plant population. In this way, we wanted to contribute to a more efficient use of the financial resources available for the analysis of genetic diversity.

In order to determine the adequate number of individuals, it was necessary to specify the number of fragments, which are required to capture most of a population's genetic diversity. Simulations of Mariette et al. (2002) showed that AFLP markers produce good estimates of genetic variation at whole genome level, but when migration rates are higher and populations exhibit low genomic heterogeneities, at least ten times more markers are necessary to reach the efficiency of microsatellites to predict the whole genetic diversity. The results for our 15 surveyed calcareous grassland species showed that reaching a number of 120 fragments (poly- and monomorphic) is sufficient to estimate a population's genetic diversity via AFLPs. Beyond this threshold, additional fragments did not significantly increase the estimates of genetic diversity. Fewer than 120 fragments resulted in significantly lower estimates of genetic diversity and therefore increased the probability of not embracing the total

population's genetic variation. This perfectly fits the results of Mittell et al. (2015) who showed that the median of 10 microsatellite loci currently used in genetic studies is sufficient for characterising a population's molecular genetic variation.

Based on these findings, we investigated our second question concerning the sufficient number of individuals to satisfactorily characterize and compare the genetic diversity of populations. To exclude bias of fragment detection, we randomly chose 120 fragments each for all following tests. The results showed that He followed a non-linear saturation curve, which ascended steeply with sample size until a plateau was reached. In other words, the curve progression culminated in a saturation point beyond which an increased sample size had no significant influence on the genetic variation estimated from the total population sample. Our compilation of 15 different calcareous grassland species revealed that an overall mean of 23.4 ± 1.20 randomly chosen individuals per population were sufficient to cover 95 % of the total genetic variance. Interestingly, similar results have been reported for other organisms such as lichens (Werth, 2011) or birds (Pruett & Winker, 2008). Our results were also corroborated by the work of McGlaughlin et al. (2015). The authors of this study determined a range of 10 to 30 individuals necessary to capture 90 % of the genetic variation estimated from the total population sample of an endangered Californian plant species, which matches with our observations. Fewer individuals are required to estimate diversity when diversity is great ($t = -2.919$, $df = 15$, p-value = 0.011, $r = -0.602$). But neither MSS nor He was dependent on the initial total number of individuals (population size). This supports the results of the review of Reisch and Bernhardt-Römermann (2014) who showed that genetic variation was independent from sample size and who concluded that the number of individuals did not influence the outcome of study comparisons.

Pollination type was the only life history trait that was significantly correlated with MSS_{95} ($r = 0.78$, $p = 0.0002$). In order to characterize the genetic variation of wind pollinated plants, more individuals had to be collected than for insect pollinated species (MSS_{90} with 17.0 ± 1.1 SE individuals vs. 12.4 ± 0.4 SE, MSS_{95} 28.4 ± 1.8 SE individuals vs. 20.6 ± 0.7 SE). Curve progressions in the two categories were almost constantly different with insect pollinated plants showing higher values of genetic variation and a steeper curve progression than wind pollinated plants. However, He was not significantly different. This can be related to the findings of Reisch & Bernhardt-Römermann (2014) who also had non-significant but higher genetic variations for dicotyledons than for monocotyledons. Except for *Sanguisorba minor*, the majority of our investigated wind-pollinated species were grasses. Considering the pollination vector the referred study divided their surveyed species into three groups with an additional category self-pollinated and found no clear results, which was explained by the difficult designation of the species' pollination vectors. This contradiction may be explained by different sample designs of the studies. Most of the surveyed articles had a large-scaled focus with a mean distance between the populations of 1,513 ± 2,529 km. We on the other hand tested individuals on a small scale of 440 meters, with an emphasis on the genetic variation within the population of a species. From this point of view, a lower genetic variation of wind-pollinated plants can be expected due to the fact, that pollen transported via wind can bridge larger distances between the individuals of one population than between insect-pollinated plant individuals. Therefore genetic differentiation of wind-pollinated individuals can be considered smaller, i.e. the individuals of this population are more genetically homogeneous. On the other hand we would predict insect-pollinated plants to display a higher genetic heterogeneity within the population because of a smaller transportation vector and a higher selectivity of the pollinators. As genetic variability rises with the number of different tested individuals, for a genetically homogeneous population a higher sample size has to be collected to gain 95 % of the total genetic variation. The homogeny leads to the fact that more individuals need to be collected over the whole population to sufficiently characterize the genetic variation.

CONCLUSIONS

Our findings demonstrate that on the whole, in most studies a sufficient number of individuals or rather just as many individuals have been used for the estimation of genetic variation resulting in diversity values located on the plateau of the progression curves. We therefore emphasise that there is no need to follow the trend to increase sample sizes, as it is an unnecessary expense in time and money. Financial resources should instead better used to analyse further populations or species.

APPENDIX

TABLE 2.5 List of studied plant species and plant traits (-=phyte): li=longevity index, sla=specific leaf area.

Species	pollination vector	clonality	mean height	life form	release height	seed mass	li	sla	dispersal vectors	flower duration
Anthyllis vulneraria	insect	no	0.200	hemicrypto-	0.150	2.450	0.03	15.91	4	5
Brachypodium pinnatum	wind	yes	0.600	geo-	0.600	3.250	0.02	28.32	2	2
Centaurea stoebe	insect	no	0.550	hemicrypto-	0.625	1.715	NA	NA	2	3
Cerastium arvense	insect	yes	0.125	chamae-	0.175	0.300	0.18	37.65	NA	6
Chamaecytisus ratisbonensis	insect	no	0.350	chamae-	0.213	5.200	NA	NA	NA	2
Dianthus carthusianorum	insect	yes	0.260	chamae-	0.300	0.875	NA	19.81	NA	3
Galium verum	insect	no	0.425	hemicrypto-	0.400	0.396	0.02	22.93	4	2
Helictotrichon pratense	wind	no	0.650	hemicrypto-	0.550	1.908	0.07	10.39	3	4
Koeleria pyramidata	wind	yes	0.325	hemicrypto-	0.550	1.300	NA	16.57	NA	3
Medicago falcata	insect	yes	0.350	hemicrypto-	0.350	2.000	0.04	NA	3	4
Phleum phleoides	wind	no	0.300	hemicrypto-	0.388	0.140	0.22	17.40	2	1
Sanguisorba minor	wind	yes	0.200	hemicrypto-	0.245	5.284	0.05	20.34	5	4
Teucrium chamaedrys	insect	no	0.225	chamae-	0.200	1.467	0.1	17.29	1	1
Teucrium montanum	insect	no	0.200	chamae-	0.160	0.950	NA	NA	1	4
Thymus pulegioides	insect	yes	0.200	chamae-	0.225	0.100	0.27	27.95	5	4

CHAPTER 3

Species distribution model reveals suitable climates for oceanic species during the Last Glacial Maximum

ABSTRACT

The peninsulas of Southern Europe, Iberia, Italy and the Balkans are traditionally viewed as refugia for temperate plant species during the Last Glacial Maximum (LGM). These assumptions are based on a latitudinal gradient representing an increasing temperature towards the south. Recently, attention has been drawn to the oceanity as a second gradient which as well might have influenced the migration of plants.

Using the software Maxent, we computed species distribution models (SMD) to exemplarily predict the present and past potential geographical distribution of the Horseshoe vetch (*Hippocrepis comosa*), a plant depending on oceanic climates. While studies of continental steppe plants mainly focused on the impact of a longitudinal gradient in eastern Europe and Asia, we demonstrate with our work that oceanic plants from Central Europe migrated to southern refugia not alone because of a temperature gradient from north to south. For these species oceanity is an equally important parameter, which decreased in Central Europe during the LGM and therefore caused a migration not only to the south but also to the west. Furthermore, the results strongly support the existence of possible cryptic refugia in northwest Europe and might also be applied to other species with oceanic habitats.

KEYWORDS

LGM, species distribution model, horseshoe vetch, *Hippocrepis comosa*

3.1 INTRODUCTION

On the geologic time scale the current and most recent period, the Quaternary (beginning 2.5 Mya) and within it the Pleistocene, was an epoch characterized by strong cyclic climatic changes (Lowe & Walker, 1997). Warm climate periods (interglacials) alternated with cold ones (glacials). During the latter, glaciers on the northern hemisphere expanded. One of the coldest periods for the last 100,000 years took place during the last glacial maximum (LGM), approximately 21,000 years ago. Studies showed that during this period northern Europe from Scandinavia to Ireland was covered by an ice shield (Lang, 1994; Ruddiman & Thomson, 2001). Since climate is considered to be the most important parameter influencing species distribution on a large geographic scale (Willis & Whittaker, 2002; Pearson & Dawson, 2003), decreasing temperatures during the glacial probably caused strong shifts in species composition and distribution mainly on the northern hemisphere (Lang, 1994; Williams et al., 2001; Ohlemüller et al., 2012; Hejcman et al., 2013). Temperate species of flora and fauna retracted to lower geographic latitudes, known as refugia, which for Europe are postulated to be three refugia located on the peninsulas of Iberia, Italy and the Balkans

(Huntley & Webb, 1989; Bennett et al., 1991; Taberlet et al., 1998). Consequently, during the following postglacial warming period the recolonization of Central and north Europe is supposed to have originated from these locations (Harrison & Prentice, 2003; Hewitt, 2004; Corlett & Westcott, 2013). In addition to this latitude-dependent shift from north to south a second driving parameter influenced the migration routes of species. Often neglected in the discussion, a longitudinal gradient in form of a decreased amount of water availability was assumed during the LGM (Lowe & Walker, 1997; Stewart et al., 2010). Demonstrated in an average model by the Palaeoclimate Modelling Intercomparison Project (PMIP3, Braconnot et al., 2012) precipitation in Central Europe was lower than today. As a further consequence, a dropping sea level exposed new land masses between continental Europe and the British Isles and in the west of France, which could have increased a more continental climate in Central Europe. Therefore, temperate species that are adapted to an oceanic climate not only had to migrate southwards to the warmer refugia but also westwards to avoid the continental climate. A technique of predicting these possible suitable refugia areas of plants for past (e.g. glacial refugia), present or future times with climate data are species distribution models (SDM). In this study we used this method to survey the potential impact of the glacial climate on the oceanic / sub-mediterranean species *Hippocrepis comosa* and its predicted refugia. Within Maxent, a machine learning application we firstly calibrated a model containing actual distribution data of *Hippocrepis comosa* in combination with a set of today's climate parameters (Elith & Leathwick, 2009). Secondly, this model was used to process climate data prevailing during the last glacial maximum to predict suitable refugia. We assume that *H. comosa* shifted westwards in the glacial periods due to the lateral expansion of continental climate, and additional to its sub-mediterranean character also southwards.

3.2 MATERIAL AND METHODS

DATA COLLECTION AND PREPARATION

Information containing georeferenced occurrences of *Hippocrepis comosa* were downloaded from the Global Biodiversity Information Facility (GBIF, http://gbif.org). The total number of downloaded data was 17,934 with about 7,000 locations clustered in the northern half of France. Therefore and because of the fact that the data set showed a mixture of grid based data (mainly in Germany, France, Spain and UK) and pinpoint occurrences, an uniform raster was created with a point distance of 2.5 minutes in an unprojected coordinate reference system (WGS84) encompassing the total distribution area of the *Hippocrepis comosa*. With this approach sampling bias can be avoided (Wisz et al., 2008). The new distribution map was reduced to 2,794 points, 38 of them were additionally added from another study focusing on the same species (see Chapter 4). Geological parameters had to be excluded from our survey, as due to our grid based approach, the occurrence data of *H. comosa* would have been linked to incorrect edaphic values. Furthermore, to our knowledge, geological maps that describe the edaphic conditions during the LGM, especially in regard to current undersea areas, are not available.

To describe the climatic circumstances of the present age and the LGM (about 22,000 years ago) we used 19 bioclimatic variables (listed in appendix Table 3.1). The variables are derived from monthly mean temperature and precipitation and represent climatic annual trends, seasonality and extreme conditions. Provided as separate climate layers at WorldClim (http://worldclim.org, Version 1.4, release 3, Hijmans et al., 2005), data were downloaded as grid (raster) format. The resolution of the data was 2.5 minutes (WGS84, unprojected). The current conditions involve interpolation of observed data from 1950 to 2000. As climatic data for the Last Glacial Maximum conditions two different reconstructions were used: CCSM4 and MPI-ESM-P. All gathered from WorldClim the original data were provided by the Coupled Model Intercomparison Project (CMIP5). The resolution was 2.5 minutes (WGS84, unprojected). Since *Hippocrepis comosa* exclusively occurs in Europe, geographic data were reduced to the European region. To avoid geographic bias data were projected to an equal area projection (Europe Albers EAC). All GIS related work was done in ArcGis 10.2.2 (ESRI, Redlands, CA, USA).

SPECIES DISTRIBUTION MODELING

Ecological niche modeling and the subsequent creation of the geographic distribution maps of *Hippocrepis comosa* at present and past time was computed with the program Maxent, version 3.3.3 (Phillips et al., 2006; Phillips & Dudik, 2008). The Maxent software uses a maximum entropy algorithm which is well suited for species habitat modelling using presence-only data (Elith et al., 2006; Elith et al., 2011). Therefore, it is a proper method for predicting species distributions for both past and future orientated scenarios (Hijmans & Graham, 2006). In a first step we calibrated the model with the actual occurrence data of *Hippo-*

crepis comosa together with all current 19 bioclimatic variables. The resulting potential species distribution model was then projected onto the climate conditions prevailing during the last glacial maximum. To validate the informative value of the model regarding species distribution we used the area under the receiver operating characteristic (ROC) curve (AUC) (Fielding & Bell, 1997), which is an implemented validation routine within Maxent. The occurrence data were randomly partitioned into 2 groups: One group containing 75 % of the data was used for the model calibration, the remaining 25 % were used for model testing (Phillips et al., 2006). High AUC values (> 0.7) indicate a good model performance (Fielding & Bell, 1997). With following exceptions we used the default settings in Maxent (Phillips & Dudik, 2008). The convergence threshold was set to 10-5, the maximum number of iterations was 5,000 and 15 replicates with the replicated run type "subsample" were made. The selection of the relevant climate data was automated. As threshold rule we chose maximum test sensitivity and specificity (MTSS) to optimize the correct discrimination of presences and pseudoabsences in the test data (Hernandez et al., 2006; Jimenez-Valverde & Lobo, 2007). The continuous logistic output of Maxent was transformed in a binary presence-absence map. The threshold value for presence was based on MTSS values which were averaged over 15 runs.

3.3 RESULTS

The following three maps show the predicted geographic distribution of *H. comosa* for present time and the two different climatic assumptions (CCSM4 and MPI-ESM-P) for the LGM. The model for today's distribution of *H. comosa* (Figure 3.1) displays a good prediction of the reported locations. Aberrations can be a result of an imprecise sampling design, due to the fact that first we had to rasterize all data and second an unsteady participation of European countries in providing occurrence data of the investigated plant species. Also, geological aspects were not included in the model. If taken into consideration they would rule out areas with no occurrences of calcareous substrates like in north Germany and the Netherlands or silicate affected subsoils for example in France or the Czech Republic. Nevertheless, when the focus lies on climatic factors only, these regions also provide suitable climatic habitats for *H. comosa*.

Out of all 19 tested bioclimatic parameters, "Precipitation of Driest Quarter" (BIO17), had the highest influence on the prediction of suitable habitats of the actual occurrence of *H. comosa*, with a contribution to the model of 45 %. Together with "Temperature Annual Range" (BIO7, percent contribution: 36 %) and the "Isothermality" (BIO3, percent contribution: 8 %, Isothermality = Mean Diurnal Range / Temperature Annual Range * 100) ranked second and third the first 3 parameters contribute with 89 % to the final model.

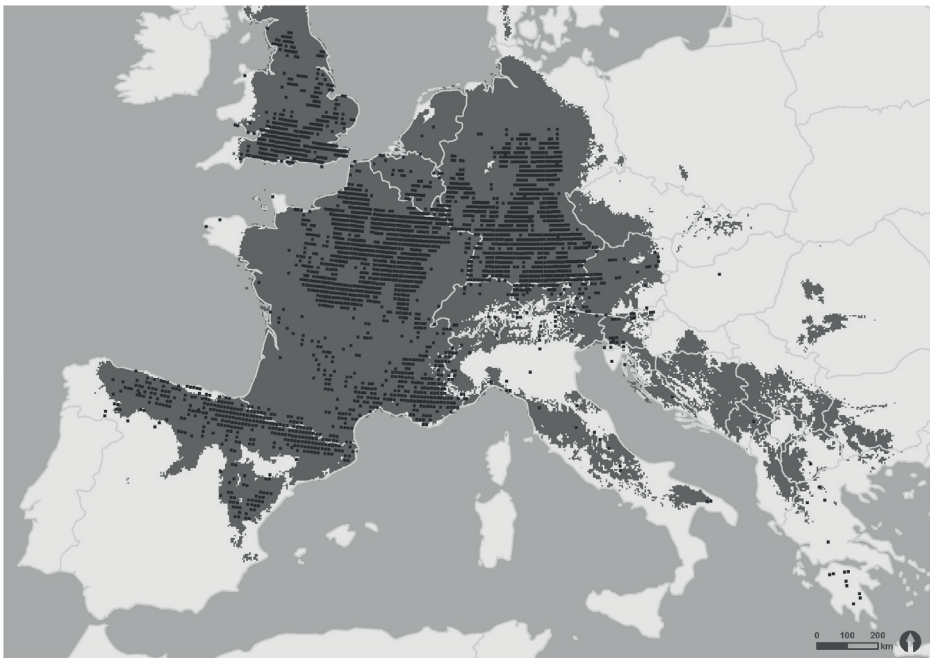


FIGURE 3.1 Distribution Model of *Hippocrepis comosa* in Europe based on current climate data. Dark grey areas indicate suitable habitats within the ecological niche, light grey area are unsuitable habitats for *H. comosa*. Black dots show the occurrence data gathered from GBIF and used for modelling. National boundaries are given.

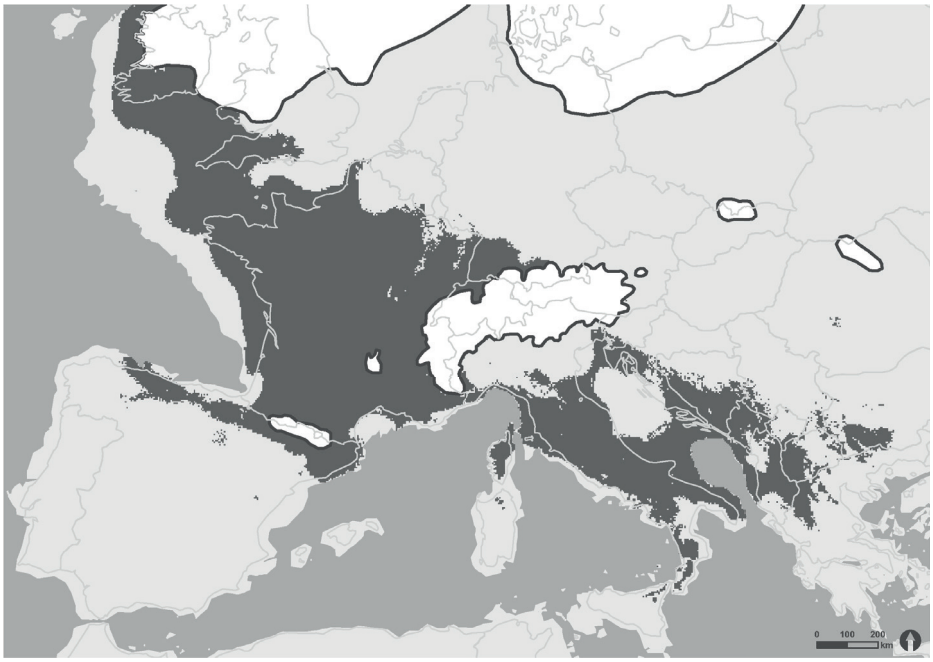


FIGURE 3.2 Species distribution model projection of *Hippocrepis comosa* at the LGM (Last Glacial Maximum, 21,000 ya) based on the output of the CCSM4 scenario. Dark grey areas indicate suitable habitats within the ecological niche, light grey area are unsuitable habitats for *H. comosa*. Ice shields are shown in white with a dark outline. National boundaries represent todays European land area.

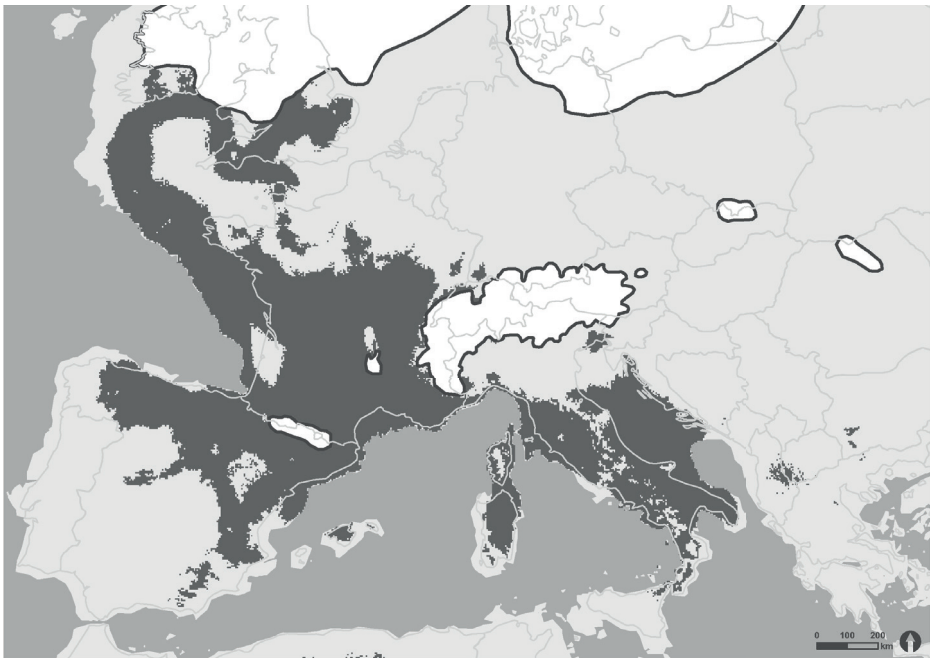


FIGURE 3.3 Species distribution model projection of *Hippocrepis comosa* at the LGM based on the output of the MPI-ESM-P scenario. Dark grey areas indicate suitable habitats within the ecological niche, light grey area are unsuitable habitats for *H. comosa*. Ice shields are shown in white with a dark outline. National boundaries represent todays European land area.

Figure 3.2 and Figure 3.3 show projected maps of the distribution model of *H. comosa* at the LGM (about 22,000 years ago). The first is based on climatic assumptions of the CCSM4 reconstruction, whereas the latter display is based on another climatic model MPI-ESM-P. Both models effected good performances with AUCs of 0.890 for CCSM4 and 0.889 for MPI-ESM-P. In both predictions of suitable climatic habitats for *H. comosa* similar distribution maps were computed. Separated by the Alps two major clusters could be distinguished: One situated in the west of Europe involving todays submersed lands west of France and the UK, France and the North of Spain and a second embracing Italy, the Adriatic Sea and parts of the Balkan Peninsula. Corresponding to the postulated refugia on the Peninsulas of Iberia, Italy and Balkan these regions formed suitable habitats for *H. comosa* during the LGM. In general, the model based on MPI-ESM-P data showed a stronger tendency of shifting suitable habitats towards the south and west than the CCSM4 model.

3.4 DISCUSSION

As the predicted present day distribution of *Hippocrepis comosa* showed a good match with the actual distribution, we considered the following SDM predictions suitable habitats during the Last Glacial Maximum as realistic. Our findings support the generally accepted assumption of northern temperate species outlasting the LGM in south located European refugia (Huntley & Birks, 1983; Bennett et al., 1991; Taberlet et al., 1998; Hewitt, 1999, 2000). Additionally, the results of the species distribution models suggest the existence of possible refugia in France and along the Atlantic coast up to the UK. Both models (CCSM4 and MPI-ESM-P) show differences in this area. While CCSM4 predicts suitable habitats for almost entire France and even parts of south west Germany (Figure 3.2) the MPI-ESM-P model draws the restriction further west (Figure 3.3). The reason lies most probably in the fact that both models make different assumptions regarding annual precipitation. While CCSM4 model only shows drier summer in central Spain and north Italy but not in Central Europe, the opposite conditions are predicted by the MPI-ESM-P model (PMIP3, Braconnot et al., 2012). Given that the main contributing parameter in our models was “Precipitation of Driest Quarter”, this assumption seems reasonable. Nevertheless, both SDM predicted vast areas of nowadays submerged land as suitable habitats for *Hippocrepis comosa* as a species adjusted to oceanic climate. The up to 110 m lower sea level (Ruddiman & Thomson, 2001) revealed ice-free land west of France and the UK of several hundred kilometres wide. Beyond climatic parameters, it is uncertain if this land masses provided the proper calcareous substrates for *H. comosa* and therefore could have served as refugia. Otherwise several studies including flora and fauna (Svenning et al., 2008; Ohlemüller et al., 2012; Boston et al., 2015) are supporting the existence of these north western potential refugia. It also remains open whether these two distinguished clusters of this work, the Italian-Balkan and the Iberian-Western Europe can withstand further investigation for example with molecular markers.

APPENDIX

TABLE 3.1 List of all climatic variables used to predict the climatic conditions in this study. The grid resolution was 2.5 min (http://worldclim.org).

	Description
BIO1	Annual Mean Temperature
BIO2	Mean Diurnal Range (Mean of monthly (max temp - min temp))
BIO3	Isothermality (BIO2/BIO7) (* 100)
BIO4	Temperature Seasonality (standard deviation *100)
BIO5	Max Temperature of Warmest Month
BIO6	Min Temperature of Coldest Month
BIO7	Temperature Annual Range (BIO5-BIO6)
BIO8	Mean Temperature of Wettest Quarter
BIO9	Mean Temperature of Driest Quarter
BIO10	Mean Temperature of Warmest Quarter
BIO11	Mean Temperature of Coldest Quarter
BIO12	Annual Precipitation
BIO13	Precipitation of Wettest Month
BIO14	Precipitation of Driest Month
BIO15	Precipitation Seasonality (Coefficient of Variation)
BIO16	Precipitation of Wettest Quarter
BIO17	Precipitation of Driest Quarter
BIO18	Precipitation of Warmest Quarter
BIO19	Precipitation of Coldest Quarter

CHAPTER 4

Insights into the European postglacial colonization of the horseshoe vetch (*Hippocrepis comosa*)

ABSTRACT

Calcareous grasslands belong to the most diverse, endangered habitats in Europe but there is still insufficient information about the origins of their plant species. In order to gain insights into the postglacial colonization of these habitats we exemplarily chose the Horseshoe vetch (*Hippocrepis comosa*). Based on an extensive AFLP analysis including 38 populations covering the species' total distribution in Europe, we describe the phylogeographic history of this plant species. We clearly demonstrate that *H. comosa* followed a latitudinal and due to its oceanity a longitudinal gradient during the Last Glacial Maximum (LGM), restricting the species to southern refugia situated on the Peninsulas of Iberia, the Balkans and Italy during the last glaciation. Furthermore, the analysis showed a distinct separation of these refugia into a western cluster embracing Iberia and an eastern group including the Balkans and Italy, which determined the postglacial recolonization of Central Europe. At the end of the LGM, *H. comosa* expanded from the Iberian refugium, to Central and Northern Europe, including the UK, Belgium and Germany. These findings remarkably match with migration routes of red deer (*Cervus elaphus*) to Central Europe. The existence of cryptic northern refugia cannot be ruled out but considering the missing genetic differentiation from other south-western populations is not directly supported that these refugia served as source populations after the LGM.

KEYWORDS

AFLP, genetic structure, grassland, phylogeography

4.1 INTRODUCTION

Calcareous grasslands are among the most species-rich ecosystems in Central Europe (Korneck et al., 1998; WallisDeVries et al., 2002; Sadlo et al., 2007) and are therefore listed as Natura 2000 Habitats (Directive 92/43/EEC). In order to understand the high species' diversity of this ecosystem and to guarantee its conservation numerous ecological studies have been performed (Cornish, 1954; Gibson & Brown, 1991; Dutoit & Alard, 1995; Poschlod & Bonn, 1998; Poschlod et al., 1998; Kahmen et al., 2002; Poschlod & WallisDeVries, 2002; Römermann et al., 2009). Notwithstanding these efforts, there is still a major gap about the origin of the associated species.

Calcareous grasslands are regarded as semi-natural landscapes (Karlik & Poschlod, 2009), created and maintained by human activities, more precisely by clearing and impeding the growth of forest, the potential natural vegetation (Poschlod & WallisDeVries, 2002; Kahmen & Poschlod, 2004; Schmidt et al., 2007). Likewise, the decline of their extent and qual-

ity since the end of the 19th century (Quinger et al., 1994) is ascribed to human behaviour, intensification or abandonment of traditional land use (Poschlod & WallisDeVries, 2002). There is a lack of archaeobotanic evidence for karst regions sediments, where stratified pollen are scarce (Bush, 1993; Dutoit et al., 2004; Poschlod & Baumann, 2010; Pokorný et al., 2015). Palynological and partly combined pedoanthracological studies reconstructed the history of calcareous grasslands, showing that man strongly shaped landscapes in Europe at least since the Mesolithic (c.f. Mesolithic: Bush (1993), Neolithic: Dutoit et al. (2009), Königsson (1968), Preece and Bridgland (1999), Bronze Age: Körber-Grohne and Wilmanns (1977), Ložek and Cílek (1995), Poschlod and Baumann (2010), Poschlod et al. (2008), Thorley (1981)). In Germany and in the Czech Republic, the extension maxima of grazed calcareous grasslands were dated to the Roman Period and Middle Ages (Pott, 1992; Knörzer, 1996; Poschlod & Baumann, 2010; Hajkova et al., 2011). These assumptions are based on charcoal deposits of woody indicator species for open grasslands like *Juniperus*, *Quercus* and *Pinus* (Schwartz et al., 2005; Dutoit et al., 2009; Poschlod & Baumann, 2010), macrofossil findings of recent calcareous grassland species e.g. *Lotus corniculatus*, *Sanguisorba minor* or *Linum catharticum* at a Neolithic lake shore sites in the Alps (Körber-Grohne, 1990) and the detection of pollen of *Juniperus* and *Helianthemum* in a peat in the Franconian Jura indicating species-rich grasslands in the Iron Age (Petrosino, 2004). *Helianthemum* pollen was also found in much older sediment in Yorkshire (England) dated to the early post-glacial, mutually with *Gentiana* and other species, suggesting the occurrence of cold or temperate grasslands (Bush, 1988). The latter study raises the question whether calcareous grasslands have already existed in Central Europe during the LGM or if they spread from glacial refugia due to climate warming. They may have existed as elements of the pleistocenic steppe-tundra vegetation during the glacial period, which is described for the periglacial zone in Central Europe (Pokorný, 2005; Kunes et al., 2008). Recent studies also described the occurrence of temperate tree species probably till 45°N latitude (Willis et al., 2000; Willis & van Andel, 2004; Magri et al., 2006; Kunes et al., 2008; Horsak et al., 2010; Tzedakis et al., 2013). In the traditional view, glacial refugia are known to be southern refugia for temperate species, plants, insects and vertebrates: The Iberian, Italian, Balkan peninsulas in southern Europe (Taberlet et al., 1998; Hewitt, 1999; Hewitt, 2000; Hewitt, 2004), where the influence of the glacial cycles was alleviated (Tzedakis et al., 2002) and species could escape from cold dry climates and persist until they could repopulate Europe in the interglacials and after the last glacial. Moreover, recent studies suggested several additional refugia for temperate species beyond these peninsulas. These cryptic northern refugia are postulated for higher latitudes than the expected southern refugia (Willis et al., 2000; Stewart & Lister, 2001; Willis & van Andel, 2004; Magri et al., 2006; Bhagwat & Willis, 2008; Bylebyl et al., 2008; Tzedakis et al., 2013) and are defined as climatic islands with favourable conditions (Stewart & Lister, 2001), surrounded by unsuitable conditions. In the case of calcareous grasslands this may have been rocky outcrops or steep sunny slopes with shallow dry soils (Ellenberg, 1988; Poschlod et al., 2009) in deeply incised valleys providing microclimates for temperate species, allowing to survive where they normally would have perished (Stewart & Lister, 2001; Flojgaard et al., 2009). As soon as climate became warmer in the post-glacial, recolonization of the surrounding steppe-tundra vegetation may have started from there. In this context it must be mentioned that the following reforestation might have been held back before the Neolithic (see Bush, 1988), either by man (fire) or by megaherbivores enlarging the potential habitats of calcareous grassland species besides naturally treeless sites like cliffs (Svenning, 2002; Pokorný et al., 2015). Consequently, both wild animals and man with domesticated animals may have contributed to species' ranges and contributions.

Stewart et al. (2010) postulated a longitudinal oceanic-continental gradient that is often ignored when speculating about recolonization of species along the latitudinal axis. The longitudinal gradient explains the expansion of steppe species and their inclusion in the Late Pleistocene steppe-tundra biome. Accordingly, the occurrence of current post-glacial steppe species is limited to eastern continental interglacial refugia which are determined by the longitudinal gradient. Occurrences in the West could therefore be interpreted as cryptic refugia (compare Kunes et al., 2008; Schmitt & Varga, 2012). On the other hand there should be counterpart examples for oceanic species, since extension of arid climates during the late Pleistocene would have been an impediment to some taxa likewise cold climates. The ranges of oceanic species would have expanded during the moister interglacials and contracted to Western Europe during the glacial periods. Together with the latitudinal gradient both work in tandem in defining suitable habitats of a species (Stewart et al., 2010).

In order to gain insight in the historic distribution and migration routes of a calcareous grassland species with a sub-mediterranean and oceanic requirement, we exemplarily chose *Hippocrepis co-*

mosa. According to Schmidt et al. (2007) the species occurs primary in natural habitats and secondary in semi-natural habitats. It also occurs in recent and ancient grasslands (Karlik & Poschlod, 2009) and seed exchange by grazing was shown possible (Müller-Schneider, 1938).

The native range of *H. comosa* covers middle and south European dry or semi-dry basi- and calciphilous grasslands or rocky cliffs (Brometalia erecti) and spring heath pine woods (Ericio-Pinetum) or alpine calcareous grasslands (Seslerietalia albicantis). Since human activities have contributed to distribution patterns at least since the early Neolithic which has been shown for crops, weeds and animals (Willerding, 1986; Poschlod & Bonn, 1998; Rosch, 1998; Beebee & Rowe, 2000; Fjellheim et al., 2006) it might be also true for grassland plants and for the horseshoe vetch. The occurrence of *H. comosa* was first time documented for the Roman age in the lower Rhine Valley (Knörzer, 1996). Therefore, the question is whether or not the species came to Central Europe via Roman settlers. As Mediterranean species there is also the possibility of spreading from Iberian or Balkan Peninsula. Exemplarily Poschlod (2017) claims the migration of dry grassland species from the Eastern Mediterranean region or south east Europe through the migration of the first farmers of the linear ware ceramic culture (LBK) to Central Europe or from Western Europe through the La Hoguette culture. It is not very likely that dry grassland species such as *H. comosa* was an element of the steppe-tundra flora during the glacial or late-glacial considering its longitudinal distribution as an oceanic element. Therefore, we assume either Western or Southern refugia but also considering glacial cryptic refugia in Central Europe. We also considered human activities that have contributed to distribution patterns, which was shown before for domestic plants, animals (Rosch, 1998; Beebee & Rowe, 2000) and weeds (Poschlod & Bonn, 1998).

In this paper we investigated *H. comosa* populations from Western Europe to Eastern Europe using AFLP markers to gain information about glacial refugia and recolonization routes of a typical calcareous grassland species to Central Europe.

4.2 MATERIAL AND METHODS

SAMPLING OF PLANT MATERIAL

The sampling of the plant material of *Hippocrepis comosa* was done throughout the species range distribution on the European continent and embraced in total 588 individuals from 38 populations (Figure 4.1, Table 4.1). Each sample encompassed multiple fresh and healthy leaves which were dried in silica gel.

TABLE 4.1 Summary of the locations of all sampled populations. Pop ID = Population identifier, longitudinal and latitudinal coordinates are given as decimal coordinates (WGS84).

Pop ID	Sample ID	Latitude	Longitude	Country
1	97	-5.945	42.932	Spain
2	99	-4.414	43.398	Spain
3	103	-3.485	41.977	Spain
4	101	-3.404	42.796	Spain
5	105	0.613	42.096	Spain
6	38	-0.107	50.901	United Kingdom
7	107	-0.399	44.762	France
8	77	1.010	49.321	France
9	32	2.182	42.875	France
10	57	4.339	44.972	France
11	62	4.609	44.802	France
12	45	4.778	50.298	Belgium
13	65	7.427	47.409	Switzerland
14	71	8.882	45.961	Switzerland
15	46	7.385	45.636	Italy
16	48	7.750	45.423	Italy
17	64	8.749	44.511	Italy
18	58	9.527	44.256	Italy
19	59	10.763	43.745	Italy
20	60	10.791	45.556	Italy
21	61	10.836	46.237	Italy
22	54	12.274	43.117	Italy
23	63	12.337	43.795	Italy
24	49	13.021	42.956	Italy
25	56	13.238	42.745	Italy
26	35	9.191	51.479	Germany
27	55	10.143	50.226	Germany
28	39	10.251	47.288	Germany
29	31	10.252	47.375	Germany
30	52	10.415	49.524	Germany
31	108	11.686	48.951	Germany
32	70	11.720	51.216	Germany
33	113	13.119	48.651	Germany
34	40	13.882	45.100	Croatia
35	37	16.368	43.894	Croatia
36	36	13.999	45.724	Slovenia
37	83	18.945	47.467	Hungary
38	34	21.655	41.368	Rep. of Macedonia

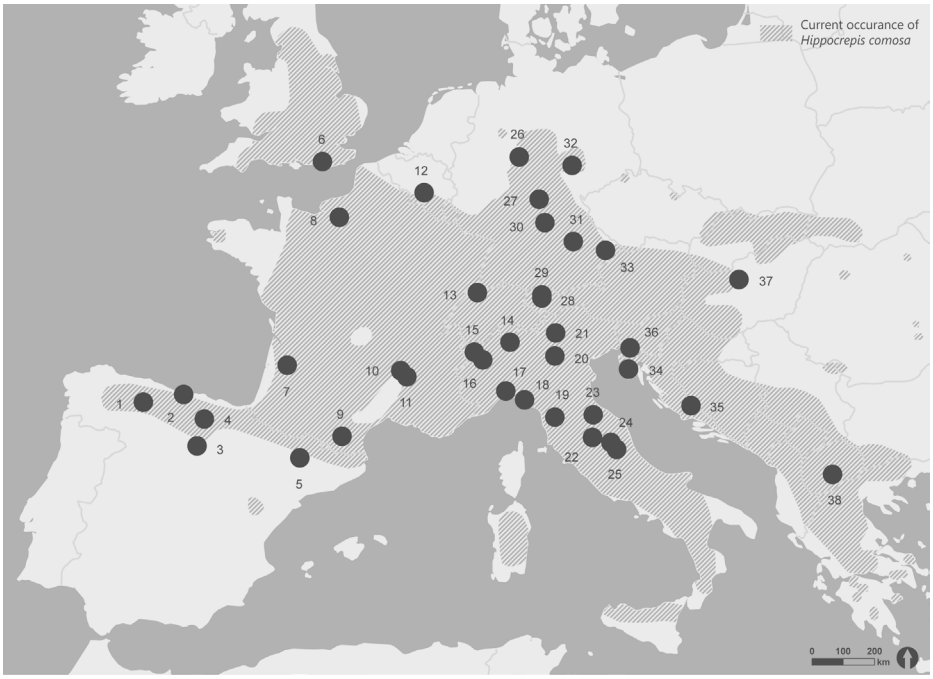


FIGURE 4.1 Distribution map of *Hippocrepis comosa* with all sampled populations.

DNA EXTRACTION AND AFLP FINGERPRINTING

DNA extraction followed CTAP protocol from Rogers and Bendich (1994) adapted by Reisch (2007) using 15 mg of the dried leaf samples. DNA contents were photometrically determined and adjusted to 7.8 ng DNA per µl H_2O. We chose the dominant marker analysis of amplified fragment length polymorphism (AFLP, Zabeau & Vos, 1993; Vos et al., 1995) to produce loci over the whole genome (standardized AFLP-protocol from Beckmann Coulter (Brea, USA)). DNA adapters from single strand DNA adapters EcoRI and MseI (MWG Biotech, Ebersberg, Germany) were combined by heating equal volumes to 95 °C for 5 min, following a 10 min cooling period at 20 °C. For a one step DNA restriction and adaptor ligation 6.4 µl adjusted DNA solution were intermingled with 3.6 µl of a restriction ligation mixture containing 2.5 U EcoRI (MBI Fermentas, St. Leon-Rot, Germany), 2.5 U MseI (MWG Biotech), 0.1 µM EcoRI and 1 µM MseI adapter pair, 0.5 U T4 Ligase with corresponding buffer (MBI Fermentas), 0.05 M NaCl and 0.5 µg BSA (New England BioLabs, Ipswich,USA). After incubation at 37 °C for 2 hours and a final enzyme termination step at 70 °C for 15 min the received products were diluted 10-fold with 1x TE buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0). 1 µl of the restriction-ligation product was used for pre-selective amplification. The reaction solution (4 µl) contained: pre-selective 0.25 U EcoRI and MseI primers (MWG Biotech), H_2O, 1x Buffer S, 0.2 mM dNTPs and 0.25 U Taq-Polymerase (PeqLab, Erlangen, Germany). PCR was run with following parameters: initial 2 min at 94 °C, 30 repeats of: 1. denaturation for 0.3 min at 94 °C, 2. annealing for 0.5 min at 56 °C, 3. elongation for 2 min at 72 °C and extra 2 min at 72 °C after finishing the cycles. 30 min at 60 °C and a cool down to 4 °C completed the procedure. The received products were diluted 20-fold with 1x TE buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0). For the selective DNA amplification we chose three pairs of primer: D2 CTA-AAC (EcoRI-MseI), D3 CAC-AGG, D4 CAC-ACA. 3 PCR one per primer pair were carried out with 0.75 µl of the pre-selective amplification product, and in total 4.25 µl of H_2O, 0.25 µM selective MseI (MWG Biotech) primer, 0.05 µM selective labelled EcoRi primer (Proligo, Paris, France), 10x Buffer S, 0.2 mM dNTP and 0.25 U Taq-Polymerase (PeqLab, Erlangen, Germany). PCR was run with following parameters: initial 2 min at 94 °C, 10 repeats of: 1. denaturation for 0.3 min at 94 °C, 2. annealing for 0.5 min at 66 °C and 3. elongation at 72 °C for 2 min. After a repeat was finished the annealing temperature was lowered by 1 °C. 25 repeats of: 1. denaturation for 0.3 min at 94 °C, 2. annealing for 0.5 min at 56 °C and 3. elongation for 2 min at 72 °C. The PCR ended with 30 min at 60 °C and a cool down to 4 °C. The products were diluted differently with 1x TE buffer (D2 1:2, D3 1:1, D4 1:3). 5 µl of each primer pair were pooled and mixed with 5 µl stop buffer (2 µl sodium acetate (3 M, pH 5.2), 2 µl Na_2EDTA (100 mM, pH 8) and 1 µl glycogen (20 mgml-1 Roche, Manheim, Germany)). DNA was precipitated and aggregated by adding 60 µl of 96 % EtOH (4 °C) and 20 min of centrifugation at 14k rpm. The DNA pellet was washed with 200 µl of 76 % eth-

anol (4 °C), centrifuged (20 min, 14k rpm) and vacuum-dried in a concentrator (Eppendorf concentrator 5301; Eppendorf, Hamburg, Germany). Prior to the sequencer run (CEQ 8000 (Beckmann Coulter, USA)), the DNA was resolved for 30 min in a mixture of 24.8 µl Sample Loading Solution (Beckmann Coulter, USA) and 0.2 µl CEQ Size Standard 400 (Beckmann Coulter, USA). Data were exported as curve-files and manually analysed for the occurrence of strong, well defined fragments in Bionumerics 6.6 (Applied Maths, Kortrijk, Belgium). The presence or absence of fragments was transformed into a binary (1-0) matrix, which served as basis for all further analysis. Individuals showing no clear banding signals were repeated or ultimately excluded.

GENETIC VARIATION

Based on the allele frequencies from the 0/1 matrix Nei's Gene Diversity (Nei, 1972), Shannon's Information Index (Shannon 1948), the number and percentage of polymorphic loci were calculated for each population using POPGENE v. 1.31 (Yeh et al., 1999). In order to make the results Nei's gene diversity more comparable an additional calculation was conducted at which the number of tested individuals was set to 12 for each population (lowest available amount). The 12 samples were chosen randomly with 50,000 iterations and a mean value was calculated. The results were plotted onto the geographic coordinates of the sample locations. A base map provided by "Natural Earth" served as background for this and all following maps.

RARITY VALUES (DW)

As an additional measure of divergence, the rarity of markers was calculated by frequency-down-weighted marker values (DW) (Schönswetter & Tribsch, 2005). The calculation of the DW values was performed via the r-script AFLPdat (Ehrich, 2006). To grant equal sample sizes, for each population 12 individuals were randomly selected with 10 iterations and a mean value of DW was calculated. The results were plotted onto the geographic coordinates of the sample locations. The value of DW is expected to be high in long-term isolated populations where rare markers should accumulate due to mutations, whereas newly established populations are expected to exhibit low values, thus helping in distinguishing old vicariance from recent dispersal (Schönswetter & Tribsch, 2005). We calculated a Pearson correlation coefficient for Nei´s Gene Diversity and DW value.

AMOVA

An analysis of molecular variance (AMOVA, Excoffier et al., 1992) should give information about the genetic variance within and between populations. The two-level AMOVA was performed within the program GENALEX v6.5 (Peakall & Smouse, 2012) and included 588 individuals of all 38 populations. Based on Euclidean pairwise genetic distances the sums of squares were calculated (SSWP) and divided by the degrees of freedom (SSWP/n-1). The resulting AMOVA-SS diversity values were also plotted onto the geographic coordinates of the sample locations. Permutation tests (9999 iterations) were conducted to show significance.

STRUCTURE

The genetic structure and group assignment of the populations was investigated with Bayesian clustering in STRUCTURE v 2.3 (Pritchard et al., 2000; Pritchard et al., 2009). The program performs a Markov chain Monte Carlo (MCMC) algorithm to assign the tested individuals into k groups based only on its genetic data, and not on population affiliation. The program was run with following parameters: no admixture ancestry model, correlated allele frequency model, k from 2 to 40, a burn-in period of 10,000 followed by 10,000 iterations, 10 replicate runs. The most likely number of groups in the data set was determined via the calculation of Δk following the method of Evanno et al. (2005). The results were plotted onto the geographic coordinates of the sample locations.

SPATIAL PCA (SPCA)

To identify spatial genetic patterns within the data set a multivariate approach was conducted using spatial Principal Component Analysis (sPCA). sPCA was carried out in R (Development Core Team, 2014) using package adegenet (Jombart et al., 2008). For this analysis, all 588 individuals of 38 populations were used. The populations' geographic coordinates (WGS 1984) were projected to ETRS 1989 LCC. To avoid same coordinates of individuals in the same population the coordinates were shifted randomly by a factor of 0.5. Unlike the analysis with STRUCTURE, the data for a sPCA does not have to meet Hardy-Weinberg expectations or linkage equilibrium. For the method, two matrices are necessary. The first one contains the relative allele frequencies of all individuals and the second embraces all spatial proximity information from the projected coordinates. The spatial proximity information matrix was gained from a connection network using Delaunay triangulation. Also, the second matrix was used to calculate a spatial autocorrelation

using Moran's I (Moran, 1948). Moran's I ranges from +1 to -1, indicating a strong positive or negative spatial autocorrelation, respectively. In case of a positive spatial autocorrelation a global structure in the data can be assumed. A Moran's I of zero indicates a totally random pattern. For a visual verification of the occurrence of spatial structures, a screeplot was drawn by plotting the variance of the sPCA against spatial autocorrelation (Moran's I). Supplementary, to statistically strengthen the previous visual findings two Monte Carlo tests with 9,999 permutations each were conducted in order to detect global or local structures in the data set. As display, the genetic differentiation of the principal components were plotted onto the geographic coordinates.

4.3 RESULTS

Within the AFLP analysis of 588 individuals with three primer combinations, 271 unambiguous fragments were selected ranging between 60 and 420 bp and of which 98.16 % were polymorphic (D2 CTA-AAC: 111 fragments, D3 CAC-AGG: 87 fragments, D4 CAC-ACA: 73 fragments).

GENETIC VARIATION

The within population genetic variation was calculated as four different measures (Table 4.2). All genetic variation values were consistently lowest in population no. 11 in France and highest in population no. 29 in Germany. Mean percentage of polymorphic loci (PPL) was 56.7 %, ranging between 46.3 and 64.7. Mean Shannon´s information index (SI) was 0.28, ranging between 0.21 and 0.32. Mean Nei's gene diversity yielded for all individuals was 0.18, ranging from 0.14 to 0.21. Mean Nei's gene diversity for 12 individuals was 0.18, ranging from 0.13 to 0.21. Higher values for genetic variation (He ≥ 0.19) were recorded only in southern populations, like the Iberian (He = 0.20 and 0.21), the Italian (He =0.19), the Balkan Peninsula (He = 0.20), south of the Alps (population no. 14, He = 0.20), except from one population in the northern Alps (population no. 29, He = 0.21) and north of the Alps in Germany (population no. 31). Lower values (He ≤ 0.16) only occurred in northern populations and on the Italian peninsula, but not in the two other peninsulas (Figure 4.2).

RARITY VALUES (DW)

The survey of the rarity of markers revealed DW-values ranging from 4.83 in an Italian population (no. 10) to 10.50 in a Spanish population (no. 2), with an average of 7.66 (SE = 0.20) (see Table 4.2). The highest values were recorded either in populations of the Iberian Peninsula (values between 8.35 and 10.50), the Balkan Peninsula (values between 8.89 and 9.89) or in some northern populations, in central Germany (DW = 10.30), Bavarian Alps (DW = 8.84 and 8.36) and the United Kingdom (DW = 8.05). Low values occurred in northern populations and on the Italian, but not on the Iberian and Balkan peninsulas (Figure 4.2). There were highly significant positive correlations be-

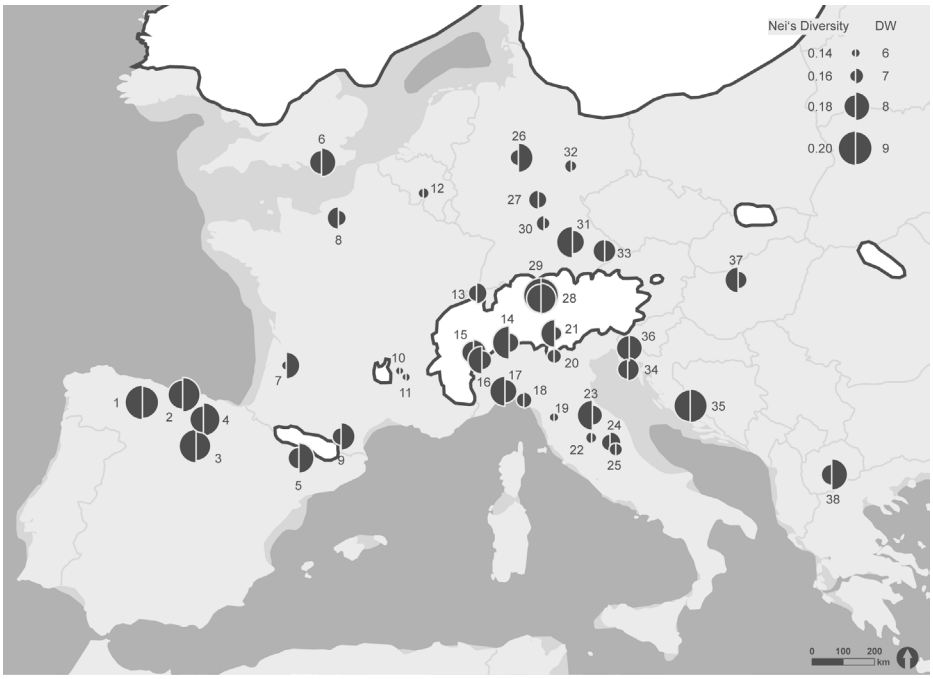


FIGURE 4.2 Map of Nei's gene diversity (left semicircle) and frequency-down-weighted marker values (DW, right semicircle) for each surveyed population. The different sizes of the circles indicate different absolute values. Ice shields are shown in white with a dark outline. National boundaries represent todays European land area.

TABLE 4.2 Genetic variation within populations of *Hippocrepis comosa*. Pop ID = Population identifier, CRY = Country of origin, N = Sample size, PL = Number of polymorphic loci, PPL = Percentage of polymorphic loci, He = Nei's gene diversity (with standard error), He_{12} = Nei's gene diversity for 12 randomly chosen individuals (with standard error), SI = Shannon-Index (with standard error), rarity value (DW_{12}), AMOVA-SS (SSWP/n-1). Total number of sampled individuals: 588, total number of loci: 271.

Pop ID	CRY	N	PL	PPL	He	He_{12}	SI	DW_{12}	SSWP/n-1
1	ES	14	171	62.9	0.21	0.208	0.32	8.965	29.95
2	ES	16	165	60.7	0.20	0.189	0.30	10.453	28.28
3	ES	15	171	62.9	0.21	0.201	0.31	8.531	30.94
4	ES	16	159	58.5	0.19	0.183	0.29	9.131	27.27
5	ES	15	159	58.5	0.18	0.176	0.27	8.842	27.40
6	UK	15	152	55.9	0.19	0.182	0.28	8.055	25.79
7	FR	15	139	51.1	0.16	0.153	0.24	7.906	22.50
8	FR	16	150	55.2	0.18	0.176	0.28	6.981	26.68
9	FR	16	153	56.3	0.18	0.173	0.27	7.944	26.35
10	FR	16	134	49.3	0.14	0.137	0.22	4.831	20.96
11	FR	16	126	46.3	0.14	0.132	0.21	4.907	20.21
12	BE	15	133	48.9	0.16	0.154	0.24	6.727	22.56
13	CH	16	152	55.9	0.18	0.171	0.27	7.484	26.81
14	CH	15	164	60.3	0.21	0.200	0.31	7.337	28.66
15	IT	16	155	57.0	0.19	0.181	0.28	7.782	27.13
16	IT	16	160	58.8	0.19	0.186	0.29	7.297	27.28
17	IT	16	174	64.0	0.20	0.195	0.31	7.760	30.28
18	IT	16	144	52.9	0.17	0.164	0.26	7.046	23.66
19	IT	16	134	49.3	0.16	0.153	0.24	6.153	22.82
20	IT	16	138	50.7	0.17	0.164	0.25	6.853	24.88
21	IT	16	159	58.5	0.19	0.185	0.29	6.829	27.07
22	IT	16	140	51.5	0.17	0.163	0.25	6.590	22.90
23	IT	16	163	59.9	0.20	0.194	0.30	7.379	28.75
24	IT	16	148	54.4	0.18	0.171	0.26	7.566	27.50
25	IT	16	159	58.5	0.17	0.163	0.26	6.762	26.05
26	DE	16	143	52.6	0.18	0.171	0.27	8.361	26.11
27	DE	16	156	57.4	0.18	0.174	0.27	7.096	27.99
28	DE	15	162	59.6	0.20	0.189	0.29	8.835	28.42
29	DE	16	176	64.7	0.21	0.206	0.32	10.339	31.96
30	DE	16	146	53.7	0.17	0.167	0.26	6.743	25.19
31	DE	16	174	64.0	0.20	0.197	0.31	7.775	30.55
32	DE	16	159	58.5	0.17	0.163	0.26	6.665	26.10
33	DE	12	147	54.0	0.18	0.181	0.27	7.578	27.79
34	HR	15	159	58.5	0.18	0.174	0.27	7.663	26.16
35	HR	14	164	60.3	0.20	0.198	0.31	9.890	27.62
36	SL	15	165	60.7	0.19	0.182	0.29	7.880	27.90
37	HU	13	153	56.3	0.19	0.183	0.28	7.180	27.67
38	MK	16	154	56.6	0.18	0.175	0.27	8.888	25.96
	mean	15.5	154	56.7	0.18 ±0.01	0.177 ±0.01	0.28 ±0.02	7.658 ±0.200	26.63 ±0.43

tween DW and all genetic variation measures ($r = 0.73$, $p < 0.0001$, $t = 6.3977$), meaning that a high number of rare markers (high DW) was associated with high genetic variation.

AMOVA

The analysis of molecular variance (AMOVA) involving all populations without classification of regions revealed a total molecular variance within the populations of 68 %. This leaves a strong differentiation among the populations with a molecular variance of 32 % (Figure 4.3). The results were highly significant ($p < 0.001$). All AMOVA-SS values are given in Table 4.2.

STRUCTURE

The result of the Bayesian clustering conducted with STRUCTURE supports a clear assignment of the individuals into two groups ($k = 2$, appendix Figure 4.6). Besides this most likely number of clusters a very small probability for 13 groups was found. Likewise,

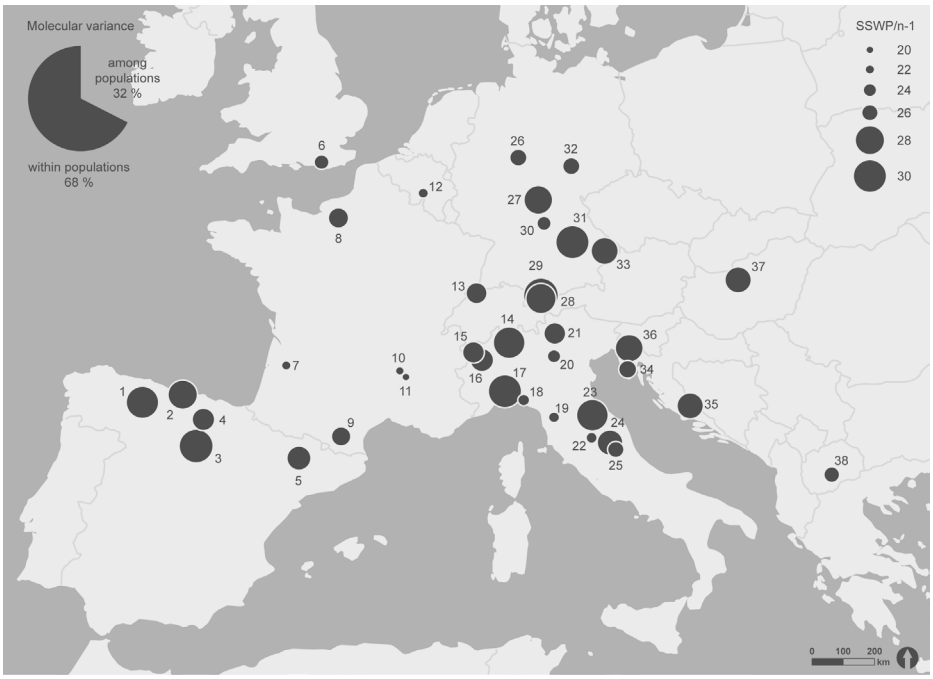


FIGURE 4.3 Map of AMOVA-SS values for each surveyed population. The different sizes of the circles indicate different absolute values of molecular variance.

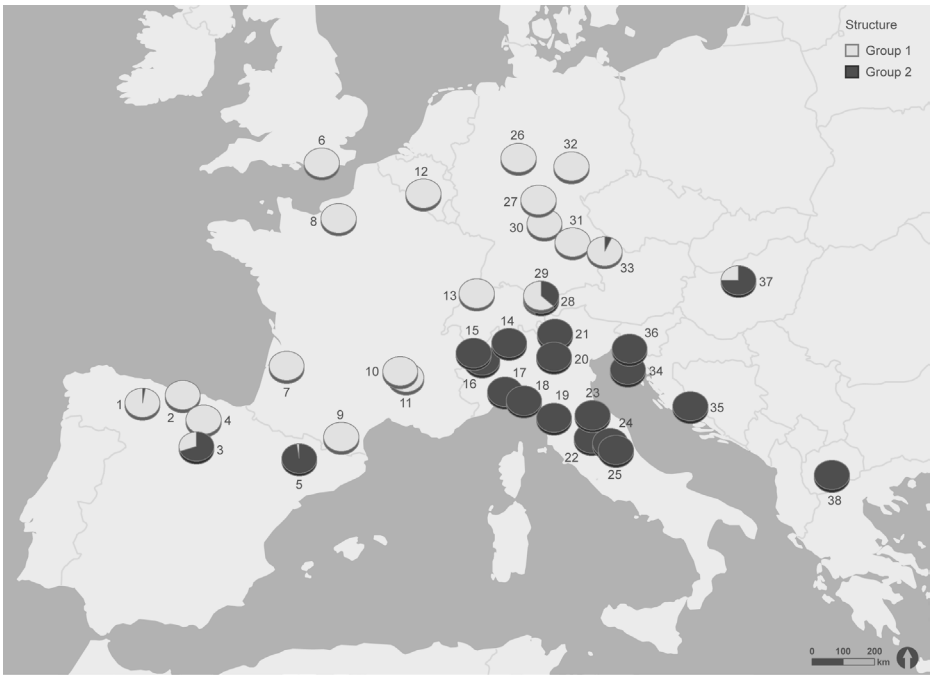


FIGURE 4.4 Results from STRUCTURE analysis plotted onto geographic coordinates of the surveyed populations of *Hippocrepis comosa*. As STRUCTURE proposed a two-group solution, each population was assigned according its associated group.

the results of the sPCA the assignment of the populations into two groups revealed a similar pattern. One group involving populations of western and central Europe (Spain, France, United Kingdom, Belgium, Switzerland, Germany) and a south eastern European group (Italy, Slovenia, Croatia, Macedonia). Only at the boundary points of the two groups in Spain (no. 3 and 5), Germany (no. 28, 29 and 33) and Hungary (no. 37) the 100 % assignment into one of the groups was diluted, resulting in some admixed populations (Figure 4.4).

SPATIAL PCA (sPCA)

All 588 individuals were included in the spatial analysis. The spatial PCA based on Delaunay triangulation as connection network. The eigenvalues of the sPCA in dependence of its Moran's I and variance are shown in a screeplot (appendix Figure 4.7). The eigenvalue of the first global score λ1 could clearly be distinguished from all other eigenvalues due to its higher levels of variance and spatial autocorrelation. This indicates the existence of spatial structures in the data, which was subsequently tested with global and local Monte Carlo tests (9,999 iterations). As the global test showed a significant ($p < 0.0001$) result and with the screeplot in mind a global spatial structure was assumed for the data set. The local Monte Carlo test was not significant.

Figure 4.5 shows the eigenvalues of the first global score plotted against geographical coordinates. Black squares indicate positive and white squares negative values of the scores. The size of the squares represents different absolute values. Therefore, large sized squares from both colours are highly differentiated, while small sized squares indicate only small differentiation. Regarding Figure 4.5 a clear distinction between two clusters can be drawn. One involving populations of Western and Central Europe (Spain, France, United Kingdom, Belgium, Switzerland, Germany) and the other one including populations from south Eastern Europe (Italy, Hungary, Slovenia, Croatia, Macedonia). A Monte-Carlo Mantel test (10,000 iterations) for a correlation between geographic and genetic distances was highly significant ($p < 0.0001$).

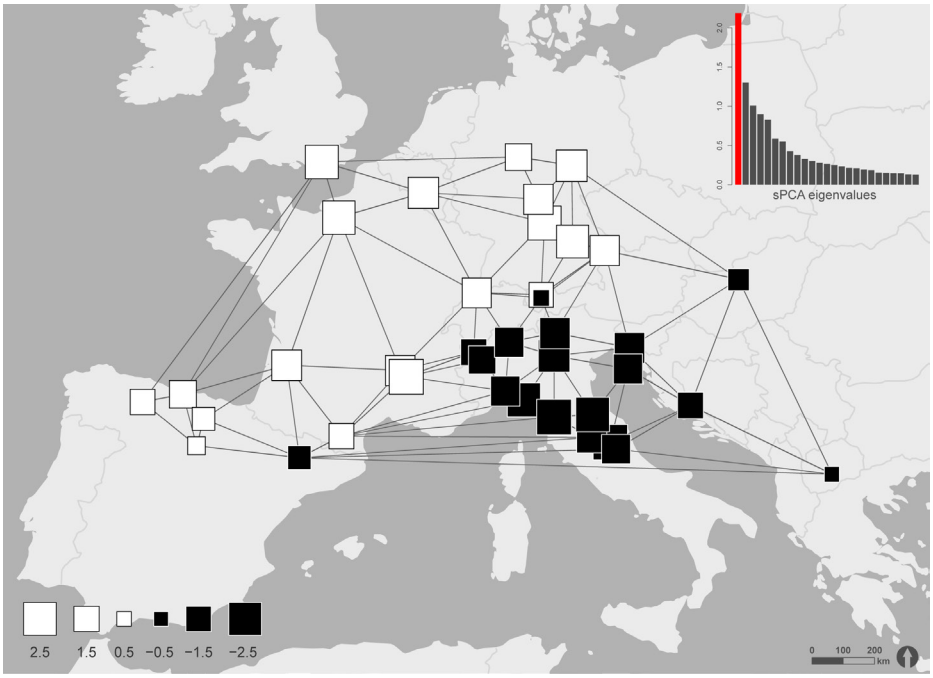


FIGURE 4.5 Graphical display of the spatial distribution of all surveyed populations with the values of the first positive (global) sPCA score. The different sizes of the squares indicate different absolute values. Large black squares are well differentiated from large white ones, while small squares show less differentiation. On the map, the genotypes differentiate in 2 distinct clusters, one in the in the northwest and one in the southeast. The used connection network based on Delaunay triangulation is shown with grey lines. On the top right position of the map, the first 25 sPCA positive scores are shown.

4.4 DISCUSSION

AFLP analysis is a powerful tool in revealing phylogeographic patterns and histories, as we show here on an example of the semi-dry grassland species *Hippocrepis comosa*. Based on 271 fragments, we assume that *Hippocrepis comosa* followed a contraction-expansion model, whereby the species was restricted to traditional southern refugia during the LGM following a latitudinal temperature and a longitudinal humidity gradient and expanded into the rest of Europe afterwards. Our results indicate additional cryptic refugia at the western shores of France and UK.

EASTERN AND WESTERN LINEAGES AND POSTGLACIAL MIGRATION

Both spatial analyses (STRUCUTRE and sPCA) identified two almost completely distinct lineages of *Hippocrepis comosa*: One group involving populations of the Iberian peninsula and western Europe (Spain, France, United Kingdom, Belgium, Switzerland, Germany) and the other including populations from the Italian and the Balkan peninsulas (Italy, Croatia, The Former Yugoslav Republic of Macedonia, Slovenia). Only a few admixed populations were detected, located in Hungary, Germany and Spain. According to these spatial results two traditional southern refugia (Taberlet et al., 1998; Hewitt, 1999; Hewitt, 2000; Hewitt, 2004) could be confirmed: Iberia as a south western refugia and Italy and the Balkans as south eastern refugia, enclosing the admixed populations in Spain, South Germany and Hungary as contact zones. Contact zones have been reported to cluster in the Alps, central Europe, northern Balkans and the Pyrenees (Taberlet et al., 1998; Hewitt, 2000), resulting in an accumulation of genotypes from both groups. Therefore, we assume that *Hippocrepis comosa* repopulated Central Europe from Iberia (Pyrenees) to France, Britain, Belgium, Switzerland, and Germany until its eastern border, where the populations admixed with populations that were migrating from Italy and the Balkans up north. It has been shown before that in contrast to Iberian lineages, Italian genomes rarely populated northern Europe, as the ice-capped Alps prevented their northward expansion (Taberlet et al., 1998; Hewitt, 2000). This barrier is regarded as explanation of the relatively low species' and genetic diversity of northern populations compared to southern populations (Hewitt, 2000). Considering the values for Nei's gene diversity and the rarity index (DW)-values, *H. comosa* populations of the Balkan Peninsula may have served as refugium from where the Italian populations were founded subsequently.

SOUTHERN AND CRYPTIC NORTHERN REFUGIA

The most important refugial areas are geographic regions where species persisted throughout several full glacial/interglacial cycles (each 100-120 kyr in duration). These so-called 'true refugia' (Stewart & Dalen, 2008) are expected to possess higher genetic variability compared with surrounding recolonized regions (Comes & Kadereit, 1998; Taberlet et al., 1998; Tzedakis et al., 2013). In contrast, recent dispersal might lead to genetic depauperation due to founder effects. Supporting this theory, the highest values for genetic variation of *Hippocrepis comosa* were recorded almost entirely in southern populations, like the Iberian, the Italian, the Balkan Peninsula and south of the Alps. Other above-average genetically diverse populations were located in the Alps and in Germany. The former can be ascribed to the above-mentioned hybridization of the western and the south-eastern lineage (Petit et al., 2003; Provan & Bennett, 2008; Tzedakis et al., 2013). We found different explanations for the high genetic variation of the latter. Firstly, it may be the result of recent genetic exchange due to grazing management, which is more prevalent in the area of the Jurassic mountains in Germany than in the northern German populations. Paun et al. (2008) criticized the use of genetic variation for identification of refugia as they may in fact reflect current processes (genetic exchange and population sizes) instead of historical processes. Secondly, the high genetic variations may also have resulted from a phalanx way of recolonization from south to north or can be interpreted as a legacy from Younger Dryas cold reversal (Hewitt, 1999; Hewitt, 2000; Hewitt, 2004). A reduction of northern populations during this cold period could have led to high diversity populations due to the mixture with recolonizing linages during the Holocene (Tzedakis et al., 2013). In order to circumvent these confusions, a second parameter, the rarity index (DW) was used, as it is a better indicator of historical processes (Paun et al., 2008). The value of DW is expected to be high in long-term isolated populations where rare fragments could accumulate due to mutations, whereas young populations are expected to show low values, thus helping in distinguishing old vicariance from recent dispersal. Refugial populations and recolonized regions would on the one hand contain identical fragments but due to drift rare fragments would accumulate and distinguish them from other refugial areas and surrounding younger populations that would be less divergent (Schönswetter & Tribsch, 2005; Provan & Bennett, 2008; Tzedakis et al., 2013). According to this, we identified additional northern populations of *Hippocrepis comosa* that

are located far from the traditional southern refugia which possessed high DW-values, supposing cryptic refugia in UK, Alps and Central Germany. Though it must not be ignored that the rarity index might be overestimated, as related southern populations with these alleles may not have been investigated or distinct gene patches might result by gene surfing on the leading edge (Tzedakis et al., 2013). Nevertheless, we found a significant correlation of the rarity index (DW) and Nei's gene diversity (He), which means that in our study rare fragments accumulation and high genetic diversity came along with each other, pointing towards 'true refugia'. The German population with a very high rarity index and relatively low genetic variation may indicate an isolated refugia during the LGM, similar to alpine populations of *Ranunculus glacialis* (Paun et al., 2008). These results also coincide with Dengler et al. (2014), who proposed a continuous existence of palearctic grassland at least since the Pleistocene (2,400 ka). During glaciations, grasslands covered most of the continent as steppe-tundra over permafrost and as xerothermic grassland further in the south. During the interglacials (Lang, 1994; Pärtel et al., 2005) grasslands were mostly replaced by forests, apart from small scale areas on soils that impede tree growth (drought, shallow ground, instability; Karlik & Poschlod, 2009; Ellenberg & Leuschner, 2010; Janišová et al., 2011) and reoccurring events like fire, wind throw (Hejcman et al., 2013), grazing by wild herbivores (Vera, 2000) or human activity since the Mesolithic (Simmons & Innes, 1981; Bush, 1988). As *Hippocrepis comosa* was described frost tolerant by Hennenberg & Bruelheide (2003) and it is growing up to 2000 m a.s.l. in the Alps, it seems reasonable to assume that the species might have occurred in Central Europe during the LGM on outcrops under favourable conditions.

LATITUDINAL AND LONGITUDINAL CONSTRAINTS OF ITS DISTRIBUTION

On a global scale, it applies for past, present and future, that wild species ranges are primarily determined by climate (Willis & Whittaker, 2002; Pearson & Dawson, 2003; Normand et al., 2011). Besides, there are additional parameters influencing the accessibility of species like life form, dispersal ability and LGM refugia location (glacial contraction), generation time, habitat adaptation, competition with established vegetation, soil development, geographical barriers and human habitat fragmentation (Normand et al., 2011), which may have resulted in a current disequilibrium (postglacial colonization) of species ranges within the actual climate (migrational lag). Regarding the distribution of *Hippocrepis comosa* in Europe we assume that it has fully expanded to its potential range. The northern distribution limitation as it is today was investigated on a regional scale by Hennenberg & Bruelheide (2003), showing a reduced fitness (reduced seed setting), which correlated with effective air temperature measured at a height of 10 cm above ground. The area of climatically suitable habitats was also shown in a present time species distribution model for this species (see Chapter 3). In addition, soil composition impedes an expansion further north and east than its present status (see GliM 2015). Therefore we depicted that the current occurrence of *H. comosa* is mainly limited due to climate and soil factors and that the distribution pattern is not limited by reduced accessibility.

The declining temperature at the beginning of the last ice age was with no doubt the main driving factor influencing migration of plants following a latitude gradient to their southern refugia (Taberlet et al., 1998). Nevertheless, likewise Stewart et al. (2010), we emphasize that not only this latitudinal gradient should be taken into consideration. The longitudinal gradient representing an increasing continental climate from west to east might have had an impact on the migration to suitable habitats especially for species classified as oceanic like *H. comosa*. Due to declining precipitations in combination with a lower sea level, which comprised great land masses between England and Europe, the oceanity of Central Europe decreased. Regarding the migration of plants at that time it is our opinion that oceanic plants were not only forced to move southwards but also westwards to maintain their climatic niche. Furthermore, the Alps have acted as natural barrier hindering exchange and migration of plants towards Italy. The north and Central European populations of *H. comosa* therefore might have followed a combination of both vectors to the south and the west pointing towards a potential refugium in Spain, which can be an explanation for our cluster including Spain, France, Belgium, UK, Switzerland and Germany. After the LGM *H. comosa* migrated to Central Europe, but no further east because of the climatic limitations and edaphic barriers. Nevertheless, according to Chapter 3 describing climatic niches during the LGM, there is also a possibility of cryptic northern refugia located in the UK and France, which could have served as additional places of origin for a recolonization.

For a successful recolonization following the LGM *Hippocrepis comosa* could rely on at least two mechanisms of seed dispersal. Endozoochory via herbivores (Müller-Schneider, 1938; Fischer et al., 1996; von Oheimb et al., 2005) and epizoochory, implying a transportation with soil material in the hooves, which

was reported for sheep and cattle (Fischer et al. 1996; Bonn and Poschlod 1998) but can most probably be transferred to other hoofed animals like red or roe deer. Therefore, we would designate *H. comosa* as a species with a high long-distance dispersal potential, especially because of the fact, that hoofed animals can bridge distances of several kilometres per day (Pépin et al., 2004). This becomes obvious when considering the work of Skog et al. (2009) and Meiri et al. (2013), presenting the recolonization and phylogeography of European red deer (*Cervus elaphus*). Based on modern and ancient DNA this study strikingly resembles our findings for *H. comosa*, showing a restriction of *Cervus elaphus* to southern refugia during the LGM and a recolonization of Western and Northern Europe originating from Iberia. This very similar phylogeographic pattern may lead to the assumption that *H. comosa* expanded to Central Europe via *C. elaphus* or other equivalent herbivores (wild horse, roe deer, see Pakeman, 2001).

Whether and to what extent human activities may have contributed to the expansion of *H. comosa* since the late stages of the Upper Palaeolithic cannot be answered. But grasslands may have continuously existed since the LGM (Bush & Flenley, 1987; Bush, 1988; Pokorný et al., 2015), also because of human practices (Poschlod, 2017). Towards the end of the last glacial period two main cultures can be distinguished in Europe. The Magdalenian culture (16,000 – 10,000 y.a.), which also expanded from the Franco-Cantabrian region up to Belgium, Switzerland, Germany and Austria (Riede, 2014) and the Epigravettian culture appearing during the same period in Italy, Balkan and Eastern Europe. The same separation appears for the Early Neolithic (8,000 – 7,000 y.a.) when the Tardenoisian culture covered Western Europe while the Printed Cardium Pottery culture was located in Italy and Balkan. While these cultures hunter-gatherers pursed the migration of herbivores, the La Hoguette culture (~ 7,500 y.a.) showed first steps towards nomadic goat and sheep breeding (Gronenborn, 2003) and therefore could have directly influenced the dispersal of *H. comosa* via epizoochory (Müller-Schneider, 1938) from southern France to Germany. These assumptions demonstrate that a more interdisciplinary approach to this subject is necessary in order to fully understand the recolonization of Central Europe by plants, animals or humans. A strengthened cooperation of phylogenetics and archaeology could deliver intriguing new insights in the genesis of our environment.

CONCLUSIONS

Based on the present phylogeographic AFLP study on the current distribution of *Hippocrepis comosa* we could demonstrate that a comprehensive climatic approach including a second driving factor can lead to a better understanding of historical and present developments. The traditional latitudinal temperature gradient as major parameter was extended by a longitudinal humidity gradient which both work in tandem defining the suitable habitats during the LGM. Of the two detected clearly distinguished phylogeographic clusters, one in Western Europe ranging from Spain to Germany and the other embracing parts of south Eastern Europe, only the western refugia contributed to the recolonization of Central Europe. As the climate became drier in Central Europe during the LGM, *H. comosa* evaded to moister climates, which prevailed in Western Europe. The results of our survey furthermore allows the existence of northern locations in France and the UK that could have served as cryptic refugia. For the postglacial recolonization, *H. comosa* could have benefited from habitats shapes by man and zoochory, which provided a long ranged dispersal to Central Europe. Thus integrated approaches incorporating multidisciplinary knowledge might be the best way to approximate and illuminate historical and present processes.

APPENDIX

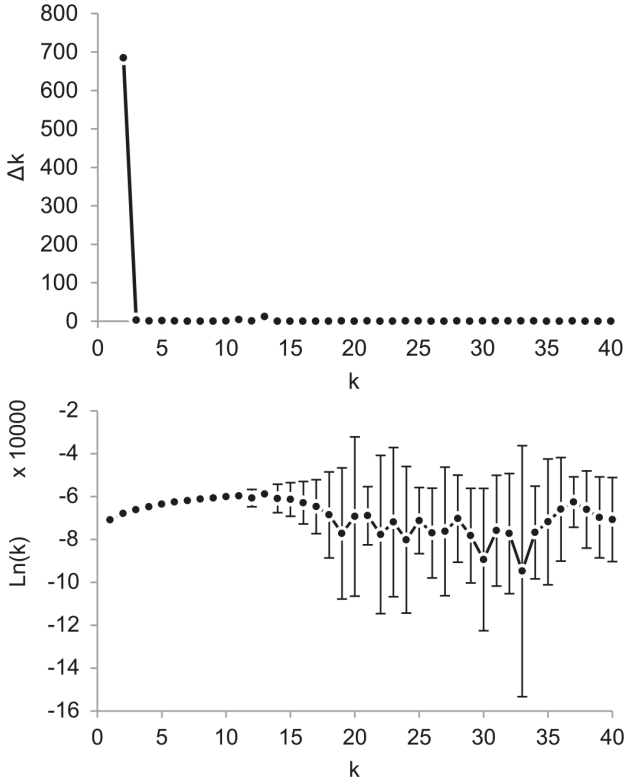


FIGURE 4.6 Analysis of the STRUCTURE runs. Relationship between the number of proposed groups k, Δk and Ln(k), respectively. The most likely number of groups with highest Δk = 684.8 was found with k = 2.

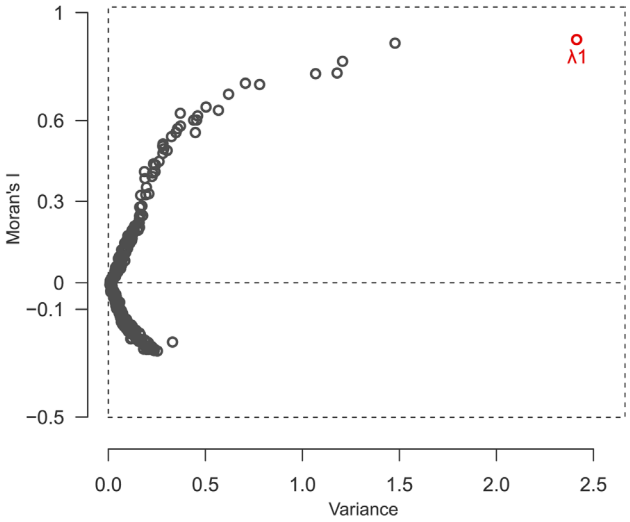


FIGURE 4.7 Screeplot of the total data set. The eigenvalues of the sPCA are arranged by its variance (x-axis) and Moran's I (spatial autocorrelation, y-axis). As the y-axis represents Moran's I, positive scores indicate global structures. Moran's I shows a high spatial autocorrelation (> 0.8). Due to its largest positive eigenvalues λ1, the first global score can be clearly distinguished from all other values and therefore was chosen for further interpretation. The maximum attainable variance from an ordinary PCA is shown as the dashed vertical line on the right side of the graph. The range of variation of Moran's I is limited by the horizontal dashed lines.

CHAPTER 5

Short communications of a seed bank for threatened plant species on the example of the Genbank Bayern Arche

5.1 INTRODUCTION

The Convention on Biological Diversity (CBD) being adopted in Rio de Janeiro in 1992 was to this date the first global attempt to stop the steadily decreasing levels of biodiversity. The treaty regulates bindingly in international law the conservation of biological diversity as one of three equal targets and was ratified by 195 states and the European Union in 1993. Due to a discrepancy in nature conservation mainly focusing on animal protection, the 1999 Gran Canaria Declaration called for a global program for plant conservation. This Declaration has built the foundation for the Global Strategy for Plant Conservation (GSPC, Decision VI/9) which was ratified in 2002 on COP6 in Den Haag. Five objectives with 16 targets in total should slow or even halt the decline of phytodiversity by the end of 2010. Among these objectives, targets 8 and 9 emphasize the need for broader *ex situ* conservation management strategies and addresses all states parties to establish collections for threatened plant species.

Although the 2010 biodiversity targets were not achieved (Secretariat of the Convention on Biological Diversity, 2010), the importance of global conservation has gained in significance. As a consequence a revised decision of the post-2010 targets for the GSPC was decided on COP10 in Nagoya, updating the targets to the strategic plan, which should be accomplished together with the Aichi Biodiversity Targets within the decade on biodiversity (2011 -2020) (CBD, 2010). The Global Biodiversity Outlook 4 published in 2015 clearly shows that the actual progress is insufficient and if no further efforts are undertaken, most of the targets will again be missed by 2020. It states that so far there is no progress regarding a reduction of habitat loss, which should at least be halved and that degradation and fragmentation are even moving away from the aimed target 5. Consequently, the status of threatened species has not improved and there is no sign overall of reduced risk of extinction across all groups of species (target 12, no progress in preventing extinction of known threatened species, moving away of improving the conservation status of threatened species). Moving from a global to a continental scale does not change the outlook. The decline of biodiversity due to persistent increases in pressures on the landscape within the European Union is not questionable (European Environment Agency, 2015) and also on a national level, in Germany, the loss of habitats along with an increasing numbers of greatly endangered plant species is significant (BfN, 2015).

This clearly shows that greater efforts are needed to accomplish the GSPC or Aichi targets. Target 8 of the GSPC stipulates that 75 % of threatened plant species should be held in *ex situ* collections, preferably in the country of origin, and at least 20 % must be available for recovery and restoration programmes. Considering the circumstances in botanical gardens that are often limited in space and therefore, may maintain only reduced genetic diversity or suffer from hybridisation of living cultures, seed banks play a prominent role for achieving target 8. They are able to store a great number and diversity of seed for a long time (Wyse Jackson & Kennedy 2009, Convention on Biological Diversity 2012, BGCI 2016).

On a regional level, one step on the way towards the updated target 8 of the GSPC was taken in 2009, when the Free State of Bavaria (total area 70,550 km^2, number of native species 2,727, 52.5 % listed on the Bavarian Red List) initiated a seed bank especially for rare and threatened wild plant species at the University of Regensburg.

Within the first 5 years the seed bank "Genbank Bayern Arche" (GBA) has collected about 30 % of

threatened Bavarian plant species with one or more accessions, which reflects the extent of these collections in other European or worldwide seed banks for rare plants (Godefroid et al., 2011; Sharrock et al., 2014). In a recent survey on seed banking including 271 institutions from 65 countries most limiting parameters towards achieving the target 8 are insufficient funding, personal shortages and a lack of infrastructures (O'Donnell & Sharrock, 2015).

Although operating and maintaining a seed bank is cost-efficient (Li & Pritchard, 2009), a steady funding is necessary. To maintain the viability of seeds and ongoing revisions of red lists to current threat status, continuous (re-)collections are necessary (Guerrant Jr & Fiedler, 2004). However, the funding of the Genbank Bayern Arche was terminated in 2015. Nevertheless, we were able to clearly identify the time and costs of every work step. The aim of this chapter, therefore, is to provide:

1. A brief illustration of each work step especially in order to point out the importance of each step for wild plant species compared to crop species.
2. We also emphasise the importance of quality assessment of seeds, which is performed via X-ray analysis within the seed bank Genbank Bayern Arche. We present short results of quality control on the level of plant families.
3. A calculation of the expenditure of time needed to run a small scale regional seed bank and an analysis of the time spent for each task performed.

5.2 METHODS AND RESULTS

WORKFLOW

To analyse the workflow within the seed bank Genbank Bayern Arche (GBA), we determined nine process steps which were applied to most of the accessions, representing the main tasks of the work sequence from seed collection to final storage: seed collection, seed cleaning & counting, quality control via X-ray, portioning, germination testing, tetrazolium testing, drying, packaging & freezing and documentation. For a more detailed differentiation, in some cases tasks were divided in multiple subsections. The time required for each process step was recorded in an access database and each entry was checked for plausibility before further analysis. For data evaluation, the average time requirement (median) for each process step over all available accessions was calculated. In the following text all steps within the work process and the particular time effort are described in chronologic order.

SEED COLLECTION

The time required for seed collection field trips strongly depends on the availability and quality of plant occurrence data. Field trips need thorough planning to enable successful collections. We neither include the time requirement for proper acquisition of information upon species identification, ecology and phenology nor for correspondence with local authorities for collecting permits. Our analysis includes seed collection trips with prior information on species location. Second or third approaches of a known population can be required in order to catch the optimum stage of seed development. On the other hand, when visiting species hot spots, synergetic effects increase the time efficiency of a collecting trip. The GBA is situated relatively centrally in Bavaria, which reduces journey times and distances (Figure 5.1). To evaluate the time spent for collection we surveyed 58 field trips performed by the two coordinators of the seed bank (Figure 5.1).

The time spent for round trips and fieldwork was summarized and divided by the amount of accessions gathered on the excursions. The calculation included approaches with immediate collection successes but also unsuccessful trips, where at least a second approach was necessary. The fieldwork encompassed

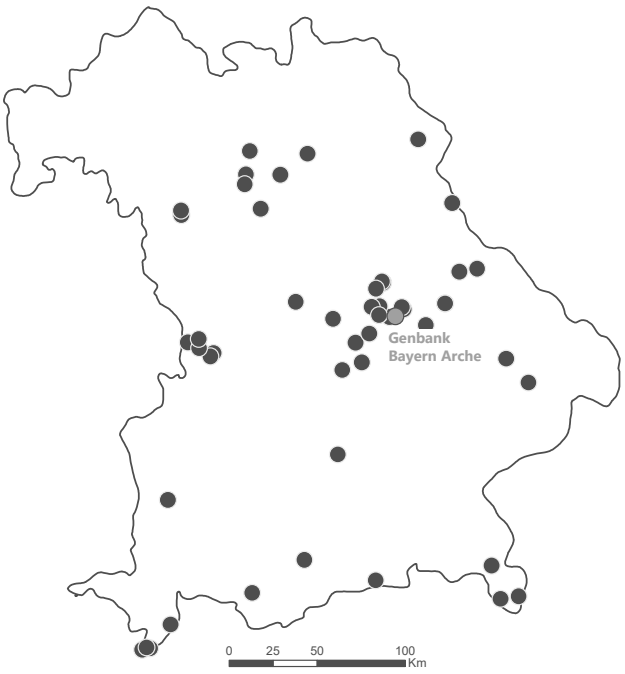


FIGURE 5.1 Map showing the shape of Bavaria, the location of the Genbank Bayern Arche (GBA) and selected collections.

the time spent for searching the population, verifying the species, seed collection and electronic documentation including the capture of geographical coordinates. Sampling strategy followed the instructions of the seed collection manual (ENSCONET, 2009).

On 58 field trips a total of 340 accessions were collected. The number of accessions ranged from zero, when the species was not found or had no fully ripened fruits to 21 the result of a two-day trip in an alpine diversity hotspot. With a median of 128.5 minutes per accession (from 21.8 to 767.0 minutes) the seed collection was the most time consuming task within the work process. As shown in Figure 5.1, the chosen field trips for the evaluation were equally distributed in geographical proximity of the seed bank (return journey < 150 km: 27 trips) and in more distant places (return journey > 150 km: 31 trips).

Although situated in central Bavaria, establishing a seed bank with only two collectors is not reasonable due to the high time expenditure. Therefore, the GBA had engaged over 30 seed collectors. Among them were employees of the University of Regensburg, freelance and voluntary botanists.

SEED CLEANING & COUNTING

Seeds need to be cleaned to lower the risks of diseases and decrease bulk density which reduces the amount of space required in the freezer (Terry et al., 2003). The wide spectrum of seed shapes showing a high diversity of appendices made a uniform treatment with automated seed cleaning machines impossible, which are often used for the purification of crop species. Therefore, most of the seeds were cleaned by hand, reducing physical damages to a minimum. Besides hand-sorting with forceps and scalpels, sieves of different mesh sizes were used to separate the seeds from remaining plant materials.

Depending on seed size, the quantity determination of the cleaned seed samples was carried out by a laser seed counter (Contador, Pfeuffer GmbH, Kitzingen). Only when the seeds were too small or had hooked appendices, which made an automated counting impossible, a portion of the seed sample was counted by hand, weighted and the number of collected seeds was estimated via the total seed mass.

With a median of 126 minutes, cleaning and counting of collections was the second most time consuming task in the seed bank. The expenditure of time was very variable and ranged from some minutes to several hours especially for huge, heavily contaminated or otherwise problematic accessions. The number of seeds stored in the GBA ranged between 1 seed of *Senecio incanus* ssp. *carniolicus* and 1.25 million seeds of *Filago lutescens*. The median number of seeds is slightly over 1000.

QUALITY CONTROL VIA X-RAY

For the X-ray analyses we used a digital radiography system (Faxitron MX-20, Faxitron X-ray Corporation, Wheeling, Illinois) with an exposure time of 9 seconds and 26 kVp. The sample size was set to 100 seeds per accession except for those with smaller quantities. The images where exported and evaluated by categorizing the seeds into five groups: “viable”, “non-viable”, “empty”, “insect infested” and “unclear result”. According to ISTA rules (1999a), seeds were classified as viable when no essential damages of the embryo or the nutritive storage tissues were recognisable. All accessions were assigned with their associated plant family based on the German Standard list (Jansen & Dengler, 2010). With this data set we created a boxplot showing all analysed plant families arranged downwards by the percentage of viable seeds (Figure 5.2).

Due to digitalized X-ray imaging the time spent for production (8.3 minutes) and export (1.2 minutes) of the pictures and the subsequent evaluation (6.5 minutes) is low. The results of a family based analysis of the percentage of viable seeds per accession (Figure 5.2) shows that 36 of the tested 57 plant families had more than 75 % viable seeds. The median viability for all families was 79 %. It should be noted that there is a wide range between 0 % (Staphyleaceae, Betulaceae) and 100 % (Droseraceae, Crassulaceae) viable seeds.

GERMINATION & TETRAZOLIUM TESTING

Alternative methods to determine the viability of seeds are germination and tetrazolium tests, which are both invasive, i.e. destroy the seeds and therefore reduce the size of the accession (ISTA, 1999a; Elias et al., 2012). On the other hand, in contrast to common crop species the knowledge of germination conditions for rare and threatened plants in Bavaria is scarce and therefore a main target of the GBA. To survey the ecological optimum of germination we could rely on 8 illuminated incubators (Type 1301, Rumed, Germany), which provide a temperature range between 0 and 30 °C. Depending on the collection size the extent of performed germination tests differed from 2 to 8 plastic petri dishes (diameter 92 mm) with 5 to 25 seeds for each replication. The seeds were sown onto two filter papers (diameter 90 mm) which were placed inside the dish and watered with pure water (4 ml). If necessary preliminary treatments (stratification, scarification, GA_3) to break dormancy were applied. The durations for these steps were summarized as ‘germination test preparation’. Germination was recorded once a week, germinated

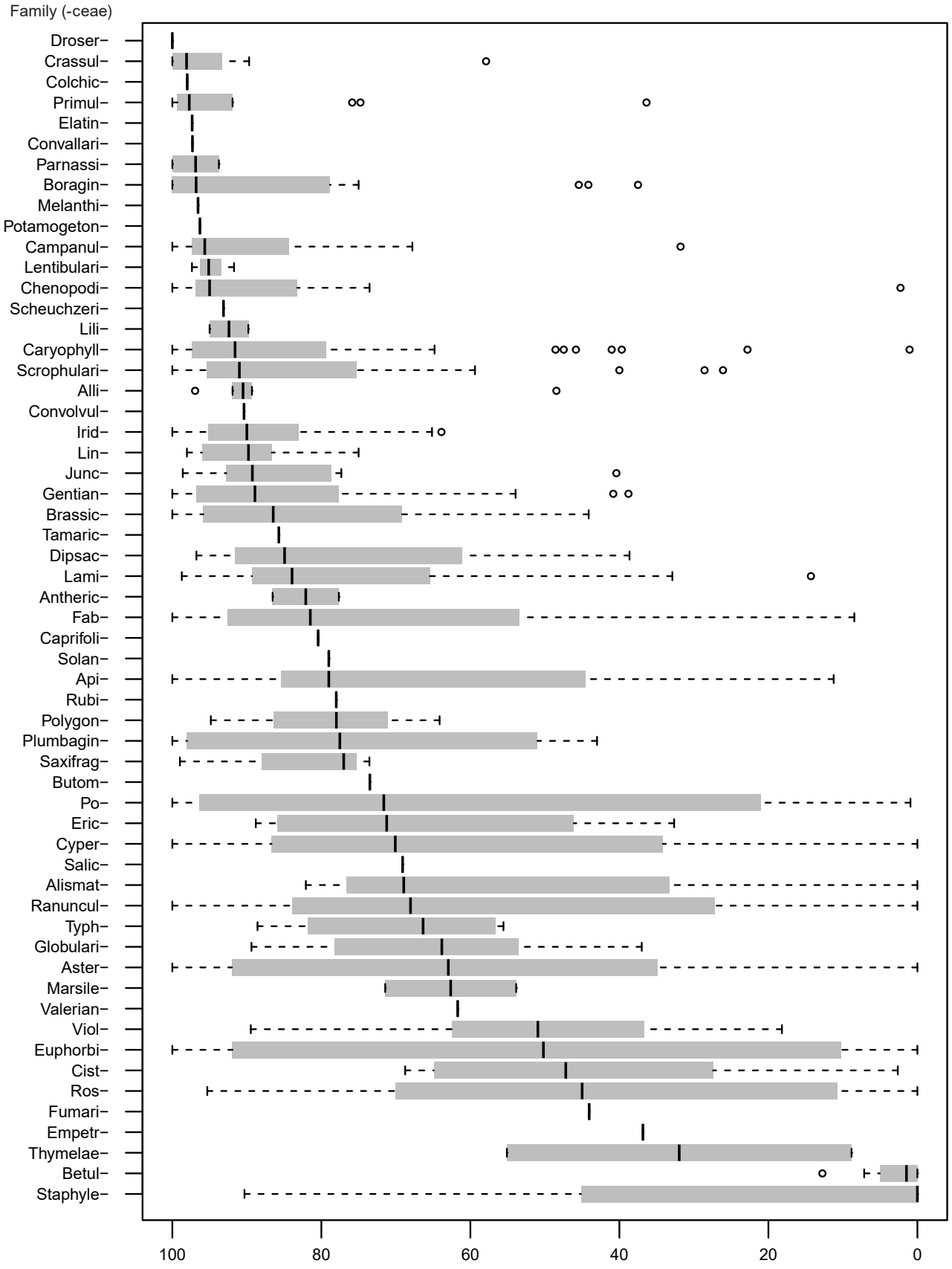


FIGURE 5.2 Percentage of full seeds for each plant family collected for the seed bank Genbank Bayern Arche (GBA).

seeds were removed and discarded or kept and cultivated in the Botanical Garden. The duration of the germination testing was generally set to 6 weeks (in some cases it was expanded to months). The "germination test evaluation" expresses the time spent for one germination tests comprising one treatment, 5 dishes and weekly inspection over 6 weeks.

Tetrazolium testing to determine viability of non-germinated seeds was conducted regularly after germination was tested. Basically the preparation followed the recommendations for tetrazolium testing published by the International Seed Testing Association (ISTA, 1999a, b). The time needed for one treatment was summarized as "tetrazolium test preparation" and included the preparation of liquid tetrazolium solution and the exposure of the inner seed tissues. For a proper evaluation of the incubated seeds a profound knowledge of the seed-morphology is decisive in order to interpret the patterns of stained and unstained tissues correctly. In case of rare and threatened plants ISTA guidelines do not provide sufficient information for evaluation. Therefore, and to produce reproducible and reliable results a set of evaluations forms was developed for every occurring embryo type.

While the preparation time spent for the germination test can vary strongly (14.4 min +/- 10 min per approach), the evaluation time was relatively constant (13.9 min +/- 3 min per approach). It is obvious that the preparation of a germination test with 20 seeds will take less time than a test with 200 seeds which all have to be scarified or undergo other pre-treatments. To what extent germination tests are carried out in a seed bank should depend on its purpose. For a simple assessment of viability 5 dishes with 20 seeds each using the best known germination temperature condition is sufficient and will take an average length of 30 minutes per accession. Nevertheless, in cases when the germination of a species regarding its ecological background is of particular interest more testing is needed. Especially for restoration of extinct or support of existing populations a high *in situ* germination rate is desirable to ensure its success. In this cases an extensive approach can involve multiple pre-treatments (scarification, different stratification times, GA_3), different temperature (constant, fluctuant, from 0 - 30 °C) and light (daylight, darkness) regimes. Therefore, a maximum approach with 24 test variables to determine a species best germination conditions can take up to 11.3 hours to complete. Additionally, when the germination speed should also be recorded two evaluations per week are recommended which doubles the necessary time for evaluation from 13.9 to 27.8 minutes.

Tetrazolium testing is also a time consuming task, which took in average 47 minutes for preparation and another 60 minutes for evaluation. Both processes are dependent on the embryo-endosperm ratio and the size of the examined seeds. As an analysis of 274 species collected and stored in the GBA showed, the median length of the seeds was relatively short with 2 mm (SD = 3.52 mm). For comparison, an analysis of 1794 European species included in the LEDA traitbase had a median length of 2.5 mm (SD = 9.4 mm). Handling small sized seeds requires additional care which directly affects the time needed for tetrazolium testing. Furthermore, the content of the seed can influence the processing time since seeds only containing an embryo can be processed faster than endospermic seeds. Also within the group of endospermic seeds, those with a starchy endosperm are easier to analyse and therefore faster to interpret than those with low amounts of starch. The latter group metabolizes tetrazolium chloride slower resulting in lighter staining of vital tissue which complicates the interpretation.

PORTIONING

To facilitate future accesses to the stored accessions, seeds were split into three fractions: a long-term storage package (base collection), which encompassed a large part of the seeds and was designated to maintain undisturbed for the longest period. A short-term storage package with up to 10 small samples for a quick access which were stored in the same glass container as the long-term package (active collection). A duplicate sample at a different location to guarantee the continuity of the accession in case of a loss of the base collection. The seed portions were packed into uncoated paper bags and afterwards placed in the drying room.

The average time (median) spent on portioning one accession was 35.6 minutes. To determine this value a total of 715 accessions processed by 27 different workers were evaluated. The strong temporal variations per accession depended on seed size, seed shape and seed appendices, which hindered machine processing via Contador in certain cases. Also the number of portions which ranged from one up to 14 bags had an impact on the elapsed time.

DRYING

Longevity of seeds can be increased by providing optimal storage conditions. As several studies have shown, drying of orthodox seeds significantly enhances longevity and therefore is at least as important as the cool storage (Ellis & Roberts, 1980; Roberts & Ellis, 1989; Probert, 2003; Ellis & Hong, 2006,

2007). Furthermore, freezing or insect damages are prevented. Since a drying room which could provide accurate conditions for drying seeds (15 °C and 15 % equilibrium relative humidity (eRH)) was not available in the first phase of the project we relayed on a multi-step seed drying procedure with silica gel. The initial conditions in the drying room were 18 - 20 °C at an eRH of 30 - 45 %. This made a second drying step inevitable: first the actual eRH of the seed sample was measured with a hygrometer (Rotronic Hygrometer AW-DIO sensor with HygroPalm 3). Subsequently seed packages from several accessions were placed in a jar and depending on the seeds' total mass silica gel was added in a weight ratio of 2:1 (seed material to silica gel). On a weekly basis the eRH of the sample was remeasured and fresh silica gel was added. This procedure was repeated until the eRH had dropped under 15 %. With the second project phase a new drying room offering optimal conditions, which are also used for crop species, was established. Therefore, no further drying with silica gel was required, which reduced expenditure of time for drying seeds to less than one minute.

Drying with silica gel in multiple steps is more time consuming than drying in a drying room. The relative humidity (eRH) must be controlled more frequently and there is a chance of over-drying by using too much silica gel. Adding all steps of drying with silica gel we had to spend 10.6 minutes per accession. On the other hand drying with silica gel is a low-price alternative to a drying room having no further running expenses. We therefore used the multi-step seed drying procedure with silica gel for the presented timeline.

PACKAGING & FREEZING

To ensure the dry state of the collections, the seeds were put in tri-laminate aluminium foil-polypropylene bags (labelled internally and externally), which provide an excellent protection against light and are highly impermeable to water vapour and gases (Hanson, 1985; Gómez-Campo, 2002; Manger et al., 2003). The bags were heat sealed in a table top vacuum chamber (TopVac, Kopp), at a low residual oxygen concentration to impede further oxidation processes. Several collections were kept in glass storage jars with a natural rubber seal and a clamped lid. For a visual control of moisture ingress in the jars self-indicating silica gel was added. For storage the jars were placed into domestic upright freezers with a temperature set to -18 °C (Ellis et al., 1985; Hanson, 1985; Rao et al., 2006).

For an active seed bank it can be useful and time saving in the long run to portion the seeds into separate bags containing small amounts. For seed requests pre-filled bags can easily be removed from the active (short-term) collection minimizing the defrosting-time for the remaining stored seeds. Furthermore when a drying room is not available seeds will not rehydrate when stored in separate bags.

The packaging and freezing takes about 10 minutes per collection. Until today we processed 1005 collections of 565 species in the GBA. To store this collection, three domestic freezers are necessary, two holding base and active collections and one storing the duplicate samples.

DOCUMENTATION

The process step documentation includes all workflow tasks concerning organizational steps such as accessioning (check-out and check-in of collection data from the geodatabase including all data documenting the accession), database entries (amount of collected seeds, thousand seed weight, number of seed per portion, dates of drying, freezing, ID of freezing container etc.), determination of portions and germination tests.

Summed up, 14.3 minutes per accession were needed to fully document one accession from arrival at the GBA to the storage in the freezers.

SUMMARY

Besides the time spent for a specific task, Table 5.1 and Figure 5.3 (next page) also display the statistical dispersion (variability). Six process steps show a wide range in their required time: seed collection, seed cleaning, portioning, germination test preparation, tetrazolium test preparation and tetrazolium test evaluation. Because of the different seed quantities, seed shapes and appendices of the collections, a task can take from some minutes to several hours. Other tasks of the workflow are less sensitive to these properties, i.e. can be processed independently from quantity or shape: quality testing via X-ray, seed drying, packaging, germination test evaluation and all database-related work.

TABLE 5.1 Average time spent (minutes) on the work steps in the seed bank Genbank Bayern Arche (GBA). min = minimum / max = maximum time spent for one step, SD = standard deviation, SE = standard error, n_{samp} = number of samples, n_{pro} = number of processors.

Process	Median staff time per collection	min	max	SD	SE	n_{samp}	n_{pro}
Seed collection	128.5	21.8	767.0	151.8	19.9	340	2
Documentation Accessioning[3]	6.3	-	-	-	-	43	1
Seed cleaning and counting	125.7	45.8	450.0	85.3	14.0	1008	37
X-ray image[1]	8.3	4.7	17.7	3.6	1.1	569	11
X-ray image export[1]	1.2	1.0	1.5	0.2	0.1	183	4
X-ray image eval[1]	6.5	3.8	14.3	5.5	3.2	219	3
Sum of 1 = Quality test via X-ray	16.0						
Documentation Database entry[3]	2.2	1.1	4.3	1.6	0.9	46	1
Portioning	35.6	17.1	112.5	24.5	4.7	715	27
Seed drying	10.6	6.7	16.2	4.1	2.1	363	4
Print labels[2]	2.4	-	-			108	1
Packaging, Freezing[2]	8.0	7.8	8.2	0.3	0.2	164	2
Sum of 2 = Packaging and Freezing	10.4						
Documentation Database entry[3]	2.0	-	-	-	-	277	2
Germination test preparation	14.4	11.7	38.0	9.7	2.9	740	11
Germination test evaluation	13.9	9.8	20.0	3.4	0.9	1622	8
Documentation Database entry[3]	3.8	2.4	3.8	0.7	0.3	377	1
Tetrazolium test preparation	47.2	19.4	80.2	18.0	4.5	202	15
Tetrazolium test evaluation	60.0	13.1	143.3	32.4	9.0	157	12
Create seed herbal entry	5.5					169	1
Sum of 3 = Documentation and Database entries	14.3						
Total (minutes)	495.8						
Total (hours)	8.3						

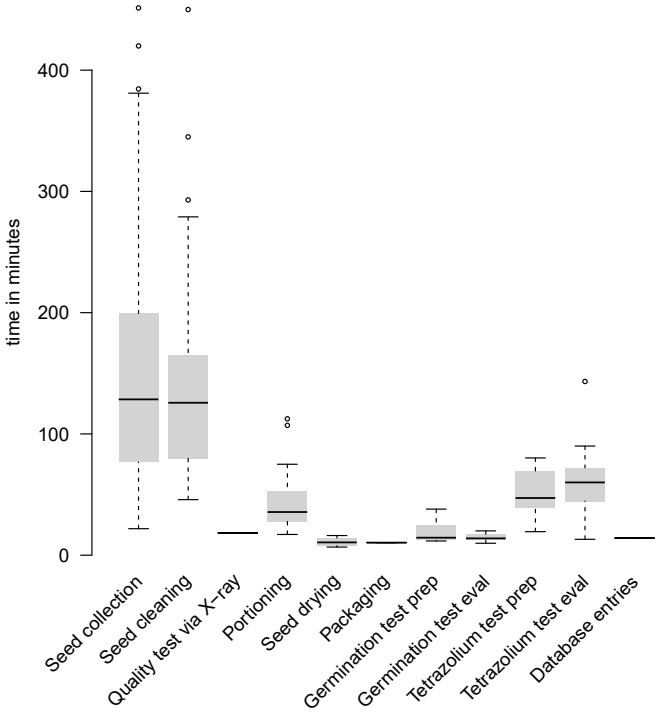


FIGURE 5.3 Boxplot of the time spent on the work steps in the seed bank Genbank Bayern Arche (GBA). prep = preparation eval = evaluation

DISUSSION, CONCLUSIONS & RECOMMENDATIONS FOR SEED BANKS

The time effort in a seed bank is strongly influenced by the species inventory as wild plant seeds often require more manual processing time. A seed bank for rare and threatened plant species must be able to handle all different seed phenotypes (appendices, shapes) as well as different qualities and quantities. Together with an unpredictable additional effort for the collection and germination testing this can be seen as the main differences to crop collections, which are often specialized on certain groups of plants.

The results of our comprehensive quality assessment via X-ray showed that seed quality differs strongly between plant families. Especially for small accessions the knowledge of the quality is crucial, as the number of potentially living seeds can be substantially lower and recollections would be necessary.

Although there is a widespread consensus in science regarding the benefits of seed banks for *ex situ* conservation (Guerrant et al., 2004), founding or continuation of seed banks depend primarily on the actual maintenance costs rather than its importance for preserving biodiversity. With this work at hand, we deliver information regarding the time required for running a small-scale seed bank to provide a calculation basis for the expenses. Glachant (1991) suggested that the costs for *ex situ* conservation of seeds depend on the size of a facility. Accordingly, each accession within the Genbank Bayern Arche (GBA) is expected to be more expensive than within the Millennium Seed Bank (MSB), probably the world's largest seed bank with conservation purpose. To our knowledge, only the MSB has given a summary of time requirements for different tasks (Terry et al., 2003). Therefore, the study at hand allows a comparison between a large-scale and a small-scale seed bank. To evaluate the obtained figures of the GBA (see Table 5.1), we compared our results (accumulated time per accession: 230.4 min) with the time calculations published by the Millennium Seed Bank (MSB) (accumulated time per accession: 219.6 min) (Terry et al., 2003). The difference for processing one accession between the two seed banks is less than 11 minutes (Table 5.2) and, therefore, can be seen as reliable basis for cost calculations and budgetary planning, regardless of the size of the facility. For this comparison the time required for seed collections was not included because no data were available from MSB. Nevertheless, it must be noted that the time for field trips can vary tremendously from seed bank to seed bank depending on the operational range (local, regional, global collections), the abundance of the target plants species and the quality of the documentation of the geographic locations.

TABLE 5.2 Comparison of the estimated processing times for collections between the Millennium Seed Bank (MSB, Terry et al., 2003) and Genbank Bayern Arche (GBA).

Process	Staff time per collection (hours)	
	MSB	GBA
Accessioning (entering collecting data)	0.22	0.24
Cleaning (with portioning)	2.32	2.69
X-ray analysis and counting	0.25	0.27
Banking (packaging, freezing)	0.17	0.17
Germination (without TZT)	0.70	0.47
Total	3.66	3.84

CHAPTER 6

Conclusions and perspectives

Facing the multiple threats of our habitats, species and diversity, many actions are necessary to preserve the remaining biodiversity (Secretariat of the Convention on Biological Diversity, 2006, 2010, 2014). The Global Strategy of Plant Conservation (GSPC) commences with understanding, documentation and recognition of plant diversity (objective I), followed by urgent and effective conservation actions (objective II). The thesis at hand aimed to contribute to meet these first two objectives (CBD, 2010).

Two of the chapters are dealing with "understanding plant diversity" (objective I) in Europe. Although a lot of research has been conducted on calcareous grasslands, one of the most species-rich habitats (Korneck et al., 1998; WallisDeVries et al., 2002; Sadlo et al., 2007) and according to Janssen et al. (2016) one of the most threatened habitats in Europe. We herein provide new information about the origin of a common calcareous grassland species, *Hippocrepis comosa*, using climate and phylogeographic modelling (Chapters 3 and 4). It yet was presumed that Central European species have endured the last glaciations at southern glacial refugia (Taberlet et al., 1998; Hewitt, 1999; Hewitt, 2000; Hewitt, 2004). Therefore, most studies concentrated on the longitudinal gradient which is associated with temperature. On the other hand the latitudinal gradient associated with continentality/oceanity has mostly been ignored (Stewart et al., 2010). We suggest that pannonic floral elements of calcareous grasslands are comparable with today's ice age relicts of the alpine flora. They are living proof for cryptic refugia: the current climate in Central Europe limits these floral elements to distinct localities, i.e. refugia. In case of another cold period these refugia could serve as origins for a colonization of Central Europe besides migration from the Alps and the pannonic floral region. Accordingly, *Hippocrepis comosa* as a submediterranean-oceanic species shows a wider distribution under the present climate conditions than during the last glacial maximum, when it shifted towards southwest and western Europe, leaving behind putative cryptic refugia in Central Europe. With our phylogeographic study, we showed that this plant that contributes to the Central European calcareous grasslands may have originated from western France or south England as there we have located some cryptic refugia, besides the traditional refugia like the Iberian Peninsula. These results can set a different light on the preservation value of northern refugia as they possibly represent very old genetic provenances where oceanic species may have survived the last glaciations.

Finally yet importantly, gained information about the origin of calcareous grassland species or glacial refugia is almost worthless if there is no substantial effort to conserve these habitats and their associated species. In 2018, the ENSOCNET database has reported the success that 62.7 % of Europe's threatened plant species have already been stored in seed banks (Rivière et al., 2018) - a big step towards fulfilling GSPC's target 8 (objective II). On a global level only a few plants in Bavaria are regarded as threatened. For example, all endemic species of the genus Sorbus are listed. Nonetheless this does not relieve of responsibility or obligation to conserve plant diversity on a regional level, as in Bavaria due to a high rate of land consumption and intensive agriculture more than half of the native plants are on the red list (Ahlmer & Scheuerer, 2003). Considering the rapid and frequently unnoticed loss of habitats and species a continuous collection of seeds as well as the maintenance of seed banks are crucial to provide safety backups (Li & Pritchard, 2009; CBD, 2010; Sharrock et al., 2014). Most often economic reasons are the limiting factor of *ex situ* facilities (O'Donnell & Sharrock, 2015). We showed that collecting and processing seeds takes about 5 hours per accession. Compared with other *ex situ* conservation actions, the storage of an accession is quite cheap (Li & Pritchard, 2009). Our list of durations for different work steps (Chapter 5) can be used to choose and to calculate only with a minimum of procession steps that are absolutely indispensable. E.g. leaving out extensive germination testing, a lot of time expenditure can be economized. However, this can only be recommended when germination requirements are known and when alternative means

to test for viability of seeds are available. A professional seed bank should be seeking to encompass all steps for a reasonable conservation.

In the field of conservation genetics, the genetic diversity (variation) of populations is frequently analysed to estimate the adaptive capacity of populations (Ellstrand & Elam, 1993; Booy et al., 2000). The results provide essential information for *in situ* and *ex situ* management of populations. In order to contribute to objective I, we aimed to find the minimum sample size for the assessment of the diversity of a plant population via AFLP markers (Chapter 2). Our work shows that in general 25 individuals are enough to cover 95 % of the population's genetic variation. Furthermore, there is a significant difference regarding the pollination vector: while for insect pollinated plants 20.6 individuals must be collected to gather 95 % of the genetic variation, 28.4 individuals are necessary for wind pollinated plants. Whether our results can be transferred to the collection of seeds is debatable. In theory, these numbers should be applicable to seed collections but depending on the species 25 individuals are probably not enough to gather sufficient seeds for *ex situ* conservation. Moreover, in Chapter 5 we could also show, that different plant families display different percentage rates of viable seeds, which have to be compensated by the collection of more individuals.

To complement the GSPC objectives and to complete necessary actions we point out objective III which states the sustainable and equitable use of plant diversity. Last but not least, it is important to provide education and awareness about biological diversity (objective IV) and finally capacities (target 16) and public engagement to implement the strategy (objective V). The last two objectives may be the biggest challenges in nature conservation. Without public and political attention all efforts made by organisations and scientists towards conserving biodiversity are pointless.

Zusammenfassung

Die vorliegende Dissertation wurde im Rahmen des Aufbaus der Genbank Bayern Arche, einer Genbank für seltene und gefährdete Pflanzenarten, angefertigt. Die einzelnen Kapitel stehen vor dem Hintergrund der Globalen Strategie zum Schutz der Pflanzen (GSPC) und sollen einen Beitrag zur effizienteren Erfassung der genetischen Diversität von Pflanzenarten und zur Feststellung der Herkunft von Kalkmagerrasenarten in Mitteleuropa leisten, sowie einen Überblick über den zeitlichen Aufwand für den Betrieb einer Genbank geben.

Kapitel 2 beschäftigte sich mit der Bestimmung der genetischen Diversität von Pflanzenarten mit unterschiedlichen Pflanzenmerkmalen (plant traits) mittels AFLP Analyse, einer in der molekularen Ökologie und Naturschutzgenetik häufig angewandten Methode. Zu diesem Zweck wurden 15 Pflanzenarten eines Kalkmagerrasenstandorts beprobt, um zum einen den Einfluss der Anzahl der untersuchten Loci und zum anderen der Anzahl an getesteten Individuen auf die Höhe der genetische Diversität hin zu untersuchen. Durch ein Generalisiertes Lineares Modell und Sättigungskurven wurde berechnet, wie viele Loci bzw. wie viele Individuen nötig waren, um 90 % respektive 95 % der gesamten genetischen Vielfalt der Populationen abzubilden. Die Ergebnisse zeigten, dass zirka 120 Loci für eine stabile Einschätzung der genetischen Diversität ausreichten. Darüber hinaus waren durchschnittlich 14 Individuen nötig um 90 % beziehungsweise 23 Individuen, um 95 % der genetischen Diversität der Populationen abzudecken. Die Untersuchung der Pflanzenmerkmale ergab lediglich für den Bestäubungstyp signifikante Unterschiede. Um 90 % beziehungsweise 95 % der genetischen Diversität der Populationen abzudecken mussten bei windbestäubten Arten mehr Proben gesammelt werden als bei insektenbestäubten Pflanzen.

In Kapitel 3 wurde die potentielle geographische Verbreitung des Hufeisenklees (*Hippocrepis comosa*) während des letzten eiszeitlichen Maximums mittels Modellierung (SDM = Species Distribution Model) untersucht. Ein Großteil der Veröffentlichungen zu diesem Thema beschrieben bisher die Iberische Halbinsel, Italien und die Balkanregion aufgrund des nach Süden zunehmenden Temperaturgradienten als Refugialorte für temperate Mitteleuropäische Pflanzenarten während der letzten Eiszeit. Die hier vorliegende Untersuchung verfolgte einen zweiten Gradienten, die Ozeanität, welche von Ost nach West zunimmt und ebenfalls einen Einfluss auf die Verbreitung von Pflanzen hat. Basierend auf der aktuellen Verbreitung des Hufeisenklees wurden unter Verwendung der Software Maxent Verbreitungsmodelle berechnet, welche die heutige und die damalige potentielle geographische Verbreitung darstellten. Diese zeigten einen großen Einfluss der Ozeanität auf diese Pflanzenart, welche neben der temperaturbedingten Migration nach Süden auch eine nach Westen zur Folge hatte. Zudem deuteten die Ergebnisse auf die Möglichkeit hin, dass in Nordwesteuropa kryptische Refugien existiert haben könnten.

In Kapitel 4 sollten die Ergebnisse aus Kapitel 3 mittels AFLP Analyse überprüft werden. Dazu wurde Pflanzenmaterial von 38 Populationen im gesamten Verbreitungsgebiet des Hufeisenklees (*Hippocrepis comosa*) in Europa gesammelt und je 15 Individuen pro Population untersucht. Die phylogeographische Analyse zeigte, dass die Migration der Art während der letzten Eiszeit aus Mitteleuropa heraus sowohl einem Breitengradienten als auch einem Längengradienten folgte. Zudem konnte eine deutliche Trennung in einen westlichen (Iberische Halbinsel) und östlichen Refugialraum (Italien und Balkanregion) festgestellt werden, wobei die westliche Region wahrscheinlich den Ausgangspunkt für die nacheiszeitliche Wiederbesiedelung Mitteleuropas bildete. Die Ergebnisse zeigten außerdem, dass Vorkommen von kryptischen Refugien nordwestlich der Alpen möglich waren.

Kapitel 5 befasste sich mit dem Betrieb der Genbank Bayern Arche, einer 2009 in Bayern gegründeten Genbank für seltene und gefährdete Pflanzenarten. Es wurde der Zeitaufwand untersucht, der für jeden Arbeitsschritt innerhalb der Genbank notwendig war. Dazu wurden alle Arbeitsschritte der Akzessionen von der Sammlung bis hin zur Einlagerung in den Gefrierschränken über 5 Jahre hinweg dokumentiert und im Anschluss ausgewertet. Die Ergebnisse zeigten, dass für eine Akzession durchschnittlich 8,3 Bearbeitungsstunden aufgewendet wurden. Im Vergleich mit der Millennium Seed Bank (UK), welche eine verkürzte

Statistik veröffentlichte, wurde für eine Akzession lediglich 11 Minuten mehr benötigt. Diese Ergebnisse können als Kalkulationsgrundlage für bestehende oder geplante Projekte dienen.

Kapitel 6 fasste die erarbeiteten Ergebnisse zusammen und stellte diese in Bezug hinsichtlich ihrer Bedeutung für den Naturschutz, aber auch bezüglich der Erfüllung der Ziele der Globalen Strategie zum Schutz der Pflanzen (GSPC).

REFERENCES

Ahlmer, W. & Scheuerer, M. (2003). Rote Liste gefährdeter Gefaßpflanzen Bayerns mit regionalisierter Florenliste. Beiträge zum Artenschutz, 24.

Beebee, T.J.C. & Rowe, G. (2000). Microsatellite analysis of natterjack toad Bufo calamita Laurenti populations: consequences of dispersal from a Pleistocene refugium. Biological Journal of the Linnean Society, 69, 367-381.

Bennett, K.D., Tzedakis, P.C. & Willis, K.J. (1991). Quaternary Refugia of North European Trees. Journal of Biogeography, 18, 103-115.

BfN (Bundesamt für Naturschutz, 2015). Artenschutz-Report 2015. Tiere und Pflanzen in Deutschland. Bonn.

Bhagwat, S.A. & Willis, K.J. (2008). Species persistence in northerly glacial refugia of Europe: a matter of chance or biogeographical traits? Journal of Biogeography, 35, 464-482.

Bonin, A., Ehrich, D. & Manel, S. (2007). Statistical analysis of amplified fragment length polymorphism data: a toolbox for molecular ecologists and evolutionists. Molecular Ecology, 16, 3737-3758.

Booy, G., Hendriks, R., Smulders, M., v. Groenendael, J. & Vosman, B. (2000). Genetic diversity and the survival of populations. Plant Biology, 2, 379-395.

Boston, E.S.M., Montgomery, W.I., Hynes, R. & Prodohl, P.A. (2015). New insights on postglacial colonization in western Europe: the phylogeography of the Leisler's bat (*Nyctalus leisleri*). Proceedings of the Royal Society B-Biological Sciences, 282 (1804), 20142605.

Braconnot, P., Harrison, S.P., Kageyama, M., Bartlein, P.J., Masson-Delmotte, V., Abe-Ouchi, A., Otto-Bliesner, B. & Zhao, Y. (2012). Evaluation of climate models using palaeoclimatic data. Nature Climate Change, 2, 417-424.

Bush, M.B. & Flenley, J.R. (1987). The age of the British chalk grassland. Nature, 329, 434-436.

Bush, M.B. (1988). Early Mesolithic disturbance: a force on the landscape. Journal of Archaeological Science, 15, 453-462.

Bush, M.B. (1993). An 11400 year paleoecological history of a British chalk grassland. Journal of Vegetation Science, 4, 47-66.

Bylebyl, K., Poschlod, P. & Reisch, C. (2008). Genetic variation of *Eryngium campestre* L. (Apiaceae) in Central Europe. Molecular Ecology, 17, 3379-3388.

CBD (Convention on Biological Diversity, 2010). Updated Global Strategy for Plant Conservation. Available at: https://www.cbd.int/gspc/ (accessed 2018).

Comes, H.P. & Kadereit, J.W. (1998). The effect of quaternary climatic changes on plant distribution and evolution. Trends in Plant Science, 3, 432-438.

Corlett, R.T. & Westcott, D.A. (2013). Will plant movements keep up with climate change? Trends in Ecology & Evolution, 28, 482-488.

Cornish, M.W. (1954). The origin and structure of the grassland types of the central North Downs. The Journal of Ecology, 359-374.

Dengler, J., Janisova, M., Torok, P. & Wellstein, C. (2014). Biodiversity of Palaearctic grasslands: a synthesis. Agriculture Ecosystems & Environment, 182, 1-14.

Durka, W., Michalski, S.G., Berendzen, K.W., Bossdorf, O., Bucharova, A., Hermann, J.M., Hölzel, N. & Kollmann, J. (2017). Genetic differentiation within multiple common grassland plants supports seed transfer zones for ecological restoration. Journal of Applied Ecology, 54, 116-126.

Dutoit, T. & Alard, D. (1995). Permanent Seed Banks in Chalk Grassland under Various Management Regimes - Their Role in the Restoration of Species-Rich Plant-Communities. Biodiversity and Conservation, 4, 939-950.

Dutoit, T., Buisson, E., Roche, P. & Alard, D. (2004). Land use history and botanical changes in the calcareous hillsides of Upper-Normandy (north-western France): new implications for their conservation management. Biological Conservation, 115, 1-19.

Dutoit, T., Thinon, M., Talon, B., Buisson, E. & Alard, D. (2009). Sampling soil wood charcoals at a high spatial resolution: a new methodology to investigate the origin of grassland plant communities. Journal of Vegetation Science, 20, 349-358.

Ehrich, D. (2006). AFLPdat: a collection of R functions for convenient handling of AFLP data. Molecular Ecology Notes, 6, 603-604.

Eidesen, P., Alsos, I., Popp, M., Stensrud, Ø., Suda, J. & Brochmann, C. (2007). Nuclear vs. plastid data: complex Pleistocene history of a circumpolar key species. Molecular Ecology, 16, 3902-3925.

Elias, S.G., Copeland, L.O., McDonald, M.B. & Baalbaki, R.Z. (2012). Seed Testing: Principles and Practices, Michigan State University Press.

Elith, J. & Leathwick, J.R. (2009). Species distribution models: ecological explanation and prediction across space and time. Annual Review of Ecology, Evolution, and Systematics, 40, 677-697.

Elith, J., Phillips, S.J., Hastie, T., Dudik, M., Chee, Y.E. & Yates, C.J. (2011). A statistical explanation of MaxEnt for ecologists. Diversity and Distributions, 17, 43-57.

Elith, J., Graham, C.H., Anderson, R.P., Dudik, M., Ferrier, S., Guisan, A., Hijmans, R.J., Huettmann, F., Leathwick, J.R., Lehmann, A., Li, J., Lohmann, L.G., Loiselle, B.A., Manion, G., Moritz, C., Nakamura, M., Nakazawa, Y., Overton, J.M., Peterson, A.T., Phillips, S.J., Richardson, K., Scachetti-Pereira, R., Schapire, R.E., Soberon, J., Williams, S., Wisz, M.S. & Zimmermann, N.E. (2006). Novel methods improve prediction of species' distributions from occurrence data. Ecography, 29, 129-151.

Ellenberg, H. (1988). Vegetation ecology of central Europe. Cambridge University Press, Cambridge.

Ellenberg, H. & Leuschner, C. (2010). Vegetation Mitteleuropas mit den Alpen in ökologischer, dynamischer und historischer Sicht. UTB, Stuttgart.

Ellis, R. & Roberts, E. (1980). Improved equations for the prediction of seed longevity. Annals of Botany, 45, 13-30.

Ellis, R. & Hong, T. (2006). Temperature sensitivity of the low-moisture-content limit to negative seed longevity–moisture content relationships in hermetic storage. Annals of Botany, 97, 785-791.

Ellis, R. & Hong, T. (2007). Seed longevity–moisture content relationships in hermetic and open storage. Seed Science and Technology, 35, 423-431.

Ellis, R., Hong, T. & Roberts, E.H. (1985). Handbook of seed technology for genebanks. Volume II. Compendium of specific germination information and test recommendations. Handbook of seed technology for genebanks. Volume II. Compendium of specific germination information and test recommendations. IBPGR, Rome.

Ellstrand, N.C. & Elam, D.R. (1993). Population Genetic Consequences of Small Population Size: Implications for Plant Conservation. Annual Review of Ecology and Systematics, 24(1), 217-242.

ENSCONET (2009). ENSCONET Seed Collecting Manual for Wild Species.

European Environment Agency (2015). State of nature in the EU. Results from reporting under the nature directives 2007–2012. EEA Technical report, 2, 1-178.

Evanno, G., Regnaut, S. & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology, 14, 2611-2620.

Excoffier, L., Smouse, P.E. & Quattro, J.M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics, 131, 479-491.

Fielding, A.H. & Bell, J.F. (1997). A review of methods for the assessment of prediction errors in conservation presence/absence models. Environmental Conservation, 24, 38-49.

Fischer, S.F., Poschlod, P. & Beinlich, B. (1996). Experimental studies on the dispersal of plants and animals on sheep in calcareous grasslands. Journal of Applied Ecology, 1206-1222.

Fjellheim, S., Rognli, O.A., Fosnes, K. & Brochmann, C. (2006). Phylogeographical history of the widespread meadow fescue (*Festuca pratensis* Huds.) inferred from chloroplast DNA sequences. Journal of Biogeography, 33, 1470-1478.

Flojgaard, C., Normand, S., Skov, F. & Svenning, J.C. (2009). Ice age distributions of European small mammals: insights from species distribution modelling. Journal of Biogeography, 36, 1152-1163.

Gaudeul, M., Till-Bottraud, I., Barjon, F. & Manel, S. (2004). Genetic diversity and differentiation in *Eryngium alpinum* L. (Apiaceae): comparison of AFLP and microsatellite markers. Heredity, 92, 508-518.

GBIF (Global Biodiversity Information Facility). GBIF Backbone Taxonomy. Available at: http://www.gbif.org/species/5353750 (accessed 2015).

Gibson, C.W.D. & Brown, V.K. (1991). The Nature and Rate of Development of Calcareous Grassland in Southern Britain. Biological Conservation, 58, 297-316.

Glachant, M. (1991). La biodiversité biologique végétale: éléments d'économie. Cerna, Paris.

Godefroid, S., Rivière, S., Waldren, S., Boretos, N., Eastwood, R. & Vanderborght, T. (2011). To what extent are threatened European plant species conserved in seed banks? Biological Conservation, 144, 1494-1498.

Gómez-Campo, C. (2002). Long term seed preservation: the risk of selecting inadequate containers is very high. Univ. Politécnica de Madrid, Madrid.

Gronenborn, D. (2003). Migration, acculturation and culture change in western temperate Eurasia, 6500–5000 cal BC. Documenta praehistorica, 30, 79-91.

Guerrant, E.O., Havens, K. & Maunder, M. (2004). *Ex situ* plant conservation: supporting species survival in the wild. Island Press, London.

Guerrant Jr, E.O. & Fiedler, P.L. (2004). Accounting for sample decline during *ex situ* storage and reintroduction. *Ex Situ* Plant Conservation: Supporting Species Survival in the Wild. Island Press, London, 365-385.

Hajkova, P., Rolecek, J., Hajek, M., Horsak, M., Fajmon, K., Polak, M. & Jamrichova, E. (2011). Prehistoric origin of the extremely species-rich semi-dry grasslands in the Bile Karpaty Mts (Czech Republic and Slovakia). Preslia, 83, 185-204.

Hanson, J. (1985). Procedures for handling seeds in genebanks. IBPGR, Rome.

Harrison, S.P. & Prentice, C.I. (2003). Climate and CO_2 controls on global vegetation distribution at the last glacial maximum: analysis based on palaeovegetation data, biome modelling and palaeoclimate simulations. Global Change Biology, 9, 983-1004.

Hejcman, M., Hejcmanova, P., Pavlu, V. & Benes, J. (2013). Origin and history of grasslands in Central Europe - a review. Grass and Forage Science, 68, 345-363.

Hennenberg, K.J. & Bruelheide, H. (2003). Ecological investigations on the northern distribution range of *Hippocrepis comosa* L. in Germany. Plant Ecology, 166, 167-188.

Hernandez, P.A., Graham, C.H., Master, L.L. & Albert, D.L. (2006). The effect of sample size and species characteristics on performance of different species distribution modeling methods. Ecography, 29, 773-785.

Hewitt, G.M. (1999). Post-glacial re-colonization of European biota. Biological Journal of the Linnean Society, 68, 87-112.

Hewitt, G.M. (2000). The genetic legacy of the Quaternary ice ages. Nature, 405, 907-913.

Hewitt, G.M. (2004). Genetic consequences of climatic oscillations in the Quaternary. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences, 359, 183-195.

Hijmans, R.J. & Graham, C.H. (2006). The ability of climate envelope models to predict the effect of climate change on species distributions. Global Change Biology, 12, 2272-2281.

Hijmans, R.J., Cameron, S.E., Parra, J.L., Jones, P.G. & Jarvis, A. (2005). Very high resolution interpolated climate surfaces for global land areas. International Journal of Climatology, 25, 1965-1978.

Hollingsworth, P.M. & Ennos, R.A. (2004). Neighbour joining trees, dominant markers and population genetic structure. Heredity, 92, 490-498.

Horsak, M., Chytry, M., Pokryszko, B.M., Danihelka, J., Ermakov, N., Hajek, M., Hajkova, P., Kintrova, K., Koci, M., Kubesova, S., Lustyk, P., Otypkova, Z., Pelankova, B. & Valachovic, M. (2010). Habitats of relict terrestrial snails in southern Siberia: lessons for the reconstruction of palaeoenvironments of full-glacial Europe. Journal of Biogeography, 37, 1450-1462.

Huntley, B. & Birks, H.J.B. (1983). An atlas of past and present pollen maps for Europe: 0-13000 years ago. Cambridge University Press, Cambridge.

Huntley, B. & Webb, T. (1989). Migration - Species Response to Climatic Variations Caused by Changes in the Earths Orbit. Journal of Biogeography, 16, 5-19.

ISTA (International Seed Testing Association, 1999a). Biochemical test for viability. Chapter 6 of the International Rules for Seed Testing 1999. Seed Science and Technology, 27, 33-35.

ISTA (1999b). Biochemical test for viability (The Topographical Tetrazolium Test). Annex to Chapter 6 of the International Rules for Seed Testing 1999. Seed Science and Technology, 27, 201-244.

Janišová, M., Bartha, S., Kiehl, K. & Dengler, J. (2011). Advances in the conservation of dry grasslands: Introduction to contributions from the seventh European Dry Grassland Meeting. Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology, 145, 507-513.

Jansen, F. & Dengler, J. (2010). Plant names in vegetation databases - a neglected source of bias. Journal of Vegetation Science, 21, 1179-1186.

Janssen, J., Rodwell, J., Criado, M.G., Arts, G., Bijlsma, R. & Schaminee, J. (2016). European red list of habitats. European Union.

Jarne, P. & Lagoda, P.J. (1996). Microsatellites, from molecules to populations and back. Trends in Ecology & Evolution, 11, 424-429.

Jimenez-Valverde, A. & Lobo, J.M. (2007). Threshold criteria for conversion of probability of species presence to either-or presence-absence. Acta Oecologica-International Journal of Ecology, 31, 361-369.

Jombart, T., Devillard, S., Dufour, A.B. & Pontier, D. (2008). Revealing cryptic spatial patterns in genetic variability by a new multivariate method. Heredity, 101, 92-103.

Kahmen, S. & Poschlod, P. (2004). Plant functional trait responses to grassland succession over 25 years. Journal of Vegetation Science, 15, 21-32.

Kahmen, S., Poschlod, P. & Schreiber, K.-F. (2002). Conservation management of calcareous grasslands. Changes in plant species composition and response of functional traits during 25 years. Biological Conservation, 104, 319-328.

Karlik, P. & Poschlod, P. (2009). History or abiotic filter: which is more important in determining the species composition of calcareous grasslands? Preslia, 81, 321-340.

Kastlwerk (2018). http://gendiv.kastlwerk.de

Kaulfuß, F. & Reisch, C. (2017). Reintroduction of the endangered and endemic plant species *Cochlearia bavarica* - Implications from conservation genetics. Ecology and Evolution, 7, 11100-11112.

Kleyer, M., Bekker, R.M., Knevel, I.C., Bakker, J.P., Thompson, K., Sonnenschein, M., Poschlod, P., Van Groenendael, J.M., Klimeš, L. & Klimešová, J. (2008). The LEDA Traitbase: a database of lifehistory traits of the Northwest European flora. Journal of Ecology, 96, 1266-1274.

Knörzer, H.K. (1996). Beitrag zur Geschichte der Grünlandvegetation am Niederrhein. Tüxenia, 16, 627-636.

Königsson, L.-K. (1968). The holocene history of the Great Alvar of Öland. Acta phytogeographica Suecica, 55.

Körber-Grohne, U. (1990). Gramineen und Grünlandvegetationen vom Neolithikum bis zum Mittelalter in Mitteleuropa. E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart.

Körber-Grohne, U. & Wilmanns, O. (1977). Eine Vegetation aus dem hallstattzeitlichen Fürstengrabhügel Magdalenenberg bei Villingen - Folgerungen aus pflanzlichen Großresten. Spingler, K: Der hallstattzeitliche Fürstengrabhügel bei Villingen im Schwarzwald, Magdalenenberg V., 51-68, Stuttgart.

Korneck, D., Schnittler, M., Klingenstein, F., Ludwig, G., Takla, M., Bohn, U. & May, R. (1998). Warum verarmt unsere Flora? Auswertung der Roten Liste der Farn- und Blütenpflanzen Deutschlands. Schriftenreihe für Vegetationskunde, 29, 299-444.

Kunes, P., Pelankova, B., Chytry, M., Jankovska, V., Pokorny, P. & Petr, L. (2008). Interpretation of the last-glacial vegetation of eastern-central Europe using modern analogues from southern Siberia. Journal of Biogeography, 35, 2223-2236.

Lang, G. (1994). Quartäre Vegetationsgeschichte Europas: Methoden und Ergebnisse. Gustav Fischer Verlag Jena, Stuttgart, New York.

Li, D.-Z. & Pritchard, H.W. (2009). The science and economics of *ex situ* plant conservation. Trends in Plant Science, 14, 614-621.

Litt, M. & Luty, J.A. (1989). A hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. American Journal of Human Genetics, 44, 397.

Lowe, J.J. & Walker, M.J.C. (1997). Reconstructing quaternary environments. Longman, London.

Ložek, V. & Cílek, V. (1995). Late Weichselian-Holocene sediments and soils in mid-European calcareous areas. Anthropozoikum, 22, 87-112.

Ludwig, G. & Schnittler, M. (1996). Rote Liste gefährdeter Pflanzen Deutschlands. Bundesamt für Naturschutz, Bonn.

Magri, D., Vendramin, G.G., Comps, B., Dupanloup, I., Geburek, T., Gomory, D., Latalowa, M., Litt, T., Paule, L., Roure, J.M., Tantau, I., van der Knaap, W.O., Petit, R.J. & de Beaulieu, J.L. (2006). A new scenario for the Quaternary history of European beech populations: palaeobotanical evidence and genetic consequences. New Phytologist, 171, 199-221.

Manger, K., Adams, J. & Probert, R. (2003). Selecting seed containers for the Millennium Seed Bank Project: a technical review and survey. Seed Conservation: turning science into practice. Royal Botanic Gardens, Kew.

Mariette, S., Le Corre, V., Austerlitz, F. & Kremer, A. (2002). Sampling within the genome for measuring within-population diversity: trade-offs between markers. Molecular Ecology, 11, 1145-1156.

McCullagh, P. & Nelder, J.A. (1989). Generalized linear models (Vol. 37). CRC press, Boca Raton.

McGlaughlin, M.E., Riley, L., Brandsrud, M., Arcibal, E., Helenurm, M.K. & Helenurm, K. (2015). How much is enough? minimum sampling intensity required to capture extant genetic diversity in *ex situ* seed collections: examples from the endangered plant *Sibara filifolia* (Brassicaceae). Conservation Genetics, 16, 253-266.

Meiri, M., Lister, A.M., Higham, T.F., Stewart, J.R., Straus, L.G., Obermaier, H., Gonzalez Morales, M.R., Marín-Arroyo, A.B. & Barnes, I. (2013). Late-glacial recolonization and phylogeography of European red deer (*Cervus elaphus* L.). Molecular Ecology, 22, 4711-4722.

Mittell, E.A., Nakagawa, S. & Hadfield, J.D. (2015). Are molecular markers useful predictors of adaptive potential? Ecology Letters, 18, 772-778.

Moran, P.A.P. (1948). The interpretation of statistical maps. Journal of the Royal Statistical Society. Series B (Methodological), 10, 243-251.

Morin, P. A., Luikart, G., & Wayne, R. K. (2004). SNPs in ecology, evolution and conservation. Trends in Ecology & Evolution, 19(4), 208-216.

Müller-Schneider, P. (1938). Über endochore Samenverbreitung durch Säugetiere. Jahrbuch Naturforschende Gesellschaft Graubünden, 75, 85-88.

Nei, M. (1972). Genetic Distance between Populations. American Naturalist, 106, 283-292.

Normand, S., Ricklefs, R.E., Skov, F., Bladt, J., Tackenberg, O. & Svenning, J.-C. (2011). Postglacial migration supplements climate in determining plant species ranges in Europe. Proceedings of the Royal Society B: Biological Sciences, rspb20102769.

Nybom, H. (2004). Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Molecular Ecology, 13, 1143-1155.

Nybom, H. & Bartish, I.V. (2000). Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. Perspectives in plant ecology, evolution and systematics, 3, 93-114.

O'Donnell, K. & Sharrock, S. (2015). Seed Banking in Botanic Gardens. Can Botanic Gardens achieve GSPC Target 8 by 2020? BGJournal, 12, 3-8.

Ohlemüller, R., Huntley, B., Normand, S. & Svenning, J.C. (2012). Potential sourc O'Donnell e and sink locations for climate-driven species range shifts in Europe since the Last Glacial Maximum. Global Ecology and Biogeography, 21, 152-163.

Pakeman, R.J. (2001). Plant migration rates and seed dispersal mechanisms. Journal of Biogeography, 795-800.

Pärtel, M., Bruun, H.H. & Sammul, M. (2005). Biodiversity in temperate European grasslands: origin and conservation: 13th International Occasional Symposium of the European Grassland Federation. In: Grassland Science in Europe, pp. 1-14. In: Integrating Efficient Grassland Farming and Biodiversity: Proceedings of the 13th International Occasional Symposium of the European Grassland Federation.

Paun, O., Schonswetter, P., Winkler, M., Tribsch, A. & Consortium, I. (2008). Historical divergence vs. contemporary gene flow: evolutionary history of the calcicole Ranunculus alpestris group (*Ranunculaceae*) in the European Alps and the Carpathians. Molecular Ecology, 17, 4263-4275.

Peakall, R. & Smouse, P.E. (2012). GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research - an update. Bioinformatics, 28, 2537-2539.

Pearson, R.G. & Dawson, T.P. (2003). Predicting the impacts of climate change on the distribution of species: are bioclimate envelope models useful? Global ecology and biogeography, 12, 361-371.

Pépin, D., Adrados, C., Mann, C. & Janeau, G. (2004). Assessing real daily distance traveled by ungulates using differential GPS locations. Journal of Mammalogy, 85, 774-780.

Petit, R.J., Aguinagalde, I., de Beaulieu, J.L., Bittkau, C., Brewer, S., Cheddadi, R., Ennos, R., Fineschi, S., Grivet, D., Lascoux, M., Mohanty, A., Muller-Starck, G.M., Demesure-Musch, B., Palme, A., Martin, J.P., Rendell, S. & Vendramin, G.G. (2003). Glacial refugia: Hotspots but not melting pots of genetic diversity. Science, 300, 1563-1565.

Petrosino, N. (2004). Zur Vegetations- und Agrargeschichte im Kelheimer Raum: archäobotanische Untersuchungen an Bodenproben vom Frauenberg oberhalb Kloster Weltenburg, Torfen aus dem Naturschutzgebiet Sippenauer Moor sowie Bodenproben und Torfen aus dem Teugner Mühlbachtal, Lkr. Kelheim.

Phillips, S.J. & Dudik, M. (2008). Modeling of species distributions with Maxent: new extensions and a comprehensive evaluation. Ecography, 31, 161-175.

Phillips, S.J., Anderson, R.P. & Schapire, R.E. (2006). Maximum entropy modeling of species geographic distributions. Ecological Modelling, 190, 231-259.

Pokorný, P. (2005). Role of man in the development of Holocene vegetation in Central Bohemia. Preslia, 77, 113-128.

Pokorný, P., Chytrý, M., Juřičková, L., Sádlo, J., Novák, J. & Ložek, V. (2015). Mid-Holocene bottleneck for central European dry grasslands: Did steppe survive the forest optimum in northern Bohemia, Czech Republic? The Holocene, 25, 716-726.

Poschlod, P. (2017). Geschichte der Kulturlandschaft. Ulmer, Stuttgart.

Poschlod, P. & Bonn, S. (1998). Changing dispersal processes in the central European landscape since the last ice age: an explanation for the actual de-

crease of plant species richness in different habitats? Acta Botanica Neerlandica, 47, 27-44.

Poschlod, P. & WallisDeVries, M.F. (2002). The historical and socioeconomic perspective of calcareous grasslands - lessons from the distant and recent past. Biological Conservation, 104, 361-376.

Poschlod, P. & Baumann, A. (2010). The historical dynamics of calcareous grasslands in the central and southern Franconian Jurassic mountains: a comparative pedoanthracological and pollen analytical study. Holocene, 20, 13-23.

Poschlod, P., Baumann, A. & Karlik, P. (2009). Origin and development of grasslands in Central Europe. Grasslands in Europe – of high nature value (ed. by Veen, P., Jefferson, R., De Smidt, J. and J., V.D.S.), pp. 15-25. KNNV Publishing, Utrecht.

Poschlod, P., Karlík, P., Baumann, A. & Wiedmann, B. (2008). The history of dry calcareous grasslands near Kallmünz (Bavaria) reconstructed by the application of palaeoecological, historical and recent-ecological methods. Human nature: studies in historical ecology and environmental history. Institute of Botany of the Czech Academy of Sciences, Brno, 130-143.

Poschlod, P., Kiefer, S., Trankle, U., Fischer, S. & Bonn, S. (1998). Plant species richness in calcareous grasslands as affected by dispersability in space and time. Applied Vegetation Science, 1(1), 75-91.

Poschlod, P., Kleyer, M., Jackel, A.-K., Dannemann, A. & Tackenberg, O. (2003). BIOPOP - a database of plant traits and internet application for nature conservation. Folia Geobotanica, 38, 263-271.

Pott, R. (1992). Die Pflanzengesellschaften Deutschlands. UTB, Stuttgart

Preece, R.C. & Bridgland, D.R. (1999). Holywell Coombe, Folkestone: A 13,000 year history of an English Chalkland Valley. Quaternary Science Reviews, 18, 1075-1125.

Pritchard, J.K., Stephens, M. & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. Genetics, 155, 945-959.

Pritchard, J.K., Wen, X. & Falush, D. (2009). STRUCTURE ver. 2.3. University of Chicago, Chicago. Available at: http://pritch. bsd. uchicago. edu (accessed 2015).

Probert, R.J. (2003). Seed viability under ambient conditions, and the importance of drying. Seed Conservation: turning science into practice. Royal Botanic Gardens, Kew.

Provan, J. & Bennett, K.D. (2008). Phylogeographic insights into cryptic glacial refugia. Trends in Ecology & Evolution, 23, 564-571.

Pruett, C.L. & Winker, K. (2008). The effects of sample size on population genetic diversity estimates in song sparrows Melospiza melodia. Journal of Avian Biology, 39, 252-256.

Quinger, B., Bräu, M. & Kornprobst, M. (1994). Lebensraumtyp Kalkmagerrasen. Landschaftspflegekonzept Bayern, 2.

R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.

Rao, N.K., Hanson, J., Dulloo, M.E., Ghosh, K. & Nowell, A. (2006). Manual of seed handling in genebanks, 8. Bioversity International.

Reisch, C. (2007). Genetic structure of *Saxifraga tridactylites* (Saxifragaceae) from natural and man-made habitats. Conservation Genetics, 8, 893-902.

Reisch, C. & Poschlod, P. (2003). Intraspecific variation, land use and habitat quality – a phenologic and morphometric analysis of *Sesleria albicans* (Poaceae). Flora - Morphology, Distribution, Functional Ecology of Plants, 198, 321-328.

Reisch, C. & Kellermeier, J. (2007). Microscale variation in alpine grasslands: AFLPs reveal a high level of genotypic diversity in *Primula minima* (Primulaceae). Botanical Journal of the Linnean Society, 155, 549-556.

Reisch, C. & Bernhardt-Römermann, M. (2014). The impact of study design and life history traits on genetic variation of plants determined with AFLPs. Plant Ecology, 215, 1493-1511.

Reisch, C., Poschlod, P. & Wingender, R. (2003). Genetic differentiation among populations of *Sesleria albicans* Kit. ex Schultes (Poaceae) from ecologically different habitats in central Europe. Heredity, 91, 519-527.

Reisch, C., Anke, A. & Rohl, M. (2005). Molecular variation within and between ten populations of *Primula farinosa* (Primulaceae) along an altitudinal gradient in the northern Alps. Basic and Applied Ecology, 6, 35-45.

Riede, F. (2014). The resettlement of Northern Europe. Cummings, V., Jordan, P. and Zvelebil, M.: The Oxford Handbook of the Archaeology and Anthropology of Hunter-Gatherers, 556-580. OUP, Oxford.

Rivière, S., Breman, E., Kiehn, M., Carta, A. & Müller, J. (2018). How to meet the 2020 GSPC target 8 in Europe: priority-setting for seed banking of native

threatened plants. Biodiversity and Conservation 27(8), 1873-1890.

Roberts, E. & Ellis, R. (1989). Water and seed survival. Annals of Botany, 63, 39-39.

Rogers, S.O. & Bendich, A.J. (1994). Extraction of total cellular DNA from plants, algae and fungi. Plant molecular biology manual, 183-190.

Römermann, C., Bernhardt-Römermann, M., Kleyer, M. & Poschlod, P. (2009). Substitutes for grazing in semi-natural grasslands – do mowing or mulching represent valuable alternatives to maintain vegetation structure? Journal of Vegetation Science, 20, 1086-1098.

Rosch, M. (1998). The history of crops and crop weeds in south-western Germany from the Neolithic period to modern times, as shown by archaeobotanical evidence. Vegetation History and Archaeobotany, 7, 109-125.

Ruddiman, W.F. & Thomson, J.S. (2001). The case for human causes of increased atmospheric CH4. Quaternary Science Reviews, 20, 1769-1777.

Sadlo, J., Chytry, M. & Pysek, P. (2007). Regional species pools of vascular plants in habitats of the Czech Republic. Preslia, 79, 303-321.

Scheffer, M., Carpenter, S., Foley, J.A., Folke, C. & Walker, B. (2001). Catastrophic shifts in ecosystems. Nature, 413, 591.

Schlötterer, C. (2004). The evolution of molecular markers - just a matter of fashion? Nature reviews genetics, 5, 63-69.

Schmidt, M., Fischer, P. & Becker, C. (2007). Zur Herkunft von Pflanzenarten anthropo-zoogener Kalk-Halbtrockenrasen Mitteleuropas-Überlegungen am Beispiel des nördlichen deutschen Hügel- und Berglandes. Hercynia-Ökologie und Umwelt in Mitteleuropa, 40, 257-267 (268).

Schmitt, T. & Varga, Z. (2012). Extra-Mediterranean refugia: The rule and not the exception? Frontiers in Zoology, 9(1), 22.

Schönswetter, P. & Tribsch, A. (2005). Vicariance and dispersal in the alpine perennial *Bupleurum stellatum* L. (Apiaceae). Taxon, 54, 725-732.

Schroeder, F.-G. (1998). Lehrbuch der Pflanzengeographie. Quelle & Meyer, Wiesbaden.

Schwartz, D., Thinon, M., Goepp, S., Schmitt, C., Casner, J., Rosique, T., Wuscher, P., Alexandre, A., Dambrine, É. & Martin, C. (2005). Premières datations directes de défrichements protohistoriques sur les chaumes secondaires des Vosges (Rossberg, Haut-Rhin). Approche pédoanthracologique. Comptes Rendus Geoscience, 337, 1250-1256.

Secretariat of the Convention on Biological Diversity (2006). Global Biodiversity Outlook 2, Montréal.

Secretariat of the Convention on Biological Diversity (2010). Global Biodiversity Outlook 3, Montréal.

Secretariat of the Convention on Biological Diversity (2014). Global Biodiversity Outlook 4, Montréal.

Sedcole, J.R. (1977). Number of Plants Necessary to Recover a Trait. Crop Science, 17, 667-668.

Semagn, K., Bjørnstad, Å. & Ndjiondjop, M. (2006). An overview of molecular marker methods for plants. African journal of biotechnology, 5(25), 2540-2568.

Sharrock, S., Oldfield, S. & Wilson, O. (2014). Plant Conservation Report 2014: A review of progress in implementation of the Global Strategy for Plant Conservation 2011-2020. Secretariat of the Convention on Biological Diversity, Montréal, Canada and Botanic Gardens Conservation International, Richmond.

Simmons, I.G. & Innes, J.B. (1981). Tree Remains in a North York Moors Peat Profile. Nature, 294, 76-78.

Sinclair, E.A. & Hobbs, R.J. (2009). Sample size effects on estimates of population genetic structure: implications for ecological restoration. Restoration Ecology, 17, 837-844.

Skog, A., Zachos, F., Rueness, E., Feulner, P., Mysterud, A., Langvatn, R., Lorenzini, R., Hmwe, S., Lehoczky, I. & Hartl, G., Stenseth, N & Jakobsen, K. (2009). Phylogeography of red deer (*Cervus elaphus*) in Europe. Journal of Biogeography, 36, 66-77.

Stewart, J.R. & Lister, A.M. (2001). Cryptic northern refugia and the origins of the modern biota. Trends in Ecology & Evolution, 16, 608-613.

Stewart, J.R. & Dalen, L. (2008). Is the glacial refugium concept relevant for northern species? A comment on Pruett and Winker 2005. Climatic Change, 86, 19-22.

Stewart, J.R., Lister, A.M., Barnes, I. & Dalen, L. (2010). Refugia revisited: individualistic responses of species in space and time. Proceedings of the Royal Society B-Biological Sciences, 277, 661-671.

Sunnucks, P. (2000). Efficient genetic markers for population biology. Trends in Ecology & Evolution, 15, 199-203.

Svenning, J.C. (2002). A review of natural vegetation openness in north-western Europe. Biological Conservation, 104, 133-148.

Svenning, J.C., Normand, S. & Kageyama, M. (2008). Glacial refugia of temperate trees in Europe: insights from species distribution modelling. Journal of Ecology, 96, 1117-1127.

Taberlet, P., Fumagalli, L., Wust-Saucy, A.G. & Cosson, J.F. (1998). Comparative phylogeography and postglacial colonization routes in Europe. Molecular Ecology, 7, 453-464.

Taheri, S., Lee Abdullah, T., Yusop, M. R., Hanafi, M. M., Sahebi, M., Azizi, P., & Shamshiri, R. R. (2018). Mining and development of novel SSR markers using next generation sequencing (NGS) data in plants. Molecules, 23(2), 399.

Terry, J., Probert, R.J. & Linington, S.H. (2003). Processing and maintenance of the Millennium Seed Bank collections. Seed Conservation: turning science into practice. Royal Botanic Gardens, Kew.

Thorley, A. (1981). Pollen analytical evidence relating to the vegetation history of the Chalk. Journal of Biogeography, 93-106.

Tilman, D., Fargione, J., Wolff, B., D'antonio, C., Dobson, A., Howarth, R., Schindler, D., Schlesinger, W.H., Simberloff, D. & Swackhamer, D. (2001). Forecasting agriculturally driven global environmental change. Science, 292, 281-284.

Tzedakis, P.C., Emerson, B.C. & Hewitt, G.M. (2013). Cryptic or mystic? Glacial tree refugia in northern Europe. Trends in Ecology & Evolution, 28, 696-704.

Tzedakis, P.C., Lawson, I.T., Frogley, M.R., Hewitt, G.M. & Preece, R.C. (2002). Buffered tree population changes in a quaternary refugium: Evolutionary implications. Science, 297, 2044-2047.

Vera, F.W.M. (2000). Grazing ecology and forest history. CABI, Wallingford.

Vitousek, P.M., Mooney, H.A., Lubchenco, J. & Melillo, J.M. (1997). Human domination of Earth's ecosystems. Science, 277, 494-499.

von Oheimb, G., Schmidt, M., Kriebitzsch, W.-U. & Ellenberg, H. (2005). Dispersal of vascular plants by game in northern Germany. Part II: Red deer (Cervus elaphus). European Journal of Forest Research, 124, 55-65.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T., Hornes, M., Friters, A., Pot, J., Paleman, J. & Kuiper, M. (1995). AFLP: a new technique for DNA fingerprinting. Nucleic acids research, 23, 4407-4414.

WallisDeVries, M.F., Poschlod, P. & Willems, J.H. (2002). Challenges for the conservation of calcareous grasslands in northwestern Europe: integrating the requirements of flora and fauna. Biological Conservation, 104, 265-273.

Werth, S. (2011). Optimal sample sizes and allelic diversity in studies of the genetic variability of mycobiont and photobiont populations. The Lichenologist, 43, 73-81.

Whitlock, R., Hipperson, H., Thompson, D., Butlin, R. & Burke, T. (2016). Consequences of in-situ strategies for the conservation of plant genetic diversity. Biological Conservation, 203, 134-142.

Willerding, U. (1986). Zur Geschichte der Unkräuter Mitteleuropas. Wachholtz, Kiel.

Williams, J.W., Shuman, B.N. & Webb, T. (2001). Dissimilarity analyses of late-Quaternary vegetation and climate in eastern North America. Ecology, 82, 3346-3362.

Willis, K.J. & Whittaker, R.J. (2002). Species diversity - scale matters. Science, 295, 1245-1248.

Willis, K.J. & van Andel, T.H. (2004). Trees or no trees? The environments of central and eastern Europe during the Last Glaciation. Quaternary Science Reviews, 23, 2369-2387.

Willis, K.J., Rudner, E. & Sumegi, P. (2000). The full-glacial forests of central and southeastern Europe. Quaternary Research, 53, 203-213.

Wisz, M.S., Hijmans, R., Li, J., Peterson, A.T., Graham, C. & Guisan, A. (2008). Effects of sample size on the performance of species distribution models. Diversity and Distributions, 14, 763-773.

Woodward, F.I. (1987). Climate and plant distribution. Cambridge University Press, Cambridge.

Woschée, R. (2009). Prioritätenliste für den botanischen Artenschutz in Bayern.

Yeh, F.C., Yang, R. & Boyle, T. (1999). POPGENE Version 1.31 - Microsoft Window-based Freeware for Population Genetic Analysis - Quick User Guide. University of Alberta, Edmonton.

Zabeau, M. & Vos, P. (1993). Selective restriction fragment amplification: a general method for DNA fingerprinting. European patent application, No. 0534858.

Zalapa, J.E., Cuevas, H., Zhu, H., Steffan, S., Senalik, D., Zeldin, E., McCown, B., Harbut, R. & Simon, P. (2012). Using next-generation sequencing approaches to isolate simple sequence repeat (SSR) loci in the plant sciences. American Journal of Botany, 99(2), 193-208.

Zane, L., Bargelloni, L. & Patarnello, T. (2002). Strategies for microsatellite isolation: a review. Molecular Ecology, 11, 1-16.

DANKSAGNUNG

Als erstes möchte ich bei meinem Doktorvater Prof. Dr. Peter Poschlod für die Betreuung und Unterstützung während der gesamten Zeit meiner Promotion bedanken. Auch für das entgegengebrachte Vertrauen und die Freiheit zur selbstständigen Arbeit am Lehrstuhl.

Ebenfalls danke ich Prof. Dr. Christoph Reisch nicht minder für die Unterstützung im Bereich der molekularen Ökologie und Naturschutzgenetik. Dank an beide für die Beharrlichkeit, Geduld und Motivation, welche nötig waren, um diese Arbeit zum Abschluss zu bringen.

Vielen Dank für die Möglichkeit, an dem Projekt Genbank Bayern Arche zu arbeiten, in dessen Rahmen diese Arbeit finanziert wurde.

Ich bedanke mich bei meinem Mentor John Dickie für die eingebrachte Fachkenntnis und die Begutachtung der Arbeit.

John Dickie, Langlois Estelle, Christine Frohnauer, Melanié Harzé, Daniela Listl, Anton Mayer, Richard Michalet, Nicola Schoenenberger, Sebastian Teufel und Molnar Zsolt danke ich für die Sammlung von Blattproben von *Hippocrepis comosa*.

Zudem danke ich allen (ehemaligen) Mitarbeitern des Lehrstuhls Poschlod sowie der Genbank Bayern Arche für die gute Zusammenarbeit. Ein herzlicher Dank geht an Markus Hirtreiter, Maria Hanauer und Philipp Glaab.

Abschließend bedanke ich mich bei meiner Familie für die großartige, jahrelange Unterstützung und besonders bei Simone Tausch. Ohne diese besondere Verbundenheit und die fortwährende intensive Zusammenarbeit wäre diese Arbeit nicht zustande gekommen.