Towards preserving threatened grassland plant species and habitats - seed longevity, seed viability and phylogeography

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CHAPTER 1

General introduction

THREATENED AND ENDANGERED HABITATS

Regarding the situation of Europe's plant species inventory, Central Europe represents the centre of endangered plant species. Germany is ranking on the upper limit: The out-dated German Red Data Book on endangered plant species lists about 40 % of its native flora as endangered, very rare or potentially endangered (Ludwig & Schnittler, 1996). The current FFH-report "Lage der Natur" shows that habitats with poor conservation status increased from 61 to 70 % from 2007 to 2014 (BfN, 2014).

The prognosis for endangerment strongly depends on the regenerative capacity of a habitat which is not only determined by the development of suitable environment conditions (nitrogen content or humidity) but also on enabling, establishing and maintaining circumstances that have led to the development of a habitat type, e.g. in Central Europe mainly agricultural habitats in an anthropogenous landscape. Correspondingly, the status of thirteen habitats in Germany has worsened within the last seven years, of which six depend on sustainable land use and maintenance (BfN, 2014).

CALCAREOUS GRASSLANDS: THREATENED CULTURAL LANDSCAPE

Calcareous grasslands are among the most species-rich ecosystems in Central Europe (WallisDeVries et al., 2002; Sádlo, 2007). Their extent and quality is declining since the end of the 19th century (Quinger et al., 1994) due to intensification or abandonment of anthropo-zoogenic usage (Poschlod & WallisDeVries, 2002). The habitat is listed in the Annex I of the Natura 2000 Habitats Directive (92/43/EEC), ranking among critically endangered (xeric grasslands) and endangered (semi dry calcareous grasslands) (Riecken et al., 2006). The current German FFH-report (2014) demonstrates an insufficient conservation status (BfN, 2014).

Correspondingly, the recoverability/substitutability of dry grasslands is of great interest (Piqueray et al., 2011). It was shown that short-term re-establishment of calcareous grasslands was successful for species with persistent soil seed banks or long distance dispersal ability (von Blanckenhagen & Poschlod, 2005). But in general, soils of calcareous grasslands exhibit low ability to buffer species extinctions by serving as donor (Thompson et al., 1997; Bekker et al., 1998a; Kalamees & Zobel, 1998; Poschlod et al., 1998; Stöcklin & Fischer, 1999; Karlik & Poschlod, 2014). In present anthropogenous landscapes calcareous grasslands are strongly fragmented and long-distance dispersal vectors such as sheep are largely missing. When using seeds for restoration, the success strongly depends on the quality of the used seed material and its autochthony (Walker et al., 2015).

A number of studies has dealt with the question about the "nearly unknown origin and history of European grasslands" (Poschlod & Baumann, 2010). Created, expanded and maintained by human activities (Poschlod & WallisDeVries, 2002; Schmidt et al., 2007) since the Neolithic periods (e.g. Bush, 1993; Tomescu, 2000; Dutoit et al., 2009), calcareous grasslands possessed expansion maxima at the Roman Period and Middle Ages (Pott, 1996; Poschlod & WallisDeVries, 2002; Poschlod & Baumann, 2010; Hájková et al., 2011). Long before human interference, their species have probably occurred naturally on rocky outcrops or steep sunny slopes with shallow dry soils that would impede tree growth (Ellenberg, 1996; Poschlod et al., 2008) but may have even survived in the non-closed forests until the beginning of human settlement (Pokorný et al., 2015). Even during (Bush, 1988) or before the early post-glacial, they may have grown at climatically favoured sites among the steppe tundra vegetation (Gradmann, 1933; Kuneš et al., 2008; Pokorný et al., 2015).

Equally as important as the knowledge about the temporal origin is the spatial origin of its species inventory, especially when facing its steady decline and attempts for restoration or *ex situ* conservation. In the traditional view, during the glacials, temperate species have retracted to the Iberian, Italian, Balkan peninsulas in southern Europe where the species could escape from cold dry climates and persist until they could repopulate Europe (Taberlet et al., 1998; Hewitt, 2000; Hewitt, 2004). To date only a small number of studies has considered the question of genetic lineages of dry grassland species (Bylebyl et al., 2008; Sutkowska et al., 2013; Harter et al., 2015). Due to limited budgets in nature conservation, the knowledge

about the (genetic) origin might become relevant when selecting valuable populations for conservation or the most suitable populations for restoration and *ex situ* conservation.

EX SITU CONSERVATION WITH SEED BANKS

Calcareous grassland habitats are only one example which clearly illustrates that direct degradation (Merritt & Dixon, 2011) and change of land use (Poschlod et al., 2005a; Maurer et al., 2006; Poschlod, 2015) strongly force the need to determine methods to preserve these valuable habitats and its species inventory. The UICN (CBD, 2010, 2015) emphasizes that *ex situ* conservation provides a "safety back-up" and an insurance policy against extinction in the wild, especially in the light of climate change. Therefore, the awareness of the importance of seed banks as a tool for *ex situ* conservation of rare and endangered plant species is increasing (Hay & Probert, 2013). In order to successfully store seeds over long periods and apply them for restoration it is crucial to understand the mechanisms and characteristics that enable seed survival, influence seed quality, dormancy and germination. Therefore, seed banking facilities with conservation background take advantage of modern tools available from agricultural science, combine it with knowledge of seed ecology and investigations of functional and evolutionary aspects to guarantee efficient seed conservation.

Endosperm, dormancy and desiccation tolerance

Seeds are the most significant innovation of vascular plants that arose in the course of plant evolution and the colonization of land approximately 350 mya (Clarke et al., 2011; Coiffard et al., 2012). This adaptation of sexual reproduction has strongly enhanced survival and dispersal, providing a selection advantage. Seeds equip the embryo with protection by a closed structure and nutrition for successful germination and establishment. These very adaptations now enable the survival of rare and threatened plant species in changing landscapes and can be successfully used for *ex situ* conservation.

Considering internal seed morphology, a seed contains the embryo, nutritive tissue (endosperm, perisperm or the haploid megagametophyte in gymnosperms) and is surrounded by the seed coat (testa) that originates from the integuments. Most seeds contain large nutritive reserves in the cotyledons or alternative storage tissue, which are composed of carbohydrates like starch, oils and storage proteins (Bewley et al., 2013). The endosperm not only serves as nutritive tissue during seed development but is also involved in seed growth, development and germination by signalling or by being a barrier for the growing radicle (Finch-Savage & Leubner-Metzger, 2006). It is either fully consumed before seed maturation and its nutrients are incorporated into the storage cotyledons or it is still present in the mature seed.

The ratio of embryo to seed size (E:S ratio) in mature seeds is largely associated with dormancy types and the relation has been analysed in several phylogenetic reconstructions (Martin, 1946; Nikolaeva, 1967; Baskin & Baskin, 1998; Forbis et al., 2002; Finch-Savage & Leubner-Metzger, 2006). Finch-Savage and Leubner-Metzger (2006) have stated that seeds of basal angiosperm species possess small E:S ratios and that in the course of evolution the E:S ratios have increased in advanced angiosperms. The evolutionary E:S ratio increase was accompanied with the evolution of dormancy (Finch-Savage & Leubner-Metzger, 2006) as morphological and physiological adaptations in response to different environmental conditions (Baskin & Baskin, 2004). The water impermeability of the coat of physically dormant seed, for instance, is assumed to be an adaptation of plants to specialized habitats (Baskin & Baskin, 2004).

The ability of seeds to tolerate desiccation is a trait of major adaptive importance to their survival and dispersal role (Dickie & Pritchard, 2002). For desiccation tolerant (orthodox) seeds, maturation drying is the terminal step in development after which they can remain viable up to several years (Bewley et al., 2013). Following drying, a number of cellular adaptations enable the preservation of cellular compartments required for continued metabolism, which control the repair of damages that occur during the dry state and germination or dormancy upon rehydration. Approximately 92 % of the spermatophyte species produce desiccation tolerant seeds (Tweddle et al., 2003). In contrast to orthodox seeds desiccation sensitive (recalcitrant) seeds are shed in a hydrated and metabolically active state and usually germinate soon after (Long et al., 2015).

Germination and dormancy

Dormancy and germination mechanisms are important to ensure that seeds will germinate when environmental conditions are suitable indicating temporal (e.g. seasons) or spatial windows (e.g. vegetation gaps) for establishment and survival (Bewley et al., 2013). In seed banks, the examination of (suitable) germination conditions not only serves as a method to detect seed viability, but it also provides knowledge for their future use in any restoration activities. In germination testing, several environmental conditions are imitated like different moisture availability, (alternating or constant) temperature, light (quantity,

daily distribution and spectral quality), availability of oxygen and chemicals (e.g. ethylene, karrikins, nitrates) that affect dormancy alleviation and germination (Finch-Savage & Leubner-Metzger, 2006; Bewley et al., 2013).

The process of germination begins with water uptake by the seed (imbibition) and is terminated with the emergence of the embryonic axis (mainly the radicle) (Bewley et al., 2013). A mature dry seed is quiescent, in a metabolical rest, until imbibition and metabolism activating conditions initiate germination (Baskin & Baskin, 2004; Bewley et al., 2013). Dormant seeds however do not germinate although imbibed and metabolically active. In other words, seed dormancy is defined as an intrinsic block to the completion of germination of a viable seed under favourable conditions for germination of the corresponding non-dormant seed (Baskin & Baskin, 2004; Finch-Savage & Leubner-Metzger, 2006; Linkies et al., 2010). Based on Nikolaeva (1967, 1977) and Baskin & Baskin (1998, 2004) the most applied classification of seed dormancy differentiates physiological (PD), morphological (MD), morphophysiological (MPD), physical (PHY) and combinational (PY + PD) dormancy from non-dormancy (ND). Physical dormancy (PHY) is broken when a seed coat disruption (e.g. scarification) allows imbibition (Baskin et al., 2000; Baskin, 2003). Seeds with MD need no special dormancy breaking treatment but time for embryo growth and germination. Depending on the level of MPD, seeds require more or less complicated (temperature sequence) pretreatments to break physiological dormancy (Baskin & Baskin, 2004). PD can be divided into three different types which are broken by after-ripening, scarification, cold or warm stratification (Baskin & Baskin, 2004). Furtheremore, seeds with PD are able to adapt their depth of dormancy on seasonal changes by dormancy cycling (Baskin & Baskin, 1998; Finch-Savage & Leubner-Metzger, 2006; Finch-Savage & Footitt, 2012, 2017; Long et al., 2015).

Seed viability

Besides seed collection and supply, a major task of seed storage facilities is to store seeds with high initial quality and viability and to maximize their longevity. In order to avoid storage of seeds with unknown viability, an assessment of viability should be performed. Several methods for viability testing are described by international rules and handbooks (ISTA, 1999, 2003; AOSA, 2010a): Preliminary viability assessments via cutting test, excised embryo test, Topographical Tetrazolium test and germination test provide useful evaluation before storage. Although a germination test is the most reliable test to determine seed viability, supposing the best germination condition of the species or accession is known, it is still very time-consuming (usually several weeks up to months for seeds requiring pre-treatments). The quicker test methods like cutting test or Tetrazolium testing are destructive and either imprecise or difficult to interpret. Because of that, mainly small seed banks forego these examinations and store seeds uncontrolled. ISTA (1999) accepts the X-ray method to distinguish full, empty, insect infested and physically damaged seeds. Despite being a promising and fast-forward method for viability detection, X-ray analysis is not yet recognized for testing viability (Elias et al., 2012).

Longevity and ageing ability

The inherent longevity of a seed can be described as "the lifespan of a seed after maturity, determined by a complex expression of physiological traits including cellular mobility, internal protective compounds and the ability of cells to resist and repair damage" (Long et al., 2015) and is strongly related to desiccation ability. It is the basis for seed persistence which itself may be influenced by abiotic and biotic factors (Kochanek et al., 2011; Abedi et al., 2014; Long et al., 2015). Aquired insights into longevity and seed ageing enables storage facilities to produce conditions that extend the viability of seeds up to hundreds of years (Walters et al., 2005a; Van Treuren et al., 2012): a dry and cold environment considerably slows down seed ageing (Walters, 1998; Smith et al., 2003; Kranner et al., 2011). Accelerated ageing methods (Smith et al., 2003; Probert et al., 2009) contribute significant knowledge to seed ageing.

Seed longevity is a quantitative plant trait that can vary between different populations within the same species based on genetic and environmental factors (Hay et al., 1997; Schoeman et al., 2010; Kochanek et al., 2011), whereas wild plant species are considered to have greater variation in longevity between different seed collections than crop species (Ellis et al., 1989; Hay et al., 1997; Kochanek et al., 2011)

Geographically large-scale examinations on seed longevity have been performed using artificial ageing to answer the question about prediction of seed longevity by phylogeny, seed traits and climate (Long et al., 2008; Probert et al., 2009; Mondoni et al., 2011; Merritt et al., 2014). The results show that seeds originating from warmer and dryer environments possess a greater longevity than those from cool, wet climates (Walters et al., 2005b; Long et al., 2008; Probert et al., 2009; Mondoni et al., 2011). While seed longevity depended at most slightly on seed size (Probert et al., 2009; Merritt et al., 2014), phylogeny (Walters et al., 2005b; Probert et al., 2009; Merritt et al., 2014), en-

dosperm proportion (Walters et al., 2005b; Probert et al., 2009; Mondoni et al., 2011; Merritt et al., 2014) and physical dormancy (Merritt et al., 2014) emerged as indicators for seed longevity. Gained knowledge has great implications for re-collection and re-testing strategies in *ex situ* conservation.

AIM OF THE THESIS AND EXPERIMENTAL APPROACH

The key elements that are addressed in this thesis are: (A) Phylogeography of calcareous grassland species on the example of *Sanguisorba minor* Scop. (B) Importance of seed traits on viability detection as well as *ex situ* and *in situ* seed survival and (C) prospects of seeds for conservation of calcareous grasslands. (D) Significance of X-ray analysis as a tool for reliable non-destructive viability detection. (E) Combining these topics, the aim of this thesis is to elucidate the origin of species of rare and threatened calcareous grasslands and to provide tools for a successful preservation of diversity via seeds.

To answer the issues of this thesis following scientific approaches were made: Phylogeographic study using AFLP (Chapter 2), X-ray analysis and tetrazolium test (Chapter 3 and 5), artificial ageing (Chapter 3), seed germination (Chapter 3 and 5), seed trait analysis (Chapter 2, 3, 4 and 5), phylogenetic constraints (Chapter 3) and soil seed bank persistence analysis (Chapter 4).

PHYLOGEOGRAPHIC STUDY

Phylogeographic studies can survey assumptions of genetic differentiation below as well as above species level without being restricted to taxonomic preconceptions based on morphological divergence (Schaal et al., 1998; Hewitt, 2001). The dominant marker AFLP (Vos et al., 1995) method was selected to study *Sanguisorba minor* as it produces many loci over the whole genome and it is a highly preferable method for the assignment of individuals to populations (Gaudeul et al., 2004). Furthermore, it has been successfully used to determine the postglacial history of plants (Schönswetter et al., 2005; Bylebyl et al., 2008; Reisch, 2008).

To identify potential glacial refugia, besides genetic variation (Comes & Kadereit, 1998; Taberlet et al., 1998; Tzedakis et al., 2013), we calculated the genetic divergence (frequency-down-weighted marker values, DW) (Schönswetter & Tribsch, 2005) as the rarity of markers has become an important criterion (Schönswetter et al., 2005; Paun et al., 2008). To survey genetic structures within all collected populations of *S. minor*, we performed a Bayesian cluster analysis (Pritchard et al., 2000; Pritchard et al., 2009) and a cluster analysis using Cavalli-Sforza & Edwards chord distances and Neighbour Joining (Schlueter & Harris, 2006). Geographic patterns in the data set were examined and displayed with a spatial Principal Component Analysis (sPCA) (Jombart et al., 2008).

SOIL SEED BANK ANALYSIS

From an ecological perspective, persistence allows seeds to disperse through time (Poschlod et al., 2005b; Poschlod et al., 2013). Soil seed persistence is important for community ecology as well as for conservation practices and management (Fischer et al., 1996; Maurer et al., 2003; Long et al., 2015). The survival of seeds in the soil is either determined by taking soil samples and examination of seed content (Thompson & Grime, 1979; Poschlod, 1993; Poschlod & Jackel, 1993; Bekker et al., 1998a; Poschlod et al., 1998; Wäldchen et al., 2005) or by seed burial trials under natural conditions (Telewski & Zeevaart, 2002; Long et al., 2008; Saatkamp et al., 2009; Abedi et al., 2014).

Soil seed bank persistence data were extracted from a compilation of Poschlod et al. (1998) that includes study results of calcareous grasslands studies (Kiefer, 1997), burial experiments (Poschlod, 1993) and the seasonal dynamics of diaspore bank and diaspore rain (Poschlod & Jackel, 1993). All these studies allowed a differentiated assignment into different categories from transient to persistent (Thompson et al., 1997; Poschlod et al., 1998). Additional persistence data was provided by the longevity index (LI) values (Thompson et al., 1997), which integrate a series of soil seed bank observations which, however, has to be carefully interpreted (Saatkamp et al., 2009).

ARTIFICIAL AGEING

To investigate seed survival under storage conditions we applied an artificial ageing test using hermetically sealed boxes with high humidity (40 %) at elevated temperature, according to the standardized protocol of Newton et al. (2009). Probert's (2009) screening of 195 species indicated a correlation between survival under rapid ageing and under gene bank conditions. Seed viability equations of rapid artificial ageing (Ellis & Roberts, 1980; Liu et al., 2008; Probert et al., 2009) are good approaches to predict the decline of seed viability.

SEED TRAITS

All chapters are connected to each other via seed traits: seed shape, seed mass, internal morphology (anatomy), seed dormancy, seed coat thickness and longevity index (LI). Seed shape and seed mass were measured and calculated (Bekker et al., 1998a) by hand, seed dormancy was determined via germination tests, anatomy and seed coat thickness were assessed by X-ray examination. As the embryo (shape, development and size) and the storage tissues (presence, absence and size of endosperm and/or perisperm) influenced the applicability of viability detection in both, X-ray and Tetrazolium testing, two seed classifications were used to group the species, based on Martin (1946), revised and extended by Finch-Savage & Leubner-Metzger (2006) and Meyer (2005). Longevity index data (Thompson et al., 1997) were sourced from the LEDA trait data base (Kleyer et al., 2008).

Moreover, external seed morphology played a crucial role for subspecies identification in *Sanguisorba minor*, which was investigated and compared with results from an excessive treatise of Nordborg (1967).

PHYLOGENETIC CONSTRAINTS

To correctly assess the influence of seed traits on the longevity of calcareous grassland species (Chapter 3), we used three phylogenetic comparative methods, to avoid misinterpretation of the results due to relatedness of species (Chapter 3). Pagel's λ (Pagel, 1999), Blomberg's K (Blomberg et al., 2003) and Fritz & Purvis' D (Fritz & Purvis, 2010) were calculated to reveal and account for phylogenetic signals.

VIABILITY DETECTION

To evaluate seed viability detection via X-ray, we compared the results with two accepted methods for viability detection, germination test and Topographical Tetrazolium test (ISTA, 2003; AOSA, 2010a).

Seeds were germinated in climate chambers on moist filter paper, at different germination conditions (constant or alternating temperatures, with or without pre-treatments like stratification or scarification).

The Tetrazolium test is based on a chemical staining reaction of seeds (Moore, 1973; Elias et al., 2012). Due to the activity of dehydrogenase enzymes during respiration, in viable seed tissues, colourless tetrazolium changes into a red dye known as formazan. To make viability determinations, the intensity and pattern of the staining as well as the physical condition of associated seed structures are evaluated. For this evaluation a certain knowledge of seed and seedling structures (e.g. shape and location of the embryo, type of storage tissues, nature of seed coats) and experience in differentiating normal and abnormal seeds is required.

The principle of X-ray testing is that X-rays are absorbed differently depending on the thickness and/or density of the seed tissue. The internal structures were subsequently digitally visualized as monochrome images and interpreted. Like the Tetrazolium test the X-ray test demands a certain expertise to interpret the results accurately. We differentiated the surveyed seeds as viable and non-viable, adopting ISTA rules on Tetrazolium testing (ISTA, 1999).

THESIS OUTLINE

Central Europe has to counter rapid habitat and species decline and still the spatial origin of many species is unexplored. The importance of seeds and their storage in *ex situ* facilities has become an object for threatened and endangered plant species. This thesis was compiled simultaneously to the establishment of the Genbank Bayern Arche (Tausch et al., 2015), a federal state *ex situ* seed bank project for threatened and endangered plant species. It comprises various aspects that are related to basic phylogeographic research and the preservation of endangered plant species and habitats on the example of species from calcareous grasslands.

Chapter 2 deals with the postglacial spread of a common calcareous grassland species, *Sanguisorba minor*. By means of molecular analysis (AFLP) a survey across the whole species range provides the opportunity to reveal migration routes after the last glaciation. Seed morphology, the main determinant of subspecies of the morphologically extremely variable species was combined with the genetic structure.

Compared to seeds of domestic plants, seeds of wild plant species are poorly explored. In particular, they possess more complicated germination characteristics than breeds of cultivated species. We were interested in the practical use of seeds for conservation purposes of calcareous grasslands. Using an artificial ageing approach, in **Chapter 3** we attempted to identify seed traits that can be associated with seed ageing and *ex situ* storage. By investigating in a single habitat, influences like climate differences that may occur and mask the significant traits, were eliminated.

Chapter 4 addresses the question whether ageing in soil and in *ex situ* storage of calcareous grassland species are correlated. We used longevity measures,

the soil seed bank persistence categories of Poschlod et al. (1998) and LI to elucidate the relationships. In addition, we attempted to illuminate the prospect of *in situ* and *ex situ* conservation under consideration of seed traits.

Due to uncontrolled environmental conditions, wild plant species produce seeds with strongly differing seed qualities and widely unknown storage ability (Gardarin et al., 2010). Therefore, besides the investigation of suitable germination conditions, a reliable and preferably non-destructive detection of initial seed viability is necessary for successful storage. In **Chapter 5** we investigated the performance of X-ray analysis to provide a tool for viability detection without destroying seeds. Comparative combined germination-Tetrazolium tests were conducted for approval and seed traits were used to examine the efficiency of X-ray analysis to detect viability and germinability of fresh seeds.

Finally, **Chapter 6** summarizes the importance of seeds considering plant origin and conservation of calcareous grasslands.

CHAPTER 2

Towards the origin of Central European grasslands: glacial and postglacial history of the Salad Burnet (*Sanguisorba minor* Scop.)

ABSTRACT

Calcareous grasslands belong to the most species rich and endangered habitats in Europe. However, little is known about the origin of the species typically occurring in these grasslands. To identify the postglacial recolonization of the widespread calcareous grassland species *Sanguisorba minor* (Scop.) in Central Europe, we investigated the spatial genetic structure of 38 populations via AFLP. Moreover, we considered the correlation of subspecies distribution (seed morphology) and spatial genetic patterns with regard to the two subspecies *minor* and *balearica*.

Our study revealed significant differences in the level of genetic variation and the occurrence of rare fragments within populations of *S. minor* from different regions of the distribution range and a distinct separation of eastern and western lineages. The analyses uncovered traditional southern but also cryptic northern refugia and point towards a broad fronted postglacial recolonization. The geographic distribution of the investigated subspecies corresponded to known references of Nordborg (1967), but not entirely reflected the genetic patterns. Genetic similarity between south-eastern and south-western subsp. *balearica* populations was lower than between northern subsp. *minor* and south-western subsp. *balearica* populations.

Based on the spatial genetic pattern we conclude that after the last glacial maximum (LGM) *S. minor* recolonized Central Europe either from Iberia or northern glacial refugia in France, Belgium or Germany. The current situation of *S. minor* subspecies depicts either incomplete lineage sorting or the existence of secondary hybrid zones, which is differently expressed in neutral marker and morphological differentiation. Our results highlight the importance of refugial areas for conservation of intraspecific variation in calcareous grassland species.

KEYWORDS

AFLP, LGM, phylogeography, plant, rare markers, recolonization, refugia, seed morphology

2.1 INTRODUCTION

Apart from contemporary genetic exchange, historical processes have strongly affected species' current ranges as well as the genetic variation and structure of their populations (Comes & Kadereit, 1998; Schaal et al., 1998; Hewitt, 2000; Soubani et al., 2014).

Regarding the landscape history of Europe, palynological and pedoanthracological studies have contributed great knowledge about the history of grasslands, but mostly by focusing on reforestation (e.g. Ložek & Cílek, 1995; Poschlod et al., 2008; Dutoit et al., 2009; Hájková et al., 2011). Due to low availability of pollen records for herbaceous plants (Poschlod & Baumann, 2010), the limited taxonomic power of pollen and the non-uniform processes of pollen deposition, such studies are only of indirect value for the reconstruction of the post-glacial distribution, abundance and history for many grassland species. It is known from several studies that small-scale natural grasslands have existed at preferable sites in the forest-dominated landscape of the post-glacial period (Ellenberg, 1996; Svenning, 2002; Karlik & Poschlod, 2009; Hájková et al., 2011; Janišová et al., 2011; Pokorný et al., 2015; Poschlod, 2015) and semi-natural grasslands have expanded at least since the Mesolithic–Neolithic transition, associated with (agricultural) activity of man (Hejcman et al., 2013; Poschlod, 2015). Whereas most agricultural species are expected to have originated and spread by man from the Fertile Crescent and the Mediterranean region (Poschlod, 2015), the origin of temperate grassland species, whether dependent or independent of man, has been analysed in fewer studies, mainly focusing on steppe species (Hensen et al., 2010; Harter et al., 2015; Kajtoch et al., 2016; Meindl et al., 2016). By the use of molecular markers, comprehensive phylogeographic studies (Avise et al., 1987; Avise et al., 1998) have provided knowledge about glacial refugia, long distance colonisations, fragmentations and isolations (Taberlet et al., 1998; Hewitt, 2001; Kadereit et al., 2004).

The Pleistocene was probably the most influential era for the composition of the present floras of the northern hemisphere. Advancing and receding ice sheets, changing temperatures and water availability caused retractions (during glacials) or expansions (during interglacials) of temperate plants and animals to areas with climatically favourable conditions. Especially during the last glacial maximum (LGM), when Northern and Central Europe were covered either by a large ice shield or permafrost soil (Lang, 1994; Ruddiman & Thomson, 2001). The vegetation of the interglacials may have resembled today's flora composition as at least the climate during interglacial periods was similar to the present Holocene (Frogley & Tzedakis, 1999). Temperate species in the western Palearctic are commonly regarded to have survived the glacials in refugia on southern Peninsulas, where the alleviated influence of the glacial cycles allowed long-term survival (Taberlet et al., 1998; Hewitt, 2000; Tzedakis et al., 2002; Hewitt, 2004). But recent phylogeographic studies have suggested additional northerly located microrefugia for temperate species within the steppe-tundra vegetation (Stewart & Lister, 2001; Magri et al., 2006; Bhagwat & Willis, 2008; Bylebyl et al., 2008; Tzedakis et al., 2013; Daneck et al., 2015). Climate modelling has supported these results (Ohlemüller et al., 2012; Boston et al., 2015). Altogether, species have shown individual responses to climate change, depending on different dispersal abilities, refugia locations and post-glacial migration trajectories (Stewart et al., 2010; Normand et al., 2011; Triponez et al., 2015).

It has been subject of debate, whether the time frames of the Quaternary climate oscillations were long enough to cause (allopatric) speciation or if they merely caused repeated range shifts and at most differentiation of isolated populations (Comes & Kadereit, 1998; Kadereit et al., 2004). Potentially, a secondary contact of divergent populations might have caused a loss of beginning differentiation or speciation, resulting in stasis (Kadereit et al., 2004), or sympatric speciation due to hybridisation as outcome of secondary contact (Stebbins, 1984; Comes & Kadereit, 1998). Moreover, it is often difficult to distinguish between hybridization and incomplete lineage sorting in sympatrically distributed lineages (Sang & Zhong, 2000), as these processes show very similar phylogenetic signatures (Wendel & Doyle, 1998). Incomplete lineage sorting is mainly observed in recently diverged populations that have retained and stochastically sorted ancestral polymorphisms (Maddison & Knowles, 2006). Especially due to a shorter life cycles of herbaceous plant species, more generations have passed since recolonization started, they are expected to have responded more quickly to climate changes than long-lived tree species (Comes & Kadereit, 1998).

To date, phylogeographic studies on plants mainly focused on trees (e.g. Petit et al., 2003; Tzedakis et al., 2013; Havrdová et al., 2015), the majority of available studies on herbaceous plants were conducted either on forest or on (arctic-) alpine plant species. Only a limited set of recent studies have demonstrated the colonisation history and the origin of Central European calcareous grassland species after the LGM (Fjellheim et al., 2006; Bylebyl et al., 2008; Meindl, 2012; Sutkowska et al., 2013; Harter et al., 2015). This is sur-

prising, since the Central European calcareous grasslands are among the most species-rich and highly endangered ecosystems (Poschlod & WallisDeVries, 2002; WallisDeVries et al., 2002). One reason for the high biodiversity lies in the fact that these grasslands are characterized by a transitional climate containing phytogeographic elements of continental, Mediterranean, Atlantic and Pannonian regions (Hejcman et al., 2013). While for sub-oceanic *Festuca pratensis* (Fjellheim et al., 2006) and (sub-) Mediterranean *Eryngium campestre* (Bylebyl et al., 2008) a re-colonisation of Central Europe originating from south-western and (south-) eastern glacial refugia was proposed, continental steppe plants in Central Europe like *Scorzonera purpurea* (Meindl, 2012) are supposed to have originated only from south-eastern European regions. However, for sub-oceanic-subcontinental *Corynephorus canescens* (Harter et al., 2015) and oceanic *Hippocrepis comosa* (Leipold et al., 2017) a migration route from Western Europe to Central Europe was presumed. Some of these studies also supported the possibility of "cryptic" northern refugia (Stewart & Lister, 2001) contributing to the explanation of contemporary genetic structures and variations of these grassland species.

To detect the glacial and post-glacial history of the widespread Central European grassland species *Sanguisorba minor* Scop. (Salad Burnet, Rosaceae), the present study investigates the genetic composition and structure of populations across Europe via AFLP (amplified fragment length polymorphism), which have been successfully used before to identify taxonomic relationships on different taxonomic levels (El Rabey et al., 2014; Wietsma et al., 2014; Reisch et al., 2015) and (post-)glacial history (Schönswetter et al., 2005; Bylebyl et al., 2008; Reisch, 2008; Daneck et al., 2015).

Phylogeographic studies analyse genetic differentiation both below and above species level without being restricted to taxonomic preconceptions based on morphological divergence (Schaal et al., 1998; Hewitt, 2001). Yet genetic variation and differentiation can be reflected in morphological characters and molecular markers (Bateman et al., 2006; Kadereit & Yaprak, 2008; Kropf, 2008; Scheepens et al., 2013). Thus we additionally investigated the connection of phylogeographic pattern of neutral marker and morphological differentiation of the challenging morphologically variable plant species (Nordborg, 1967; Tutin et al., 1968; Dahlgren, 1995). Our study considers the two of three subspecies with distribution in Central and Southern Europe, subsp. *minor* (Scop.) and subsp. *balearica* (Bourg. ex Nyman) Muñoz Garm. & C. Navarro, that can in fact be differentiated via seed (hypanthia) morphology, but of which inhomogeneous hybrid swarms and intermediate individuals due to hybridization have frequently been described (Nordborg, 1967; Tutin et al., 1968; Muñoz Garmendia & Navarro, 1999).

We aimed to answer the following questions: (1) Where were putative glacial refugia of *S. minor* located and what were the post-glacial recolonization routes? (2) Does the genetic structure of *S. minor* reflect its intraspecific morphological descriptions and the geographical distribution of the subspecies?

2.2 MATERIALS AND METHODS

SPECIES DESCRIPTION AND FIELD SAMPLING

The Salad Burnet, *Sanguisorba minor* (Rosaceae), is a regular element in dry, semi-dry grasslands or lowland hay meadows. It grows on waysides, railroad embankments, gravel, steep slopes and rocks up to 2200 m. The species is described as a suboceanic-subcontinental floristic element, which is widely distributed in the Western, Central and Southern European, the Western Asian and the Northern African flora and has been introduced to large parts of Northern America (Dahlgren, 1995).

For the genetic analysis of *S. minor*, due to financial limitations, we decided in favour of collecting a high number of populations in place of high sample size within population. Fresh leaf material from 5 plant individuals of 38 populations in 14 countries (see Figure 2.1) was collected and immediately dried with silica gel. Where available, seeds (nutlets) were collected for a proper delimitation of subspecies.

S. minor subspecies are morphologically extremely variable considering vegetative and generative traits (Tutin et al., 1968; Dahlgren, 1995; Muñoz Garmendia & Navarro, 1999). Along with the absence of reproductive barriers, taxon delimitation is difficult (Dahlgren, 1995) and the low correspondence of traits within a single plant or population has led to inconsistent taxonomic designations. For instance, according to the online data base of Flora Iberica (2015), 36 heterotypic synonyms had been assigned alone to the subspecies *S. minor* subsp. *minor*.

In her extensive treatise, Nordborg (1967) differentiated five subspecies of *S. minor*: subsp. *minor* (Scop.), subsp. *lasiocarpa* (Boiss. & Hausskn.) Nordborg, subsp. *magnolii* (Spach) Nordborg, subsp. *muricata* (Spach) Nordborg and subsp. *rupicola* (Boiss. &Reut.) Nordborg. The more recent classification in the Flora Iberica (Muñoz Garmendia & Navarro, 1999)

considers subsp. *magnolii* and subsp. *rupicola* as distinct species *S. verrucosa* (Link ex G. Don) Ces. and *S. rupicola* (Boiss. & Reut.) A. Braun & C.D. Bouché. and subsp. *muricata* as subsp. *balearica* (Bourg. ex Nyman) Muñoz Garm. & C. Navarro. In the investigated area of this study, covering the latitude from 8 °W to 22 °E and the longitude from 38 to 55 °N (see Table 2.1), two subspecies have been described (Nordborg, 1967; Tutin et al., 1968): subsp. *minor* and subsp. *balearica*. The remaining subsp. *lasiocarpa* occurs in temperate Asia was not investigated in this study. Moreover, as the subspecies possess no sterility barriers, intermediate forms that may be attributed to hybridization, can be observed between subsp. *balearica* and subsp. *minor* or subsp. *balearica* and *S. verrucosa*, which makes delimitation partially difficult (Nordborg, 1967; Tutin et al., 1968; Dahlgren, 1995).

The investigated subspecies were differentiated based on hypanthium morphology according to Nordborg (1967) and (Muñoz Garmendia & Navarro, 1999):1. *S. minor* subsp. *minor*: hypanthium with ridges, faces reticulate. 2. *S. minor* subsp. *balearica*: hypanthium winged, faces with irregular sculptures. We moreover differentiated two form series of subsp. *balearica* according to the form series of subsp. *muricata* of Nordborg (1967): A. platylopha-series: distinctly winged hypanthia with irregularly structured faces, referred to as subsp. *balearica* (platylopha). B. stenolopha-series: wings ridge-like and faces with reticulate sculptures, referred to as subsp. *balearica* (stenolopha). Co-occurring *S. verrucosa* can mainly be differentiated by its strongly verrucose or brain like hypanthium without ridges or wings. *S. rupicola* occurs beyond our study area in south-eastern Iberia and northern Morocco and is distinguished by habitus and ellipsoid hypanthium.

Based on this identification key, six populations (1, 2, 3, 5, 15 and 17) with available seeds were identified as subsp. *minor* (Table 2.1). Seven populations (25, 27, 29, 31, 35, 38) were assigned to as subsp. *balearica* (platylopha) and two populations (24 and 33) to subsp. *balearica* (stenolopha). Populations 21, 22 and 23 were composed of seeds assigned to subsp. *balearica* (stenolopha) and to subsp. *balearica* (platylopha). Population 26 contained subsp. *balearica* (stenolopha) and subsp. *balearica* (platylopha) individuals as well as individuals with wingless verrucose seeds assigned to *S. verrucosa*. Population 20 contained strongly intermediate verrucose winged seeds, which were referred to as *balearica* x *verrucosa* and *S. verrucosa*-individuals.

S. minor subsp. *minor* occurs throughout most of the species' range, mainly present in Central and Northern Europe. Subsp. *balearica* is native to Southern Europe and according to Tutin et al. (1968) naturalised in Central Europe. According to Nordborg (1967), in overlapping regions these subspecies are isolated either by altitude or ecological niche. In Southern Europe, subsp. *minor* is confined to altitudes above 1,000 m and replaced by subsp. *balearica* in altitudes below 1,000 m. Growing within one habitat, subsp. *minor* and subsp. *balearica* occupy different ecological niches but they occasionally occur together (Nordborg, 1967). While subsp. *minor* grows on stony slopes with relatively few other species, subsp. *balearica* grows in dense meadow vegetation. *S. verrucosa* can be found in the Mediterranean region and commonly occurs in macchia, garigue and phrygana (Nordborg, 1967).

DNA ISOLATION AND AFLP FINGERPRINTING

Genomic DNA was extracted from 15-20 mg dry leaf samples using the CTAB-method (Rogers & Bendich, 1994) following an adapted protocol outlined in Reisch & Kellermeier (2007). The DNA contents of each sample were measured photometrically and adjusted at 7.8 ng/µl.

The dominant marker AFLP (Vos et al., 1995) method was selected as it produces many loci over the whole genome and it is a highly preferable method for the assignment of individuals to populations (Gaudeul et al., 2004). The AFLPs were conducted adopting the protocol from Beckmann Coulter as described previously (Bylebyl et al., 2008; Reisch, 2008). Following a wider pre-screening, for the subsequent selective DNA amplification two primer pairs produced clear band patterns (M-CAC/D2-E-ACC, M-CAA/D3-E-AGG, Beckman Coulter, Krefeld).The selective PCR products were appropriately diluted with 1x TE0.1 buffer for AFLP to receive optimal results with fluorescent dyes of the selective primers. Pelleted DNA was re-dissolved in a mixture of 24.8 µl sample loading solution and 0.2 µl CEQ Size Standard 400 (both Beckman Coulter). The fluorescence-labelled selective amplification products were separated by capillary gel electrophoresis on an automated sequencer (CEQ 8000, Beckman Coulter). Raw data were collected and analysed with the CEQ Size Standard 400 using the CEQ 8000 software (Beckman Coulter). Results were analysed in Bionumerics 6.6 (Applied Maths). After manually scoring AFLP profiles, the presence or absence of bands for every particular fragment size and individual was transformed into a binary (1-0) matrix, serving as basis for all further analysis. For individuals with no clear banding signals AFLPs were repeated.

DATA ANALYSIS

Refugia are expected to possess higher genetic variation and can therefore be distinguished from surrounding recolonized regions, where recent dispersal might have led to genetic depauperation due to founder effects (Hewitt, 1996; Comes & Kadereit, 1998; Taberlet et al., 1998; Tzedakis et al., 2013). Therefore, within-population genetic variation as Nei´s Gene Diversity He (Nei, 1972), Shannon´s Information Index (SI) and the number and percentage of polymorphic loci (PL and PPL) were calculated using POPGENE v. 1.31 (Yeh et al., 1999).

Besides higher genetic variation, rare fragments have become an important criterion for identifying refugia (Schönswetter et al., 2005; Paun et al., 2008). As measure of divergence, the rarity of markers by frequency-down-weighted marker values (DW) was calculated (Schönswetter & Tribsch, 2005) with the r-script AFLPdat (Ehrich, 2006) in R v.3.1.1 (Development Core Team, 2014). The value of DW is expected to be high in long-term isolated populations (Paun et al., 2008) where rare markers should accumulate due to mutations whereas newly established populations are expected to exhibit low values, thus DW values assist in distinction between old vicariance from recent dispersal (Schönswetter & Tribsch, 2005). According to Paun et al. (2008) rare fragments (DW values) are better indicators for locating glacial refugia than patterns of genetic diversity, which rather mirror contemporary processes like connectivity of populations and population sizes. He and DW values were plotted onto the geographic coordinates of the sample locations.

Single He, SI, PPL and DW values of each population were opposed to the average of all populations using alternative one sample t-tests in order to categorize them as below (↓) or above average (↑) in R (R Core Team, 2013).

To survey the existence of a genetic structure within all collected populations of *Sanguisorba minor*, a Bayesian cluster analysis in STRUCTURE v 2.3 was performed (Pritchard et al., 2000, 2009). Assuming that all individuals originate from one population and based on the band frequencies in multiple loci, the program calculates a k value by multiple Markov chain Monte Carlo (MCMC) algorithm, an estimate of the number of genetically related groups in the data set. Individuals are assigned to one or several groups, unbiased by their original population. The no admixture ancestry and correlated allele frequency model was used to investigate k from 2 to 40, performing a burn-in period of 10,000 followed by 10,000 iterations with 10 replicate runs. Δk was calculated to assess the most likely number of groups following the method of Evanno et al. (2005). The results were plotted onto the geographic coordinates of the sample locations.

To identify geographic patterns in our data set we performed a spatial Principal Component Analysis (sPCA) using the package adegenet (Jombart et al., 2008) in R v.3.1.1 (Development Core Team, 2014). Geographical coordinates of each population originally recorded in the reference system WGS 1984 were projected to ETRS 1989 LCC. In order to avoid identical coordinates of individuals belonging to one population, the coordinates were modified by random shifts by the smallest possible factor of 0.5. Geographical data were used to generate a spatial weighting matrix that contains all spatial proximity information, derived from Delaunay triangulation as connection network. Moran's I (Moran, 1948) was then calculated to measure the spatial structure in the band frequencies of AFLP data. 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, whereas negative values indicate a local structure and zero a totally random pattern. For the assessment and interpretation of local and global structures, a screeplot was drawn by plotting the variance of the sPCA against Moran's I. To statistically test for presence of spatial structures, Monte Carlo tests were performed using 9999 permutations. The sPCA-screeplot revealed an apparent differentiation of the eigenvalues of the first and the second global scores λ1 and λ2 in terms of geographical and genetic variance and a significant global ($p < 0.001$) but no local ($p = 1$) spatial structure (Monte-Carlo test structure).The genetic differentiation of the principal components was therefore plotted for the first two global scores λ1 and λ2 against geographical coordinates.

Moreover, we performed a cluster analysis based on the 38 studied populations of *S. minor* in FAMD v.1.30 (Schlueter & Harris, 2006) using Cavalli-Sforza & Edwards chord distances and Neighbour Joining (NJ). The unrooted tree was displayed with Tree View (Page, 1996).

To investigate the variation within and between the surveyed populations and to quantify the differentiation between regions, we performed analyses of molecular variance (AMOVA, Excoffier et al., 1992), using GENALEX v6.5 (Peakall & Smouse, 2012), using resultant sPCA-groups and NJ-clusters as regions. Based on Euclidean pairwise genetic distances between the individuals (ΦPR), populations (ΦPT) and regions (ΦRT), the sums of squares were calculated (SSWP) and divided by the degrees of freedom (SSWP/n-1).He and DW values within detected regions

were additionally compared using ANOVAs and subsequent Tukey-HSD-tests in R (R Core Team, 2013).

CORRELATION BETWEEN GENETIC PATTERNS AND HYPANTHIUM MORPHOLOGY

To combine the genetic patterns with the morphology of hypanthia (subspecies delimitation), the subspecies (subsp. *minor*, subsp. *balearica* and *balearica* x *verrucosa*) were presented graphically on the unrooted NJ tree.

2.3 RESULTS

The AFLP analysis of 190 individuals with two primer combinations resulted in 166 fragments (D2: 97, D3: 69 fragments), ranging between 60 and 420bp, of which 94.58 % were polymorphic. Nei's Gene Diversity (He) ranged between 0.06 and 0.22 (mean 0.16 ± 0.01), Shannon Index (SI) between 0.09 and 0.33 (mean 0.23 ± 0.01) and the percentage of polymorphic loci (PPL) between 16.87 % and 57.83 % (mean 41.47 ± 1.56, Table 2.1, Figure 2.1). Firstly, some of the most diverse populations were located north of the southern peninsulas in Belgium (population 08: PPL = 57.8 %, He = 0.22 ± 0.21, SI = 0.33 ± 0.30), the Pyrenees (population 03: PPL = 51.8 %, He = 0.19 ± 0.21, SI = 0.29 ± 0.30), western France (population 04: PPL = 51.2 %, He = 0.22 ± 0.22, SI = 0.31 ± 0.31), Southern Germany (population 13: PPL = 48.2 %, He = 0.19 ± 0.21, SI = 0.28 ± 0.31) and Switzerland (population 11: PPL = 48.8 %, He = 0.19 ± 0.21, SI = 0.28 ± 0.30). Similar high levels of genetic diversity were also found in Southern France (population 26: PPL = 53.0 %, He = 0.19 ± 0.20, SI = 0.29 ± 0.29) and on the southern peninsulas in Croatia (population 31: PPL = 48.2 %, He = 0.19 ± 0.22, SI = 0.28 ± 0.31), Gulf of La Spezia (population 27: PPL = 46.4 %, He = 0.19 ± 0.22, SI = 0.27 ± 0.31) and northern Spain (population 21: PPL = 50.0 %, He = 0.18 ± 0.21, SI = 0.27 ± 0.29). Accordingly, populations with the lowest diversity were located as well north of as on the southern peninsulas, e.g. in Germany (population 16: PPL = 17.5 %, He = 0.06 ± 0.14, SI = 0.09 ± 0.21 and population 17: PPL = 16.9 %, He = 0.06 ± 0.15, SI = 0.09 ± 0.21), the Czech Republic (population 18: PPL = 28.9 %, He = 0.10 ± 0.17, SI = 0.15 ± 0.25) or in central Spain (population 22: PPL = 25.9 %, He = 0.10 ± 0.18, SI = 0.15 ± 0.26).

DW values ranged between 3.07 and 7.15 (mean 4.66 ± 0.14). The lowest DW values were located in Greece (population 37: DW = 3.07) and in the Czech Republic (population 18: DW = 3.32), the highest in Belgium (population 08: DW = 7.15) and in northern Spain (population 01: DW = 7.12). Significantly higher DW values than on average were found in Atlantic regions of northern Spain and western France (population 01: DW = 7.12 and population 04: DW = 4.97), in Mediterranean regions at the Gulf of La Spezia (population 27: DW = 5.10, Italy), Central Italy (population 29: DW = 5.85) and north-western Croatia (population31: DW = 5.71), in the Continental region of south-eastern Serbia (population 38: DW = 6.13) and in Sub-oceanic-subcontinental regions of Wallonia (population 08: DW = 7.15, Belgium), North Rhine-Westphalia (population 12: DW = 5.14, Germany), Lower Franconia (population 14: DW = 5.31, Germany) and Upper Palatinate (population 16: DW = 5.13, Germany).

The results with spatial reference indicated a strong separation of the investigated area in an eastern and western lineage or a north-western (NW), south-western (SW) and south-eastern (SE) lineage (see Figure 2.2, Figure 2.3). Bayesian cluster analysis conducted with STRUCTURE assigned the investigated individuals to three distinct genetic groups (k = 3, appendix Figure 2.5). Figure 2.2 shows an unambiguous placement of group 1 individuals into populations of the south-eastern study area and a combination of group 2 and 3 genotypes in the centre, northwest and southwest of the study area. Populations involving only group 1 genotypes were located in Southeast Europe (Greece, Republic of Macedonia, Serbia), southeast Central Europe (Croatia, Hungary, Slovenia), the Apennines and southern Alps in Italy. Exceptions from the almost genetically uniform south-eastern study area were population 27 (Italy), containing additional genotypes assorted to group 2, populations 32 (Croatia), 36 (Republic of Macedonia), 37 (Greece), containing also genotypes assorted to group 3 and population 19 (Slovenia) with genotypes assorted to all three groups. Group 2 and 3 individuals occurred in varying proportions in populations of the Iberian Peninsula (Spain), north-western Europe (South England, France, Belgium) and Central Europe (Germany, Switzerland, Czech Republic).

An even clearer east-west division appeared in the sPCA analysis (first global score λ1, Figure 2.3A, appendix Figure 2.6). Populations in the south-western (Iberian Peninsula) and the central study area (France, Great Britain, Belgium, Germany, Switzerland and Czech Republic) were separated from populations located south-east of the Alps in the eastern study area (Italy, Balkan Peninsula, Greece, Hungary). The weaker second global score λ2 presented a distinction between most Mediterranean populations (all populations on the Iberian Peninsula and in Southern France, population 27 and 30 in Italy, population 37 in Greece and population 36 in the Republic of Mac-

edonia and population 32 in Croatia) and all populations north and east of the Mediterranean region plus populations 29 (Italy), 31 and 33 (both Croatia; Figure 2.3B).

The neighbour joining tree generated by FAMD revealed three distinct clusters, corresponding their spatial distribution, embracing populations in the north-western (NW), south-western (SW) or south-eastern (SE) study area (Figure 2.4). Populations mainly originating from north-western and Central Europe (England, France excluding Mediterranean but including Pyrenees, Belgium, Germany, Switzerland, Czech Republic and Slovenia) were assigned to cluster NW. Cluster SW represents populations from the western Mediterranean region (Southern France, North-western Italy and Iberia excluding the northernmost populations) and cluster SE eastern Mediterranean and eastern European populations. It was difficult to assign population 27 (Italy) and 19 (Slovenia) to either cluster.

The distinct south-eastern populations of the sPCA (first global score λ1) were entirely congruent with the cluster SE of the NJ tree, except from population 26 (Italy). Regarding the second global score λ2 of the sPCA, the NJ tree and sPCA produced slightly different results concerning the affiliation of the Iberian Peninsula. While in the NJ tree, the northern Spanish and Pyrenean populations were assigned to the northern cluster (NW), the sPCA separated all populations located on the Iberian Peninsula and the Pyrenees from all northern populations.

The analysis of molecular variance (AMOVA) encompassing all populations without further classification revealed a total molecular variance of 65 % within and 35 % among populations (Table 2.2). Considering the sPCA-groups, the molecular variation between northern and southern populations was 6 %, whereas the variation between eastern and western populations was 10 %. The highest genetic variation of 16 % was detected between the three NJ clusters, NW, SW and SE, with a differentiation between the clusters SE and SW of 19 % and a lower differentiation between NW and SE as well as between NW and SW of 15 and 14 %, respectively. The mean genetic variation (He) was not significantly different between the NJ clusters (NW, SW and SE; $F = 0.499$, $N = 38$, $p = 0.611$) and the sPCA-regions north-south ($F = 0.377$, $N = 38$, $p = 0.543$) or east-west ($F = 1.532$, $N = 38$, $p = 0.224$). Equally, the mean DW values did not differ significantly between the NJ clusters ($F = 0.089$, $n = 38$, $p = 0.915$) and the sPCA-regions north-south ($F = 2.249$, $n = 38$, $p = 0.142$) or east-west ($F = 0.028$, $n = 38$, $p = 0.867$).

Comparing phylogeographic patterns with subspecies assignment of *S. minor*, Figure 2.4 shows that all six populations of subsp. *minor* (populations 1, 2, 3, 5, 15 and 17) were placed within the NJ cluster NW. Homogenous subsp. *balearica* (platylopha) populations were found in cluster SW (populations 25) and in cluster SE (populations 27, 29 ,31 ,35 ,38). The two subsp. *balearica* (stenolopha) populations (population 24 and 33) occurred in the two southern clusters SE and SW. Inhomogeneous populations composed of the subspecies subsp. *balearica* (stenolopha) and

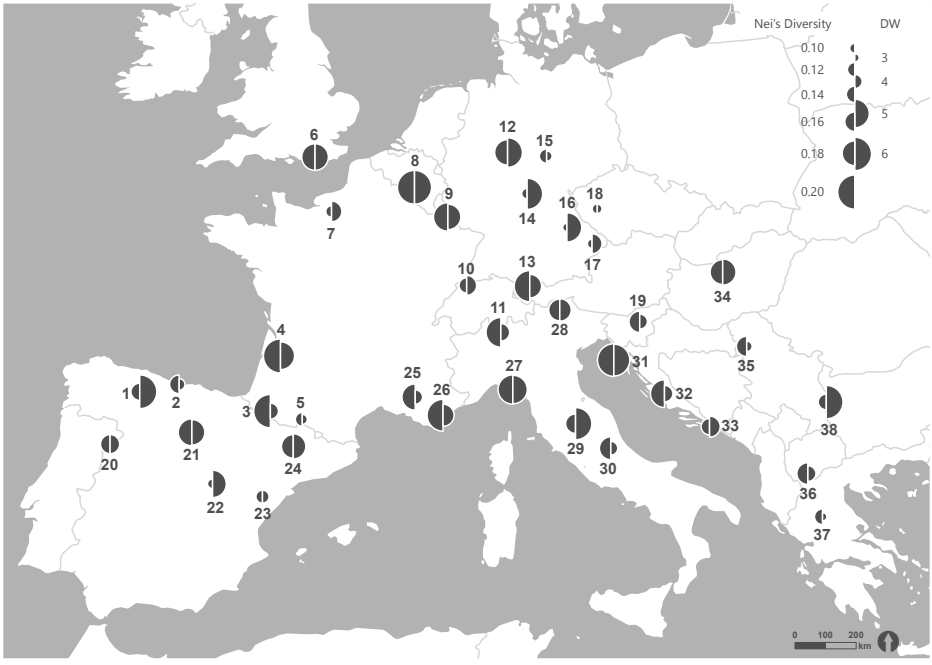


FIGURE 2.1 Nei's gene diversity (He) and frequency-down-weighted marker values (DW) of *Sanguisorba minor* populations in Europe surveyed via AFLP. Different circle sizes indicate different absolute values.

subsp. *balearica* (platylopha; populations 21, 22 and 23) were only found in cluster SW and were placed on the same branch together with the subsp. *balearica* (stenolopha) population 24. At the same time, the only subsp. *balearica* (stenolopha) population 33 within the cluster SE was placed on the same branch

TABLE 2.1 Genetic variation within populations of *Sanguisorba minor* based on AFLP fragments. ID (population identifier), Cnty (country code according to ISO 3166), Ssp (Subspecies assignment of 19 S. minor populations based on hypanthium morphology. A: subsp. *minor*, B: subsp. *balearica* (stenolopha), C: subsp. *balearica* (platylopha), D: *S. verrucosa*. He (Nei's gene diversity), SI (Shannon-Index), PL (number of polymorphic loci), PPL (Percentage of polymorphic loci) and DW (rarity value), ↓ (values below average), ↑ (values above average). Significance levels: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. Sample size of each population 5, total number of sampled individuals 190 and total number of loci 166.

ID	Latitude	Longitude	Cnty	Ssp	He	He SD		I	I SD		PL	PPL		DW	
1	2741516	1960198	ES	A	0.156	0.202	↓	0.230	0.289	↓	69	41.57	↑	7.12	↑***
2	2871155	1984391	ES	A	0.157	0.191	↑	0.239	0.275	↑	78	46.99	↑***	3.76	↓***
3	3169159	1898007	FR	A	0.195	0.209	↑***	0.288	0.297	↑***	86	51.81	↑***	4.17	↓***
4	3203281	2077812	FR		0.215	0.223	↑***	0.311	0.315	↑***	85	51.20	↑***	4.97	↑*
5	3274552	1870185	FR	A	0.119	0.186	↓***	0.178	0.268	↓***	55	33.13	↓***	3.63	↓***
6	3315581	2728755	UK		0.184	0.214	↑***	0.268	0.304	↑***	76	45.78	↑**	4.89	↑
7	3370234	2550604	FR		0.113	0.186	↓***	0.167	0.267	↓***	50	30.12	↓***	4.56	↓
8	3641322	2629779	BE		0.223	0.210	↑***	0.328	0.298	↑***	96	57.83	↑***	7.15	↑***
9	3750301	2532697	FR		0.187	0.218	↑***	0.270	0.309	↑***	75	45.18	↑*	4.82	↑
10	3812006	2308901	CH		0.133	0.196	↓***	0.196	0.281	↓***	58	34.94	↓***	4.46	↓
11	3924404	2155997	CH		0.189	0.210	↑***	0.278	0.300	↑***	81	48.80	↑***	4.32	↓*
12	3945726	2744271	DE		0.182	0.210	↑***	0.267	0.299	↑***	78	46.99	↑***	5.14	↑***
13	4018618	2306096	DE		0.192	0.217	↑***	0.279	0.307	↑***	80	48.19	↑***	4.66	
14	4009876	2609473	DE		0.109	0.185	↓***	0.161	0.266	↓***	47	28.31	↓***	5.31	↑***
15	4074132	2732207	DE	A	0.121	0.185	↓***	0.181	0.268	↓***	56	33.73	↓***	3.67	↓***
16	4139951	2498091	DE		0.060	0.143	↓***	0.090	0.207	↓***	29	17.47	↓***	5.13	↑**
17	4221974	2444927	DE	A	0.063	0.151	↓***	0.093	0.216	↓***	28	16.87	↓***	4.43	↓
18	4239679	2560361	CZ		0.099	0.172	↓***	0.149	0.249	↓***	48	28.92	↓***	3.32	↓***
19	4378267	2190396	SL		0.163	0.205	↑	0.240	0.293	↑	71	42.77	↑	4.13	↓***
20	2645308	1789442	ES	D, CxD	0.157	0.200	↓	0.233	0.287	↑	71	42.77	↑	4.36	↓*
21	2913028	1827554	ES	B, C	0.184	0.205	↑***	0.273	0.293	↑***	83	50.00	↑***	4.83	↑
22	2980194	1658641	ES	B, C	0.103	0.184	↓***	0.150	0.264	↓***	43	25.9	↓***	4.90	↑
23	3143957	1617416	ES	B, C	0.115	0.186	↓***	0.171	0.268	↓***	51	30.72	↓***	3.88	↓***
24	3244727	1781566	ES	B	0.177	0.217	↑**	0.257	0.306	↑**	73	43.98	↑	4.73	↑
25	3643963	1942374	FR	C	0.184	0.204	↑***	0.273	0.291	↑***	83	50.00	↑***	4.09	↓***
26	3734857	1884488	FR	B, C, D	0.193	0.200	↑***	0.287	0.288	↑***	88	53.01	↑***	4.62	↓
27	3963289	1967082	IT	C	0.188	0.216	↑***	0.274	0.307	↑***	77	46.39	↑**	5.10	↑**
28	4117431	2227780	IT		0.167	0.203	↑	0.246	0.292	↑	73	43.98	↑	4.65	↓
29	4168674	1855975	IT	C	0.161	0.201	↑	0.239	0.288	↑	72	43.37	↑	5.85	↑***
30	4283470	1775774	IT		0.168	0.199	↑*	0.250	0.287	↑*	76	45.78	↑**	3.94	↓***
31	4294181	2064660	HR	C	0.194	0.216	↑***	0.282	0.307	↑***	80	48.19	↑***	5.72	↑***
32	4459280	1954615	HR		0.184	0.204	↑***	0.273	0.293	↑***	81	48.80	↑***	4.23	↓**
33	4606595	1846122	HR	B	0.143	0.205	↓*	0.208	0.292	↓**	59	35.54	↓***	4.60	↓
34	4650717	2352434	HU		0.181	0.210	↑***	0.266	0.299	↑***	78	46.99	↑***	4.89	↑
35	4728781	2108665	RS	C	0.163	0.191	↑	0.246	0.277	↑	79	47.59	↑***	3.71	↓***
36	4928849	1692140	MK		0.166	0.195	↑	0.249	0.283	↑*	77	46.39	↑**	4.15	↓***
37	4976423	1551289	GR		0.138	0.188	↓**	0.207	0.272	↓**	66	39.76	↓	3.07	↓***
38	4988778	1927190	RS	C	0.137	0.197	↓**	0.202	0.282	↓**	60	36.14	↓***	6.13	↑***

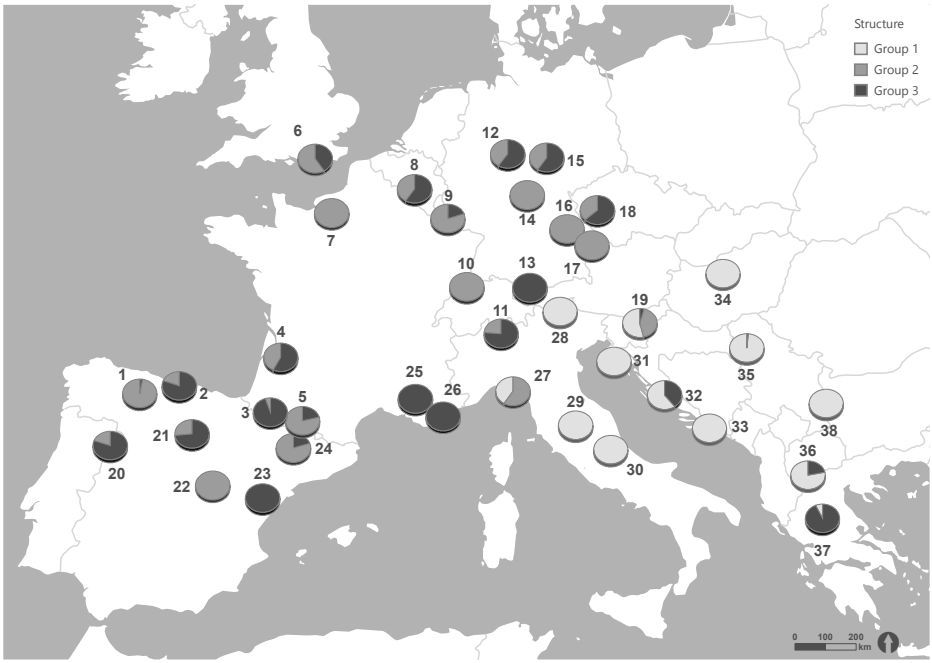


FIGURE 2.2 Results from the STRUCTURE analysis plotted onto geographic coordinates of the surveyed populations of *Sanguisorba minor* for K = 3.

with subsp. *balearica* (platylopha)-populations 29, 31, 35 and 38. Two populations in the NW cluster were intermingled with individuals of *S. verrucosa* as inhomogeneous hybrid swarms either together with a potential hybrid (*balearica* (platylopha) x *verrucosa*; population 20) or with individuals of subsp. *balearica* (stenolopha) and subsp. *balearica* (platylopha; population 26). These populations showed close genetic relationship in the NJ tree and were located on the same branch together with the homogenous subsp. *balearica* (platylopha) population 25.

From the morphology of available seeds it became apparent that the south-eastern study area, dominated by group 1 genotypes (sPCA), was mainly composed of subsp. *balearica* (platylopha)-populations (27, 29, 31, 35, 38) with the exception of population 33, which was assigned to subsp. *balearica* (stenolopha). At the same time, populations with different proportions of group 2 and group 3 genotypes in the south-western and north-western study area were not associated with morphological differentiation of subspecies. For instance, population 26, containing a mixture subsp. *balearica* (stenolopha), subsp. *balearica* (platylopha) and *S. verrucosa*, as well as population 25, which only included subsp. *balearica* (platylopha) individuals, exhibited only group 3 genotype.

2.4 DISCUSSION

We were able to reconstruct patterns of postglacial colonisations of the widespread grassland species *S. minor* compiling a phylogeographic study, the most effective way to gain insights into the origin of species and of the composition of present floras (Avise et al., 1987). We found evidence that *S. minor* may have survived the last glacial maximum (LGM) in both, northern and southern glacial refugia by analysing genetic divergence (rarity index DW) as well as genetic diversity. Moreover, spatial analyses and graphical representations of genetic differentiation revealed genetic differences between populations in the west and east of the study area. Despite the complicated subspecies delimitation of *S. minor*, we were able to contribute to the understanding of the correspondence of genetic, geographic and morphologic patterns.

POTENTIAL GLACIAL REFUGIA OF *S. MINOR*

Granted that north-western and Central European lineages of *S. minor* have derived from southern glacial refugia, they are expected to exhibit reduced diversity (Hewitt, 1996; Hallatschek & Nelson, 2008). In contrast to many previous studies, we observed no latitudinal decline of genetic diversity within populations. Such a geographical gradient of genetic diversity from south to north is normally ascribed to continuous founder effects and the related genetic drift during the postglacial range expansion (Sebasky

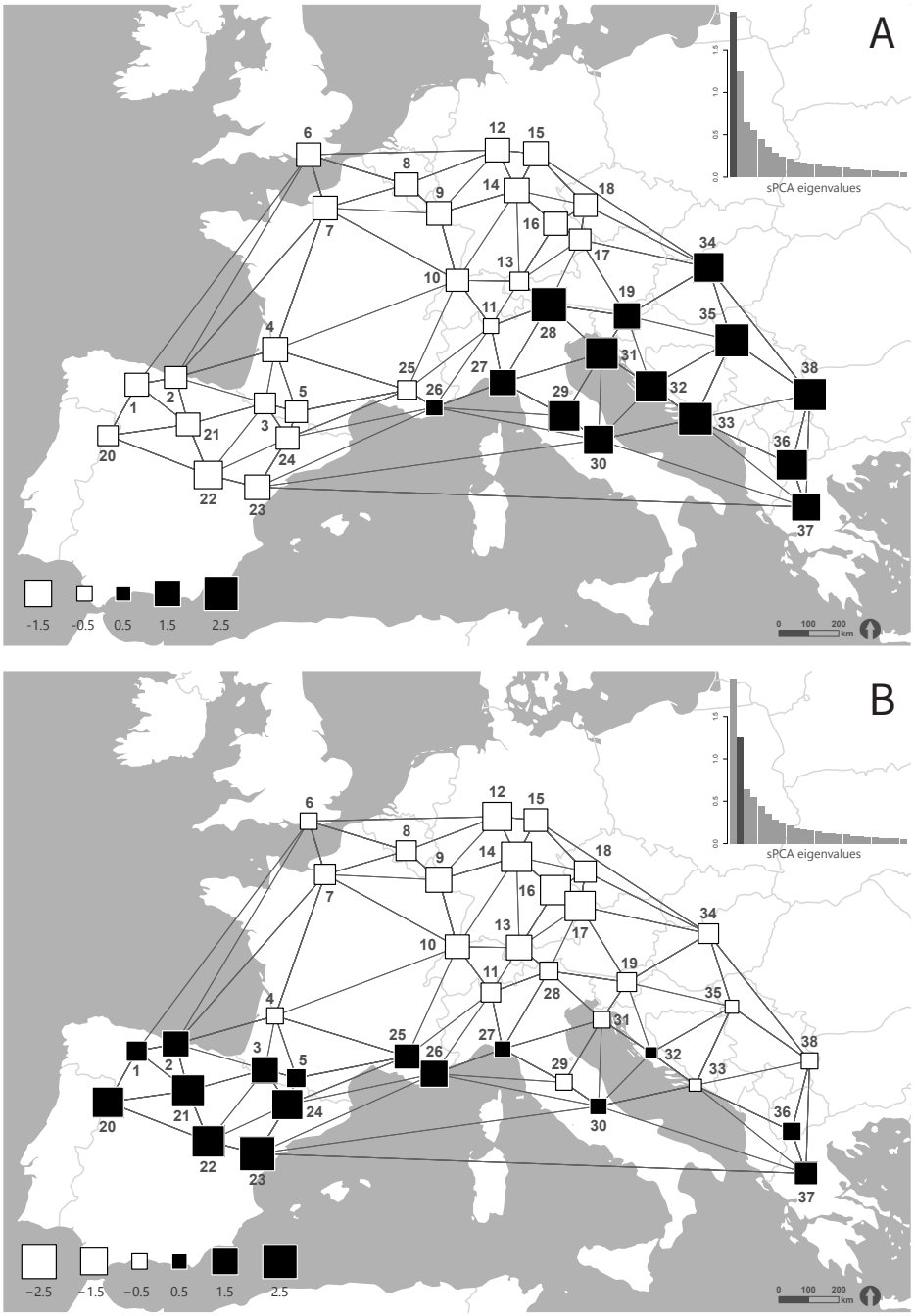


FIGURE 2.3 Spatial distribution of all surveyed populations of *Sanguisorba minor* with values of the A) first (λ1) and the B) second (λ2) positive (global) sPCA scores. Different square sizes indicate different absolute values. Large black squares are distinct from large white ones, while small squares show less differentiation. Grey lines show the used connection network based on Delaunay triangulation. On the top right position of the maps, the first 25 sPCA positive scores are given.

et al., 2016). The underlying mechanism of reduced genetic diversity in recently colonized habitats is described as "gene surfing" (Edmonds et al., 2004; Klopfstein et al., 2006), which means that founder individuals at the leading front are more successful in passing their genes to the new habitat. Consequently, their progeny will be advantaged and pioneer genes attain high frequencies. Due to random effects, some of these lineages might go extinct or not be able to keep up with the wave front, which causes the number of leading lineages to decline until one remaining lineage dominates the colonization process.

Albeit populations of *S. minor* with high genetic diversity were located on the southern peninsulas, e.g. on the Istrian peninsula (population 31, Croatia), in the Gulf of La Spezia (population 27, Italy) and in the north of the Iberian Peninsula (population 21, Spain), further diverse populations were located in Southern France (population 26) and far from the Mediterranean region, the Pyrenees (population 03), Southern Germany (population 13) and Switzerland (population 11). Even the two most diverse populations were found beyond these southern peninsulas, in Belgium (population 08) and western France (population 04).

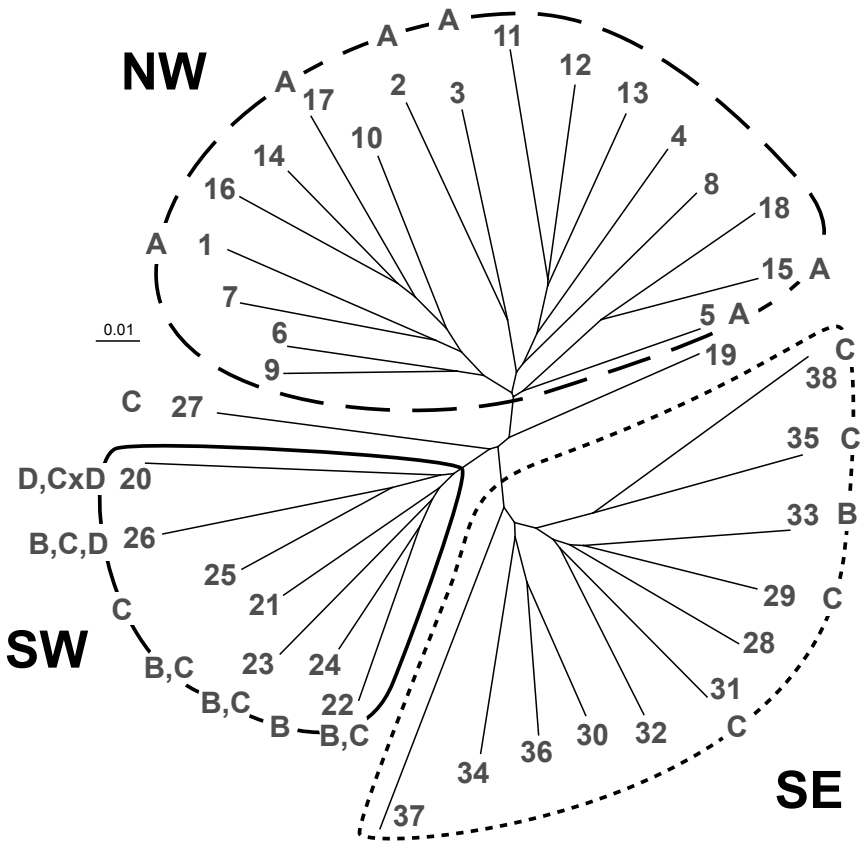


FIGURE 2.4 Unrooted cluster analysis of 38 *Sanguisorba minor* populations based on 166 amplified fragment length polymorphism (AFLP) fragments using Cavalli -Sforza & Edwards chord distances and the neighbour-joining (NJ) algorithm. Broken lines indicate the separation of regions in the north-western (NW), south-western (SW) or south-eastern (SE) study area.
A: subsp. *minor*,
B: subsp. *balearica* (stenolopha),
C: subsp. *balearica* (platylopha),
D: *S. verrucosa*

Highly variable populations both in the southern and northern regions of the distribution range suggest a fast and broad fronted post glacial recolonization as previously reported for other plant species (Alsos et al., 2009; Jadwiszczak et al., 2011; Jiménez-Mejías et al., 2012; Windmaißer et al., 2016). The fact that northerly populations partially possess higher genetic diversity than expected may on the other hand determine them as "cryptic" northern refugia (Stewart & Lister, 2001). However, these populations could also be considered secondary contact zones of southerly derived lineages meeting postglacially in the north, where hybridisation may also have caused a higher genetic diversity (see Petit et al., 2003; Havrdová et al., 2015). As genetic diversity is also dependent on contemporary processes like genetic exchange (Paun et al., 2008), identifying refugia solely based on genetic diversity is delicate. Therefore, we additionally consulted the rarity of markers by frequency-down-weighted marker values (DW), as the detection of rare marker accumulation facilitates the location of putative glacial refugia (Hewitt, 1996; Petit et al., 2003; Paun et al., 2008; Daneck et al., 2015). Because of their continuous existence, glacial refugia have maintained more private haplotypes and were more divergent (Stewart & Lister, 2001; Ohlemüller et al., 2012). Half of the *S. minor* populations with significantly higher DW values than average were placed in periglacial northern locations like Guyenne (population 04, France), Wallonia (population 08, Belgium), North Rhine-Westphalia, Lower Franconia and Upper Palatinate (populations 12, 14 and 16, Germany). High DW values of populations beyond the southern refugia emphasise their continuous persistence in northerly Europe rather than a colonization from southern refugia. Significantly higher DW values indicating refugia on the southern peninsulas, applying the traditional view of southern refugia (Taberlet et al., 1998), were detected in northern Spain (population 01), at the Gulf of La Spezia, Umbria (populations 27 and 29, Italy), the Istrian peninsula (population 31, Croatia) and south-eastern Serbia (population 38).

Considering divergence and diversity, putative glacial refugia possessing both high DW- and diversity values, which indicate continuous persistence and sufficient population size were located in southern Europe at the Gulf of La Spezia (population 27, Italy) or on the Istrian peninsula (population 31, Croatia) but definitely also northerly in western France (population 04), Belgium (population 08) or Germany (population 12). Other potential refugia that may have recently lost genetic diversity e.g. due to isolation of populations (Paun et al., 2008) occurred in northern Spain (population 01), Umbria (population 29, Italy),

TABLE 2.2 Genetic variation within and between studied populations and regions of *Sanguisorba minor* detected by analysis of molecular variance (AMOVA). df (degree of freedom), SS (sum of squares), MS (mean squares), Est. Var. (estimated variation), % (proportion of genetic variation). NW (north-western study region), SE (south-eastern study region), SW (south-western study region). Significance levels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Molecular variation	Df	SS	MS	Est. Var.	%	ΦPT	ΦRT	ΦPR	p
A. Between and within populations- without regions									
Between pop.	37	2338.3	63.20	9.24	35	0.352			***
Within pop.	152	2586.8	17.02	17.02	65				
B. Between and within sPCA-regions east-west (first global score λ1)									
Between reg.	1	297.1	297.10	2.81	10		0.101		***
Between pop. within reg.	36	2041.2	56.70	7.94	29	0.387			***
Within pop.	152	2586.8	17.02	17.02	61			0.318	***
C. Between and within sPCA-regions north-south (second global score λ2)									
Between reg.	1	206.2	206.19	1.59	6		0.059		***
Between pop. within reg.	36	2132.2	59.23	8.44	31	0.371			***
Within pop.	152	2586.8	17.02	17.02	63			0.332	***
D. Between and within NJ clusters NW, SE, SW									
Between clusters	2	606.6	304.31	4.28	16		0.154		***
Between pop. within clusters	35	1731.7	49.48	6.49	23	0.388			***
Within pop.	152	2586.8	17.02	17.02	61			0.276	***
E. Between and within NJ clusters NW, SE									
Between clusters	1	364.9	364.93	4.12	15		0.149		***
Between pop. within clusters	29	1480.7	51.06	6.82	24	0.39			***
Within pop.	124	2104.8	16.97	16.97	61			0.287	***
F. Between and within NJ clusters NW, SW									
Between clusters	1	236.8	236.76	3.70	14		0.138		***
Between pop. within clusters	23	1162.6	50.55	6.85	26	0.393			***
Within pop.	100	1628.8	16.29	16.29	61			0.296	***
G. Between and within NJ clusters SE, SW									
Between clusters	1	290.99	290.99	5.39	19		0.187		***
Between pop. within clusters	18	820.02	45.56	5.51	19	0.337			***
Within pop.	90	1440.0	18.00	18.00	62			0.234	***

eastern Serbia (population 38) and Upper Palatinate (populations 14 and 16, Germany). Populations with high genetic diversity but relatively low DW values might not represent glacial refugia but meeting points of different lineages (Havrdová et al., 2015), for instance in the southern Pyrenees (population 03), Southern France (population 26), Switzerland (population 11) and in Istria (population 31, Croatia). Several authors have already proposed that besides temperate-boreal species of the steppe-tundra (Kajtoch et al., 2016), also temperate trees (Stewart & Lister, 2001; Tzedakis et al., 2013) and temperate or sub-Mediterranean grassland species (Bylebyl et al., 2008) may have survived in Central Europe or the French Atlantic coast (Pfeifer et al., 2009; Harter et al., 2015) in small-scaled climatically sheltered areas with sufficient water supply (Dobrowski, 2011).

COLONIZATION PATTERNS OF *S. MINOR*

Our study showed a clear phylogeographic differentiation between the investigated eastern and western populations of *S. minor*, which most likely represents the genetic structure and variation that has resulted from particular glacial refugia and glacial-interglacial migration histories. The genetic east-west delimitation was unambiguously demonstrated by sPCA (first global score λ1, Figure 2.3) and STRUCTURE analysis (Figure 2.2) and partially by the distinct north-eastern cluster in the NJ tree (Figure 2.4). Genetic differentiation between eastern and western regions is typical for Mediterranean species (Escudero et al., 2010; Santiso et al., 2016) but similar patterns of east-west differentiation have recently been reported for species with a distribution range reaching Central or even Northern Europe such as the calcareous grassland species *Hippocrepis comosa* (Leipold et al., 2017), the sandy grassland species *Corynephorus canescens* (Harter et al., 2015), the meadow grass *Festuca pratensis* (Fjellheim et al., 2006) or the weed *Silene vulgaris* (Sebasky et al., 2016). Differentiation between eastern and western lineages and homologous patterns of recolonization have also been reported for the red deer (Meiri et al., 2013). The observed separation of eastern and western lineages in temperate species can clearly be attributed to the postglacial recolonization from different eastern and western refugia located in southern Europe on the Iberian, Appennine and Balkan Peninsulas (Comes & Kadereit, 1998; Taberlet et al., 1998; Hewitt, 2004).

As geographic borders such as mountain ranges play a significant role as physical barriers for genetic exchange (Hewitt, 2004), the Alps and the Carpathian Mountains (Cieślak, 2014) as well as the Mediterranean Sea most likely have until today prevented a notable gene flow between the eastern and the western populations of *S. minor*. Thus, the south-eastern study area encompassed almost exclusively group 1 genotypes. At the same time, genetic exchange between the Italian and Balkan Peninsula may have occurred due to a lower sea level during the LGM (Ruddiman & Thomson, 2001). Populations in the Gulf of La Spezia (population 27, Italy) and Slovenia (population 19) could hardly be assigned to any group in the NJ analysis (Figure 2.4) and showed concurrent group 2 and group 3 genotypes in the STRUCTURE analysis (Figure 2.2), therefore representing potential meeting points with lineages of the western study area. While we furthermore considered population 27 as glacial refugia due to its high divergence and diversity values, population 19 possibly represents a classical meeting point showing gene flow within the Pannonian Basin along the Danube River through the Vienna Basin.

The Pyrenees seemed to have less impact. Although we found evidence for genetic differentiation between *S. minor* populations in the south-western and north-western study area in the NJ and in the sPCA (second global score λ2), STRUCTURE analysis as well as the first global score λ1of the sPCA and the AMOVA results indicated a significantly lower differentiation than between eastern and western populations. However, we cannot exclude that south-eastern population have contributed to the re-colonization of the northern study area.

Notwithstanding, we conceive two plausible scenarios for the revealed phylogeographic pattern of *S. minor*. While the eastern populations have originated from populations that outlasted the Pleistocene on the Italian or Balkan Peninsulas, all western populations have descended from refugia on the Iberian Peninsula or alternative northern refugia. According to this, genotype 2 and 3 groups represent separate lineages that have either derived from Iberian refugia or each from northern and southern refugia and have contributed to the founding of new populations. Western populations with sympatric distribution of different genotype 2 and 3 proportions therefore indicate (1.) hybrid zones of post-glacially spreading south-western or south-western and north-western lineages. This secondary contact may have prevented significant differentiation of south-western and north-western populations. At the same time it would be expected to have caused populations with higher genetic diversity (see Petit et al., 2003; Havrdová et al., 2015). However, these potential meeting points do not consistently inhibit high genetic diversity even tough genetic diversity is not the optimal basis for discussion as mentioned above. Moreover, as these meeting points seem to be unlikely frequent, we consider another scenario, in which (2.) incomplete lineage sorting (Maddison & Knowles, 2006) has contributed to the presented phylogenetic pattern. Refugial populations on the Iberian Peninsula as well as in France, Belgium or Germany, may have retained ancestral polymorphisms and stochastically sorted these, resulting in populations sharing genotype groups 2 and 3 and variable genotype proportions. Yet, we would not exclude that additionally, after the LGM, northern and south-eastern populations may have expanded in all directions, causing close genetic relationship of the north-western and south-western study area due to secondary contact (Kadereit et al., 2004).

As mentioned above, highly variable populations both in the southern and northern regions of the dis-

tribution range suggest a fast and broad fronted post glacial recolonization, which is moreover supported by the fact that *S. minor* is well dispersed by animals via ectozoochory (Fischer et al., 1996) and endozoochory (Poschlod et al., 2003). It has already been postulated before that especially the postglacial recolonization of grassland species may be related to the migration of hoofed animals (Leipold et al., 2017). Interestingly, the phylogeography of *Cervus elaphus* strongly resembles the pattern of *S. minor*. European red deer was restricted to southern refugia and recolonized Western and Northern Europe originating from Iberia (Meiri et al., 2013). Moreover, exchange between human populations might as well have affected the recolonization speed, routes and genetic exchange of *S. minor*. Using simulated climate and archaeological population patterns, Tallavaara et al. (2015) have shown, that although human population dynamics was also driven by major climate fluctuations, even during the peak of the LGM, contiguous European human populations extended from central France to the lowlands in southern Germany and to Eastern Europe. At least the La Hoguette culture (~7,500 y.a.) started nomadic goat and sheep breeding (Gronenborn, 2003) and could, therefore, have affected the zoochorous dispersal of *S. minor* from southern France to Germany. It should, however, not be concealed that the observed pattern of genetic variation could have been obscured in historic times by the transport of seeds via large scale sheep flock migrations within the so-called Southwest German transhumance covering the eastern part of France and Germany since the medieval times (Hornberger, 1959).

MORPHOLOGICAL AND GENETIC VARIATION

We further investigated whether the detected phylogeographic patterns of *S. minor* were also reflected in morphological seed characters and molecular markers of the two subspecies subsp. *minor* and subsp. *balearica*. Firstly, the geographical distribution of the investigated subspecies was in accordance with the descriptions of Nordborg (1967; see also appendix Figure 2.8).

In the treatise of Nordborg (1967) and in the Flora Europaea (Tutin et al., 1968) it was stated that subsp. *balearica* (stenolopha) has resulted from hybridization with subsp. *minor*, which has also been confirmed by inspection of pollen morphology. To complicate the intraspecific relationships, intermediate populations that we delimited as subsp. *balearica* (stenolopha) might also have resulted from hybridization of subsp. *minor* and subsp. *balearica* (platylopha; Tutin et al., 1968). We are therefore not entirely sure about the correct assignment of subspecies or intermediates in our study, but can safely assume that the populations of subsp. *balearica* (stenolopha) are strongly liaised with subsp. *minor*.

Homogenous intermediate populations of *S. minor* have commonly been observed by Nordborg (1967) not only between subsp. *minor* and subsp. *balearica* but also between subsp. *balearica* and *S. verrucosa*, which was then considered as another subspecies of *S. minor*, subsp. *magnolii* (but not between subsp. *minor* and *S. verrucosa*). We found obvious inhomogeneous hybrid swarms containing *S. verrucosa* with potential hybrid individuals (*balearica* (platylopha) x *verrucosa*; population 20) or with individuals of subsp. *balearica* (stenolopha) and subsp. *balearica* (platylopha; population 26) in the south-western study area (Figure 2.4). At least the hybrid swarm in Southern France (population 26) was congruent with observations of Nordborg (1967). Genetic evidence for a hybrid swarm might be given for this population by its high genetic diversity and relatively low divergence, (but it only exhibited group 3 genotype).

Considering only the NJ results (Figure 2.4), the distribution of genetic lineages of *S. minor* was mirrored in the distribution of the subspecies. We could easily conclude that populations of subsp. *minor* have emerged from distinct refugia in the north-western study area, subsp. *balearica* (platylopha) from the south-eastern and subsp. *balearica* (stenolopha) from the south-western study area, as they predominate these clusters. Alternatively, *S. minor* has only survived in southern refugia and a south-western lineage has given rise to subsp. *minor* during the recolonization of north-western Europe or southern populations on higher altitudes (see Nordborg, 1967).

These clear results become more complex as only the south-eastern study area was almost entirely genetically delimited in all remaining analyses, STRUCTURE, sPCA and AMOVA. The exclusive range of group 1 genotype in the south-eastern study area and the finding that it was predominated by subsp. *balearica* (platylopha) may be connected with bounded migration options and restricted genetic exchange due to geographic and climatic limitations. However, these relations present no compelling evidence that seed morphology differentiation is congruent with genetic differentiation. This became evident as although subsp. *minor* clearly dominated the north-western area, group 2 and group 3 genotypes occurred over the entire western study area. Additionally, variable proportions of these genotypes were not associated with subspecies delimitation or the occurrence of intermediate populations (see Figure 2.2 and Figure 2.4). Moreover, the differentiation between north-western

and south-western populations was lower than between south-western and south-eastern population (Table 2.1), which were both mainly identified as subsp. *balearica*.

We assume that the visible phenotypic divergence in seed morphology of *S. minor* resulted from variation in only a small number of genes (Gottlieb, 1984; Bradshaw et al., 1995) and therefore was only marginally reflected in neutral marker differentiation. By contrast, the older division between the (south-) western and south-eastern lineages of *S. minor* exhibited stronger neutral differentiation but did not reflect differentiation in seed morphology (see Schaal et al., 1998).

Yet, secondary contact of postglacially spreading north-western (subsp. *minor*) and south-western (subsp. *balearica*) lineages might explain the occurrence of intermediates and the reduced genetic differentiation between north-western and south-western. But again, the sympatric distribution of two genetic lineages might depict incomplete lineage sorting.

Adaptive radiation can cause rapid speciation and morphological evolution given their suspected time of origin (Schaal et al., 1998) without showing strong genetic divergence (e.g. Witter & Carr, 1988) and or reproductive barriers to hybridization (Berry & Calvo, 1994). The morphology of subsp. *minor* seeds (wingless and even reticulate faces), may somehow represent adaptions to northerly refugia as well as refugia at higher altitudes on the southern peninsulas (see Nordborg, 1967). Given that *S. minor* has only survived in southern refugia and that a south-western lineage has recolonized north-western Europe or southern populations on higher altitudes, the subsp. *minor* morphology may represent adaption to enhancement of seed propagation.

2.5 CONCLUSION AND PERSPECTIVES

To our knowledge, this study presents the first comprehensive phylogeographic analysis of *Sanguisorba minor* that encompasses the genetic structure over most of the native range and concurrently considering the correspondence of hypanthia morphology of its subspecies.

The presence of high DW values suggests the occurrence of putative northern refugia for this typical calcareous grassland species in western France, Belgium and Germany as well as southern glacial refugia on the Iberian Peninsula, in the Gulf of La Spezia or on the Istrian peninsula. We assume that older vicariance has led to a separation between the western and south-eastern populations investigated, observed in a strong neutral marker differentiation, which was not reflected in seed morphology. We could reveal that the visible phenotypic divergence in seed morphology of *S. minor* is only marginally reflected in neutral marker differentiation. Therefore, the current situation of *S. minor* depicts either incomplete lineage sorting or the existence of secondary hybrid zones, which is differently expressed in genetic and morphological characteristics.

We conclude that investigating multiple genetic markers with different mode of inheritance (and an increased number of individuals) might help to detect the visible phenotypic divergence and at the same time enable distinguishing between recent admixture and incomplete lineage sorting (Maddison & Knowles, 2006; Twyford & Ennos, 2012) revealed by sympatric distribution of two genetic lineages in the western clusters. To improve our understanding of the complex intraspecific relations within the *S. minor* complex, the investigations should be widened on further species, subspecies and populations with available seeds. Especially to extend collections into the range of *S. verrucosa* (southern Iberian Peninsula and southern Greece) should allow a comprehensive discussion of the relations of this (sub-)species concerning the location of glacial refugia and of postglacial re-colonization as well as the degree of (sub-)speciation and hybridization with other (sub-)species. Nonetheless, we support the utility of using along morphological characters and molecular markers for supporting phylogeographic reconstructions.

The study at hand provides further evidence for climatically sheltered northern glacial refugia for a temperate grassland species (e.g. Bylebyl et al., 2008; Harter et al., 2015, Leipold et al., 2017). Due to their likely long history, these highly divergent and diverse populations possess a high conservation value and may point to periglacial origin of other calcareous grassland species. We therefore emphasise the need for further investigations on additional calcareous grassland species especially under consideration of these potential northern refugia to identify calcareous grassland areas with conservation priority. Provided that these northern areas have facilitated long-term persistence throughout climatic oscillations, they could also ensure survival in future climate change, unless the potential localities and populations have not been destroyed (Bhagwat & Willis, 2008).

APPENDIX

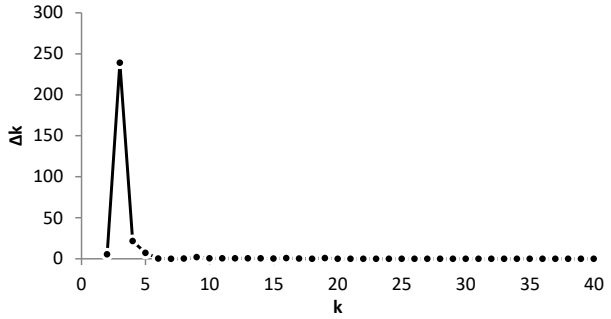


FIGURE 2.5 Results from STRUCTURE analysis. Relationship between the number of proposed groups k and Δk respectively. The most likely number of groups with highest Δk = 238.9 was found with k = 3.

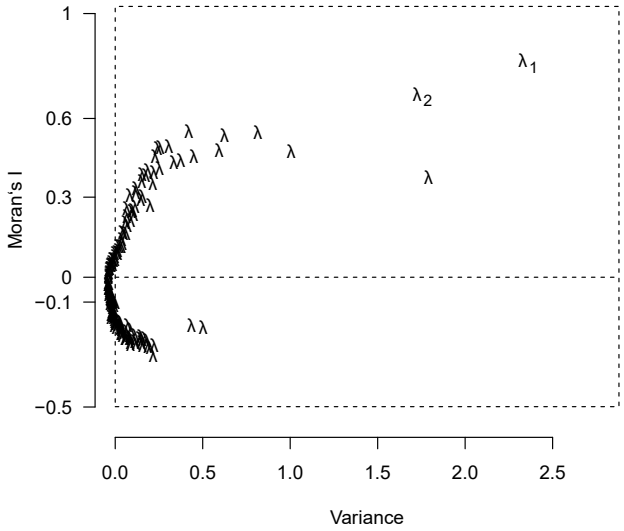


FIGURE 2.6 Screeplot of the total data set. The eigenvalues of the sPCA are arranged by its variance (x-axis) and Moran's I (spatial autocorrelation, y-axis). As the y-axis represents Moran's I, positive scores indicate global structures. Due to its positive eigenvalues λ1 and λ2 can be clearly distinguished from all other values and therefore were 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 3

A habitat-scale study of seed lifespan in artificial conditions examining seed traits

ABSTRACT

We investigated the seed longevity of 39 calcareous grassland species in order to assess the prospects of *ex situ* storage of seeds originating from a single habitat. Seed longevity (p_{50}) was determined by artificially ageing the seeds under rapid ageing conditions (45 °C and 60 % eRH), testing for germinability and calculating survival curves. We consulted seed and germination traits that are expected to be related to seed longevity.

P50 values strongly varied within calcareous grassland species. The p_{50} values ranged between 3.4 and 290.2 days. We discovered significantly positive effects of physical dormancy and endosperm absence on p_{50}. Physiological dormancy was negatively correlated with longevity. Seed mass, seed shape and seed coat thickness were not associated with longevity. These relationships remained significant when accounting for phylogenetic effects.

We therefore recommend more frequent viability assessments of stored endospermic, non-physically and physiologically dormant seeds.

KEYWORDS

Ageing, calcareous grassland, LiCl, longevity, seed, trait

3.1 INTRODUCTION

The awareness of the importance of seed banks as a tool for *ex situ* conservation of rare and endangered plant species is increasing (Hay & Probert, 2013). Its subsequent use for landscape and restoration management is becoming apparent, regionally (Tausch et al., 2015) as well as globally (Godefroid et al., 2011; Merritt & Dixon, 2011).

Besides the initial viability of a seed lot (see Chapter 5), knowledge about seed lifespan in storage is essential, as viability decline may not only result in a reduced seedling amount but also in a loss of genetic diversity. In *ex situ* storage facilities seeds are being preserved under conditions that can extend seed persistence considerably up to hundreds of years (Walters et al., 2005a; Van Treuren et al., 2012). More precisely, freezing seeds with low water content (Smith et al., 2003) reduces metabolic activity, delays degenerative processes and therefore slows down seed ageing (Walters, 1998; Kranner et al., 2011). This is valid for orthodox seeds, which are prevalent in the Central European flora (Hay & Probert, 2013), while recalcitrant seeds are not resistant to drying. Desiccation tolerant seeds possess intrinsic mechanisms to preserve cellular components as water is removed, like non-reducing sugars, oligosaccharides and LEA proteins (Bewley et al., 2013). However, also orthodox seeds cannot endure infinitely in these preferable conditions (Walters et al., 2005b). As well as seeds of

different species persist different time spans in the soil (Kiefer & Poschlod, 1996; Bekker et al., 1998a; Saatkamp et al., 2009), banked seeds strongly differ in their storage longevity (Pritchard & Dickie, 2003; Walters et al., 2005b; Long et al., 2008; Probert et al., 2009; Mondoni et al., 2011; Merritt et al., 2014). Therefore, prioritising species is not only a matter for the selection of target species for collection (Godefroid et al., 2011; Griffiths et al., 2015) but also for choosing species for regeneration and re-collection in certain time intervals (Hay & Probert, 2013).

Information about seed bank longevity can be gathered by monitoring and detecting viability decrease of stored seeds over decades (Crawford et al., 2007; Probert et al., 2009; Godefroid et al., 2010) or, more timesaving, by using artificial ageing methods (Newton et al., 2009). Another method is a statistical modelling approach (Ellis & Roberts, 1980) that incorporates species constants that are, however, mainly known for crop species (Hay & Probert, 2013), which makes this approach less feasible for wild species. The artificial ageing method induces accelerated seed death by the use of warm and moist conditions, which are virtually inverse to life extending conditions utilized in *ex situ* facilities. Germinability and viability loss is measured in regular intervals and the p_{50} value (time until viability has reached 50 % of the initial viability) is determined to enable comparability of seed longevity data (Long et al., 2008; Probert et al., 2009). The method was validated by Probert et al. (2009) showing a highly significant correlation between viability decline of seeds after 20 years in seed bank storage and the mean p_{50} in artificial ageing.

Therefore, the accelerated ageing method is applied to gain a better understanding of the underlying physiological and biochemical mechanisms of deterioration and repair in plant cells during ageing, which are complex and still not fully understood (Nagel et al., 2014). Higher temperature, humidity and oxygen concentration increase the amount of free radicals and reactive oxidative species (ROS), which accumulate during seed ageing (Bailly, 2004). These molecules lead to damages of macromolecules such as nucleic acids, lipids, enzymatic and structure proteins, especially in combination with a lowered antioxidant enzyme activity due to ageing (Walters, 1998; Bernal-Lugo et al., 2000; Bailly, 2004; Kranner et al., 2011; Nagel et al., 2014). Such detailed cellular examinations of viability loss are mainly performed by research departments of plant breeding industry or agricultural seed banks, on one or different genotypes of one (model) species (Walters, 1998; Bailly, 2004; Kranner et al., 2011; Nagel et al., 2014).

Recently published large comparative longevity studies on wild plant species focus on the influence of the climate of the provenance and seed or plant traits on seed longevity (Long et al., 2008; Probert et al., 2009; Mondoni et al., 2011; Merritt et al., 2014). These characteristics are used to predict seed longevity and assess the prospects of storing seeds in seed banks. It was found that seeds sourced from plants of warmer and drier environments were more long-lived in dry storage (Walters et al., 2005b; Probert et al., 2009) and rapid ageing (Long et al., 2008; Probert et al., 2009; Mondoni et al., 2011) than those from cooler and wetter climates. For example seeds collected from alpine populations (with cool wet conditions) were short lived in comparison to seeds from (related taxa of) lowland populations (Mondoni et al., 2011). Merritt et al. (2014) confirmed a weak correlation of temperature and p_{50} for Australian species, but they also found a contradictory result in form of a negative correlation of annual precipitation and p_{50}. As in addition, the correlations of Mondoni et al. (2011) and Probert et al. (2009) were relatively weak, rainfall appears to be an unsuitable predictor so far (Merritt et al., 2014). Regarding the influence of seed traits on seed persistence, seed size and shape as well as dormancy and seed coat thickness have turned out to be promising predictors for soil seed bank persistence (Thompson et al., 1993; Bekker et al., 1998a; Hodkinson et al., 1998; Funes et al., 1999; Peco et al., 2003; Thompson et al., 2003; Moles & Westoby, 2006; Gardarin et al., 2010; Schwienbacher et al., 2010; Saatkamp et al., 2011; Zhao et al., 2011). However, longevity in *ex situ* facilities did not depend on seed size (Probert et al., 2009), or only a slightly positive correlation was found (Merritt et al., 2014). Moreover, *Arabidopsis thaliana* showed a negative correlation of dormancy and longevity (Nguyen et al., 2012). Additional seed traits, endosperm presence or embryo-endosperm ratio, emerged as indicators for *ex situ* seed longevity (Walters et al., 2005b; Probert et al., 2009; Mondoni et al., 2011; Merritt et al., 2014) and also phylogeny proofed to be of significance (Walters et al., 2005b; Probert et al., 2009; Merritt et al., 2014).

The missing influence of seed morphological traits such as seed size on *ex situ* storage may be explained by the huge geographic range of the investigated species which might cover the effect and alter the significance of these seed traits on longevity (Long et al., 2015). Other traits like seed coat thickness and seed shape have not been investigated yet, although they have shown to be correlated with soil seed bank persistence. To eliminate climatic effects, a study of seed persistence in a single habitat might reveal the main drivers for *ex situ* seed persistence. To our knowledge,

comparative studies on the longevity of seeds in a single habitat, as performed by Tuckett et al. (2010) for temporal wet grasslands, are quite rare.

In the present study we explored the seed longevity of 39 calcareous grassland species. We aimed to reveal information about the influence of seed traits (mass, shape, seed coat thickness, endosperm presence and dormancy) on seed longevity. As recent studies showed no correlation with oil content and carbohydrate composition (Pritchard & Dickie, 2003; Walters et al., 2005b; Probert et al., 2009) and the availability of appropriate data is sparse for wild plant species, we neglected these potential correlates in our analyses. Furthermore, we considered phylogenetic influences on the investigated data to account for relatedness of species.

Considering this background, our study focuses on following questions: (1) Is there a basic characterisation of seed longevity of calcareous grassland species? (2) Which seed traits influence seed longevity and do significant effects remain when statistically testing and accounting for phylogenetic relationships?

3.2 MATERIAL AND METHODS

SEEDS OF CALCAREOUS GRASSLAND SPECIES OF CENTRAL EUROPE

Seeds of 39 calcareous grassland species were collected in 2012 in the area of the Jurassic Mountains of the Franconian Alb (Bavaria, southern Germany). The climate can be characterised as a transition climate, with intermediate conditions between mild oceanic climate of western Germany and subcontinental climate in the east (Herbst et al., 2014). Mean annual precipitation is 648 mm with summer and winter rains, including heavy snowfalls. Annual mean temperature with 8.4 °C can be described as mild but events like freezing or cold air blockage may take place in winter and significantly reduce temperature (Herbst et al., 2014).

Species were selected to represent both, the habitat and a broad variation in German plant families. Most seeds were stored for 3 months at 4 °C before testing and seed viability was assessed via X-ray prior to the ageing experiments. Table 3.1 provides an overview of the 39 species from 18 plant families and 13 orders and their origin. Additionally, we used seeds of *Ranunculus sceleratus* L. as a marker species for short lived seeds (Newton et al., 2009), with a known p_{50} published by Probert et al. (2009).

CONTROLLED AGEING TEST

Controlled ageing tests were conducted according to the protocol for comparative seed longevity testing (Newton et al., 2009; Probert et al., 2009). Firstly, for rehydration and humidity adjustment, seeds were placed in glass vials in a thermoplastic enclosure box (30 x 40 x 10.2 cm; Ensto, Finland) at 20 °C for 14 days over a non-saturated solution of LiCl (EMSURE® ACS, Reag. Ph Eur, Merck, Germany) of 47 % RH (1l distilled water and 385 g LiCl). Secondly, a controlled ageing environment was arranged by placing the seeds in another box in a drying oven at 45 ± 1 °C over a LiCl-solution with 60 % RH (1 l distilled water and 300 g LiCl). A sample of 50 seeds was regularly withdrawn and used for germination experiments.

The eRH of a dummy sample and the solutions were regularly controlled via hygrometer (Hygropalm-AW1 - AW-DIO, Rotronic, Germany). If necessary, the LiCl-solution was adjusted by adding distilled water.

GERMINATION TESTING

Prior to germination testing seeds were X-rayed (Faxitron MX 20, Faxitron Bioptics, LLC, Tucson, USA) to guarantee that none of the seeds were empty or infested. Two replicates of 25 seeds each were germinated under appropriate conditions (see Table 3.1) sown on two layers of moist (deionised water) filter paper in petri dishes and placed in climate chambers (Rumed, type 1301, Rubarth Apperate GmbH, Laatzen, Germany) or in a cooling room (4°C), when pre-chilling was required. The incubators were run with a photoperiod of 14 h light (cool white fluorescent tubes, ± 10 000 lux; approx. ± 250 µmol·m-2·s-1 PPFD) and 10 h darkness. The particular alternating temperatures are shown in Table 3.1. Light was provided during the warm temperature phase. Four species required additional treatment with GA_3 (250 mg·l-1; Sigma-Aldrich Company Ltd, Dorset, UK) and 11 species with physically dormant seeds were scarified with a scalpel before germination. Seeds were regularly checked for germination and considered viable when germinated - e.g. a radicle protrusion of ≥ 2 mm occurred and a development of "normal seedlings" was ascertained (Black et al., 2006; Bewley et al., 2013). Germination run time was at least 42 days, tests were finished after a 14 days period without germination. At the end of the germination tests Tetrazolium tests were performed to confirm that the remaining seeds were dead.

SEED TRAITS

Seed mass was determined as thousand seed weight (TSW) by averaging the weights of eight samples of 100 seeds each. Seed dimensions were measured on five replicate seeds per species. Seed shape (VS) was used as the variance of seed dimensions, which was calculated according to Bekker et al. (1998a):

$$V_s = \frac{\sum(x_i - \bar{x})^2}{n} \quad (1)$$

where x1 = length/length, x2 = height/length and x3 = width/length, n = 3. Seed shape is a dimensionless trait that varies between 0 in perfectly rounded and 0.2 in disk- or needle-shaped seeds.

Endosperm presence/absence was determined by X-ray analysis, dissection and the classification according to Martin (1946), revised and extended by Finch-Savage & Leubner-Metzger (2006). Seeds with peripheral embryo were assorted to non-endospermic seeds, as they had a higher embryo to seed ratio than seeds with abundant endosperm (endospermic basal embryo types B1, B3 and B4, phylogenetically more advanced endospermic seeds LA, MA). Prior germination tests allowed us to identify whether seeds possessed physical or physiological dormancy (see Table 3.1).

Seed coat thickness was determined as mean seed coat thickness (MCT) of five seeds using X-ray photographs in an image processing program. We were not able to measure seed coat thickness of four species (*Dianthus carthusianorum*, *Bromus erectus*, *Melica ciliata* and *Phleum phleoides*), as the seed coat or testa plus pericarp were not visible. These species therefore had to be excluded from some statistical analyses.

DATA ANALYSIS

When not specified differently, all statistical analyses were performed in R version 3.1.1 (R Development core team).

VIABILITY CURVES AND ASSESSMENT OF P_{50} VALUES

For the calculation of the time taken for seed viability to fall to 50 % (p_{50}) two different approaches were made. The first was a probit analysis that fits the seed viability equation of Ellis & Roberts (1980):

$$\upsilon = K_i - p/\sigma \quad (2)$$

where υ is the viability in normal equivalent deviates (NED) at time p (days); Ki is the initial viability (NED) and σ is the standard deviation of the normal distribution of seed deaths in time. The probit analysis was performed using both the statistics software Genstat 11th edition (Payne et al., 2008) and the drc package in R (Ritz & Streibig, 2005), especially for drawing the viability curves.

As a second approach we fitted curves using the formula (3) of Long et al. (2008), that provides the fitted initial germination percentage (100-α), the rate of viability loss in the rapidly declining section of the curve (β), the accumulated time in the ageing environment (t) and the p_{50} value (c). Not for all species negative logistic (sigmoidal) curves were suitable but exponential curves with the formula (4) gave a good fit, where a is the initial germination percentage and b is the rate of viability loss.

$$Germination\ (\%) = \frac{(100 - \alpha)}{1 + e^{-\beta(t-c)}} \quad (3)$$

$$Germination\ (\%) = ae^{-bx} \quad (4)$$

PHYLOGENY

The phylogenetic tree required for the phylogenetic analysis was constructed using Phylomatic v3 (Webb & Donoghue, 2005) based on the megatree R20120829 APG III (2009). Nodes of the phylogeny were then dated according to Wikstrom et al. (2001) and attached to the phylogeny using BLADJ, returning a new phylogeny with adjusted branch lengths (Webb et al., 2008).

TRANSFORMATIONS AND PHYLOGENETIC SIGNALS OF SEED TRAITS AND P_{50}

Due to non-normality (Shapiro-Wilk-tests), p_{50}, seed mass (TSW), seed shape (VS) and mean seed coat thickness (MCT) were log10- transformed in order to gain normal distributed data.

As closely related species tend to share phenotypic similarities, which they inherited from ancestors, direct correlation studies that treat each species as an independent data point may increase the risk of Type I errors and thus lead to incorrect rejection of the null hypothesis (Freckleton et al., 2002). Therefore, it is advised to account for dependencies due to relatedness of species by using phylogenetic comparative methods (Garland & Ives, 2000; Freckleton et al., 2002; Garland et al., 2005).

To quantify for phylogenetic signals in our continuous variables, we used two alternative parameters: Pagel's λ (Pagel, 1999) and Blomberg's K (Blomberg et al., 2003). In addition, for the binary traits endosperm presence, physical dormancy and physiological dormancy, we used Fritz & Purvis' D (Fritz & Purvis, 2010). All three phylogenetic parameters evaluate the signal in a trait against a Brownian motion model of trait evolution. In the Brownian motion model, trait evolution follows a random walk along the branches of the phylogenetic tree, with time being represented by branch lengths and the trait being directly proportional to the branch length/time (Revell et al., 2008). For continuous valued traits under a pure Brownian motion evolution, the expected covariance between the trait values of species at the tips of the phylogeny is proportional to the lengths of shared branch lengths (off-diagonals, Ives & Garland, 2010).

For λ and K a value of 0 reveals that the variation of a trait is modelled as a function of independent evolution (star phylogeny, no phylogenetic signal), while values of 1 show that the variation of a trait is as expected under the Brownian model (strong phylogenetic signal). K can exceed 1, which indicates a greater degree of trait similarity among related taxa than expected under Brownian motion. K and λ were calculated using the phylosig function in the phytools package (Revell, 2012) and λ was additionally estimated using the pgls function in the caper package (Orme et al., 2012) with a maximum likelihood approach.

D statistic was carried out with the phylo.d function in caper. Here, 0 indicates that a trait evolves on a tree following the Brownian model and 1 indicates that the trait evolves following a star phylogeny. A negative D indicates a trait that is more conserved than predicted by the Brownian model. Additionally we conducted a simulation (1000 permutations) to test whether an estimated D was significantly different from the predictions of a random or a Brownian evolution.

CONVENTIONAL STATISTICAL ANALYSIS OF SEED TRAIT CORRELATES OF P_{50}

For our first set of analyses, we used non-phylogenetic methods that assume species to be related by a star phylogeny e.g. that there is no phylogenetic structure and all species being equally related (Felsenstein, 1985; Perry & Garland, 2002; Blomberg et al., 2003). Relationships between p_{50} and the seed traits seed mass, seed shape, mean coat thickness, endosperm presence/absence, physical dormancy presence/absence and physiological dormancy presence/absence were examined through generalized least-squares regression analyses, using maximum likelihood estimation. Models were compared using the small unbiased Akaike Information Criterion (AIC_c) and the Akaike weight (w_i). Finally, we computed the model-averaged predictions as weighted means, where w_i served as model probabilities (Burnham & Anderson, 2002). We also compared p_{50} values of non-dormant, physically and physiologically dormant seeds, using two one-way ANOVAs and subsequent Tukey's HSD post hoc analyses.

PHYLOGENETIC ANALYSIS OF SEED TRAIT CORRELATES OF P_{50}

Hence we have found phylogenetic signals, we used a phylogenetic generalized least squares model, (Grafen, 1989; Pagel, 1999; PGLS; Freckleton et al., 2002) to correct for phylogenetic non-independence. PGLS is capable of evaluating multiple predictor variables and incorporating polytomies (Pagel, 1992) and is regarded as the most general robust way of correcting for non-independence in data (Freckleton et al., 2002). Here, estimated λ was used not only for measuring strength of phylogenetic signal, but also for optimising internal branch length transformation using maximum likelihood. Model comparison was conducted likewise the non-phylogenetic models.

3.3 RESULTS

SEED VIABILITY DECLINE OF CALCAREOUS GRASSLAND SPECIES IN CONTROLLED AGEING

Seed viability loss curves by time of the examined species showed different curve progressions, as shown in Figure 3.1. 24 species were best fitted (lowest AIC) using sigmoidal curves from probit function while for 13 species the alternative sidmoidal function was the best approach. For 2 species a exponential function gave the best fit (Figure 3.1, Table 3.1). The variation in p_{50} of calcareous grassland species ranged from 3.4 ± 0.2 days for *Rhinanthus minor* to 290.2 ± 26.6 for *Trifolium arvense*. In general, seeds that were scarified prior to germination ranked higher than non-scarified seeds (Table 3.1). Three Fabaceae species, *Anthyllis vulneraria*, *Medicago lupulina* and *Trifolium arvense* had not yet reached p_{50} when the experiment ended after 210 days (Figure 3.1). In these cases extrapolated p_{50} values resulting from curve fitting served as approximations. Some species showed perfect sigmoidal curves like *Arenaria serpyllifolia* (54.2 ± 1.5) and *Dianthus carthusianorum* (42.4 ± 1.2), other species such as *Seseli annuum* (5.3 ± 0.6) and *Thymus pulegioides* (13.9 ± 1.6) showed very steep viability losses (Figure 3.1).

Plant orders can be assorted following increasing seed longevity (p_{50}): Gentianales (14.4 ± 0.6, n = 2), Dipsacales (18.3 ± 00, n = 1), Apiales (20.5 ± 15.4, n = 3), Poales (21.5 ± 3.1, n = 5), Lamiales (27.4 ± 6.6, n = 8), Ranunculales (31.6 ± 0, n = 1), Malpigiales (37.1 ± 7.0, n = 2), Liliales (40.6 ± 0, n = 1), Asterales (47.0 ± 13.7, n = 5), Caryophyllales (51.3 ± 4.6, n = 3), Brassicales (71.3 ± 0, n = 1), Malvales (154.9 ± 0, n = 1) to Fabales (176.4 ± 34.0, n = 6). Within the Apiaceae (n = 3) a large variation of p_{50} was observed, with *Daucus carota* being relatively long-lived (51.3 ± 4.2), *Pimpinella saxifraga* and *Seseli annuum* being very short-lived (4.8 ± 0.4 and 5.3 ± 0.6). In contrast, there was a low variation within the Caryophyllales (n = 3) with relatively consistent values for *Arenaria serpyllifolia* (54.2 ± 1.5), *Cerastium arvense* (57.4 ± 0.8) and *Dianthus carthusianorum* (42.4 ± 1.2). The reference species *Ranunculus sceleratus* possessed a p_{50} of 10.4 ± 6 days.

PYLOGENETIC SIGNALS

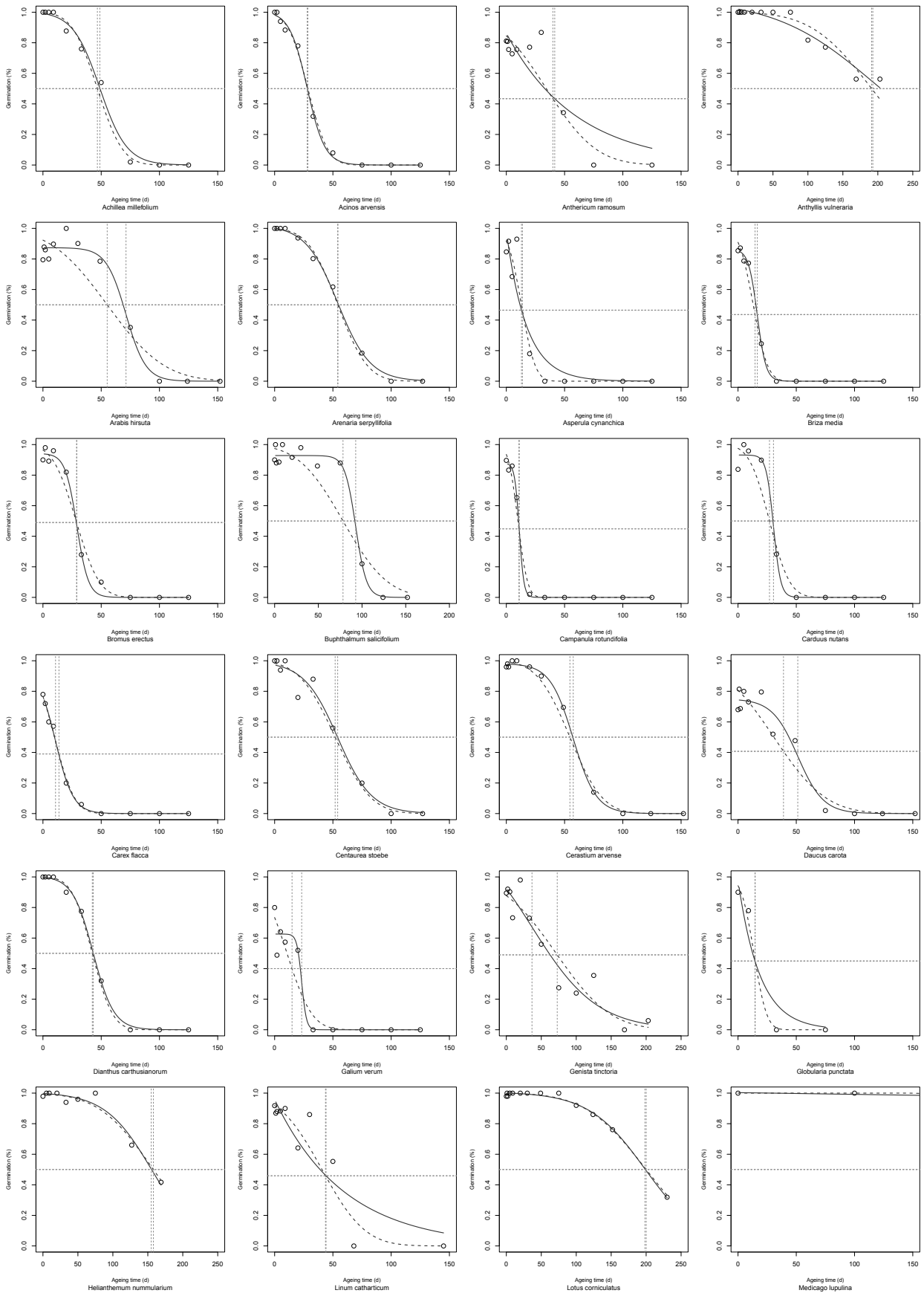
The survey of phylogenetic signals revealed phylogenetic influences in both, dependent and independent variables. All binary traits showed significant phylogenetic signals. Except from seed coat thickness, all continuous seed traits showed relatively high phylogenetic signals although the outputs were significantly different from a Brownian motion model and not significantly different from a star phylogeny considering λ (Table 3.2).

INFLUENCE OF SEED TRAITS ON P_{50} OF CALCAREOUS GRASSLAND SPECIES

The comparison of all non-phylogenetic models to analyse the influence of seed traits on p_{50} revealed the best fit for the model including all seed traits (AICc = 25.5, wi = 0.93, Table 3.3). The average model of the non-phylogenetic analysis showed significant effects of endosperm presence/absence, physiological dormancy and physical dormancy on p_{50} (see Table 3.4). With a mean p_{50} of 20.25 ± 3.77 days (n = 14) endospermic seeds were significantly shorter-lived than non-endospermic seeds which had a mean p_{50} of 78.83 ± 14.67 days (n = 25; one-way ANOVA, F = 20.25, p < 0.001). Even after removing physically dormant seeds the p_{50}–values remained significantly different (one-way ANOVA F = 7.937, p = 0.009) with 18.58 ± 4.70 days (n = 11) in endospermic and 42.07 ± 5.24 days (n = 17) in non-endospermic seeds. Within non-endospermic seeds dormancy had a highly significant influence on p_{50} (one-way ANOVA F = 15.87, p < 0.001, Figure 3.2 A): physically dormant seeds were significantly longer-lived than non-dormant or physiologically dormant seeds (post-ANOVA Tukey HSD, p < 0.001 for both comparisons) but there was no significant difference between physiologically dormant and non-dormant seeds (post-ANOVA Tukey HSD, p = 0.502). Within endospermic seeds non-dormant seeds were significantly longer-lived than physiologically dormant seeds (one-way ANOVA F = 5.45, p = 0.038, Figure 3.2 B).

Seed shape differed between 0.019 in *Lotus corniculatus* and 0.179 in *Bromus erectus*. Seed mass varied between 0.053 mg in *Campanula rotundifolia* and 5.132 mg in *Bromus erectus* and seed coat thickness between 0.021 mm in *Thymus pulegioides* and 0.173 mm in *Teucrium chamaedrys*. P_{50} was influenced neither by seed mass or shape nor by seed coat thickness (Table 3.4).

AIC_c comparison of all phylogenetic models ranked the model including all seed traits highest (AIC_c = 22.1, w_i = 0.79) but it was not significantly different from the model only including endosperm presence/absence (see Table 3.3). Consideration of phylogeny in the average model showed no significant difference to the non-phylogenetic model (see Table 3.4).


Germination (%)
Ageing time (d)
Achillea millefolium
Acinos arvensis
Anthericum ramosum
Anthyllis vulneraria
Arabis hirsuta
Arenaria serpyllifolia
Asperula cynanchica
Briza media
Bromus erectus
Buphthalmum salicifolium
Campanula rotundifolia
Carduus nutans
Carex flacca
Centaurea stoebe
Cerastium arvense
Daucus carota
Dianthus carthusianorum
Galium verum
Genista tinctoria
Globularia punctata
Helianthemum nummularium
Linum catharticum
Lotus corniculatus
Medicago lupulina

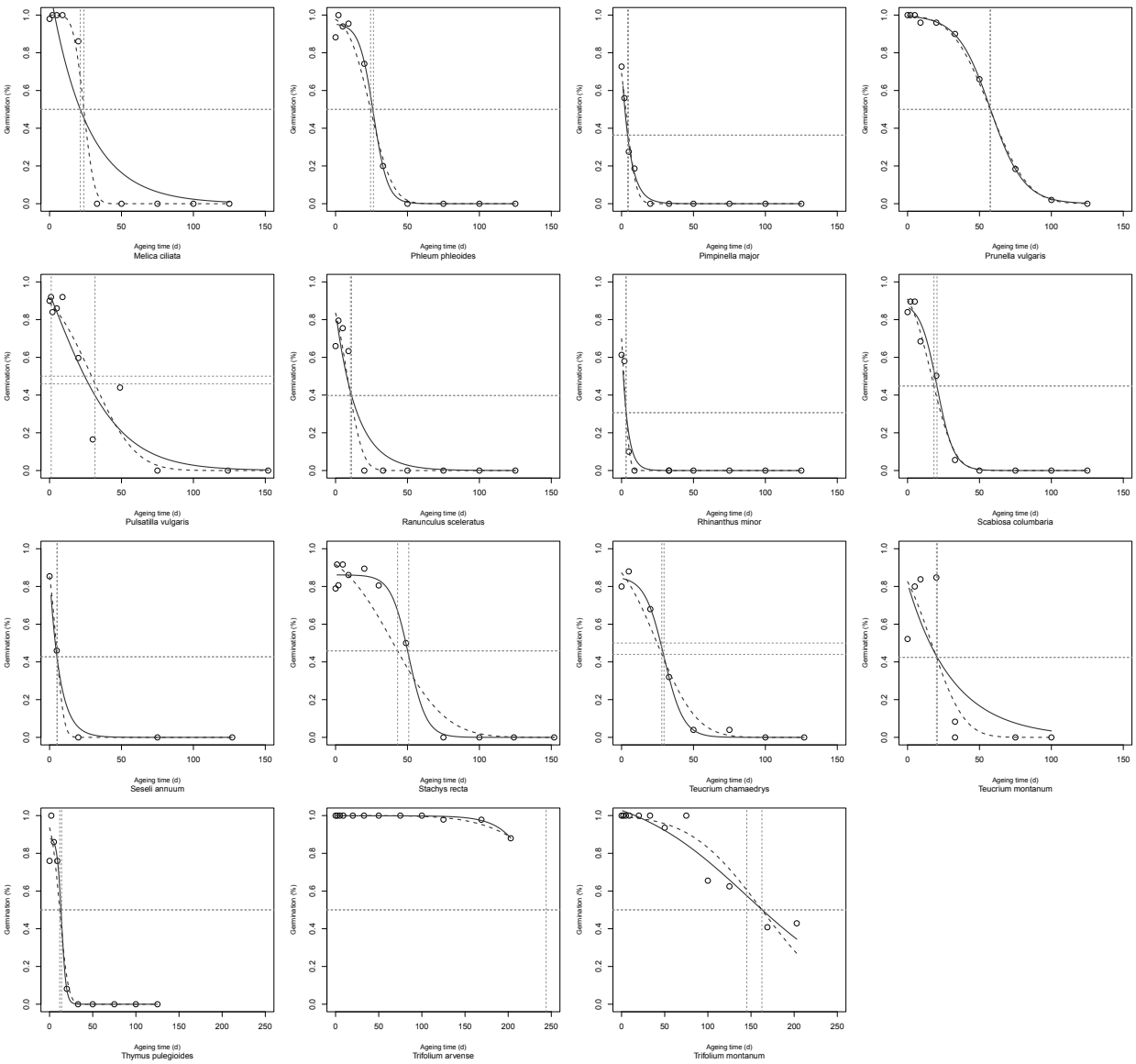


FIGURE 3.1 Seed survival curves of calcareous grassland species in controlled ageing at 60% relative humidity and 45°C. Curves were fitted by probit analysis (dashed lines) or by sigmoidal or exponential curve fitting (continuous lines).

TABLE 3.1 Calcareous grassland species used for controlled ageing. Plant families, orders and endosperm presence/absence (N = little or non-endospermic (embryo types FA1-FA4, P); E = abundant endosperm (MA, LA, B1-B4), following Finch-Savage and Leubner-Metzger (2006)) are given. Seed longevity is expressed as p_{50} (the time to 50 % viability loss) for seeds aged at 45°C and 60 % RH. Seed longevity for each species is ranked as 1–39, with 1 being the longest-lived species. Pre-treatment refers to the treatment used to break dormancy. SCAR = scarification, STRAT = stratification for 6 weeks at 4°C. Germination temperature (Germ. Temp.) refers to the constant or daily alternating (14/10 h) temperature regime and parallel light/darkness fluctuations used for germination testing.

Species	Family (-aceae)	Order (-ales)	Endo-sperm	pre-treatm.	Germ. Temp	p_{50} ± SE (days)	p_{50} Model	Rank
Achillea millefolium	Aster-	Aster-	N	-	22/22	46.7 ± 1.3	Probit	15
Acinos arvensis	Lami-	Lami-	N	-	22/14	28.6 ± 1.0	Probit	25
Anthericum ramosum	Asparag-	Lili-	E	STRAT	22/14	40.0 ± 2.0	Probit	18
Anthyllis vulneraria	Fab-	Fab-	N	SCAR	22/14	191.2 ± 5.9	Probit	4
Arabis hirsuta	Brassic-	Brassic-	N	-	22/14	71.3 ± 2.6	sigmoidal	8
Arenaria serpyllifolia	Caryophyll-	Caryophyll-	N	-	22/14	54.2 ± 1.5	Probit	11
Asperula cynanchica	Rubi-	Gentian-	E	STRAT	22/14	13.80 ± 0.7	Probit	34
Briza media	Po-	Po-	E	-	22/14	14.6 ± 0.7	Probit	30
Bromus erectus	Po-	Po-	E	-	22/14	28.9 ± 1.0	Probit	24
Buphthalmum salicifolium	Aster-	Aster-	N	-	26/18	92.8 ± 2.4	sigmoidal	7
Campanula rotundifolia	Campanul-	Aster-	E	-	22/14	10.8 ± 0.5	Probit	35
Carduus nutans	Aster-	Aster-	N	-	22/14	30.4 ± 1.4	sigmoidal	21
Carex flacca	Cyper-	Po-	E	STRAT	22/14	13.8 ± 0.9	Probit	32
Centaurea stoebe	Aster-	Aster-	N	-	22/22	54.1 ± 3.8	sigmoidal	12
Cerastium arvense	Caryophyll-	Caryophyll-	N	-	14/6	57.4 ± 0.8	sigmoidal	9
Daucus carota	Api-	Api-	E	-	22/14	51.3 ± 4.2	sigmoidal	13
Dianthus carthusianorum	Caryophyll-	Caryophyll-	N	-	22/14	42.4 ± 1.2	Probit	17
Galium verum	Rubi-	Gentian-	E	STRAT	22/14	14.9 ± 1.0	Probit	33
Genista tinctoria	Fab-	Fab-	N	SCAR	22/14	36.7 ± 54.80	sigmoidal	19
Globularia bisnagarica	Plantagin-	Lami-	N	STRAT	22/14	14.8 ± 1.10	Probit	29
Helianthemum nummularium	Cist-	Malv-	N	SCAR	22/14	154.9 ± 4.70	sigmoidal	5
Hypericum perforatum	Clusi-	Malpighi-	N	-	22/14	30.1 ± 1.0	Probit	22
Linum catharticum	Lin-	Malpighi-	N	GA_3	22/14	44.1 ± 1.9	exponential	16
Lotus corniculatus	Fab-	Fab-	N	SCAR	22/14	200.1 ± 6.2	Probit	2
Medicago lupulina	Fab-	Fab-	N	SCAR	22/14	194.8 ± 29562.6	Probit	3
Melica ciliata	Po-	Po-	E	-	22/14	23.8 ± 0.6	Probit	27
Phleum phleoides	Po-	Po-	E	-	22/14	26.4 ± 0.8	sigmoidal	26
Pimpinella saxifraga	Api-	Api-	E	STRAT	22/14	4.8 ± 0.4	Probit	38
Prunella grandiflora	Lami-	Lami-	N	-	18/10	57.3 ± 1.6	Probit	10
Pulsatilla vulgaris	Ranuncul-	Ranuncul-	E	-	26/18	31.6 ± 1.4	Probit	20
Rhinanthus minor	Scrophulari-	Lami-	E	STRAT	22/14	3.40 ± 0.2	Probit	39
Scabiosa columbaria	Dipsac-	Dipsac-	N	-	22/14	18.3 ± 0.9	Probit	36
Seseli annuum	Api-	Api-	E	STRAT	22/14	5.3 ± 0.6	Probit	37
Stachys recta	Lami-	Lami-	N	GA_3	22/14	50.9 ± 1.5	sigmoidal	14
Teucrium chamaedrys	Lami-	Lami-	N	GA_3	22/14	29.5 ± 1.2	sigmoidal	23
Teucrium montanum	Lami-	Lami-	N	GA_3	22/14	20.7 ± 1.0	exponential	28
Thymus pulegioides	Lami-	Lami-	N	-	22/14	13.9 ± 1.6	sigmoidal	31
Trifolium arvense	Fab-	Fab-	N	SCAR	18/10	290.2 ± 26.6	Probit	1
Trifolium montanum	Fab-	Fab-	N	SCAR	22/14	145.1 ± 20.8	sigmoidal	6

TABLE 3.2 Tests of the phylogenetic signals in seed traits and seed longevity for 35 species. Values of λ and K close to 1 indicate a strong phylogenetic signal; values close to 0 indicate absence of phylogenetic signal in the trait. Values of D close to 0 indicate a strong phylogenetic signal, negative values show a stronger conservation than predicted by the Brownian model.

		Pagel's lambda			Blomberg's K		Fritz & Purvis' D		
			Difference from				p		
Trait	n	λ	0	1	K	P	D	star	BM
Seed shape	35	0.633	0.143	0.002	0.497	0.076			
Seed mass	35	0.837	0.152	0.051	0.568	0.038			
Seed coat thickness	35	0.000	1.000	0.001	0.447	0.165			
p50	35	0.693	<0.001	0.005	0.675	0.005			
Endosperm presence	35						-0.946	0.000	0.928
Non-dormancy	35						0.233	0.021	0.345
Physical dormancy	35						-2.185	0.000	0.999
Physiological dormancy	35						-1.422	0.000	0.970

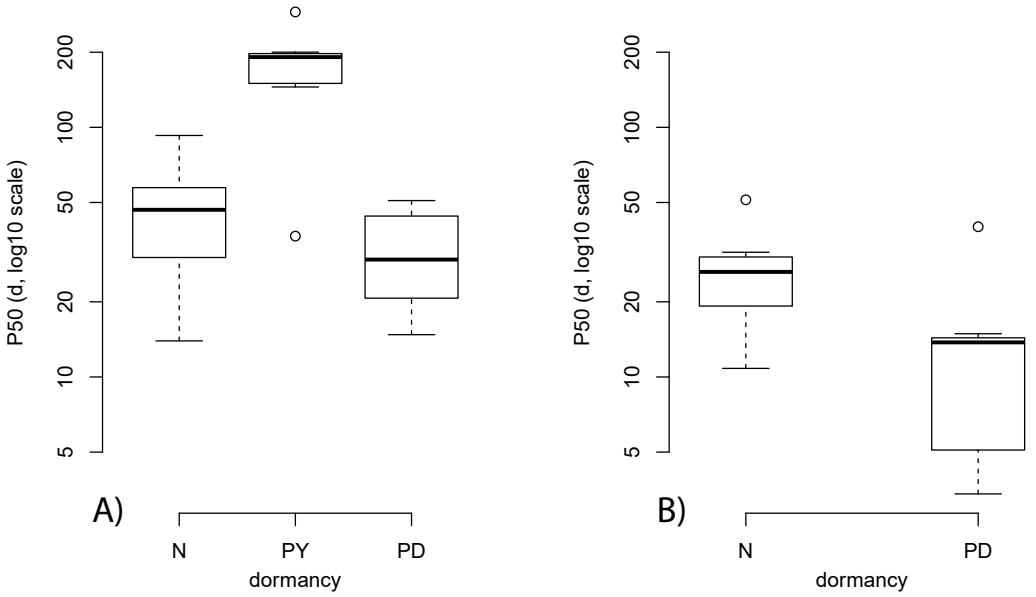


FIGURE 3.2 Box plots of p_{50} values of (A) non-endospermic seeds (non-dormant N, n = 13; physically dormant PY, n = 5; physiologically dormant PD, n = 7) and (B) endospermic seeds (non-dormant N, n = 7; physiologically dormant PD, n = 7). Boxplots show the 25–75th percentiles, whiskers span the 10 and 90th percentiles and circles span the 5 and 95th percentiles.

TABLE 3.3 Non-phylogenetic and phylogenetic candidate models to explain variation for the p_{50} values of 35 calcareous grassland species by seed traits compared to the null model (i.e. no explanatory variables). In the phylogenetic analysis, λ was used for optimising internal branch length transformation using maximum likelihood. The number of estimated parameters in each model (K), AIC_c values for each model, differences in AIC_c between each model and the best-fit model (Δi) and the Akaike weight (w_i) are displayed. Seed shape (V_S), seed mass (TSW) and mean coat thickness (MCT) were log10-transformed. Endo = endosperm presence/absence, PY = physical dormancy, PD = physiological dormancy.

candidate model	λ	K	logLik	AIC_c	Δi	w_i
non-phylogenetic analysis						
V_S, TSW, MCT, endo, PD, PY		8	-1.96	25.5	0.00	0.93
PY		3	-12.28	31.3	5.88	0.05
MCT, PY		4	-12.27	33.9	8.42	0.01
endo		3	-14.62	36.0	10.56	0.01
PD		3	-16.44	39.7	14.20	0.00
V_S		3	-20.89	48.6	23.10	0.00
Null model		2	-22.91	50.2	24.74	0.00
V_S, TSW		4	-20.86	51.0	25.59	0.00
MCT		3	-22.83	52.4	26.98	0.00
TSW		3	-22.88	52.5	27.08	0.00
phylogenetic analysis						
V_S, TSW, MCT, endo, PD, PY	0.000	7	-1.96	22.1	0.00	0.79
endo	0.502	2	-10.44	25.3	3.19	0.16
PY	0.000	2	-12.28	28.9	6.87	0.03
PD	0.668	2	-12.90	30.2	8.12	0.01
MCT, PY	0.000	3	-12.27	31.3	9.25	0.01
V_S	0.658	2	-15.57	35.5	13.45	0.00
Null model	0.693	1	-16.73	35.6	13.52	0.00
MCT	0.688	2	-16.49	37.4	15.29	0.00
TSW	0.693	2	-16.73	37.8	15.77	0.00
V_S, TSW	0.660	3	-15.56	37.9	15.82	0.00

TABLE 3.4 Regression results for the non-phylogenetic and phylogenetic general least squares models for p_{50} of 35 calcareous grassland species computed by model averaging. The estimates, standard errors of the estimates, z values and estimated p values (Pr(>|z|)) are given. Seed shape (V_S), seed mass (TSW) and mean coat thickness (MCT) were log10- transformed. In the phylogenetic analysis, λ was used for optimising internal branch length transformation using maximum likelihood.

model averaged coefficients	Estimate	SE	z value	Pr(>|z|)	
non-phylogenetic analysis					
(Intercept)	1.077	0.559	1.847	0.065	.
V_S	-0.380	0.275	1.323	0.186	
TSW	0.028	0.149	0.181	0.856	
MCT	0.120	0.291	0.394	0.694	
non-endo-spermic	0.324	0.130	2.389	0.017	*
PY	0.419	0.212	1.908	0.056	.
PD	-0.355	0.140	2.432	0.015	*
phylogenetic analysis					
(Intercept)	1.100	0.523	2.106	0.035	*
V_S	-0.380	0.275	1.383	0.167	
TSW	0.028	0.149	0.189	0.850	
MCT	0.120	0.291	0.413	0.680	
non-endo-spermic	0.365	0.162	2.251	0.024	*
PY	0.410	0.205	1.998	0.046	*
PD	-0.356	0.140	2.546	0.011	*

3.4 DISCUSSION

In artificial ageing conditions, seed longevity (p_{50}) of calcareous grassland species varied from 3.4 to 92.77 days (290.2 days including hard-coated seeds). Our results were consistent with the longevity p_{50} values of Northern Italian species from different habitats that ranged from 4.7 to 95.4 days (Mondoni et al., 2011). However, in two large studies with Australian species (Merritt et al., 2014) or with a global scope (Probert et al., 2009), species' longevities reached p_{50} values of 588.6 and 771 days, respectively. Also Long et al. (2008) discovered higher longevities for Australian than for Western European species. Obviously, warmer and drier climates are bearing larger proportions of long-lived seeds (Walters et al., 2005b; Long et al., 2008; Probert et al., 2009; Mondoni et al., 2011). Likewise, on a smaller geographic scale, climatic traits (precipitation and temperature) influence seed longevity, e.g. alpine populations possessed more short-lived seeds than lowland populations (Mondoni et al., 2011). As our results have shown, this cannot be transferred to an even smaller scale, in such way that species originating from a single habitat comprise the same seed longevities. Based on a logarithmic scale to categorize species according to their relative longevity, the majority of 30 species could be classified as having medium-lived seeds in artificial ageing, three as short-lived and six as long-lived (Mondoni et al. (2011): 'very short': $p_{50} \leq 1$, 'short': $1 < p_{50} \leq 10$, 'medium': $10 < p_{50} \leq 100$, 'long': $100 < p_{50} \leq 1000$, 'very long': $p_{50} > 1000$). However, within the group of medium-lived seeds, p_{50} values differed strikingly, which indicates that these species do not rely solely upon long-lived seeds for persistence, as complementary mechanisms for regeneration in plant communities are the basis for maintaining species richness (Thompson & Grime, 1979). Habitats with a high disturbance frequency or an unpredictable environment can lead to the formation of persistent seed banks (Hodkinson et al., 1998; Saatkamp et al., 2014). Calcareous grasslands are exposed to fluctuations of temperature and moisture, especially in the upper layer of the soil, but these are more of predictable character (Saatkamp et al., 2014). On the other hand, this grazed habitat is frequently disturbed and therefore at least some species should be adapted by forming long-lived soil seed banks. As only persistent seeds can be dispersed through time, enabling germination at favourable conditions, these mostly non-competitive calcareous grassland species depend on germination niches provided by disturbance (Venable & Brown, 1988; von Blanckenhagen & Poschlod, 2005). In fact, the majority of typical calcareous grassland species were shown to be transient and short-term persistent in the soil (Bekker et al., 1998b; Poschlod et al., 1998).

Regarding plant families or orders, our p_{50} values confirmed the descriptions of other studies for Apiaceae (Walters et al., 2005b; Merritt et al., 2014), Campanulaceae and Poales (Probert et al., 2009; Mondoni et al., 2011) possessing relatively short lived and Caryophyllaceae or Fabales (Probert et al., 2009; Merritt et al., 2014) possessing long-lived seeds. Nevertheless, most other families produced species with wide-ranging longevities. These studies imply a phylogenetic basis of seed persistence and capture also the well-known variations in seed persistence according to the pre-harvest environment of the provenance (Ooi et al., 2009; Probert et al., 2009; Kochanek et al., 2011; Mondoni et al., 2011; Walck et al., 2011). This can lead to the phenomenon, that even seeds of the same species possess variable seed longevities when growing in different environments (Kochanek et al., 2011; Mondoni et al., 2011). Therefore, on account of the differences in seed longevity of species originating from one habitat, potential explanatory factors can become clearer by reducing the influence of climate.

We herein investigated the influence of seed mass, seed shape, seed coat thickness, seed dormancy, endosperm presence/absence and additionally considered phylogenetic constraints on p_{50}. P_{50} itself showed a strong phylogenetic signal, indicating that seed longevity is determined by traits that possess a high phylogenetic signal themselves. This applied to seed endosperm presence, physical and physiological dormancy, seed mass and seed shape, which all showed dependencies due to relatedness of species. While endosperm is more abundant in basal plant groups, Finch-Savage & Leubner-Metzger (2006) showed that gain and loss of physiological dormancy occurred several times and at several levels of seed evolution. The strong influences of endosperm, physiological dormancy and physical dormancy on p_{50} were still existent when we corrected for phylogenetic non-independence. This again indicates that although these traits exhibit phylogenetic signals, they can also be highly variable in shared clades. It becomes evident, as abundant endosperm is existent as well in basal endospermic plant families such as Poaceae and Ranunculaceae as in more advanced endospermic plant families like Apiaceae and Scrophulariacae. Probert et al. (2009) and Merritt et al. (2014) also considered the role of endosperm showing that non-endospermic seeds persist longer. Seeds with small embryos and endosperm are basal among angiosperms (Forbis et al., 2002; Finch-Savage & Leubner-Metzger, 2006) which led Probert et al. (2009) to

the assumption that the moist environment of the early angiosperms accounts for the poor longevity of endospermic seeds as seeds did not have to rely on long-term survival in a dry state. As a consequence of increasing seasonality and aridity or invasion into hotter and drier environments, competitive seeds with larger embryos and an orthodox (dessication tolerant) behaviour might have evolved (Kranner et al., 2010). Surprisingly, this strong effect has not been reported for the 69 species set of alpine and lowland species in the study of Mondoni et al. (2011).

Unlike shown for soil seed banks (Gardarin et al., 2010), seed coat thickness did not influence p_{50}, whereas physically dormant seeds stood out due to their high p_{50} values. Merritt et al. (2014) even showed that not water impermeability of the seed coat itself contributed to high longevity of physically dormant seeds, as in their study the investigated seeds were scarified prior to artificial ageing. These findings support the assumption of the evolution of non-endospermic seeds together with hard water impermeable seed coats and a high intrinsic longevity. Whereas physical dormancy proofed to be effective in extending seeds' longevity, physiologically dormant seeds possessed reduced longevity, which was significant for endospermic seeds. This result is contradictory to the results of soil seed bank persistence studies, which showed prolonged germination time of dormant seeds extends persistence (Saatkamp et al., 2011). However, our results are in agreement with a recent QTL-study on *Arabidopsis thaliana*, which demonstrated that seed dormancy and seed longevity QTLs were co-located and negatively correlated, using both, artificially and naturally aged seeds (Nguyen et al., 2012). In accelerated ageing, seed water contents of 75 to 100 % RH enable enzyme activity and metabolism (Bewley et al., 2013). But as antioxidant and regeneration mechanisms are only sufficiently active in fully imbibed seeds, reactive oxygen species (ROS) accumulate uncontrolledly as byproducts of aerobic metabolism (Bailly, 2004; Bailly et al., 2008). When imbibed for germination, excessive ROS amounts lead to oxidative damages and finally death in aged seeds (Bailly et al., 2008; Bewley et al., 2013). In seeds that have not been exposed to ageing, a balanced increasing ROS level is correlated with germination and dormancy release, which is ascribed to an interaction with dormancy releasing hormones (Bailly et al., 2008). Moreover, simultaneously, cell repair is activated (Bewley et al., 2013) and germinating (non-dormant) seeds produce protective antioxidants that counteract this excessive ROS activity (Haslekas et al., 2003). Dormant seeds do not produce these germination specific antioxidants. In dormant aged seeds, where ROS is already elevated, this may be fatal even before germination is initiated. These findings may not obligatorily affect all dormant seeds stored in seed banks as it has repeatedly been shown that some seeds may overcome dormancy by cold storage temperatures (Perez-Garcia et al., 2007; Mira et al., 2011; Van Treuren et al., 2012). Although in stored dry seeds integrated cellular processes like respiration, ATP generation, transcription, translation and enzyme activity are at least reduced or completely halted (Bewley et al., 2013), ROS accumulates as a main factor of seed ageing in stored seeds (Bailly, 2004). As seeds with deep dormancy need prolonged time to gradually overcome dormancy and germinate, we would suspect these seeds to be transient in the soil (survival < 1 year). On the other hand, in nature, dormancy cycling (Nguyen et al., 2012) and wet-dry cycling (Long et al., 2011) may extend the life span of seeds by enabling repair mechanisms.

Considering seed size measures, seed mass and seed shape, we found no influence on seed longevity. While in context of *ex situ* longevity seed shape has not been studied so far, the lack of influence of seed mass was consistent with findings of Probert et al. (2009) and Walters et al. (2005b). However, Merritt et al. (2014) found a significant slight correlation of seed mass and p_{50} and speculated that their evidence was found due to their greater sample of species with larger seeds compared to other studies. In soil seed banks seed shape as well as seed mass have been proofed to be of significance (e.g. Bekker et al., 1998a, b). Seed mass may play a role in the soil as predation is more likely and additionally, the trade-off between seed size and seed number may reduce the detection of bigger seeds and therefore causes misinterpretation (Saatkamp et al., 2009). These factors are irrelevant in artificial ageing conditions, although one might assume that oxidative damage may be higher in large, flattened seeds (Kranner et al., 2010) due to stressful conditions of high temperature and humidity and may additionally overburden repair mechanisms during imbibition.

3.5 CONCLUSIONS

As p_{50} values differed strongly within one habitat, there is no potential for a general advice to curators of storage facilities for an adequate storage of species of calcareous grasslands. By investigating in a single habitat, calcareous grasslands, we attempted to eliminate the potential influence of climate differences that may have masked the significance of

traits in other studies. However, we showed that at least two seed traits can provide guidance: physical dormancy (e.g. Fabaceae) and endosperm absence significantly promote storage ability. We therefore confirmed previous results of geographically more large-scale studies (Walters et al., 2005b; Probert et al., 2009; Mondoni et al., 2011; Merritt et al., 2014), implicating the major influence of intrinsic seed characters exceeding the importance of climate. Although physiologically dormant seeds were shorter-lived than non-dormant seeds, this trait may be less relevant as seeds may overcome dormancy in prolonged storage. Viability assessment and recollection of stored seeds possessing one or more of these characteristics can be postponed in favour of species with different features. According to FAO (2013) viability should be checked regularly in 5-year intervals to enable regeneration or recollection of seeds. Seeds that are expected to have rapid deterioration rates should also be considered for cryostorage. In face of our results seed bank curators must be aware of the fact, that longevity of different accessions of one species can be variable due to the predispersal environment (Kochanek et al., 2011).

CHAPTER 4

Seed survival in the soil and at artificial storage: Implications for the conservation of calcareous grassland species

ABSTRACT

Calcareous grassland plants exhibit a relatively low potential for regeneration from the soil seed bank. In order to comprehensively investigate seed survival of 39 calcareous grassland species in the soil and at artificial storage, we consulted longevity information gained from artificial ageing trials and two soil seed bank persistence measures, the soil seed bank persistence categories of Poschlod et al. (1998) and the longevity index LI of Thompson et al. (1998). We moreover investigated seed and germination traits that may be advantageous or disadvantageous for seed survival. For ¾ of the surveyed species soil seed bank persistence and survival at artificial ageing were correlated, what we assigned to an inherent species-specific seed longevity. Remaining contradictory results of species with long-lived soil seed banks but low survival at artificial ageing and vice versa revealed the complexity of seed decline. We interpret inconsistencies with unpredictable effects that operate on seeds in the soil like germination, regeneration of buried seeds by wet-dry and dormancy cycling, predation and the seed size-seed number trade-off, which may cover the actual inherent longevity. While soil seed bank persistence has mainly been associated with seed mass, we found evidence for an additional correlation with endosperm presence and dormancy traits that were mainly connected with *ex situ* longevity. The apparent correlation of seed survival has a strong implication for conservation and increases the pressure of safeguarding healthy plant populations of species with short-lived seeds but it also demands proper *ex situ* storage as it nonetheless extends the life span.

KEYWORDS

Ageing, calcareous grassland, LiCl, longevity seed, soil seed persistence, trait

4.1 INTRODUCTION

CALCAREOUS GRASSLANDS AND THEIR SOIL SEED BANKS

Semi-dry calcareous grasslands are among the most species-rich habitats in Central Europe, comprising many rare plant taxa (WallisDeVries et al., 2002; Dengler, 2005). Due to change of land use, calcareous grasslands have been decreasing during the last decades (Poschlod et al., 2005). Soil seed banks of former calcareous grasslands showed low ability to buffer species extinctions by serving as donor for re-colonization, as the seeds of most species were proven to be short-lived in the soil (Thompson et al., 1997; Bek-

ker et al., 1998a; Kalamees & Zobel, 1998; Poschlod et al., 1998; Stöcklin & Fischer, 1999; Karlik & Poschlod, 2014). One approach to re-introduce species is sowing of seed mixtures (Kiehl & Pfadenhauer, 2007; Hedberg & Kotowski, 2010; Walker et al., 2015). Such management tools can become crucial as besides the low soil seed bank persistence, seed dispersal is strongly limited in a fragmented landscape (Poschlod et al., 1996; Poschlod et al., 1998). Long-term *ex situ* storage of seeds is therefore regarded as a valuable tool for plant conservation and reintroduction (Merritt et al., 2014; Walters, 2015). At the same time, the success reintroduction strongly depends on the quality of the seed material (Walker et al., 2015).

IN SITU SURVIVAL MECHANISMS

Seed persistence in the soil defines the survival of seeds until germination, predation, pathogen attack, ageing or death occur (Long et al., 2015). It is associated with seed traits like dormancy and germination characteristics, inherent longevity, defence mechanisms and extrinsic factors such as climate and soil conditions (Kochanek et al., 2011; Pakeman et al., 2012; Crawley, 2013; Abedi et al., 2014; Colbach, 2014; Long et al., 2015). Accordingly, oceanic humid climate was linked to higher soil seed bank densities than a continental dry climate (Karlik & Poschlod, 2014) and fen meadows contained higher numbers of seeds in the seed bank than dry-mesophilous meadows (Valko et al., 2011). Disturbance intensities within the habitat can also influence the formation of persistent soil seed banks (Saatkamp et al., 2014). Seeds in the soil seed bank of wetter habitats are exposed to wet-dry cycling, which enables re-instating antioxidant capacity and therefore extends persistence, as it was exemplarily shown for seeds of *Avena sterilis* (Long et al., 2011). In the long time, dry soil does not allow frequent regeneration in the imbibed state, moreover, oxidative damages accumulate and gradually overburden antioxidative rescue and therefore reduce persistence in the soil.

EX SITU SURVIVAL MECHANISMS

Seed longevity in *ex situ* storage facilities is mainly determined by physiological traits such as cellular mobility, internal protective components, cell damage resistance and repair mechanisms (Long et al., 2015). Under *ex situ* conditions, the influence of extrinsic factors of the environment (predation, pathogen attack, soil properties, water availability) are reduced and the ageing ability of seeds become crucial. It was found that seeds sourced from warmer and drier environments were more long-lived in *ex situ* storage then those from cooler and wetter climates (Walters et al., 2005b; Long et al., 2008; Probert et al., 2009; Mondoni et al., 2011). In *ex situ* storage, seeds are prevented from germination by dehydration, whereas in the soil seeds may acquire or lose dormancy and exit the soil seed bank by successful or fatal germination. In storage, seeds are frozen with low water content, which generally lowers metabolic activity, delays degenerative processes and therefore reduces seed ageing of orthodox seeds (Walters, 1998; Kranner et al., 2011). Desiccation tolerant seeds possess intrinsic mechanisms to preserve cellular components as water is removed, like non-reducing sugars, oligosaccharides and LEA proteins (Bewley et al., 2013). Nonetheless, oxidative and peroxidative damage gradually accumulate during storage (Bailly et al., 2008). As soon as seeds become imbibed, repair processes and antioxidant reactions can compensate accumulated oxidative and peroxidative damage - as long as a certain level has not been passed (Bewley et al., 2013). *Ex situ* storage conditions are highly effective in extending seed longevity for orthodox seeds especially when stored under reduced oxygen concentrations.

IN SITU AND *EX SITU* SEED SURVIVAL DETECTION METHODS

One way to determine soil seed bank persistence is based on taking soil samples and identifying the included seeds visually or via seedling emergence (Thompson & Grime, 1979; Poschlod, 1993; Poschlod & Jackel, 1993; Bekker et al., 1998a; Poschlod et al., 1998; Wäldchen et al., 2005). For this method, the vertical distribution of the seeds in the soil plays a crucial role (Bekker et al., 1998a). Another more precise but time-consuming and therefore rarely used approach to access seed survival of a known seed amount are burial experiments under natural conditions (Telewski & Zeevaart, 2002; Long et al., 2008; Long et al., 2009; Saatkamp et al., 2009; Abedi et al., 2014). Seed persistence is usually categorized in three groups: transient (< 1 year), short-lived (1 to 5 years) and long-lived (> 5 years, Thompson et al., 1998). Poschlod et al. (1998) used a more detailed classification with four categories: transient seeds (soil seed bank persistence category = 1) are seeds which survived less than 2 years and persistent seeds are splitted into three groups (soil seed bank persistence categories = 2, 3 and 4) with 2-5 years, 6-20 years and over 20 years. Thompson et al. (1997) introduced a longevity index which comprises the results of various longevity studies carried out by different methods. Saatkamp et al. (2009) criticized the longevity index for pooling results of different methods, since soil seed bank sampling methods are correlated with seed production and seed burial studies are not (seed

size- seed number trade-off), consequently leading to misinterpretations of seed persistence.

Due to the brief history of seed banking of wild plant species the number of publications describing *ex situ* long term survival is still low (Walsh et al., 2003; Probert et al., 2009) compared to those of cultivated species (Walters et al., 2005b; Niedzielski et al., 2009; Van Treuren et al., 2012). A time-efficient method is the meanwhile established method of seed longevity assessment by artificial ageing following the standardized protocol of Newton et al. (2009). Seed death is accelerated by warm and moist conditions, the primary factors that reduce seed viability and enhance ageing (Smith et al., 2003). Probert et al. (2009) showed in a large study that the time of the viability to drop to 50 % (p_{50}) correlates with the longevity of seeds stored under dry and cold conditions.

IN SITU AND *EX SITU* SEED SURVIVAL-TRAITS

Besides extrinsic factors, several seed anatomical and morphological adaptations may contribute to seed survival. It is still unsolved if seed size and/or seed shape influence seed persistence in the soil (Thompson et al., 1993; Bekker et al., 1998a; Hodkinson et al., 1998; Leishman & Westoby, 1998; Funes et al., 1999; Moles et al., 2000; Peco et al., 2003; Holzel & Otte, 2004; Traba et al., 2006; Zhao et al., 2011). Moreover, seed longevity in *ex situ* facilities also proofed to be independent of seed size or shape (see chapter 3, Probert et al., 2009) or only a slightly positive correlation was found (Merritt et al. 2014). These latter studies however showed a significant influence of the embryo-endosperm ratio or endosperm presence/absence and longevity traits that have not been examined for soil seed banks so far. Other parameters that influenced persistence in soil seed banks such as seed coat thickness (Gardarin et al., 2010; Saatkamp et al., 2011) or concentration of aromatic alcohols in the coats (Hendry et al., 1994) did not correlate with *ex situ* longevity in the surveys in this thesis (Chapter 3) or of Walters et al (2005b). Decreasing amounts of seeds in the soil are not only a result of seed death, decay or predation but also of germination, which is another important aspect of soil seed bank persistence (Saatkamp et al., 2011). Seeds germinating quickly, at dark and/or constant temperatures do not contribute to the soil seed bank. However, investigations of seed dormancy and germination traits yielded differing results (Grime, 1989; Noronha et al., 1997; Thompson et al., 2003; Baskin & Baskin, 2006; Saatkamp et al., 2011).

CORRELATION OF *IN SITU* AND *EX SITU* SEED SURVIVAL

Two previous studies found either no correlation between soil seed bank persistence and longevity in storage (Walters et al., 2005b) or significant correlations between *in situ* persistence and p_{50} (Bekker et al., 2003; Long et al., 2008). The comparatively low soil seed bank potential of dry calcareous grasslands emphasises the need for *ex situ* storage but simultaneously gives reason to further investigate the relationship of seed survival *in situ* and *ex situ*.

Using a dataset of 39 species originating from a single habitat we aimed to (1) summarize information about seed survival of calcareous grassland species in order to provide recommendations for conservation of single species or groups of species and (2) provide a better understanding of mechanisms and seed traits that contribute to seed survival.

4.2 MATERIAL AND METHODS

SEED LONGEVITY DATA OF 39 CALCAREOUS GRASSLAND SPECIES

Seed longevity values (p_{50}) for 39 calcareous grassland species were available from a previous examination of artificial seed ageing behaviour (Chapter 3). The seeds were collected in the Jurassic Mountains of the Franconian Alb (Bavaria, southern Germany). Soil seed bank persistence data were extracted from a compilation of Poschlod et al. (1998) that includes study results of fallow and afforested calcareous grasslands studies (Kiefer, 1997), burial experiments (Poschlod, 1993) and diaspore bank-diaspore rain comparisons (Poschlod & Jackel, 1993). Soil seed bank persistence was therein categorized as followed: 1 = generative diaspore population transient; < 2 yrs; 2 = persistent over ca. 2-5 yrs; 3 = persistent over ca. 6-20 (25) yrs; 4 = persistent over > 20 yrs.

As additional persistence data, we used the longevity index values of Thompson et al. (1997). The LI integrates a series of soil seed bank observations, in which seeds were categorized in transient (< 1 year), short-term persistent (1-4 years) and long-term persistent (> 4 year). The ratio of the counts of persistent seeds (short-term and long-term) and all seed bank records is generated to build the overall mean of mainly heterogeneous data. Values close to 1 imply a strictly persistent and values close to 0 a strictly transient soil seed bank. This rough grouping is an approach to account for heterogeneity of data and to prevent underestimation of persistence (Bekker

et al., 1998a). Data were sourced from the LEDA trait data base (Kleyer et al., 2008) and the longevity index (LI) was calculated via the formula:

$$LI = \frac{(R_{SP} + R_{LP})}{(R_T + R_{SP} + R_{LP})} \quad (1)$$

where R is the number of records of short-term persistent (SP), long-term persistent (LP) and transient (T) seeds. For *Globularia bisnagarica* and *Centaurea stoebe* no LI value was available, consequently all analyses considering LI are lacking these two species.

GERMINATION DATA

As soil seed bank persistence can be evaluated by the potential of seeds to germinate in the soil, we compiled germination results from literature (Grime et al., 1981; Kew, 2015), unpublished germination data (Beier 1991 and own data see appendix Table 4.6) and results from an unpublished seed burial study (Hahn, 1993) to assess the germinability at darkness and at constant or fluctuating temperatures.

SEED TRAITS

Seed mass was determined as thousand seed weight (TSW) by averaging the weights of eight samples of 100 seeds each. Seed shape (V_s) was calculated with five replicate seeds according to the formula described by Bekker et al. (1998a):

$$V_s = \frac{\sum(x_i - \bar{x})^2}{n} \quad (2)$$

where x1 = length/length, x2 = height/length and x3 = width/length, n = 3.

Seed coat thickness was determined as mean seed coat thickness (MCT) of five seeds using X-ray photographs in an image processing program. We were not able to measure seed coat thickness of four species (*Dianthus carthusianorum*, *Bromus erectus*, *Melica ciliata* and *Phleum phleoides*), as the seed coat or testa plus pericarp were not visible. These species therefore had to be excluded from some statistical analyses.

Endosperm presence/absence was determined using X-ray analysis, dissection and classification according to Martin (1946), revised and extended by Finch-Savage and Leubner-Metzger (2006). Seeds with peripheral embryo were assorted to non-endospermic seeds, as they had higher embryo to seed ratio than seeds with abundant endosperm (endospermic basal embryo types B1, B3 and B4, phylogenetically more advanced endospermic seeds LA, MA; see Table 4.1). Prior germination tests allowed us to identify whether seeds possessed physical (PY) or physiological dormancy (PD).

DATA ANALYSIS

An overview table was generated to compile seed survival data for the 39 investigated calcareous grassland species, including longevity at artificial ageing (p_{50}), soil seed bank persistence categories, LI, germination and seed trait data. The species were assorted in two soil seed persistence groups, "short-lived" (soil seed bank persistence categories 1 + 2, 0-5 years) and "long-lived" (categories 3 + 4, > 5 years). Seeds that possessed lower p_{50} values than average were categorized as "short-lived" (≤ 34 days) and seeds with higher longevities than average as "long-lived" (> 34 days). LI, seed mass (TSW), mean coat thickness (MCT) and seed shape (V_s) were opposed to the average of all species using alternative one sample t-tests in order to categorize them as below (↓) or above average (↑).

We furthermore performed statistical tests to investigate the correlation of seed survival in the soil and at artificial ageing and the influence of seed traits on seed survival. All statistical analyses were performed in R version 3.1.1 (R Development core team).

SOIL SEED BANK PERSISTENCE AND *EX SITU* LONGEVITY

We statistically compared survival at artificial ageing (p_{50}), longevity index values (LI) and soil seed bank persistence categories. The two continuous/metric variables LI and p_{50} were analysed by a Pearson's product moment correlation. Two one-way ANOVAs were used for the examination of the correlation of seed bank persistence categories with LI and p_{50} respectively. Thereby, non-significant soil seed bank persistence categories were aggregated, resulting either in a separation of "0-5 years" (categories 1 + 2) and "> 5 years" (categories 3 + 4) or "transient"(categroy 1) and "persistent" (categories 2 + 3 + 4).

SOIL SEED BANK PERSISTENCE, SEED AND GERMINATION TRAITS

We investigated the influence of different seed traits that may be related to seed survival (TSW, endosperm presence, seed hard-coatedness, V_s, MCT) on soil seed bank persistence (soil seed bank persistence categories and LI) by comparing a series of models using the small sample unbiased Akaike Information

Criterion (AIC_c) and the Akaike weight (w_i; Burnham & Anderson, 2002). We generated a full model including all traits and models with only single traits as well as models that combine traits that are related to seed size (V_s + TSW) or seed coat (MCT + hard-coatedness). In the analysis of the soil seed bank persistence categories we used the aggregated levels that proved to be significantly different in the prior correlations of soil seed bank persistence and LI ("0-5 years" and "> 5 years") or p_{50}. ("transient" and "persistent") as binary responses of a series of single or multiple logistic regressions. For the investigation of LI, we calculated generalized least-squares regression analyses, using maximum likelihood estimation. Similar to the analyses of seed traits we calculated several models examining the influence of seed germination traits on soil seed bank persistence. These models included the binary variables darkness (germination at darkness), physiological dormancy (PD) and physical dormancy (PY) as explanatory variables. As most species germinated similarly at fluctuating or constant temperatures, we excluded this trait from the analyses. We finally calculated three average models each to present the results. An additional one-way ANOVA was performed to check for the correlation of endosperm presence/absence and TSW.

Due to non-normality (Shapiro-Wilk-tests), p_{50}, TSW, V_s and MCT were log_{10}- transformed and LI was square root-transformed in order to gain normal distributed data.

The seeds of five species were short-lived in the soil but long-lived at artificial ageing and the seeds of eight species were long-lived in the soil but short-lived at artificial ageing (Table 4.1). For *Globularia bisnagarica* no soil seed bank data was available.

The statistical analysis showed that the two seed survival measures, soil seed bank persistence categories and longevity index (LI) were significantly correlated (one-way ANOVA, $F = 7.94$, $p < 0.001$, $df = 22$). Seeds included in the aggregated survival category "0-5 years" (soil seed bank persistence categories 1 and 2) possessed significantly lower LI values than seeds within the aggregated category "> 5 years" (Figure 4.1 A, soil seed bank persistence categories 3 and 4; mean(1 + 2) = 0.079 ± 0.027, n = 10, mean(3 + 4) = 0.332 ± 0.056, n = 16; ANOVA $F = 15.83$, $p = < 0.001$, $df = 24$). Moreover, soil seed bank persistence categories and seed longevities at artificial ageing (p_{50}) were significantly correlated (one-way ANOVA, $F = 3.66$, $p = 0.040$, $df = 22$). Seeds within the aggregated survival category "persistent" (soil seed bank persistence categories 2, 3 and 4, mean = 68.75 ± 14.25, n = 20), were significantly more long-lived than "transient" seeds (Figure 4.1 B, soil seed bank persistence category 1, mean = 15.85 ± 5.39, n = 6, one-way ANOVA, $F = 10.68$, $p = 0.003$, $df = 24$).

Additionally, we found a slight correlation between LI and p_{50} (Figure 4.2, $R = 0.339$, $p = 0.040$, $df = 35$).

4.3 RESULTS

CORRELATION OF SOIL SEED BANK PERSISTENCE AND SEED LONGEVITY

Seed survival in the soil and in artificial ageing were in agreement for 25 out of 39 species (see Table 4.1). *Medicago lupulina* possessed the most long-lived seeds (p_{50} = 194.8 days, soil seed bank persistence category 4; mean(p_{50}) = 56.5 ± 10.6), *Rhinanthus minor* the most short-lived (p_{50} = 3.4 days, soil seed bank persistence category 1). Longevity indices varied between 0 for transient seeds like *Anthericum ramosum*, *Dianthus carthusianorum*, *Genista tinctoria*, *Melica ciliata*, *Pulsatilla vulgaris*, *Seseli annuum* and *Teucrium montanum* and 0.45 for *Arabis hirsuta* (mean(LI) = 0.15 ± 0.60, N = 38). Seeds of 11 species were relatively short lived (in relation to the median) as well in the soil (< 5 years) as at artificial ageing ($p_{50} \leq 34$ days), 14 were relatively long-lived in the soil (> 5 years) and in artificial ageing ($p_{50} > 34$ days).

TABLE 4.1 (NEXT PAGE) Compilation of seed survival data for 39 calcareous grassland species. p_{50}: seed survival at artificial ageing (a: see chapter 3). Soil: soil seed bank persistence. Cat.: soil seed bank categories (c: Kiefer 1997: 1 = transient, 2 = persistent; 3a = permanently unsteady; 3b = permanently steady; b: Poschlod et al. 1998: 1 = generative diaspore population transient; < 2 yrs; 2 = persistent over ca. 2-5 yrs; 3 = persistent over ca. 6-20 (25) yrs; 4 = persistent over > 20 yrs). LI: longevity index (d: Thompson et al. 1997, calculated from LEDA trait data base, Kleyer et al. 2008). Germination: relations between germination at fluctuating (f) or constant (c) temperatures, light (L) or darkness (D). 1) Beier (1991), 2) own germination trials (Table 4.6); 3) Grime et al. (1981); 4) Baskin & Baskin (1998); 5) Seed Information Database Kew (2015); 6) Seed burial results of Hahn (1993). dorm: dormancy type: PD = physiological; PY = physical dormancy and ND = non-dormant. endo: N = little or non-endospermic (embryo types FA1-FA4, P); E = abundant endosperm (MA, LA, B1-B4; e: Finch-Savage & Leubner-Metzger 2006), TSW = thousand seed weight, V_s = seed shape index and MCT = mean coat thickness. Relation of a value to the mean: lower (↓) or higher (↑). Significance levels P: *** < 0.001, ** < 0.01, * < 0.05, n.s. > 0.05.

TABLE 4.1

		Soil			Seed traits						
	P_{50}	Cat.		LI	germination			endo			
species	a	b	c	d	f/c	L/D	dorm	e	TSW	V_s	MCT
I. short-lived in soil and at artificial ageing											
Rhinanthus minor	3.4	1	1	↓	f > c^5		PD	E	↑***	↑***	↓*
Pimpinella saxifraga	4.8	1	2	↓***	f = c^5	L > $D^{1,6}$	PD	E	↑n.s.	↑n.s.	↑***
Seseli annuum	5.3	-	-	↓***			PD	E	↓*	↓*	↓***
Asperula cynanchica	13.8	1	2	↓		L > D^1	PD	E	↓n.s.	↓***	↓**
Briza media	14.6	1	-	↓***	f = c^5	L > D^4	ND	E	↓***	↑***	↓***
Scabiosa columbaria	18.3	1	2	↓*	f = c^5	L > D^1	ND	N	↑n.s.	↑***	↓***
Teucrium montanum	20.7	2	1	↓***	f = c^5	L > D^1	PD	N	↓**	↓***	↑***
Melica ciliata	23.8	-	-	↓***	f = c^5		ND	E	↓*	↑***	
Bromus erectus	28.9	1	1	↓***	f = c^5	L = $D^{1,6}$	ND	E	↑***	↑***	
Carduus nutans	30.4	-	2	↑		L > D^4	ND	N	↑***	↑***	↑***
Pulsatilla vulgaris	31.6	2	-	↓***	c^5	L = $D^{1,2}$	ND	E	↑***	↑***	↑***
II. long-lived in soil and at artificial ageing											
Linum catharticum	44.1	4	3b	↑***	f = $c^{2,5}$	L > $D^{1,2,4,6}$	PD	N	↓***	↑**	↓***
Achillea millefolium	46.7	3	2	↓***	f > c^2	L > $D^{2,4}$	ND	N	↓***	↓*	↓***
Stachys recta	50.9	3	3a	↓***	c^5	L = D^1	PD	N	↑*	↓***	↑***
Daucus carota	51.3	4	3a	↑*	f = c^5	L > $D^{1,4,6}$	ND	E	↑n.s.	↑n.s.	↓n.s.
Centaurea stoebe	54.1	4	-		f = $c^{2,5}$	L >> D^2	ND	N	↑*	↑***	↑***
Arenaria serpyllifolia	54.2	4	3a	↑***	f = $c^{2,5}$	L >> $D^{2,4}$	ND	N	↓***	↓***	↓***
Prunella grandiflora	57.3	3	1	↓.		L > D^1	ND	N	↓*	↓**	↑**
Cerastium arvense	57.4	-	-	↑**	f = c^2	L > D^2	PD	N	↓***	↓***	↓n.s.
Arabis hirsuta	71.3	3	-	↑***	f = $c^{2,5}$		ND	N	↓***	↑***	↓***
Buphthalmum salicifolium	92.8	3	3a	↑***	f = c^2	L >> $D^{1,2}$	ND	N	↓***	↑***	↓***
Trifolium montanum	145.1	-	-	↑***	f = c^5		PY	N	↓n.s.	↓***	↓n.s.
Medicago lupulina	194.8	4	3b	↑**	f = $c^{3,5}$	L = $D^{3,4}$	PY	N	↑*	↓n.s.	↑n.s.
Lotus corniculatus	200.1	3	3a	↓**	f = c^5	L = $D^{1,6}$	PY	N	↓n.s.	↓***	↓n.s.
Trifolium arvense	290.2	-	-	↑***	f = $c^{3,5}$	L = D^3	PY	N	↓***	↓***	↓**
III. short-lived in soil but long-lived at artificial ageing											
Genista tinctoria	36.7	-	-	↓***			PY	N	↑***	↓*	↑n.s.
Anthericum ramosum	40	1	1	↓***		L = $D^{1,6}$	PD	E	↑***	↓***	↓n.s.
Dianthus carthusianorum	42.4	1	-	↓***	c^5	L = D^1	ND	N	↓n.s.	↑***	
Helianthemum nummularium	154.9	2	2	↓***	c^5	L = D^1	PY	N	↓n.s.	↓***	↑n.s.
Anthyllis vulneraria	191.2	2	1	↓***	f = c^5		PY	N	↑***	↓***	↓n.s.
IV. long-lived in soil but short-lived at artificial ageing											
Campanula rotundifolia	10.8	3	3a	↑**	f = $c^{2,3}$	L >> $D^{2,3}$	ND	E	↓***	↑**	↓***
Carex flacca	13.8	4	3b	↑*		L > $D^{1,6}$	PD	E	↓n.s.	↓***	↑***
Thymus pulegioides	13.9	4	3a	↑**	c^5	L > D^1	ND	N	↓***	↓***	↓***
Galium verum	14.9	3	3a	↓***	f = c^5	L = D^1	PD	E	↓***	↓***	↓***
Phleum phleoides	26.4	-	-	↑**	f = c^5		ND	E	↓***	↑***	
Acinos arvensis	28.6	-	-	↑***	f > c^2	L >> D^2	ND	N	↓***	↑n.s.	↓n.s.
Teucrium chamaedrys	29.5	3	3a	↓***	f = c^5	L > D^1	PD	N	↑n.s.	↓***	↑***
Hypericum perforatum	30.1	4	3b	↑***	f = c^5	L > $D^{1,4}$	ND	N	↓***	↓n.s.	↓***
V. no soil seed bank data											
Globularia bisnagarica	14.8	-	-		c^5		PD	N	↓**	↑n.s.	↓***

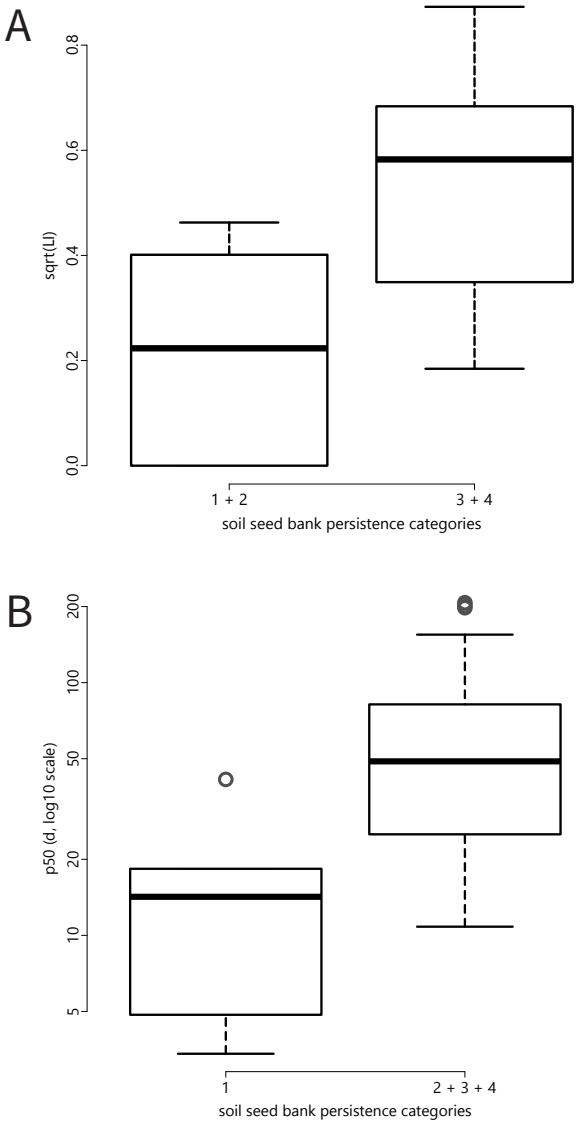


FIGURE 4.1 Box plots of soil seed bank persistence categories and longevity index LI (A) and soil seed bank persistence categories and survival at artificial ageing (p_{50}) (B). Aggregations of soil seed bank persistence categories: "0-5 years" (1+2; n=10); ">5 years" (3+4; n=16); "transient" (1; n=6); "persistent" (2+3+4; n=20). p_{50} was log10- and LI square root-transformed. Boxplots show the 25–75th percentiles, whiskers span the 10 and 90th percentiles and circles span the 5 and 95th percentiles.

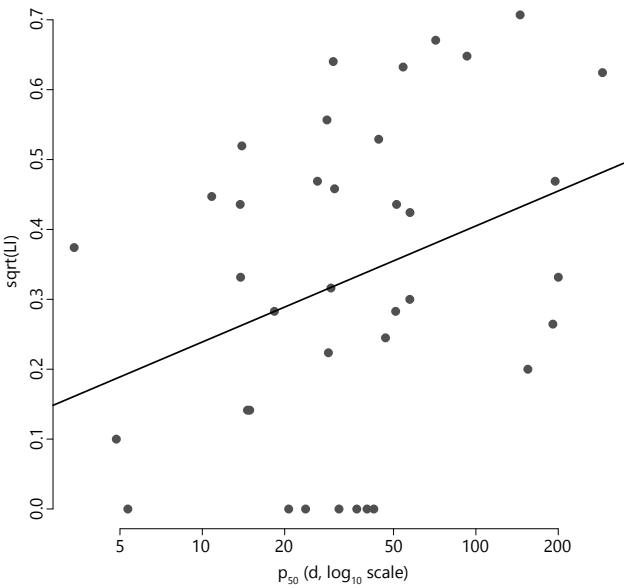


FIGURE 4.2 Correlation analysis of controlled ageing p_{50} values and seed LI (longevity index, Thompson et al., 1997). p=0.040, R=0.33, df=35.

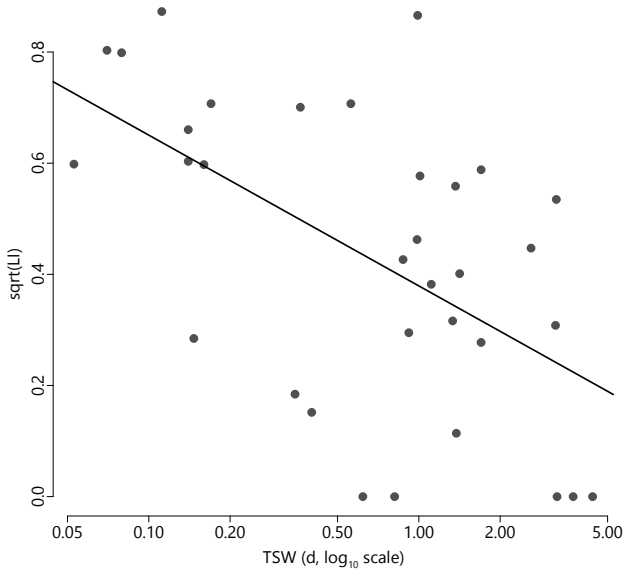


FIGURE 4.3 Correlation analysis of longevity index (LI) and seed mass (TSW). TSW was log10- and LI square root-transformed. R=-0.553, p<0.001, df=31

SOIL SEED BANK PERSISTENCE AND SEED TRAITS

Seeds that were long-lived in the soil possessed predominantly lower seed weights (TSW) than average (Table 4.1). At the same time, seeds that were short-lived in the soil were predominantly heavier than the average. Endospermic seeds were more prevalent in short-lived seeds than in long-lived seeds. These results were underpinned by the statistical analysis.

Using the aggregated survival categories "0-5 years" and ">5 years" as dependent binary variables, the average model showed a significant effect of TSW, whereas endosperm presence/absence showed to be influencial using the aggregated survival categories "transient" and "persistent" (see Table 4.3, for candidate model comparison see Table 4.2). Heavier seeds (mean=1.851±0.388 g) were more likely to survive only "0-5 years" in the soil, lighter seeds (mean=0.672±0.155 g) were more likely to persist

"> 5 years". Non-endospermic seeds were more likely to be found in the "persistent" group and endospermic seeds occurred more frequently in the "transient" group (Table 4.3).

The average model for LI showed a highly significant negative correlation with TSW (see Table 4.3, Figure 4.3, for candidate model comparison see Table 4.2).

TABLE 4.2 Candidate models to explain variation for soil seed bank persistence of calcareous grassland species by seed traits compared to the null model. Soil seed bank persistence was investigated as soil seed bank persistence categories (Poschlod et al. 1998) and LI (longevity index, Thompson et al. 1997). Aggregations of soil seed bank persistence categories: "0-5 years" (1+2); "> 5 years" (3+4); "transient"(1); "persistent" (2+3+4). Number of estimated parameters in each model (K), AIC_c value for each model, the difference in AIC_c between each model and the best-fit model (Δi) and Akaike weight (w_i) are displayed. Seed shape (V_S), seed mass (TSW) and mean coat thickness (MCT) were log10-, LI was square root-transformed. endo: endosperm presence/absence, PY: physical dormancy.

candidate model	K	logLik	AIC_c	Δi	w_i
soil seed bank persistence: "0-5 years" and "> 5 years" (n = 26)					
TSW	2	-12.7	30.0	0.00	0.660
V_S + TSW	3	-12.4	32.0	2.00	0.243
endo	2	-15.7	36.0	5.99	0.033
Null model	1	-17.3	36.8	6.82	0.022
PY	2	-16.7	38.0	8.00	0.012
MCT	2	-16.8	38.1	8.13	0.011
V_S TSW	2	-17.3	39.0	9.05	0.007
V_S + TSW + MCT + endo + PY	6	-11.4	39.3	9.30	0.006
MCT + PY	3	-16.3	39.6	9.65	0.005
soil seed bank persistence: "transient" and "persistent" (n = 26)					
endo	2	-10.7	25.9	0.00	0.557
TSW	2	-12.1	28.8	2.89	0.131
V_S + TSW	3	-11.0	29.0	3.15	0.115
Null model	1	-14.0	30.3	4.39	0.062
V_S	2	-13.2	31.0	5.14	0.043
V_S + TSW + MCT + endo + PY	6	-7.2	30.8	4.97	0.047
MCT	2	-14.0	32.5	6.61	0.020
PY	2	-14.0	32.6	6.71	0.019
MCT + PY	3	-14.0	35.0	9.16	0.006
soil seed bank persistence: LI (n = 33)					
TSW	3	2.733	1.4	0.00	0.445
V_S + TSW + MCT + endo + PY	7	8.222	2.0	0.67	0.318
V_S + TSW	4	3.235	3.0	1.60	0.201
endo	3	-0.480	7.8	6.42	0.018
MCT	3	-1.170	9.2	7.80	0.009
Null model	2	-3.290	11.0	9.63	0.004
MCT + PY	4	-1.100	11.6	10.28	0.003
V_S	3	-2.830	12.5	11.13	0.002
PY	3	-3.270	13.4	12.00	0.001

TABLE 4.3 Average models to explain variation for soil seed bank persistence of calcareous grassland species by seed traits. Persistence was investigated as soil seed bank persistence categories (Poschlod et al. 1998) and LI (longevity index, Thompson et al. 1997). Aggregations of soil seed bank persistence categories: "0-5 years" (1+2); "> 5 years" (3+4); "transient"(1); "persistent" (2+3+4). Estimates, standard errors of the estimates, z values and estimated p values (Pr(>|z|)) are given. Seed shape (VS), seed mass (TSW) and mean coat thickness (MCT) were log10-, LI was square root-transformed. Endo: endosperm presence/absence, PY: physical dormancy.

model averaged coefficients	Estimate	SE	z value	Pr (>|z|)	
soil seed bank persistence: "0-5 years" and "> 5 years" (n = 26)					
(Intercept)	-0.284	1.671	0.163	0.870	
TSW	-3.146	1.410	2.118	0.034	*
V_S	-1.416	1.971	0.681	0.496	
endo absence	1.471	0.916	1.522	0.128	
PY	-1.032	1.182	0.825	0.409	
MCT	-0.885	2.444	0.346	0.729	
soil seed bank persistence: "transient" and "persistent" (n = 26)					
(Intercept)	0.216	2.926	0.072	0.943	
TSW	-2.517	1.625	1.478	0.139	
V_S	-2.810	2.662	1.000	0.317	
endo absence	2.728	1.248	2.072	0.038	*
PY	-0.063	1.697	0.035	0.972	
MCT	5.123	5.243	0.941	0.347	
soil seed bank persistence: LI (n = 33)					
(Intercept)	0.597	0.374	1.560	0.119	
TSW	-0.294	0.092	3.066	0.002	**
V_S	0.314	0.217	1.404	0.160	
endo absence	0.142	0.090	1.502	0.133	
PY	0.213	0.116	1.753	0.080	
MCT	0.144	0.245	0.567	0.571	

SOIL SEED BANK PERSISTENCE AND GERMINATION TRAITS

For most species, we found homogenous records for germination at fluctuating and constant temperatures (see Table 4.1). Within the species that were short lived in the soil but long-lived in artificial ageing there was an accumulation of seeds that germinated at higher percentages at darkness than at light, whereas most other species germinated better at light (Table 4.1). We found no influence of germination traits on soil seed bank persistence category (Table 4.4 and Table 4.5). The average model for the influence of germination traits on LI showed a slight negative influence of darkness and a highly significant negative influence of physiological dormancy (Table 4.5, for model comparison see Table 4.4). Physiologically dormant seeds (mean = 0.134 ± 0.053, n = 9) were significantly shorter-lived than seeds without physiological dormancy (mean = 0.304 ± 0.047, n = 18; Figure 4.4 A, one-way ANOVA, F = 5.579, p = 0.026). This effect remained relevant when removing physically dormant seeds from the data set (one-way ANOVA, F = 6.678, p = 0.017). There was a trend for seeds that germinated at darkness (mean = 0.147 ± 0.063, n = 8) to be more short-lived than seeds only germinating at light (mean = 0.290 ± 0.063, n = 19; Figure 4.4 B, one-way ANOVA, F = 3.503, p = 0.073).

TABLE 4.4 Candidate models to explain variation for soil seed bank persistence of calcareous grassland species by germination traits compared to the null model. Persistence was investigated as soil seed bank persistence categories (Poschlod et al. 1998) and LI (longevity index, Thompson et al. 1997). Aggregations of soil seed bank persistence categories: "0-5 years" (1+2); ">5 years" (3+4); "transient"(1); "persistent" (2+3+4). Number of estimated parameters in each model (K), the AIC_c value for each model, the difference in AIC_c between each model and the best-fit model (Δi) and the Akaike weight (w_i) are displayed. LI was square root-transformed. d: darkness, PD: physiological dormancy, PY: physical dormancy.

candidate model	K	logLik	AIC_c	Δi	w_i
soil seed bank persistence: "0-5 years" and ">5 years" (N = 23)					
Null model	1	-14.860	31.9	0.00	0.457
PY	2	-14.622	33.8	1.93	0.174
d	2	-14.718	34.0	2.12	0.158
PD	2	-14.754	34.1	2.20	0.152
d + PD	3	-14.608	36.5	4.57	0.047
d + PD + PY	4	-14.419	39.1	7.15	0.013
soil seed bank persistence: "transient" and "persistent" (n = 23)					
Null model	1	-12.042	26.3	0.00	0.443
PD	2	-11.690	28.0	1.70	0.189
d	2	-11.868	28.3	2.06	0.158
PY	2	-12.028	28.7	2.38	0.135
d + PD	3	-11.514	30.3	4.02	0.059
d + PD + PY	4	-11.350	32.9	6.65	0.016
LI (N = 33)					
d + PD	4	4.552	0.7	0.00	0.347
d + PD + PY	5	5.911	1.0	0.32	0.296
PD	3	2.665	1.7	1.00	0.210
d	3	1.715	3.6	2.90	0.081
Null model	2	-0.055	4.6	3.90	0.049
PY	3	0.083	6.9	6.16	0.016

TABLE 4.5 Average models to explain variation for soil seed bank persistence of calcareous grassland species by germination traits. Persistence was investigated as soil seed bank persistence categories (Poschlod et al. 1998) and LI (longevity index, Thompson et al. 1997). Aggregations of soil seed bank persistence categories: "0-5 years" (1+2); ">5 years" (3+4); "transient"(1); "persistent" (2+3+4). Estimates, standard errors of the estimates, z values and estimated p values (Pr(>|z|)) are displayed. LI was square root-transformed. d: darkness, PD: physiological dormancy, PY: physical dormancy.

model averaged coefficients	Estimate	SE	z value	Pr(>|z|)	
soil seed bank persistence: "0-5 years" and ">5 years" (n = 23)					
(Intercept)	0.729	0.520	1.324	0.185	
PY	-0.774	1.127	0.647	0.518	
d	-0.487	0.946	0.485	0.628	
PD	-0.415	0.886	0.441	0.659	
soil seed bank persistence: "transient" and "persistent" (n = 23)					
(Intercept)	0.729	0.520	1.324	0.185	
PY	-0.774	1.127	0.647	0.518	
d	-0.487	0.946	0.485	0.628	
PD	-0.415	0.886	0.441	0.659	
LI (n = 33)					
(Intercept)	0.527	0.0648	7.825	<2e-16	***
PY	0.189	0.1284	1.395	0.163	
d	-0.211	0.1072	1.885	0.059	.
PD	-0.203	0.0907	2.130	0.033	*

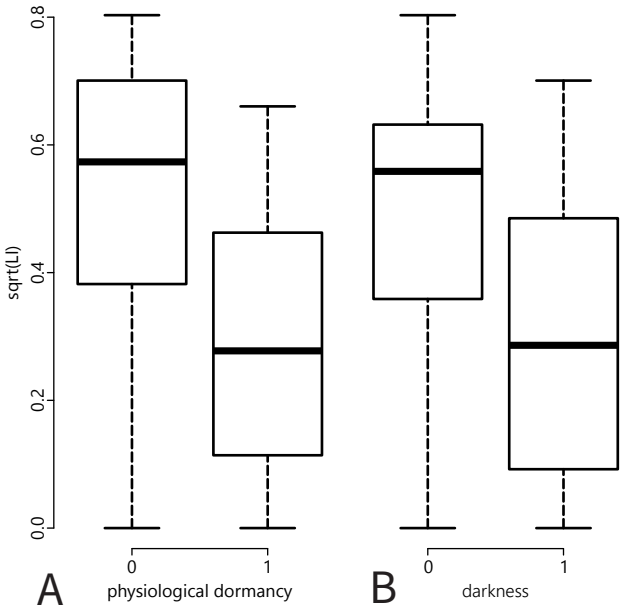


FIGURE 4.4 Box plots of longevity index (LI) and physiological dormancy (A) or germination at darkness (B). Seeds without physiological dormancy (1, n = 18) were significantly more long-lived than physiologically dormant seeds (2, n = 9; one-way ANOVA, F = 5.579, p = 0.026). Seeds that germinated at darkness (1, n = 8) were by trend more short-lived than seeds only germinating at light (0, n = 19; one-way ANOVA, F = 3.503, p = 0.073). Boxplots show the 25–75th percentiles, whiskers span the 10 and 90th percentiles.

4.4 DISCUSSION

The study at hand revealed that in general, transient seeds (soil seed bank persistence category 1, survival < 2 years) possessed significantly lower seed longevity at artificial conditions (p_{50}) than persistent seeds (soil seed bank persistence categories 2-4, > 2 years). Accordingly, LI and p_{50} values were significantly positively correlated. For almost 2/3 of the investigated species, survival at artificial ageing and in the soil seed bank was consistently high or low. Seed and germination traits relevant for soil seed bank persistence were seed weight, endosperm presence/absence, physiological dormancy and germination at darkness.

CORRELATION BETWEEN *EX SITU* AND *IN SITU* SURVIVAL

The consulted soil seed bank data of Poschlod et al. (1998) integrate several repeated studies in calcareous grasslands (seed bank studies along successional series, depth distribution, burial experiments and diaspore bank-diaspore rain comparisons), which were compared and validated with germination traits, resulting in a very detailed classification of soil seed banks of calcareous grasslands. With calculation of the LI (Thompson et al., 1998), we summarized seed bank persistence and soil seed survival literature data, without regard of survey methods. That way, burial survival and LI of annual weeds were not correlated (Saatkamp et al., 2009), indicating a limited reliability of the LI due to the pooling of different survey methods, especially of seed bank persistence estimates correlated to seed production. However, focusing on calcareous grasslands, the quality of the integrated available literature was apparently high enough, leading to accordance of the soil seed bank persistence categories of Poschlod et al. (1998) and LI, insofar as seeds that survived between 0 and 5 years (categories 1 and 2) possessed significantly lower LI values than seeds that survived more than five years (categories 3 and 4).

This shows the complexity and difficulty of estimating seed survival potential of species and its dependence on the source and origin of both, species and data. Whereas our investigation confirmed the results of Long et al. (2008), who found corresponding survival in the soil and at artificial ageing for 13 Australian weed species, it contrasted those of Walters et al. (2005b), who reported no correlation between *ex situ* longevity and soil seed persistence (based on Duvel's buried seed experiment in Toole & Brown, 1946) for 107 crop and wild species originating from different provenances. Walters et al. (2005b) explained the differences by diverse environmental parameters acting on seeds *in situ* and *ex situ*, with seeds from moist habitats showing low survival rates under banking conditions (dry conditions) and high persistence in the soil (wet conditions). This indicates that seeds adapted to arid climates possess enhanced intrinsic ability to cope with these slowly occurring damages at dry storage. Likewise it was found that seeds sourced from warmer and drier environments were more long-lived in dry storage (Walters et al., 2005b; Probert et al., 2009) and rapid ageing (Long et al., 2008; Probert et al., 2009; Mondoni et al., 2011) than those from cooler and wetter climates.

In artificial ageing, seeds are exposed to humid and warm conditions that accelerate oxidative as well as hydrolytic damage in all species, but no interim repair intervals occur until the final imbibition for germination, where it emerges whether excessive oxidative damage has already overburdened repair mechanisms and seeds may germinate or die (Bewley et al., 2013). Nonetheless, for Australian weed (Long et al., 2008) and calcareous grassland species, soil seed bank persistence and survival at artificial ageing

were correlated. Within single habitats the resistance of a single seed against artificial ageing is proportionally related to the ability to regenerate and survive in the soil, indicating an intrinsic longevity. Conclusions about the survival *ex situ* and *in situ* can therefore only be made for species originating from one habitat and a comparison over a large number of habitats is not useful to find patterns and make predictions.

SEED MORPHOLOGICAL TRAITS RELATED TO *EX SITU* AND *IN SITU* SURVIVAL OF CALCAREOUS GRASSLAND SEEDS

Seed mass showed to be significantly related with soil seed bank persistence. Whereas long-lived seeds possessed lower seed mass and by trend lower seed shape, short-lived seeds had opposite characteristics. Seed mass and seed shape were considered the most important traits that can be related to soil seed bank persistence (e.g. Bekker et al., 1998a), but showed to be not influential in artificial ageing (Chapter 3). Although there is no consensus whether small or large seeds are more affected by predation (see Hulme, 1998; Moles et al., 2003; Traba et al., 2006) buried seeds are less exposed than seeds on the surface (Crawley, 2013) and as large seeds are less incorporated into the soil (Bekker et al., 1998a), they may be strongly threatened by predators (Hulme, 1998). Additionally, as small seeds are better incorporated into the soil and soil water content elevates even within low depths, small seeds may stronger benefit from reinitiated repair mechanisms in the imbibed state. However, this does not exclude long-term survival of bigger seeds when incorporated into the soil. A further aspect is the potentially lower detectability of bigger seeds by soil sampling methods due to the seed number seed size trade off, which could cause underestimation of soil persistence (Saatkamp et al., 2009).

Moreover, endospermic seeds were significantly associated with a transient soil seed bank (category 1), whereas non-endospermic seeds were predominantly persistent (categories 2-4). Endosperm presence was previously shown to be of significance for *ex situ* longevity (Probert et al., 2009; Merritt et al., 2014). We therefore assume that endospermic seeds tend to quicker accumulate damage and/or possess less efficient repair mechanisms to fight free radical damage- during imbibition in the soil or germination after artificial ageing. Another important seed trait related to soil seed bank persistence, seed coat thickness (Gardarin et al., 2010), showed no significant effect on seed survival of calcareous grassland species.

GERMINATION TRAITS RELATED TO *EX SITU* AND *IN SITU* SURVIVAL OF CALCAREOUS GRASSLAND SEEDS

It is well-known that soil seed bank persistence is strongly connected to germination behaviour Saatkamp et al., 2011). The examination of a correlation between seed germination traits and soil seed bank persistence categories showed no effect of darkness, physiological or physical dormancy on soil seed bank persistence. However, LIs were significantly lower for seeds that were physiologically dormant and there was a trend for lower LIs for seeds that germinated at darkness. Physiologically dormant seeds also proofed to be more short-lived at artificial ageing (see Chapter 3).

In dormant seeds, oxidative damage is not countervailed by protective germination specific antioxidants, which are produced in germinating seeds (Haslekas et al., 2003). Due to this absent protection, dormant seeds may die before germination because of an accumulation of oxidative damage. This contradicts the general assumption that dormancy is a requirement for soil seed persistence. Notwithstanding, compared to extremely transient seeds (e.g. recalcitrant seeds), with immediate germination (Daws et al., 2005), dormant seeds are more long-lived as they need to overcome dormancy first, which delays germination (Saatkamp et al., 2011).

In a review, Thompson et al. (2003) already showed that dormant seeds are not per se long-lived and all combinations of dormancy and persistence exist. Accordingly, we found physiologically dormant seeds in all groups (see Table 4.1). This may be explained by the ability of some seeds to perform dormancy cycling (see e.g. Finch-Savage and Footitt 2017) and thereby repair damage in the non-dormant state, whereas other species are missing this capability. Latter is most probably true for the most transient, all physiologically dormant species (*Rhinanthus minor, Pimpinella saxifraga, Seseli annuum* and *Asperula cynanchica*). Seeds of these species are prone to death when no germination occurs immediately after winter stratification (see Donelan and Thompson (1980).

Another germination behaviour enhancing seed persistence by preventing fatal germination in deep soil is the ability to detect light and fluctuating temperatures (Thompson & Grime, 1983). While most of the investigated species possessed no detection mechanism for fluctuating temperatures, they germinated to a higher extent at light. Three Fabaceae species were not dependent on light for germination. Possibly, these species never developed a light detec-

tion mechanism as these seeds rely on the protective effect of a hard and water-impermeable seed coat for persistence. As long as the seed coat remains intact, seeds exhibit extended longevity and no germination occurs. As soon as the seed coat becomes permeable ("natural" scarification), seeds will germinate or if the conditions are insufficient die. Although not statistically verifiable, physically dormant seeds were the most long-lived species in our study. As well, at artificial ageing physical dormancy was a major parameter to prolong seed survival (Merritt et al 2014, see Chapter 3), even in scarified seeds (Merritt et al., 2014).

INCONSISTENCIES BETWEEN *IN SITU* AND *EX SITU* SURVIVAL IN CALCAREOUS GRASSLANDS

Species that were short-lived *in situ* but long-lived at artificial ageing were mainly non-endospermic and even physically dormant - both factors previously associated with high intrinsic longevity. Most probably due to larger seed size and an affinity to germinate in the dark, these species with high intrinsic longevity do not exhibit long soil seed bank persistence. We suggest that considering this group as short-lived would strongly underestimate the intrinsic longevity, especially as these findings may also result from methodological errors in detection. Seven of the investigated species were considered long-lived in the soil but short-lived at artificial ageing. At first sight it is difficult to explain how seeds with low intrinsic longevity could survive over 6 to over 20 years in the soil (Poschlod et al., 1991). Nevertheless, this low intrinsic longevity could be correlated with endosperm presence or physiological dormancy. Therefore, these mainly small-sized seeds possibly rely on dormancy cycling (Nguyen et al., 2012) and/or wet-dry cycling (Long et al., 2011) in the soil to compensate low intrinsic seed longevity and antagonise accumulated damages, which is impossible at artificial ageing.

4.5 CONCLUSIONS AND IMPLICATIONS FOR CONSERVATION

The present study on seeds of 39 calcareous grassland species revealed significant similarities between soil seed bank persistence and survival at artificial ageing. We found a correlation between *in situ* and *ex situ* seed survival, based on an intrinsic longevity that allows at least a placement of single species relatively to each other. We moreover emphasise the comparison of longevity within single habitats to detect influencing traits.

Endosperm presence/absence was associated with survival at artificial ageing and could be transferred on soil seed bank survival for at least 2/3 of the investigated species. For the remaining species, we assume that this seed trait was masked by other parameters. Moreover, we believe that the observation of a negative correlation of soil seed bank persistence and seed size does not necessarily indicate reduced intrinsic longevity of larger seeds, as such relation has not been detected in artificial ageing of the same set of species. Larger seeds may possess great intrinsic longevity but as they become less incorporated into the soil, they are more vulnerable to predation, germination, have less opportunities to repair oxidative damages or these seeds are rarely detected due to low seed production (Saatkamp et al., 2009).

Non-endospermic seeds seem to possess the most effective repair mechanisms or a slowed accumulation of oxidative damage. In combination with smaller seed size, which enables wet-dry cycling in the soil, long survival in both, the soil seed banks of abandoned calcareous grasslands and *ex situ* storage, is very likely. In our opinion, field collection or retesting and replenishing stored seeds of this group should be of least priority for seed bank curators and conservation. In contrast, endospermic seeds probably inhabit less effective repair mechanisms, which results in reduced longevity *in situ* and *ex situ*. This group of species eventually never developed mechanisms for long-term survival and therefore should be considered for *ex situ* storage including frequent regeneration. Seeds with low soil seed bank persistence but high longevity in artificial ageing must also be prioritised for *ex situ* storage as long term survival in the soil is not guaranteed. When longevity in the soil was high although low intrinsic longevity was determined by artificial ageing, we consider wet-dry cycling or dormancy cycling as the reason for enhanced persistence in the soil which do not function at artificial ageing. The potential of these species for restoration from the soil seed bank was clearly underestimated by artificial ageing in the laboratory. Species with consistently low survival probably rely naturally on alternative strategies for persistence like being long-lived or clonal (*Pimpinella saxifraga*, grasses like *Bromus erectus*). Notwithstanding, even for those species, habitat loss indicates *ex situ* storage and besides, longevity can be highly extended by suitable *ex situ* storage conditions. SID-database (Liu et al., 2008) research showed, that for the least long-lived species *Rhinanthus minor*, no significant viability decline was

detected after 14 years of *ex situ* storage (initial germination 94 %, 93 % after 14 years). However, as viability can decline rapidly after a certain threshold time has been reached (Walters et al., 2010), short-lived seeds should be handled with caution.

APPENDIX

TABLE 4.6 Germination results (%) for selected calcareous grassland species. n = number of seeds used for each trial. Germination percentage at each trial. c = constant and f = fluctuating temperatures, D = darkness, L = light.

		Final germination percentage			
Species	n	c + D	c + L	f + D	f + L
Achillea millefolium	100	57	77	54	90
Acinos arvensis	100	13	81	21	100
Arabis hirsuta	50		100		87
Arenaria serpyllifolia	200	41	95	55	85
Buphthalmum salicifolium	100	22	73	0	63
Campanula rotundifolia	100	2	73	39	73
Centaurea stoebe	100	13	86	27	75
Cerastium arvense	100	40	88	44	84
Linum catharticum	100	48	95	51	94
Melica ciliata	100		60		58
Pulsatilla vulgaris	100			68	74

CHAPTER 5

How precise can X-ray predict the viability of wild flowering plant seeds?

ABSTRACT

The detection of seed viability is an important procedure step in seed production industry, as well as in conservation and restoration practice. Due to random natural events seed quality and viability of wild flowering species vary substantially and hence a quick and reliable method for seed viability assessment is desirable. X-rays provide information about the internal structures of a seed and therefore show promise for detection of viability and germination capacity. In the study at hand plant seeds of 207 accessions of 176 wild flowering plant species were X-rayed and the viability results were compared with combined germination-TZ tests. Of special interest was for which plant families X-ray is an appropriate method for viability detection, considering correlations with seed internal morphological aspects, seed mass and/or shape. The comparison revealed a strong analogy of viability determination by combined germination-TZ tests and X-ray analysis. According to the taxonomy and seed type two main groups could be distinguished, that differed significantly in the viability detection by X-ray and combined germination-TZ test. Whereas the evaluation of little/non-endospermic seeds gave approximately same results, endospermic seed evaluation was different for both methods. Especially for non/little endospermic seeds X-ray analysis can provide a useful non-destructive and quick tool for viability detection, whereas for endospermic seeds further research is needed.

KEYWORDS:

Seed, viability, X-ray, germination, tetrazolium, seed banks, *ex situ* conservation

5.1 INTRODUCTION

The exact determination of seed quality and viability is essential for crop seed production but also for *ex situ* conservation of seeds in seed banks and for restoration. Within the framework of the first German federal seed bank for rare and endangered plant species (Genbank Bayern Arche, Tausch et al., 2015), the research objectives are to improve seed viability testing in terms of resources and time. As rare species' populations often lack larger quantities of seeds due to small population sizes or reduced seed production (Godefroid et al., 2011), an ideal viability test should be non-destructive.

Several methods can be used to achieve information about seed viability and quality. Their implementations are described by international rules and handbooks (ISTA, 2003; AOSA, 2010a, b). The most direct method for viability assessment is a germination test. However, conclusions about the viability of seeds via germination tests can only be drawn when germination conditions as well as dormancy breaking treatments are well known. Alternatively, subsequent chemical Tetrazolium (TZ) tests are commonly used in order to detect viability of non-germinated mostly

dormant seeds (Baskin & Baskin, 1998; ISTA, 1999). Both tests are very time-consuming and the interpretation of the TZ test is considered to be partly difficult and needs a lot of experience regarding different plant families (Moore, 1973; Copeland & McDonald, 1995).

A quick and non-destructive alternative method for indirect detection of seed viability is the X-ray analysis. ISTA acknowledges this method only to detect full, empty, insect infested and physically damaged seeds (Simak & Gustafsson, 1953; ISTA, 1999). The use of X-rays provides an insight into the internal structures of a seed. Different attenuation of X-rays in a seed is determined by the type of the penetrated tissue (atom composition, density and thickness) and the energy of the X-rays (tube voltage and filtering). Since dense and thick tissues attenuate X-rays more than thin tissues, an image is generated where transmission differences can be seen as different luminescence nuances.

So far, X-ray analyses have already been used with some success to predict seedling morphology, germination performance and/or quality, but only on agricultural crops such as *Lycopersicon esculentum* (van der Burg et al., 1994), *Zea mays* (Carvalho et al., 1999), *Oryza sativa* (Menezes et al., 2012), *Solanum melongena* (Neumann Silva et al., 2012), *Capsicum annuum* (Dell'Aquila, 2007; Gagliardi & Marcos-Filho, 2011), *Ricinus communis* (Carvalho et al., 2010) and some tree species (Goodman et al., 2005; Socolowski et al., 2011; Sturiao et al., 2012). Being aware of the importance that the seed viability has to be known for reasonable conservation of seeds in a seed bank and also for the restoration of plant populations, a reliable method for quick and non-destructive viability assessment is required. Considering the lack of information for wild flowering species we surveyed the X-ray method for viability assessment with 207 fresh wild flowering plant seed accessions. The results were compared with combined germination-TZ tests. Of special interest was if the results correspond to each other or not and if the findings are correlated to the internal anatomy of seeds, seed mass and/or shape. The results should provide a quick overview for which kind of plant species X-ray analysis can be used for a reliable viability assessment of wild flowering plant seeds.

5.2 MATERIALS AND METHODS

PLANT MATERIAL

Plant seeds of 207 accessions of 176 wild flowering plant species were collected throughout Bavaria (Southeast Germany) between 2009 and 2012 within the framework of the seed bank "Genbank Bayern Arche" (see Table 5.1). The species were collected randomly and represent about ten per cent of the plant species in Bavaria (without microspecies and hybrids, Scheuerer & Ahlmer, 2003). Seeds were dried at room temperature and were stored at 4 °C until the examination, which took place in average three months after collection. 100 seeds were used for each examination, except for accessions with smaller seed quantities.

SEED GERMINATION

Seeds were germinated in the laboratory in petri dishes on moist filter paper, 5 replicates with 20 seeds at different germination conditions (constant or alternating temperatures, with or without pre-treatments like stratification or scarification). The results of the best germination conditions for each species were used for statistical analyses. The trials were performed in climate chambers (Rumed, type 1301, Rubarth Apperate GmbH, Laatzen, Germany) and in a cooling room (4 °C), where chilling was required. Seeds were regularly checked for germination and were considered to be viable when germinated - e.g. a radicle protrusion of ≥ 2 mm occurred and a development of "normal seedlings" was ascertained (Black et al., 2006; Bewley et al., 2013).

TETRAZOLIUM TESTING

A subsequent topographical Tetrazolium test was carried out with the non-germinated seeds (Grabe, 1970). The test allows a differentiation between living and dead seed tissues as in living cells the colourless 2,3,5-triphenyl-tetrazolium chloride is metabolized into red 1,3,5-triphenylformazan by dehydrogenase activity (Lakon, 1948). Seeds were prepared and soaked in a 1 % Tetrazolium solution for 24 to 48 hours at 22 °C. Depending on the plant family, the embryo anatomy and presence or absence of endosperm, essential parts had to be stained and firm in order to be called "viable". Different staining pattern evaluations were developed for each plant family by adapting ISTA (1999) and AOSA (2010b) Tetrazolium testing rules.

TABLE 5.1 Seed classification of the investigated plant families. Seed type classification and seed type categories (cat.) according to Martin (1946) and Finch-Savage and Leubner-Metzger (2006). Nutritive storage tissues given by Meyer (2005). + found in mature seed, - not found in mature seed. Nutritive storage tissue category (cat) defined as follows: UE for (by Tetrazolium) unstained seeds with significant Endosperm, SE for (by Tetrazolium) stained seeds with significant endosperm, P for seeds with considerable Perisperm, LE for seeds with medium to little endosperm, VLE for seeds with very little to no endosperm, N for seeds without nutritive tissue. Differing Meyer (2005) Juncaceae, Iridaceae and Liliaceae were classified as endospermic instead of perispermic. Plant families with missing descriptions were classified via X-ray and Watson and Dallwitz (1992 onwards) (italicized letters). Numbers of used plant families are also given.

family (-aceae)	seed type		nutritive storage tissue			no of accessions
	cat.	Embryo characterization	perisperm	endosperm	cat.	
Junc-	B2	broad	–	+	UE	5
Cyper-	B3	capitate	–	+	UE	15
Po-	B4	lateral	–	+	UE	11
Caryophyll-	P	peripheral	+	–/very little	P	23
Chenopodi-	P	peripheral	+	–/very little	P	1
Papaver-	B1	rudimentary	–	+	SE	2
Ranuncul-	B1	rudimentary	–	+	SE	8
Rubi-	FA1	spatulate axile, cotyledons thin/thick	–	+	SE	1
Cist-	FA3	folded axile, cotyledons thin	–	+	SE	3
Amaryllid-	LA	linear axile	–	+	SE	1
Api-	LA	linear axile	–	+	SE	12
Asparag-	LA	linear axile	–	+	SE	2
Dipsac-	LA	linear axile	–	+	SE	2
Irid-	LA	linear axile	–	+	SE	3
Plantagin-	LA	linear axile	–	+	SE	2
Primul-	LA	linear axile	–	+	SE	3
Viol-	LA	linear axile	–	+	SE	1
Campanul-	MA	seed miniature axile, dwarf or micro	–	+	SE	13
Gentian-	MA	seed miniature axile, dwarf or micro	–	+	SE	4
Scrophulari-	MA	seed miniature axile, dwarf or micro	–	+	SE	9
Polygon-	P	peripheral	–	+	SE	1
Boragin-	FA4	investing axile, cotyledons thick	–	+/–	LE	5
Plumbagin-	FA4	investing axile, cotyledons thick	–	+/–	LE	1
Aster-	FA1	spatulate axile, cotyledons thin/thick	–	–/very little	VLE	28
Hyperic-	FA1	spatulate axile, cotyledons thin/thick	–	–/very little	VLE	1
Lin-	FA1	spatulate axile, cotyledons thin/thick	–	–/very little	VLE	5
Tamaric-	FA1	spatulate axile, cotyledons thin/thick	–	–/very little	VLE	1
Brassic-	FA2	bent axile, cotyledons thick	–	–/very little	VLE	17
Fab-	FA2	bent axile, cotyledons thick	–	–/very little	VLE	10
Betul-	FA4	investing axile, cotyledons thick	–	–/very little	VLE	1
Lami-	FA4	investing axile, cotyledons thick	–	–/very little	VLE	11
Ros-	FA1	spatulate axile, cotyledons thin/thick	–	–	N	5

X-RAY ANALYSES

X-ray analyses were carried out by a digital radiography system (Faxitron MX-20, Faxitron X-ray Corporation, Wheeling, Illinois) with an exposure time of 9 seconds and 26 kV. Likewise the Tetrazolium examination for the analysis of the X-ray pictures the same four groups "viable", "non-viable", "empty" and "insect infested" were used to categorize the examined seeds. Seeds were determined as "viable" when no essential part of the embryo and the nutritive storage tissue was damaged and the tissue density was high, e.g. according to ISTA rules (1999), all essential tissues for germination were present.

SEED CLASSIFICATION AND TRAIT ANALYSIS

Internal anatomy of seeds (as described below) and two morphological seed traits, seed mass and seed shape, were used to identify their influence on the comparability of the two viability assessment methods. Seed mass was determined as thousand seed weight. Seed dimensions were measured on five replicate seeds per species. Seed shape was used as variance of seed dimension, which was calculated according to the formula described by Bekker et al. (1998a):

$$V_s = \frac{\sum(x_i - \bar{x})^2}{n} \quad (1)$$

with x1 = length/length, x2 = height/length and x3 = width/length, n = 3. The dimensionless seed shape had spans from a minimum value 0.0004 in perfectly spherical seeds (*Lathyrus palustris*) to a maximum value of 0.2277 in needle- and disc-shaped seeds (*Betula nana*).

As the seed anatomy concerning the embryo (shape, development and size) and the storage tissues (presence, absence and size of endosperm and/or perisperm) seemed to influence the applicability of viability detection in both, X-ray and Tetrazolium testing, two seed classifications were used to group the species (see Table 5.1). The first classification (= seed type classification) is based on Martin (1946) and has been revised and extended by Finch-Savage and Leubner-Metzger (2006). Eleven seed types (B1 to B4, FA1 to FA4, LA, MA and P) can be divided into basal seeds with abundant endosperm and a tiny embryo (B1 to B4), more advanced endospermic seeds with linear axile embryos (LA), endospermic miniature or dwarf seeds (MA), seeds with peripheral embryos (P) and little or non-endospermic seeds with foliate axile embryos (FA1 to FA4). The second classification (= nutritive storage tissue type classification), which is based on Meyer's (2005) review, only relates to the type of nutritive storage tissues (endosperm or perisperm) and its size in relation to the embryo. For statistical analysis six categories were assigned to Meyer's groups, that reflect the most evident characteristics: UE for seeds with (by Tetrazolium) unstained significant endosperm, SE for seeds with (by Tetrazolium) stained significant endosperm, P for seeds with considerable perisperm, LE for seeds with medium to little endosperm, VLE for seeds with very little to no endosperm, N for seeds without any nutritive storage tissue. Missing families in both classification systems were classified autonomously via X-ray and/or Tetrazolium.

DATA ANALYSIS

In order to compare the two viability assessment methods, X-ray and combined germination-TZ test, the seed viability difference SVD was calculated:

(2)

$$SVD = \frac{(\%\ viable\ seeds_{GER,TZ} - \%\ viable\ seeds_{x-ray})}{100}$$

SVD was calculated as the percentage of viable seeds assessed by combined germination-TZ test (% viable $seeds_{GER,TZ}$) minus the percentage of viable seeds assessed by X-ray analysis (% viable $seeds_{x\text{-}ray}$), divided by 100. The closer to zero the value for SVD is the more equal the results of the two seed viability assessment methods are.

Firstly, the impact of the three parameters thousand seed weight, seed shape and seed classification on SVD was analysed. Two ANCOVA maximal models were fitted. The first model included seed type classification, thousand seed weight and seed shape as explanatory variables, plus interactions between these factors and SVD as response variable. In the second model nutritive storage tissue classification, thousand seed weight and seed shape were used as explanatory variables, plus interactions between these factors and SVD as response variable. Subsequently, two minimal adequate models for each classification system, that contained significant terms only (p value < 0.05) and had minimal AIC, were created by stepwise model simplification (Crawley, 2007). Beforehand, inter-correlation of thousand seed weight and seed shape was excluded by a correlation test (Pearson product-moment correlation). The fitted models were checked for goodness with respect to heteroscedasticity, evidence of curvature, temporal correlation and non-normality of errors (Crawley, 2007).

Secondly, as only the two seed classifications showed a significant effect on SVD, the factor levels of the two seed classifications were examined in detail, to see which gave better results for viability difference SVD. Hence two one-way ANOVAs were calculated and model simplification was conducted by aggregation of non-significant factor levels of seed type and nutritive storage tissue type in a stepwise a posteriori procedure.

Thirdly, the newly aggregated seed type and the nutritive storage tissue groups were used to carry out a type II regressions MA, because in both of the methods, X-ray and combined germination-TZ analysis, measuring errors might occur (Köhler et al., 2002). The percentages of viable seeds from X-ray and combined germination-TZ analysis were tested against each other for the aggregated seed type and the nutritive storage tissue categories. Homogenous variances and normal distribution of the residuals were proved. The R-package lmodel2 (Legendre, 2013) was used for the statistical data analyses, the MA-residuals were gained following the R-script of Bergmann (2012). All analyses were conducted in R v.2.14.1.

5.3 RESULTS

The seed viability difference (SVD) between the two compared viability assessment methods for all analysed accessions was distributed as follows: 16 % had a SVD < 1 %, 39 % had a SVD < 5 %, 57 % had a SVD < 10 %, 43 % had a SVD > 10 % (cumulative values). That means that in more than 50 % of the species, the two viability assessment methods produce identical or more or less similar results. It must be considered that these results include all seed types. As following results show, the correspondence of the two viability assessment methods strongly depends on the seed classification types. Table 5.2 shows the detailed results for single plant families.

The ANCOVA revealed that in both models the seed classification (seed type or the nutritive storage tissue) was the only parameter that had a highly significant influence on the viability difference SVD (see Table 5.3), whereas seed shape and thousand seed weight did not have a significant effect, as well as interactions between the factors, which were consequently removed from the maximal model. The beforehand Pearson correlation revealed no significant inter-correlation of thousand seed weight and seed shape ($N = 177$, $t = -1.5319$, $r = -0.1160$, $p = 0.1274$).

In the following one-way ANOVAs the initial eleven levels of the seed type classification and the six levels of the nutritive storage tissue classification were aggregated each into two groups by stepwise aggregation (Crawley, 2007). The resulting aggregated groups for the seed type classification were P.FA1.FA2.FA3.FA4.B4 and B1.B2.B3.LA.MA and for the nutritive storage tissue classification P.LE.VLE.N and UE.SE respectively. This means that for both classifications all seeds with significant endosperm (B1.B2.B3.LA.MA and UE.SE) and all perispermic, little to non-endospermic seeds (P.FA1.FA2.FA3.FA4.B4 and P.LE.VLE.N) were aggregated, with the exception of Poaceae seeds (B4), which were assigned to the group of little/non-endospermic seeds in the seed type classification (P.FA1.FA2.FA3.FA4.B4). For both seed classifications, the average SVDs of the two newly aggregated groups were significantly different from each other (see Figure 5.1 and Table 5.4). The means of SVD over the little/non endospermic seeds were not significantly different from zero in each case. In contrast the means of SVD over the seeds with significant endosperm, just as the mean of all species showed a significant difference from zero.

Also, the MA-regressions for each seed classification showed a significant relation between combined germination-TZ test and X-ray analysis (see Figure 5.2, Table 5.5). For perispermic, little/non-endosper-

mic seeds (P.FA1.FA2.FA3.FA4.B4 and P.LE.VLE.N), by the coefficient of determination (R^2), 72 % respectively 74 % of the total variance could be explained with regard to a statistical correlation ($p < 0.001$). For seeds with significant endosperm (B1.B2.B3.LA.MA and UE.SE) only 25 % respectively 28 % could be explained ($p < 0.001$). This weaker correlation lowers the overall result of the regression model for all accessions ($R^2 = 0.45$, $p < 0.001$). The regression lines given by the model for little/non-endospermic seeds showed slopes close to 1 and intercepts close to 0, whereas the regression lines for the model for seeds with significant endosperm showed weaker slopes and an intercept more distant from zero.

TABLE 5.2 Seed viability differences SVD between combined germination-TZ and X-ray examinations. Number of analysed accessions, median and standard error of SVD are given.

family	N	Median (SVD)	SE (SVD)
Amaryllidaceae	1	0.002	NA
Apiaceae	12	-0.086	0.082
Asparagaceae	2	-0.018	0.068
Asteraceae	28	-0.023	0.026
Betulaceae	1	-0.002	NA
Boraginaceae	5	-0.031	0.113
Brassicaceae	17	-0.031	0.032
Campanulaceae	13	-0.047	0.059
Caryophyllaceae	23	0	0.027
Chenopodiaceae	1	-0.025	NA
Cistaceae	3	-0.021	0.111
Cyperaceae	15	-0.143	0.092
Dipsacaceae	2	-0.044	0.036
Fabaceae	10	0	0.03
Gentianaceae	4	-0.493	0.182
Hypericaceae	1	0	NA
Iridaceae	3	0.004	0.058
Juncaceae	5	-0.219	0.125
Lamiaceae	11	-0.014	0.048
Linaceae	5	-0.022	0.016
Papaveraceae	2	-0.647	0.303
Plantaginaceae	2	-0.159	0.079
Plumbaginaceae	1	-0.02	NA
Poaceae	11	0	0.06
Polygonaceae	1	0	NA
Primulaceae	3	-0.474	0.138
Ranunculaceae	8	-0.088	0.077
Rosaceae	5	-0.04	0.048
Rubiaceae	1	-0.202	NA
Scrophulariaceae	9	-0.071	0.062
Tamaricaceae	1	-0.19	NA
Violaceae	1	-0.497	NA

TABLE 5.3 Impact on viability difference SVD for the seed type and the nutritive storage tissue classification. Seed type classification and seed type categories according to Martin (1946) and Finch-Savage and Leubner-Metzger (2006), nutritive storage tissues given by Meyer (2005). The ANCOVA table (minimal adequate model) includes degrees of freedom (df), sums of squares (SS), means squares (MS), F value and corresponding p value for the significant explanatory variables. Thousand seed weight and seed shape as explanatory variables and all interactions have been removed from the full model.

	df	SS	MS	F	p value	
seed type	10	1.374	0.137	3.186	<0.001	***
error	196	8.457	0.043			
nutritive storage tissue	5	0.937	0.187	4.235	0.001	**
error	201	8.894	0.044			

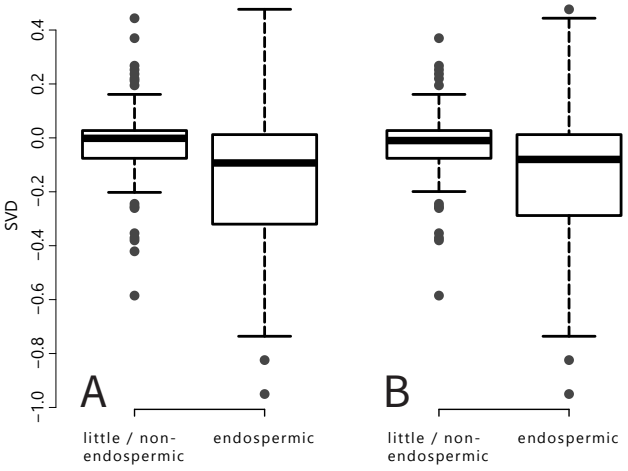


FIGURE 5.1 Boxplots of SVD for the different viability assessment methods combined germination-TZ test and X-ray analysis. A: Seed type classification after Martin (1946) and Finch-Savage and Leubner-Metzger (2006). B: Nutritive storage tissue classification after Meyer (2005). Comparison of little/non-endospermic (A: P.FA1-FA2. FA4.B4 and B: P.LE.VLE.N) with endospermic seeds (A: B1.B2.B3.LA. MA and B: UE.SE). Boxes and central bars represent the interquartile range and median, dashed lines represent the range of sample and dots are outliers.

TABLE 5.4 Seed classifications influence on viability difference SVD. Presented are the summary statistics of the one-way analyses of variance using SVD as the dependent variable and the factors of the two seed classifications as explanatory variables. Seed type classification after Martin (1946) and Finch-Savage and Leubner-Metzger (2006). Nutritive storage tissue classification after Meyer (2005). The two respective main groups containing little/non-endospermic (P.FA1-FA2.FA4.B4 and P.LE.VLE.N) or endospermic seeds (B1.B2.B3.LA.MA and UE.SE) had been gained by aggregation of non-significant factor levels. The table includes mean percentage, standard error of SVD, number of accessions for each group and for all accessions, t- and corresponding p values for the difference to zero. Model statistics for seed type classification: $r^2 = 0.12$, adjusted $r^2 = 0.12$, F value = 28.11 on 1 and 205 DF, p value < 0.001. Model statistics for nutritive storage tissue classification: $r^2 = 0.08$, adjusted $r^2 = 0.08$, F value = 18.45 on 1 and 205 DF, p value < 0.001.

	n	mean	SE	t val.	p val.	
All accessions (intercept)	207	-0.086	0.015	-5.644	< 0.001	***
seed type						
little/non-endospermic (intercept)	125	-0.024	0.018	-1.329	0.185	
endospermic (intercept)	82	-0.179	0.023	-7.899	< 0.001	***
difference little/non-endospermic - endospermic (intercept)		0.155	0.030	5.302	< 0.001	***
nutritive storage tissue						
little/non-endospermic (intercept)	109	-0.026	0.020	-1.310	0.192	
endospermic (intercept)	98	-0.152	0.021	-7.162	< 0.001	***
difference little/non-endospermic - endospermic (intercept)		0.125	0.029	4.296	< 0.001	***

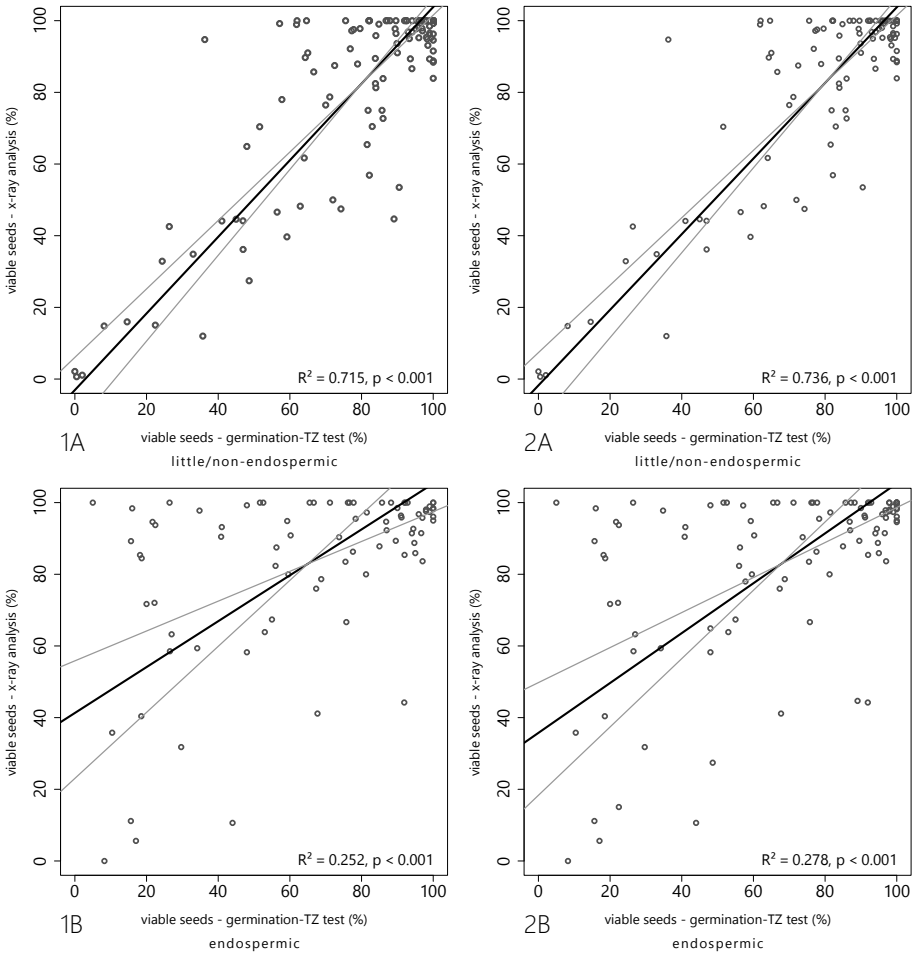


FIGURE 5.2 Relationships of viability assessment between combined germination-TZ tests and X-ray analysis. 1: Seed type classification after Martin (1946) and Finch-Savage and Leubner-Metzger (2006). 2: Nutritive storage tissue classification after Meyer (2005). Comparison of little/non-endospermic (1A: P.FA1-FA2.FA4.B4 and 2A: P.LE.VLE.N) with endospermic seeds (1B: B1.B2.B3.LA.MA and 2B: UE.SE). R^2 and p value of the regression line are given. Regression line is shown as red, confidence intervals of the slope are shown as grey lines. Full results (intercept and slope) are shown in Table 5.4.

TABLE 5.5 Comparison of MA relationships of X-ray analysis and combined germination-TZ tests for the two seed classifications. Seed type classification after Martin (1946) and Finch-Savage and Leubner-Metzger (2006) and nutritive storage tissue classification after Meyer (2005). The two respective main groups little/non-endospermic (P.FA1-FA2.FA4.B4 and P.LE.VLE.N) or endospermic seeds (B1.B2.B3.LA.MA and UE.SE) were tested against each other. For each relationship, R^2, p values, intercept estimate, upper and lower confidence interval of the intercept estimate, slope estimate, upper and lower confidence interval of the slope estimate and the one-tailed permutational propability for the slope estimate are given.

				Intercept			Slope			
	N	R^2	p value	Est.	LowCI	UppCI	Est.	LowCI	UppCI	p perm
All accessions	207	0.446	<0.001	18.928	8.419	28.145	0.86	0.736	1.002	0.01
seed type										
little/non-endospermic	125	0.715	<0.001	-3.071	-13.35	6.044	1.069	0.955	1.197	0.01
endospermic	82	0.252	<0.001	41.297	23.04	55.843	0.64	0.416	0.921	0.01
nutritive storage tissue										
little/non-endospermic	109	0.736	<0.001	-1.782	-12.12	7.378	1.055	0.795	1.001	0.01
endospermic	98	0.278	<0.001	35.744	18.327	49.774	0.696	0.489	0.953	0.01

5.4 DISCUSSION

The present data reveal a strong analogy of viability determination by combined germination-TZ tests and X-ray analysis in fresh seeds (R^2 for all species 0.45, $p < 0.001$). Two groups could be distinguished that differed significantly in their applicability for viability detection, following the corresponding seed classification type. In agreement with other studies on selected agricultural species (van der Burg et al., 1994; Dell'Aquila, 2007; Neumann Silva et al., 2012), our findings underline at an extended scale of 179 plant species of 32 different plant families that X-raying is a very useful method for little or non-endospermic seeds which enables a quick, non-destructive and thus economic viability determination of seeds.

SEED MASS, SEED SHAPE AND SVD

It was found that thousand seed weight and seed shape had no significant impact on the viability difference SVD. This means that both methods, X-ray and combined germination-TZ test, evaluate seeds with different weights and shapes analogously. Although Miller (2005) described the evaluation of large embryos with Tetrazolium as complex because deterioration can be hidden in critical places in the case of insufficient intersections, this does not apply to the X-ray analysis. Here it is possible to determine deteriorated tissue and exclude damage in essential tissue areas like radicle, critical juncture or larger areas of the cotyledons, especially in larger seeds. Nevertheless size seems to impact viability detection - not in terms of seed size, but in terms of the proportion of the embryo size to the nutritive storage tissue.

SEED TYPE AND NUTRITIVE STORAGE TISSUE CLASSIFICATION AS SIGNIFICANT DETERMINING FACTOR

Evaluation of Tetrazolium staining patterns strongly depends on the embryo morphology and nutritive storage tissue type and these are highly related to the taxonomic family (Miller, 2005). This is also true for X-ray analysis, as only a profound knowledge about seed's morphology allows a correct interpretation of the black and white nuances of the radiograph. In our statistical analyses seed classification categories were assorted to two main groups, endospermic and little/non-endospermic seeds. In the model with the seed type classification Poaceae (B4) were assigned to little/non-endospermic seeds. The two groups differ significantly in the viability difference SVD, e.g. evaluation of endospermic seeds was more difficult than evaluation of little/non-endospermic seeds. These findings either imply overestimation of seed viability by X-ray analysis or underestimation by combined germination-TZ tests. In many cases the distinction of embryonic and endospermic tissue is not explicitly possible via X-ray in endospermic seeds such as Apiaceae. According to Socolowski et al. (2011) it is not required to identify the embryo in

order to detect seed viability. These findings can only conditionally be confirmed in our observations. Seed testing rules state that for instance in MA-type seeds the whole embryo needs to be sound/stained to be called viable (AOSA, 2010b; ISTA, 1999). In contrast the embryo of Poaceae can be visualized more easily (Carvalho et al., 1999; Menezes et al., 2012), what accounts for the assignment of Poaceae to the group of little/non- endospermic seeds. On the other hand, many of the endospermic seeds can exhibit morphological or morphophysiological dormancy (Finch-Savage & Leubner-Metzger, 2006) and germination sometimes lasts for several weeks (Baskin & Baskin, 2004). Since for many rare species germination requirements are unknown, we might have not discovered the optimal conditions in our investigations. Due to prolonged germination time imbibed seeds may have deteriorated, lowering the total viability result (Hegarty, 1978). Moreover misinterpretation of staining patterns cannot be excluded completely (Moore, 1973). This assumption can be confirmed by the fact that seeds of Poaceae, which do not possess morphophysiological dormancy, produced lower SVD- values (closer to zero) than e.g. Ranunculaceae or Apiaceae. For endospermic seeds with morphological dormancy or morphophysiological dormancy, it is still necessary to perform germination tests and consequent viability tests such as Tetrazolium tests as long as dormancy breaking needs are not completely understood.

IMPLICATIONS FOR GENE BANKS AND SEED INDUSTRY

It is a huge challenge for seed banks to insure high levels of viability in stored seeds. For many wild flowering plant species, little is known about the right method for viability assessment. In general, seed testers can choose between various methods, which all show advantages and disadvantages (Gosling, 2003). Miller (2005) emphasized that in the flowering industry, Tetrazolium tests (TZ) should be used as viability indicator in addition to germination tests. At the same time, some authors (Gosling, 2003; Milosevic et al., 2010) postulated that Tetrazolium tests overestimate vitality, as they are not able to predict the development of a typical or abnormal seedling. Krämer (2010) however, proved the opposite and showed that abnormalities can be detected by use of Tetrazolium. Authors like Carvalho et al. (2010) and van der Burg et al. (1994) have already shown on single agricultural species that the detection of abnormalities and prediction of seedling performance is also possible with X-ray analysis.

Using seeds of a variety of wild plant species, the study at hand demonstrates that X-ray analysis represents a useful method for viability detection for many wild plant species and that the use of X-ray analysis goes clearly beyond quantifying and upgrading seed quality by removing empty seeds (Keefe & Davis, 2012). Especially for non-endospermic, little endospermic or perispermic seeds, X-ray analysis can predict germinability/viability and therefore substitute for time-consuming germination and Tetrazolium testing. Yet, viability interpretation for endospermic seeds is more difficult with both methods and requires further research.

Comparable in the quality of results, Tetrazolium test and X-ray analysis detect viability of non-dormant as well as dormant seeds and can distinguish them from dead seeds. In contrast, a germination test can only reveal viability, when the dormancy breaking treatments are known. Regarding the average time needed for viability testing and interpretation, it differs from several weeks for germination tests, two days for a Tetrazolium test and minutes for an X-ray analysis. Whereas preparation of seeds for the TZ test is very time-consuming and takes from two to ten times longer than germination test performance (Miller, 2005), preparation time for X-raying is insignificant. But still, for the investigation of the germination ecology and suitable germination requirements germination testing is essential.

McDonald and Kwong (2005) proclaimed that in the seed producing and breeding industry seed viability testing procedures are not sensitive enough to differentiate seed lots of varying quality. Instead vigour tests should be preferred which are used to predict germination performance at suboptimal conditions (Geneve, 2005), with the aim of producing high yield plants. Such tests are less important for wild plant species especially in the field of seed banking. For conservation and restoration concerns, the primary target is the preservation of seeds with a broad natural genetic variability in order to maintain a maximum ability of adaptation to a changing environment. Assessing seed viability related to internal morphology via X-ray analysis is a quick and accurate viability assessment method that can be performed without wasting valuable material. As a second step in commercial seed production, vigour testing can be performed with high quality seed lots.

CHAPTER 6

Seed dispersal in space and time - origin and conservation of calcareous grasslands

Tremendously variable in morphology and size, seeds contain the entire genetic information of a plant, holding the capacity to outgrow even a gigantic tree. Defying heat, cold, fire or digestion, seeds are able to persist for many, even thousands of years until the environment offers suitable conditions for germination. Seeds as dispersal units have contributed the most to today's plant distribution. The majority of these small packages contain supplies that assist successful germination and establishment of a new plant. Facing the increasing habitat loss on a global level, a new focus is put on wild crop relatives and wild plants (Hay & Probert 2013). Yet seeds may be the resource for restoring depauperated habitats and landscapes destroyed by man.

The thesis at hand is dealing with seeds as dispersal units through space and time within its *in situ* and *ex situ* environment, keeping focus on endangered calcareous grasslands and its conservation and restoration perspectives. The aim of this thesis was to elucidate the origin of species of rare and threatened calcareous grasslands and to provide tools for a successful preservation of diversity with seeds. The origin of calcareous grassland species was exemplarily investigated by retracing the immigration route of *Sanguisorba minor* Scop. (Chapter 2), taking into account the seed morphological aspects of this species' subspecies. By persisting long-term in the soil (dispersal in time), seeds can contribute to the future (genetic) composition of plant communities and provide material for restoration of degraded sites (Karlik & Poschlod 2009; Kiefer & Poschlod 1996; Mitlacher 2009; Poschlod et al. 2013; Poschlod et al. 1996; Poschlod et al. 1998; Walck et al. 2011). Following chapters concentrate on this dispersal of seeds in time: Chapter 3 uncovers seed characters that influence *ex situ* storage longevity of seeds. In Chapter 4 we gathered common patterns of *in situ* seed persistence and *ex situ* storage longevity to provide prospects for conservation of calcareous grasslands via seeds. Due to habitat destruction, abandonment of land use, missing dispersal vectors and climate change, *ex situ* conservation with seed banks - an artificial method to disperse seeds through time - will gain more importance for future reintroductions. In order to preserve high quality seeds in seed banks for future reintroduction, we investigated the use of X-ray analysis to support the quick identification of viability proportion by focusing on seed traits (Chapter 5).

POST-GLACIAL ORIGIN OF CALCAREOUS GRASSLAND SPECIES

It is impossible to study the origin and distribution of plant species without considering dispersal as it influences distribution in space and time (see also Gillespie et al. 2012; Hendry et al. 1994). It is often invoked that constrained dispersal during postglacial range expansions, e.g. due to rarity of long-distance seed dispersal events or geographical barriers, has even resulted in discrepancies between actual and potential geographic distribution under current climate conditions (Hampe 2011; Normand et al. 2011). The morphology of a seed or dispersal unit is routinely linked with the dispersal syndrome (fleshy fruits imply endozoochory, hooks imply epizoochory). However, studies have shown that long-distance dispersal (LDD) is poorly related with morphology but rather with multiple processes or extreme events (Heydel et al. 2014; Higgins et al. 2003; Poschlod et al. 2005b; Tackenberg et al. 2003) such as meteorological weather conditions, transportation by animals or humans (Nathan et al. 2008; Poschlod et al. 2005b). For calcareous grasslands, long-distance seed dispersal by domestic animals and wind are known to be of central importance for plant colonization (Fischer et al. 1996; Tackenberg et al. 2003). Moreover, postglacial recolonization may be related to the migration of wild hooved animals (Leipold et al. 2017; Poschlod & Bonn 1998).

Results from Chapter 2 indicate that *S. minor* has outlasted the LGM in southern refugia but also in northern localities in France, Belgium or Germany. Independently whether populations survived in northern regions, close genetic similarity between northern and south-western populations indicate genetic exchange and closer relatedness, while older vicariance caused by the Alps and Carpathians has led to

a separation between the western and south-eastern populations. A constant exchange between human populations might as well have affected the recolonization speed, routes and genetic exchange of *S. minor* seeds after the LGM (Gronenborn 2003), during interglacials and even during the LGM (Tallavaara et al. 2015). It is known that at least since the medieval times (Hornberger 1959), transhumance favoured the transport and exchange of seeds over hundreds of kilometres between the eastern part of France and Germany (Poschlod & Bonn 1998). The endozoochorous and epizoochorous dispersal by goat and sheep (Bugla & Poschlod 2005; Fischer et al. 1996) as well as the utilization of *S. minor* as medicinal, vegetable and fodder plant (likewise *S. officinalis*), emphasize the potential dispersal of this species by man, wild animal or domestic livestock.

Considering our results, it is likely that *S. minor* has achieved post-glacial northern expansion through local dispersal from northern populations rather than long-distance dispersal from southern glacial refugia. The potential availability of northern refugia for calcareous grassland species (see also Bylebyl et al. 2008) has a number of implications for understanding the present and future distribution as well as conserving calcareous grasslands in Central Europe.

RELEVANCE OF SEED TRAITS FOR CALCAREOUS GRASSLAND CONSERVATION

Ecological studies often consult seed traits related to dispersal in space and time to investigate past, current and future species composition of habitats (Walck et al. 2011). Seed characteristics that have been associated with dispersal in space are size/mass, shape and its relation to diaspore production, surface, coats and their appendices, falling velocity, buoyancy, attachment capacity and digestion tolerance (Poschlod et al. 2005b; Römermann et al. 2005). Regarding natural dispersal in time (soil seed persistence), former studies have mainly focused on traits such as seed mass and seed shape (Bekker et al. 1998a; Hodkinson et al. 1998; Moles et al. 2000; Peco et al. 2003; Thompson et al. 1993; Zhao et al. 2011), seed coat thickness (Gardarin et al. 2010) and dormancy (Thompson et al. 2003).

The study at hand has shown that, for species originating from the same habitat, survival at artificial ageing could be transferred on soil seed bank persistence at least for 2/3 of the surveyed species. Our study confirmed previous results of geographically large-scaled studies (Merritt et al. 2014; Probert et al. 2009), implicating the major influence of inherent seed characters exceeding the importance of climate. Although the relationships turned out to be complicated, we were able to explain similarities and differences between *ex situ* and *in situ* survival of calcareous grassland species based on seed morphology and germination traits (see Chapter 4).

Seed shape and seed coat thickness were neither associated with *ex situ* survival nor *in situ* soil persistence. Seed mass however, proved to be irrelevant for prediction of *ex situ* longevity but seeds with lower mass tended to possess increased soil seed bank persistence in calcareous grasslands. While we were able to corroborate extended *ex situ* survival of physically dormant seeds, this characteristic was partially correlated with short-lived seeds in the soil. Such contradictory results could be explained with unpredictable effects that operate on the soil seed bank like germination, regeneration of buried seeds by wet-dry cycling, predation and the seed size-seed number trade-off, which may cover the actual inherent longevity (see Chapter 4).

Both, *ex situ* longevity and soil seed bank persistence showed correlations with endosperm presence and partially physiological dormancy (see Chapter 3 and 4). We assume that endospermic as well as physiologically dormant seeds without the capacity of dormancy cycling possess less capable repair mechanisms, quicker accumulate damage or repair mechanisms are overburdened because of the time necessary to break dormancy, respectively. These findings may be connected with phylogenetic relations as an adaption for dispersal through time for phylogenetically younger seeds (Probert et al. 2009, see also Chapter 3). At the same time, viability assessment via X-ray showed that neither seed shape nor seed mass had a significant impact on the precision of viability detection (Chapter 5). The ratio of endosperm however strongly determined the prognosis of viability detection and we therefore consider non-endospermic seeds to be suitable for quick viability detection via X-ray. Results from Chapter 3, 4 and 5 highlight the importance of endosperm presence/absence as a mostly ignored seed trait for seed survival *ex situ*, *in situ* and for the detection of seed viability of calcareous grassland species. It has been investigated in physiology, biochemistry (e.g. Donohue 2009; Finch-Savage & Leubner-Metzger 2006; Yan et al. 2014) and phylogenetic research (e.g. Finch-Savage & Leubner-Metzger 2006; Forbis et al. 2002; Martin 1946) but has rarely been considered in ecology, which is indicated by its absence in ecological plant trait databases like LEDA

(Kleyer et al. 2008), BIOPOP (Poschlod et al. 2003) or BiolFlor (Klotz et al. 2002). Nonetheless, being aware of the fact, that endospermic seeds cause difficulties regarding germination and viability detection and possess lower longevity, further scientific research concentrating on affected plant families is crucial (e.g. Apiaceae, Poaceae, Ranunculaceae).

In summary, at least in calcareous grasslands, species producing endospermic seeds should be prioritized for conservation, as their prognosis for seed survival is low in both, *ex situ* and *in situ*. Moreover, a reliable viability assessment of endospermic seeds is difficult and needs combined examinations via X-ray, Tetrazolium or germination tests. The absence of endosperm indicates good perspectives for *in situ* and *ex situ* survival as well as for viability detection. Due to their water impermeable seed coat, physically dormant seeds (e.g. Fabaceae) are bearing good prerequisites for long-term survival. On the other hand in their *in situ* environment, when combined with large seed size, these seeds may be preferred by predators and therefore, safeguarding in seed banks is suggested.

PERSPECTIVES FOR RESEARCH AND CONSERVATION

The thesis at hand strongly advises that for target-oriented safeguarding of threatened calcareous grassland species, both should be considered, the *in situ* as well as the *ex situ* environment.

The possibility that calcareous grassland species like *Sanguisorba minor* may have originated from putative northern “cryptic” refugia makes these areas very valuable for conservation priorities as they possess high genetic variation and are differentiated from other populations. Provided that these northern areas have facilitated long-term persistence throughout climatic oscillations, they may become important for the long term persistence and conservation of calcareous grasslands in a changing climate (Bhagwat & Willis 2008). We therefore emphasise the need for further investigations on additional calcareous grassland species especially under consideration of these potential northern refugia to identify calcareous grassland areas with conservation priority.

The example of *S. minor* also puts focus on another rather modern dispersal in space. *S. minor* ssp. *balearica* is described as a German neophyte (Hetzel 2006), which is often observed in seed mixtures originating from Southern Europe and used for the greening of roadside embankments (Frank & John 2007; Klotz 2012). It is known that genetic exchange can occur in a reticulate manner between populations, even between well-established and morphologically distinct species (Schaal et al. 1998). As this widespread phenomenon in plants (Hewitt 2001; Longo et al. 2014) was also observed between *S. minor* subspecies, attention must be paid before introducing subspecies or varieties on roadside plantings as they may quickly intermingle with autochthonous plants. This points out that although the habitat of calcareous grasslands is mainly threatened by intensification or abandonment of anthropo-zoogenic usage (Poschlod & WallisDeVries 2002), careless plantation subsequent to construction works can result in artificial bastardisation and may even accelerate species extinction. The use of viable autochthonous seed material is therefore indispensable (Frank & John 2007; LfU 2002), especially when plantings are located closely to highly valuable populations.

Considering *in situ* conservation, short-term re-establishment was shown to be more successful in species with persistent soil seed banks and long distance dispersal ability (von Blanckenhagen & Poschlod 2005). Likewise, even within highly fragmented calcareous grasslands, many species that have a high long distance dispersal potential could still persist locally under the presence of grazing disturbance (Purschke et al. 2012). But in general, calcareous grasslands are expected to possess soil seed banks with low persistence (Bekker et al. 1998a; Kalamees & Zobel 1998; Karlik & Poschlod 2014; Poschlod et al. 1998; Stöcklin & Fischer 1999; Thompson et al. 1997) and in the present cultural landscape, the function of grazing animals as dispersal vectors has become marginal (Jedicke 2015). Considering the recoverability of endangered habitats, the potential offered by *ex situ* conservation facilities has so far been ignored.

Especially when the *in situ* environment can neither provide dispersal in space, nor dispersal in time via persistent soil seed banks, efforts need to be made to specifically conserve species and maintain genepools *ex situ*. In total our investigation on *ex situ* and *in situ* seed persistence encompassed 39 calcareous grassland species. *In situ* data were available for 26 of these species, of those 10 species are expected to survive 0-5 years and 16 species more than 5 years in the soil (see Chapter 4). Regarding *ex situ* longevity based on a logarithmic scale (Mondoni et al. 2011), 30 out of 39 species could be classified as having medium-lived seeds in artificial ageing, three as short-lived and six as long-lived (see Chapter 5). Although longevity of different accessions of one species depends on the pre-dispersal environment (Kochanek et al. 2011), our research has determined and con-

firmed seed traits that can be of value when considered for *ex situ* conservation. In view of limited time and financial resources, the results of this thesis can be used to primarily focus on seeds with characters that are expected to possess short longevity *in situ*. Within the scope of upcoming conservation actions, even for endospermic seeds, where the prognosis for *ex situ* seed survival is comparatively low, frozen seed material can serve at least as mid-term backup and provide great restoration potential. Moreover, preceding long term storage, our research has shown that at least for non-endospermic seeds, the initial viability of a seed lot can be quickly assessed via X-ray, without wasting valuable material. This facilitates the use of viable seeds for the restoration or the support of populations and reduces waste of public funds by ineffective application of non-viable seeds.

Undoubtedly, it is a huge challenge for seed banks to insure high levels of viability in stored seeds. But we strongly appeal for integrative strategies as *ex situ* preservation not only provides means to safeguard viable seeds for many decades, but also offers tools to improve the success of restoration efforts with seeds of known quality and autochthonous origin (Smith et al. 2011).

Summary

The thesis at hand dealt with the topic of preservation of calcareous grasslands by comprising various aspects, which range from phylogeographic research to seed science. The aim of this thesis was to elucidate the origins of rare and threatened calcareous grasslands species and to provide tools for a successful conservation of biodiversity via seeds.

Chapter 1 presented an introduction by reporting on threatened and endangered landscapes in Germany, the current conservation status of calcareous grasslands, their known restoration potential and provided knowledge about the origin of calcareous grassland species. Moreover the issue of *ex situ* conservation via seed banks was addressed, detailing important subjects of seed science such as seed morphology, germination and dormancy, viability and longevity. The aims of all chapters were listed and the used scientific approaches were explained.

Chapter 2 focused on the postglacial spread of a common calcareous grassland species, *Sanguisorba minor* Scop., to Central Europe. Furthermore, seed morphology was included, as it is the main determinant of subspecies of the morphologically extremely variable species. To achieve these objectives, leaf and seed material from 38 populations throughout Europe were collected and examined in a two-step analysis. Firstly, the spatial genetic structure was investigated via AFLP and secondly, the findings were combined with the seed morphology.

The phylogeographic analysis revealed a distinct separation of eastern and western lineages and rare markers pointed towards a traditional southern but also a potential northern refugia. Genetic similarity between south-eastern and south-western subsp. *balearica* populations was lower than between northern subsp. *minor* and south-western subsp. *balearica* populations. It was concluded that *S. minor* recolonized Central Europe from Iberia or northern glacial refugia in France, Belgium or Germany. The present situation of the subspecies depicts either incomplete lineage sorting or the existence of secondary hybrid zones, which is differently expressed in neutral marker and morphological differentiation.

In **Chapter 3** seed traits associated with seed ageing and *ex situ* storage and their prospects for *ex situ* conservation were evaluated. Seeds of 39 calcareous grassland species were collected and the seed longevity was determined by artificially ageing under rapid ageing conditions. The results showed that the seed longevity values strongly varied. Physical dormancy and endosperm absence had significantly positive effects and physiological dormancy was negatively correlated with seed longevity. Seed mass, seed shape and seed coat thickness were not associated with longevity. Calcareous grassland species therefore do not solely rely on seed longevity for long-term persistence and regeneration. The influence of physical dormancy and endosperm presence were discussed with regard to the evolution from endospermic to non-endospermic seeds. Low seed longevity of physiologically dormant seeds was explained by the lack of germination specific antioxidants that otherwise counteract oxidative damages in non-dormant aged seeds.

Chapter 4 addressed the question whether seed longevity of calcareous grassland species in the soil and in *ex situ* storage are correlated. Therefore, longevity information gained from artificial ageing trials was compared with soil seed bank persistence on the basis of soil seed bank persistence categories of (Poschlod et al., 1998) and the longevity index LI of Thompson et al. (1997). For three-quarter of the surveyed species soil seed bank persistence and survival of artificially aged seeds were correlated, which was explained by an inherent species-specific seed longevity. Regarding seed traits, a correlation with endosperm presence and physiological dormancy was confirmed for soil seed bank persistence. Remaining contradictory results of species with long-lived soil seed banks but low survival at artificial ageing and vice versa were interpreted with unpredictable effects that operate on seeds in the soil like germination, seed regeneration by wet-dry cycling, predation and the seed size-seed number trade-off, which may cover the actual inherent longevity.

In **Chapter 5** the assessment of seed viability via X-ray was investigated. X-rays provide information about the internal structures of a seed and therefore show promise for detection of viability and even germination capacity without destroying valuable

seeds. Combined germination-Tetrazolium tests and X-ray images of 176 wild flowering plant species from 207 accessions were compared in order to examine the efficiency of X-ray analysis to detect viability. The comparison revealed a strong analogy of viability determination of both methods. Whereas the evaluation of little/non-endospermic seeds gave approximately same results, endospermic seed evaluation differed. Therefore especially for non/little endospermic seeds X-ray analysis can provide a useful and quick tool for viability detection and prediction of germinability, whereas for endospermic seeds additional research is needed.

Chapter 6 summarized the gained results with regard to *ex situ* and *in situ* seed dispersal in space and time. Seeds have contributed the most to species current geographical distribution (dispersal in space). It was pointed out that for calcareous grassland species such as *S. minor* long distance seed dispersal by domestic animals are of importance for colonization. Even during the LGM, contiguous European human populations extended from central France to the lowlands in Southern Germany and to Eastern Europe and may have caused direct and indirect carriage of *S. minor*. It was suggested that the discovered northern refugia of *S. minor* might be of importance as a source for future safeguarding calcareous grassland species. Due to present-day limited dispersal in space, it was postulated that at least dispersal in time needed to be guaranteed by integrative strategies. Maintaining genepools on site but additionally preserve viable seeds in seed banks was proposed to be necessary to safeguard calcareous grasslands plant inventory and improve the success of restoration efforts. In the following the relevance of seed traits for calcareous grassland conservation (dispersal in time) was discussed. The importance of the seed trait “endosperm presence” for ecological studies was emphasized as it points towards seed longevity and provides information about the feasibility of quick viability assessment via X-ray analysis.

Zusammenfassung

Die vorliegende Dissertation thematisiert den Erhalt von Kalkmagerrasen anhand vielfältiger Aspekte, welche phylogeographische sowie samenmorphologische und samenökologische Untersuchungen umfassen. Die Arbeit sollte zum einen klären, wie die Arten des gefährdeten Habitats Kalkmagerrasen Mitteleuropa kolonialisiert haben und zum anderen Möglichkeiten für deren erfolgreichen Erhalt mittels Samen aufzeigen.

In **Kapitel 1** wurden die derzeitige Situation von Kalkmagerrasen, Möglichkeiten zur Wiederherstellung und der aktuelle Wissensstand bezüglich der Herkunft der ihn auszeichnenden Arten dargestellt. Zudem wurden Genbanken als Instrumente des Ex-situ-Naturschutzes und deren Bedeutung im Rahmen der Saatgutforschung (Untersuchungen zur Samenmorphologie, -keimung und -dormanz, Lebensfähigkeit und Langlebigkeit) hervorgehoben. Die Zielsetzungen der jeweiligen Kapitel und die verwendeten Untersuchungsmethoden schließen die allgemeine Einleitung ab.

Kapitel 2 beschäftigte sich mit der nacheiszeitlichen Ausbreitung der häufigen Kalkmagerrasenart *Sanguisorba minor* Scop., nach Mitteleuropa. Die Untersuchung umfasste auch die Samenmorphologie dieser morphologisch extrem variablen Pflanzenart, das Hauptbestimmungsmerkmal der Unterarten. Zu diesem Zweck wurde Pflanzen- und Samenmaterial von 38 Populationen in ganz Europa gesammelt, zunächst die genetische Struktur mittels AFLP untersucht und anschließend die Ergebnisse mit der Samenmorphologie in Zusammenhang gebracht. Die Ergebnisse der phylogeographischen Untersuchung zeigten eine deutliche Trennung in östliche und westliche genetische Abstammungslinien, welche zusammen mit gefundenen seltenen Markern auf traditionelle südliche Refugien, aber auch auf potenzielle nördliche Rückzugsgebiete hinweisen. Die genetische Ähnlichkeit zwischen der südöstlichen und südwestlichen subsp. *balearica*-Populationen erwies sich niedriger als zwischen nördlichen subsp. *minor* und westlichen subsp. *balearica*-Populationen. Daraus wurde geschlossen, dass die Besiedlung Mitteleuropa durch *S. minor* von der Iberischen Halbinsel oder von nördlichen Refugien in Frankreich, Belgien oder Deutschland ausging. Die derzeitige Situation der Unterarten weist entweder auf eine unvollständige Aufspaltung der Abstammungslinien oder die Existenz von sekundären Hybridisierungszonen hin, was sich in der unterschiedlichen genetischen und morphologischen Differenzierung zeigte.

In **Kapitel 3** wurden Samenmerkmale, welche mit Samenalterung und der Fähigkeit zur Ex-situ-Einlagerung in Verbindung gebracht werden, bezüglich ihrer Eignung als Indikatoren für den Ex-situ-Naturschutz überprüft. Samen von 39 Pflanzenarten des Lebensraums Kalkmagerrasen wurden gesammelt und die Langlebigkeit der Samen durch künstliche Alterung bestimmt. Die Ergebnisse zeigten große Unterschiede in der Langlebigkeit von Kalkmagerrasenarten. Während physikalische Dormanz und das Fehlen von Endosperm einen signifikant positiven Effekt auf die Langlebigkeit von Samen hatte, wirkte sich physiologische Dormanz negativ aus. Samengewicht, -form und -schalendicke hingegen zeigten keinen Zusammenhang mit der Langlebigkeit. Des Weiteren wurde der Einfluss von physikalischer Dormanz und Endospermhaltigkeit unter Berücksichtigung der Entwicklungsgeschichte diskutiert. Die Ergebnisse führten zu der Empfehlung, in Genbanken eine häufigere Überprüfung der Lebensfähigkeit von Samen mit endospermhaltigen, nicht physikalisch und physiologisch dormanten Samen durchzuführen.

In **Kapitel 4** wurde untersucht, ob Langlebigkeit von Kalkmagerrasenarten im Boden und bei der Ex-situ-Einlagerung miteinander korrelieren und welche Samenmerkmale diesbezüglich einen Einfluss haben. Hierzu wurden die Ergebnisse von eigenen künstlichen Alterungsversuchen mit den Kategorien der Samenbankuntersuchungen von Poschlod et al. (1998) und dem Longevity Index (LI) von Thompson et al. (1997) verglichen. Bei Dreiviertel der untersuchten Arten korrelierte die Überlebensfähigkeit bei künstlicher Alterung mit der in der Diasporenbank, was einer angeborenen artspezifischen Samen-Langlebigkeit zugeschrieben wurde. Während bislang die Überlebensfähigkeit im Boden vor allem mit der Samenmasse assoziiert worden war, wurde der Einfluss von Endosperm und Dormanz bestätigt.

Nicht übereinstimmende Ergebnisse wurden dahin gehend interpretiert, dass unvorhersehbare Einflüsse, die nur in-situ auf Samen einwirken, nicht aber ex-situ, die angeborene Langlebigkeit maskieren.

Kapitel 5 untersuchte die Möglichkeit zur Beurteilung der Samenlebensfähigkeit mittels Röntgenuntersuchung. Durch Röntgenstrahlen können schnell und präzise die inneren Strukturen von Samen dargestellt werden, wodurch sich die Methode vielversprechend für den Einsatz in der Untersuchung der Lebensfähigkeit und der Keimfähigkeit darstellt, ohne dabei das Saatgut zu schädigen. Kombinierte Keimungs- und Tetrazoliumtests wurden mit Röntgenaufnahmen von 176 Wildpflanzen aus 207 Akzessionen verglichen, um die Wirksamkeit der Röntgenanalyse zu untersuchen. Das Ergebnis zeigte zum Teil eine starke Übereinstimmung beider Methoden zur Bestimmung der Lebensfähigkeit. Während die Untersuchungen bei Samen mit wenig oder keinem Endosperm dieselben Ergebnisse lieferten, unterschieden sich die Ergebnisse bei Samen mit einen hohen Endospermanteil. Die Röntgenmethode bietet deshalb für Samen ohne oder nur mit wenig Endosperm eine schnelle und einfache Möglichkeit zur Überprüfung der Lebens- und Keimfähigkeit.

Kapitel 6 fasste die erarbeiteten Ergebnisse hinsichtlich der Bedeutung von Samen als Ausbreitungsvektoren in Raum und Zeit und für den In-situ- und Ex-situ-Schutz von Kalkmagerrasen zusammen. Es wurde darauf eingegangen, dass für Arten der Kalkmagerrasen wie *S. minor* der Fernausbreitung der Samen durch (Haus-)Tiere eine große Bedeutung zukommt und möglicherweise selbst während der letzten Eiszeit eine kontinuierlich in Mitteleuropa lebende Bevölkerung direkt oder indirekt zur Ausbreitung von *S. minor* beigetragen hat. Es wurde betont, dass aufgrund der aktuell stark limitierten Möglichkeit zur Ausbreitung von Samen im Raum eine verstärkte Notwendigkeit für integrative Methoden des Naturschutzes besteht, welche auch der Ausbreitung in der Zeit eine größere Beachtung schenkt. Um das Arteninventar der Kalkmagerrasen effektiv schützen und bei Bedarf wiederherstellen zu können, sollte neben der Erhaltung des In-situ-Genpools zusätzlich der Ex-situ-Erhalt in Genbanken gewährleistet sein. Es wurde auf die Merkmale eingegangen, welche sowohl für den Ex-situ- als auch den In-situ-Erhalt von Kalkmagerrasenarten von Bedeutung sind. Dabei wurde die große Bedeutung des Samenmerkmals Endospermhaltigkeit betont, welche auch als Indikator für Eignung für Röntgenuntersuchungen von Samen dient.

REFERENCES

Abedi, M., Bartelheimer, M. & Poschlod, P. (2014). Effects of substrate type, moisture and its interactions on soil seed survival of three *Rumex species*. Plant and Soil, 374, 485-495.

Alsos, I.G., Alm, T., Normand, S. & Brochmann, C. (2009). Past and future range shifts and loss of diversity in dwarf willow (*Salix herbacea* L.) inferred from genetics, fossils and modelling. Global Ecology and Biogeography, 18, 223-239.

AOSA (2010a). Association of Official Seed Analysts. Tetrazolium Testing Handbook. AOSA, Washington.

AOSA (2010b). Association of Official Seed Analysts. AOSA rules for testing seeds. AOSA, Washington.

APG III (2009). An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. Botanical Journal of the Linnean Society, 161, 105–121.

Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A. & Saunders, N.C. (1987). Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annual review of ecology and systematics, 489-522.

Avise, J.C., Walker, D. & Johns, G.C. (1998). Speciation durations and Pleistocene effects on vertebrate phylogeography. Proceedings of the Royal Society of London B: Biological Sciences, 265, 1707-1712.

Bailly, C. (2004). Active oxygen species and antioxidants in seed biology. Seed Science Research, 14, 93-107.

Bailly, C., El-Maarouf-Bouteau, H. & Corbineau, F. (2008). From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. Comptes Rendus Biologies, 331, 806-814.

Baskin, C.C. (2003). Breaking physical dormancy in seeds - focussing on the lens. New Phytologist, 158, 229-232.

Baskin, C.C. & Baskin, J.M. (1998). Seeds. Ecology, biogeography,and evolution of dormancy and germination. Academic Press, San Diego.

Baskin, C.C. & Baskin, J.M. (2006). The natural history of soil seed banks of arable land. Weed Science, 54, 549-557.

Baskin, J.M. & Baskin, C.C. (2004). A classification system for seed dormancy. Seed Science Research, 14, 1-16.

Baskin, J.M., Baskin, C.C. & Li, X. (2000). Taxonomy, anatomy and evolution of physical dormancy in seeds. Plant Species Biology, 15, 139-152.

Bateman, R.M., Hilton, J. & Rudall, P.J. (2006). Morphological and molecular phylogenetic context of the angiosperms: contrasting the 'top-down' and 'bottom-up' approaches used to infer the likely characteristics of the first flowers. Journal of Experimental Botany, 57, 3471-3503.

Beier, B. (1991). Keimungsbiologische Untersuchungen an Kalkmagerrasenpflanzen und ihre Bedeutung für die Interpretation der Samenbank. Universität Hohenheim.

Bekker, R.M., Bakker, J.P., Grandin, U., Kalamees, R., Milberg, P., Poschlod, P., Thompson, K. & Willems, J.H. (1998a). Seed size, shape and vertical distribution in the soil: indicators of seed longevity. Functional Ecology, 12, 834-842.

Bekker, R.M., Schaminée, J.H.J., Bakker, J.P. & Thompson, K. (1998b). Seed bank characteristics of Dutch plant communities. Acta Botanica Neerlandica, 47, 15-26.

Bekker, R.M., Bakker, J.P., Ozinga, W.A. & Thompson, K. (2003). Seed traits: essential for understanding seed longevity. Aspects of Applied Biology, 69, 1-10.

Bergmann, P.J. (2012). Bivariate Regression. Available at: http://www.clarku.edu/faculty/pbergmann/biostats/RMA_function.txt (accessed 2015).

Bernal-Lugo, I., Camacho, A. & Carballo, A. (2000). Effects of seed ageing on the enzymic antioxidant system of maize cultivars. Seed biology: Advances and applications (ed. by Black, M., Bradford, K.J. & Vazquez-Ramos, J.), 151–160. CABI Publishing, Wallingford.

Berry, P.E. & Calvo, R.N. (1994). An overview of the reproductive biology of *Espeletia* (Asteraceae) in the Venezuelan Andes. Tropical Alpine Enviroments (ed. by Rundel, P.W., Smith, A.P. and Meinzer, F.C.), 229-248. Cambridge University Press, Cambridge.

Bewley, J.D., Bradford, K.J., Hilhorst, H.W.M. & Nonogaki, H. (2013). Seeds: Physiology of development, germination and dormancy, 3 edn. Springer, New York.

BfN (2014). Die Lage der Natur in Deutschland - Ergebnisse von EU-Vogelschutz und FFH-Bericht. 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.

Black, M., Bewley, J.D. & Halmer, P. (2006). The encyclopedia of seeds. Science, technology and uses. CABI Publishing, Wallingford.

Blomberg, S.P., Garland, T. & Ives, A.R. (2003). Testing for phylogenetic signal in comparative data: Behavioral traits are more labile. Evolution, 57, 717-745.

Boston, E.S.M., Montgomery, W., Hynes, R. & Prodöhl, 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 of London B: Biological Sciences, 282, 2014-2605.

Bradshaw, H.D., Wilbert, S.M., Otto, K.G. & Schemske, D.W. (1995). Genetic mapping of floral traits associated with reproductive isolation in Monkeyflowers (*Mimulus*). Nature, 376, 762-765.

Bugla, B. & Poschlod, P. (2005). Biotopverbund für die Migration von Pflanzen – Förderung von Ausbreitungsprozessen statt „statischen" Korridoren und Trittsteinen. Das Fallbeispiel „Pflanzenarten der Sandmagerrasen" in Bamberg, Bayern. 101-117 in H. Reck, K. Haenel, M. Böttcher, and A. Winter, editors. Lebensraumkorridore für Mensch und Natur. Bundesamt für Naturschutz, Bad Godesberg.

Burnham, K.P. & Anderson, D.R. (2002). Model selection and multimodel inference: a practical information-theoretic approach. Springer, New York.

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

Bush, M.B. (1993). An 11 400 year paleoecological history of a British chalk grassland. Journal of Vegetation Science, 47-66.

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

Carvalho, M.L.M., van Aelst, A.C., van Eck, J.W. & Hoekstra, F.A. (1999). Pre-harvest stress cracks in maize (*Zea mays* L.) kernels as characterized by visual, X-ray and low temperature scanning electron microscopical analysis: effect on kernel quality. Seed Science Research, 9, 227-236.

Carvalho, M.L.M., Alves, R.A. & Oliveira, L.M. (2010). Radiographic analysis in castor bean seeds (*Ricinus communis* L.). Revista Brasileira de Sementes, 32, 170-175.

CBD (2010). Strategic Plan for Biodiversity 2011–2020, Decision X/17, Nagoya. Available at: http://www.cbd.int/decision/cop/default.shtml?id=13178 (accessed 2015).

CBD (2015). Decisions - COP 10 Available at: www.cbd.int/doc/decisions/cop-10/cop-10-dec-17-en.pdf (accessed 2015).

Cieślak, E. (2014). Phylogeography of pontic-pannonian species in Central Europe. Polish Botanical Studies, 30, 1-53.

Clarke, J.T., Warnock, R. & Donoghue, P.C.J. (2011). Establishing a time scale for plant evolution. New Phytologist, 192, 266-301.

Coiffard, C., Gomez, B., Daviero-Gomez, V. & Dilcher, D.L. (2012). Rise to dominance of angiosperm pioneers in European Cretaceous environments. Proceedings of the National Academy of Sciences, 109, 20955-20959.

Colbach, N. (2014). The functional role of the soil seed bank in agricultural ecosystems. Seeds: the ecology of regeneration in plant communities (ed. by Gallgher, R.S.), 235-262. CABI Publishing, Wallingford.

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.

Copeland, L.O. & McDonald, F. (1995). Principles of Seed Science and Technology. Springer, New York.

Crawford, A.D., Steadman, K.J., Plummer, J.A., Cochrane, A. & Probert, R.J. (2007). Analysis of seed-bank data confirms suitability of international seed-storage standards for the Australian flora. Australian Journal of Botany, 55, 18-29.

Crawley, M.J. (2007). The R Book. Wiley & Sons, UK.

Crawley, M.J. (2013). Seed predators and plant population dynamics. Seeds: The Ecology of Regen-

eration in Plant Communities (ed. by Gallagher, R.S.). CABI Publishing, Wallingford.

Dahlgren, G. (1995). Sanguisorba. Gustav Hegi. Illustrierte Flora von Mitteleuropa. Band IV Teil 2B: Spermatophyta: Angiospermae: Dicotyledones 2 (3) (Rosaceae, 2. Teil) (ed. by Scholz, H.). Blackwell, Berlin/Vienna.

Daneck, H., Fér, T. & Marhold, K. (2015). Glacial survival in northern refugia? Phylogeography of the temperate shrub *Rosa pendulina* L. (Rosaceae): AFLP vs. chloroplast DNA variation. Biological Journal of the Linnean Society, DOI: 10.1111/bij.12619

Daws, M.I., Garwood, N.C. & Pritchard, H.W. (2005). Traits of recalcitrant seeds in a semi-deciduous tropical forest in Panama: some ecological implications. Functional Ecology, 19, 874-885.

Dell'Aquila, A. (2007). Pepper seed germination assessed by combined X-radiography and computer-aided imaging analysis. Biologia Plantarum, 51, 777-781.

Dengler, J. (2005). Zwischen Estland und Portugal Gemeinsamkeiten und Unterschiede der Phytodiversitätsmuster europäischer Trockenrasen. Tuexenia, 25, 387-405.

Dickie, J.B. & Pritchard, H.W. (2002). Systematic and evolutionary aspects of desiccation tolerance in seeds. Desiccation and survival in plants: drying without dying. (ed. by Black, M. and Pritchard, H.W.), 239-259. CABI Publishing, Wallingford.

Dobrowski, S.Z. (2011). A climatic basis for microrefugia: the influence of terrain on climate. Global Change Biology, 17, 1022-1035.

Donelan, M. & Thompson, K. (1980). Distribution of Buried Viable Seeds Along a Successional Series. Biological Conservation, 17, 297-311.

Donohue, K. (2009). Completing the cycle: maternal effects as the missing link in plant life histories. Philosophical Transactions of the Royal Society B-Biological Sciences, 364, 059-1074.

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.

Edmonds, C.A., Lillie, A.S. & Cavalli-Sforza, L.L. (2004). Mutations arising in the wave front of an expanding population. Proceedings of the National Academy of Sciences of the United States of America, 101, 975-979.

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

El Rabey, H.A., Al-Malki, A.L., Abulnaja, K.O., Ebrahim, M.K., Kumosani, T. & Khan, J.A. (2014). Phylogeny of ten species of the genus *Hordeum* L. as revealed by AFLP markers and seed storage protein electrophoresis. Molecular Biology Reports, 41, 365-372.

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

Ellenberg, H. (1996). Vegetation Mitteleuropas mit den Alpen: In ökologischer, dynamischer und historischer Sicht, 5 edn. Ulmer Eugen Verlag, Stuttgart.

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

Ellis, R.H., Hong, T.D. & Roberts, E.H. (1989). A comparison of the low-moisture-content limit to the logarithmic relation between seed moisture and longevity in twelve species. Annals of Botany, 601-611.

Escudero, M., Vargas, P., Arens, P., Ouborg, N.J. & Luceño, M. (2010). The east-west-north colonization history of the Mediterranean and Europe by the coastal plant *Carex extensa* (Cyperaceae). Molecular Ecology, 19, 352-370.

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.

FAO (2013). Genebank standards for plant genetic resources for food and agriculture, Rome.

Felsenstein, J. (1985). Phylogenies and the comparative method. American Naturalist, 125, 1-15.

Finch-Savage, W.E. & Leubner-Metzger, G. (2006). Seed dormancy and the control of germination. New Phytologist, 171, 501-523.

Finch-Savage, W.E. & Footitt, S. (2012). To germinate or not to germinate: a question of dormancy relief not germination stimulation. Seed Science Research, 22, 243-248.

Finch-Savage, W.E., & Footitt, S. (2017). Seed dormancy cycling and the regulation of dormancy mechanisms to time germination in variable field environments. Journal of experimental botany, 68(4), 843-856.

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, 33, 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.

Flora Iberica (2015). Synonyms of *Sanguisorba minor* subsp. *minor* Scop. Available at: http://www.floraiberica.es/eng/PHP/cientificos2.php?gen=Sanguisorba&espe=minor&infrank=subsp.&infra=minor&autabre=Scop.&familia=Rosaceae (accessed 2015).

Forbis, T.A., Floyd, S.K. & de Queiroz, A. (2002). The evolution of embryo size in angiosperms and other seed plants: Implications for the evolution of seed dormancy. Evolution, 56, 2112-2125.

Frank, D. & John, H. (2007). Bunte Blumenwiesen–Erhöhung der Biodiversität oder Verstoß gegen Naturschutzrecht. Mitteilungen zur floristischen Kartierung in Sachsen-Anhalt 12.

Freckleton, R.P., Harvey, P.H. & Pagel, M. (2002). Phylogenetic analysis and comparative data: A test and review of evidence. American Naturalist, 160, 712-726.

Fritz, S.A. & Purvis, A. (2010). Selectivity in mammalian extinction risk and threat types: a new measure of phylogenetic signal strength in binary traits. Conservation Biology, 24, 1042-1051.

Frogley, M.R. & Tzedakis, P.C. (1999). Climate variability in northwest Greece during the last interglacial. Science, 285, 1886-1889.

Funes, G., Basconcelo, S., Diaz, S. & Cabido, M. (1999). Seed size and shape are good predictors of seed persistence in soil in temperate mountain grasslands of Argentina. Seed Science Research, 9, 341-345.

Gagliardi, B. & Marcos-Filho, J. (2011). Relationship between germination and bell pepper seed structure assessed by the X-ray test. Scientia Agricola, 68, 411-416.

Gardarin, A., Durr, C., Mannino, M.R., Busset, H. & Colbach, N. (2010). Seed mortality in the soil is related to seed coat thickness. Seed Science Research, 20, 243-256.

Garland, T. & Ives, A.R. (2000). Using the past to predict the present: Confidence intervals for regression equations in phylogenetic comparative methods. American Naturalist, 155, 346-364.

Garland, T., Bennett, A.F. & Rezende, E.L. (2005). Phylogenetic approaches in comparative physiology. Journal of Experimental Biology, 208, 3015-3035.

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.

Geneve, R.L. (2005). Vigour Testing in Flower Seeds. Flower Seeds- Biology and Technology (ed. by Mcdonald, M.B. & Kwong, F.Y.), 311-332. CABI Publishing, Oxfordshire, UK & Cambridge, USA.

Gillespie, R.G., Baldwin B.G., Waters J.M., Fraser, C.I., Nikula, R. & Roderick, G.K. (2012). Long-distance dispersal: a framework for hypothesis testing. Trends in Ecology & Evolution, 27, 47-56.

Godefroid, S., Van de Vyver, A. & Vanderborght, T. (2010). Germination capacity and viability of threatened species collections in seed banks. Biodiversity and Conservation, 19, 1365-1383.

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.

Goodman, R.C., Jacobs, D.F. & Karrfalt, R.P. (2005). Evaluating desiccation sensitivity of Quercus rubra acorns using X-ray image analysis. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere, 35, 2823-2831.

Gosling, P.G. (2003). Viability testing. Seed conservation: turning science into practice (ed. by Smith, R.D., Dickie, J.B., Linington, S.H., Pritchard, H.W. & Probert, R.J.), 445-481. Kew Publishing, Kew.

Gottlieb, L.D. (1984). Genetics and morphological evolution in plants. American Naturalist, 123, 681-709.

Grabe, D.F. (1970). Tetrazolium testing handbook for agricultural seeds. Contribution No. 29 to the Handbook on Seed Testing. AOSA.

Gradmann, R. (1933). Die Steppenheidetheorie. Geographische Zeitschrift, 39, 265-278.

Grafen, A. (1989). The phylogenetic regression. Philosophical Transactions of the Royal Society of London, 326, 119–157.

Griffiths, K.E., Balding, S.T., Dickie, J.B., Lewis, G.P., Pearce, T.R. & Grenyer, R. (2015). Maximizing the phylogenetic diversity of seed banks. Conservation Biology, 29, 370–381.

Grime, J.P. (1989). Seed banks in ecological perspective. Ecology of soil seed banks (ed. by Leck, M.A., Parker, V.T. & Simpson, R.L.). London Academic Press, London.

Grime, J.P., Mason, G., Curtis, A.V., Rodman, J., Band, S.R., Mowforth, M.A.G., Neal, A.M. & Shaw, S. (1981). A Comparative-Study of Germination Characteristics in a Local Flora. Journal of Ecology, 69, 1017-1059.

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

Hahn, J. (1993). Untersuchungen zur Dauerhaftigkeit der Diasporenbank ausgewählter Arten der Kalkmagerrasen-Ein Vergleichsexperiment. Universität Hohenheim, Hohenheim.

Hájková, P., Roleček, J., Hájek, M., Horsák, M., Fajmon, K., Polák, M. & Jamrichová, E. (2011). Prehistoric origin of extremely species-rich semi-dry grasslands in the Bílé Karpaty Mts (Czech Republic and Slovakia). Preslia, 83, 185-204.

Hallatschek, O. & Nelson, D.R. (2008). Gene surfing in expanding populations. Theoretical population biology, 73, 158-170.

Hampe, A. (2011). Plants on the move: The role of seed dispersal and initial population establishment for climate-driven range expansions. Acta Oecologica-International Journal of Ecology, 37, 666-673.

Harter, D.E.V., Jentsch, A. & Durka, W. (2015). Holocene recolonisation, central–marginal distribution and habitat specialisation shape population genetic patterns within an Atlantic European grass species. Plant Biology, 17, 684-693.

Haslekas, C., Viken, M.K., Grini, P.E., Nygaard, V., Nordgard, S.H., Meza, T.J. & Aalen, R.B. (2003). Seed 1-cysteine peroxiredoxin antioxidants are not involved in dormancy, but contribute to inhibition of germination during stress. Plant Physiology, 133, 1148-1157.

Havrdová, A., Douda, J., Krak, K., Vít, P., Hadincová, V., Zákravský, P. & Mandák, B. (2015). Higher genetic diversity in recolonized areas than in refugia of *Alnus glutinosa* triggered by continent-wide lineage admixture. Molecular Ecology, 24, 4759-4777.

Hay, F.R. & Probert, R.J. (2013). Advances in seed conservation of wild plant species: a review of recent research. Conservation Physiology, 1, cot030.

Hay, F.R., Probert, R.J. & Smith, R.D. (1997). The effect of maturity on the moisture relations of seed longevity in foxglove (*Digitalis purpurea* L.). Seed Science Research, 7, 341-350.

Hedberg, P. & Kotowski, W. (2010). New nature by sowing? The current state of species introduction in grassland restoration, and the road ahead. Journal for Nature Conservation, 18, 304-308.

Hegarty, T.W. (1978). The physiology of seed hydration and dehydration, and the relation between water stress and the control of germination: a review. Plant, Cell & Environment, 1, 101-119.

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

Hendry, G.A.F., Thompson, K., Moss, C.J., Edwards, E. & Thorpe, P.C. (1994). Seed Persistence - a Correlation between Seed Longevity in the Soil and Ortho-Dihydroxyphenol Concentration. Functional Ecology, 8, 658-664.

Hensen, I., Kilian, C., Wagner, V., Durka, W., Pusch, J. & Wesche, K. (2010). Low genetic variability and strong differentiation among isolated populations of the rare steppe grass *Stipa capillata* L. in Central Europe. . Plant Biology, 12, 526-536.

Herbst, B., Schillinger, C. & Fickert, T. (2014). Gefährliche Felsböschungen im Nördlichen Frankenjura-Geologischer Rahmen, Gefährdungspo-

tenzial und mögliche Sicherungsmaßnahmen. Mitteilungen der Fränkischen Geographischen Gesellschaft Band, 59, 59–74.

Hetzel, G. 2006. Die Neophyten Oberfrankens. Floristik, Standortcharakteristik, Vergesellschaftung, Verbreitung, Dynamik. Page 156. Universität Würzburg, Würzburg.

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

Hewitt, G.M. (1996). Some genetic consequences of ice ages, and their role in divergence and speciation. Biological Journal of the Linnean Society, 58, 247-276.

Hewitt, G.M. (2001). Speciation, hybrid zones and phylogeography - or seeing genes in space and time. Molecular Ecology, 10, 537-549.

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

Heydel, F., Cunze, S., Bernhardt-Romermann, M. & Tackenberg, O. (2014). Long-distance seed dispersal by wind: disentangling the effects of species traits, vegetation types, vertical turbulence and wind speed. Ecological Research, 29, 641-651.

Higgins, S. I., Nathan, R. & Cain, M. L. (2003). Are long-distance dispersal events in plants usually caused by nonstandard means of dispersal? Ecology 84, 1945-1956.

Hodkinson, D.J., Askew, A.P., Thompson, K., Hodgson, J.G., Bakker, J.P. & Bekker, R.M. (1998). Ecological correlates of seed size in the British flora. Functional Ecology, 12, 762-766.

Holzel, N. & Otte, A. (2004). Assessing soil seed bank persistence in flood-meadows: The search for reliable traits. Journal of Vegetation Science, 15, 93-100.

Hornberger, T. (1959). Die kulturgeographische Bedeutung der Wanderschäferei in Süddeutschland. Süddeutsche Transhumanz. Forschungen zur Deutschen Landeskunde, 109, 1-173.

Hulme, P.E. (1998). Post-dispersal seed predation and seed bank persistence. Seed Science Research, 8, 513-519.

ISTA (1999). International Seed Testing Association. International Rules for Seed Testing. Seed Science and Technology, 27, 340

ISTA (2003). International Seed Testing Association. Working Sheets on Tetrazolium, Zurich.

Ives, A.R. & Garland, T. (2010). Phylogenetic logistic regression for binary dependent variables. Systematic Biology, 59, 9-26.

Jadwiszczak, K.A., Banaszek, A., Jablonska, E. & Sozinow, O.V. (2011). Could *Betula humilis* have survived the last glaciation at a current margin of its distribution? Testing the hypothesis of a glacial refugium using nuclear microsatellites. Plant Systematics and Evolution, 297, 147-156.

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, 145, 507-513.

Jedicke, E. (2015). “Lebender Biotopverbund” in Weidelandschaften. Naturschutz und Landschaftsplanung, 47, 257-262.

Jiménez-Mejías, P., Luceño, M., Lye, K.A., Brochmann, C. & Gussarova, G. (2012). Genetically diverse but with surprisingly little geographic structure: the complex history of the widespread herb *Carex nigra* (Cyperaceae). Journal of Biogeography, 39, 2279-2291.

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.

Kadereit, G. & Yaprak, A.E. (2008). “*Microcnemum coralloides*” (Chenopodiaceae-Salicornioideae): an example of intraspecific East-West disjunctions in the Mediterranean region. Anales del Jardín Botánico de Madrid, 65(2), 415-426.

Kadereit, J.W., Griebeler, E.M. & Comes, H.P. (2004). Quaternary diversification in European alpine plants: pattern and process. Philosophical Transactions of the Royal Society B: Biological Sciences, 359, 265-274.

Kajtoch, Ł., Cieślak, E., Varga, Z., Paul, W., Mazur, M.A., Sramkó, G. & Kubisz, D. (2016). Phylogeographic patterns of steppe species in Eastern Central Europe: a review and the implications for conservation. Biodiversity and Conservation, 25, 2309-2339.

Kalamees, R. & Zobel, M. (1998). Soil seed bank composition in different successional stages of a species rich wooded meadow in Laelatu, western Estonia. Acta Oecologica, 19, 175-180.

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.

Karlik, P. & Poschlod, P. (2014). Soil seed-bank composition reveals the land-use history of calcareous grasslands. Acta Oecologica, 58, 22-34.

Keefe, R.F. & Davis, A.S. (2012). Marked, biased, filter (MBF): use of digital X-radiography and mark-recapture to partition seed lots based on sampled individual seed quality attributes. New Forests, 43, 169-184.

Kew, R.B.G. (2015). Seed Information Database (SID). Available at: http://data.kew.org/sid/ (accessed 2015).

Kiefer, S. (1997). Untersuchungen zur Wiederherstellung brachgefallener oder aufgeforsteter Kalkmagerrasen. Philipps-Universität Marburg, Marburg.

Kiefer, S. & Poschlod, P. (1996). Restoration of fallow or afforested calcareous grasslands by clear-cutting. Pages 209-218 in Settele, J., Margules, C., Poschlod, P. & Henle. K., editors. Species Survival in Fragmented Landscapes. Kluwer, Dordrecht.

Kiehl, K. & Pfadenhauer, J. (2007). Establishment and persistence of target species in newly created calcareous grasslands on former arable fields. Plant Ecology, 189, 31-48.

Kleyer, M., Bekker, R.M., Knevel, I.C., Bakker, J.P., Thompson, K., Sonnenschein, M., Poschlod, P., van Groenendael, J.M., Klimes, L., Klimesova, J., Klotz, S., Rusch, G.M., Hermy, M., Adriaens, D., Boedeltje, G., Bossuyt, B., Dannemann, A., Endels, P., Gotzenberger, L., Hodgson, J.G., Jackel, A.K., Kuhn, I., Kunzmann, D., Ozinga, W.A., Romermann, C., Stadler, M., Schlegelmilch, J., Steendam, H.J., Tackenberg, O., Wilmann, B., Cornelissen, J.H.C., Eriksson, O., Garnier, E. & Peco, B. (2008). The LEDA Traitbase: a database of life-history traits of the Northwest European flora. Journal of Ecology, 96, 1266-1274.

Klopfstein, S., Currat, M. & Excoffier, L. (2006). The fate of mutations surfing on the wave of a range expansion. Molecular Biology and Evolution, 23, 482-490.

Klotz, J. (2012). *Pimpinella peregrina* und *Sanguisorba minor* subsp. *polygama* bei Regensburg: Ausbreitung durch Rasenansaaten. Hoppea, 73, 127-134.

Klotz, S., Kühn, I., & Durka, W. (2002). BIOLFLOR - Eine Datenbank zu biologisch-ökologischen Merkmalen der Gefäßpflanzen in Deutschland. Bundesamt für Naturschutz, Bonn.

Kochanek, J., Steadman, K.J., Probert, R.J. & Adkins, S.W. (2011). Parental effects modulate seed longevity: exploring parental and offspring phenotypes to elucidate pre-zygotic environmental influences. New Phytologist, 191, 223-233.

Köhler, W., Schachtel, G. & Voleske, P. (2002). Biostatistik - Eine Einführung für Biologen und Agrarwissenschaftler. Springer, New York.

Krämer, S. (2010). Relationship between Tetrazolium and Germination tests. Presentation. In: ISTA Annual Meeting 2011 (ed. Ista). ISTA, Zurich, Switzerland.

Kranner, I., Minibayeva, F.V., Beckett, R.P. & Seal, C.E. (2010). What is stress? Concepts, definitions and applications in seed science. New Phytologist, 188, 655-673.

Kranner, I., Chen, H., Pritchard, H.W., Pearce, S.R. & Birtić, S. (2011). Inter-nucleosomal DNA fragmentation and loss of RNA integrity during seed ageing. Plant growth regulation, 63, 63-72.

Kropf, M. (2008). Intraspecific patterns of European mountain plants: a morphometric analysis confirms molecular results in the submediterranean oreophyte *Anthyllis montana* L. (Fabaceae). Taxon, 57, 511-524.

Kuneš, P., Pelánková, B., Chytrý, M., Jankovská, V., Pokorný, 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.

Lakon, G. (1948). The topographical tetrazolium method for determining the germinating capacity of seeds. Plant Physiology, 24, 389-394.

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

Legendre, P. (2013). lmodel2: Model II Regression. R package version 1.7–1. Available at: http://

CRAN.R-project.org/package=lmodel2 (accessed 2014).

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, 1919-1935.

Leishman, M.R. & Westoby, M. (1998). Seed size and shape are not related to persistence in soil in Australia in the same way as in Britain. Functional Ecology, 12, 480-485.

LfU. (2002). Gräser und Kräuter am richtigen Ort. Begrünung mit regionalem Samenmaterial als Beitrag zur Erhaltung der naturraumeigenen Pflanzenarten und genetischen Typen. 4 in Naturschutz, LfU Baden-Württemberg, Karlsruhe.

Linkies, A., Graeber, K., Knight, C. & Leubner-Metzger, G. (2010). The evolution of seeds. New Phytologist, 186, 817-831.

Liu, K., Eastwood, R.J., Flynn, S., Turner, R.M. & Stuppy, W.H. (2008). Seed information database (release 7.1). Available at: http://www.kew.org/data/sid (accessed 2015).

Long, R.L., Panetta, F.D., Steadman, K.J., Probert, R., Bekker, R.M., Brooks, S. & Adkins, S.W. (2008). Seed persistence in the field may be predicted by laboratory-controlled aging. Weed Science, 56, 523-528.

Long, R.L., Steadman, K.J., Panetta, F.D. & Adkins, S.W. (2009). Soil type does not affect seed ageing when soil water potential and temperature are controlled. Plant Soil, 320, 131-140.

Long, R.L., Kranner, I., Panetta, F.D., Birtic, S., Adkins, S.W. & Steadman, K.J. (2011). Wet-dry cycling extends seed persistence by re-instating antioxidant capacity. Plant and Soil, 338, 511-519.

Long, R.L., Gorecki, M.J., Renton, M., Scott, J.K., Colville, L., Goggin, D.E., Commander, L.E., Westcott, D.A., Cherry, H. & Finch-Savage, W.E. (2015). The ecophysiology of seed persistence: a mechanistic view of the journey to germination or demise. Biological Reviews, 90, 31-59.

Longo, D., Lorenz-Lemke, A. P., Mäder, G., Bonatto, S.L. & Freitas, L.B. (2014). Phylogeography of the *Petunia integrifolia* complex in southern Brazil. Botanical Journal of the Linnean Society, 174, 199-213.

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.

Maddison, W.P. & Knowles, L.L. (2006). Inferring phylogeny despite incomplete lineage sorting. Systematic Biology, 55, 21-30.

Magri, D., Vendramin, G.G., Comps, B., Dupanloup, I., Geburek, T., Gömöry, D., Latałowa, M., Litt, T., Paule, L. & Roure, J.M. (2006). A new scenario for the Quaternary history of European beech populations: palaeobotanical evidence and genetic consequences. New Phytologist, 171, 199-221.

Martin, A.C. (1946). The comparative internal morphology of seeds. American Midland Naturalist, 36, 513-660.

Maurer, K., Durka, W. & Stöcklin, J. (2003). Frequency of plant species in remnants of calcareous grassland and their dispersal and persistence characteristics. Basic and Applied Ecology, 4, 307-316.

Maurer, K., Weyand, A., Fischer, M. & Stöcklin, J. (2006). Old cultural traditions, in addition to land use and topography, are shaping plant diversity of grasslands in the Alps. Biological Conservation, 130, 438-446.

McDonald, M.B. & Kwong, F.Y. (2005). Introduction to Flower Seeds and the Flower Seed Industry. Flower Seeds - Biology and Technology (ed. by Mcdonald, M.B. & Kwong, F.Y.), 1-6. CABI Publishing, Oxfordshire, UK & Cambridge, USA.

Meindl, C. (2012). New aspects in plant conservation- Phylogeography, population dynamics, genetics and management of steppe plants in Bavaria. Universität Regensburg, Regensburg.

Meindl, C., Brune, V., Listl, D., Poschlod, P. & Reisch, C. (2016). Survival and postglacial immigration of the steppe plant *Scorzonera purpurea* to Central Europe. Plant Syst Evol, 302, 971-984.

Meiri, M., Lister, A.M., Higham, T.F.G., 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.

Menezes, N.L., Cicero, S.M., Villela, F.A. & Bortolotto, R.P. (2012). Using X rays to evaluate fissures in rice seeds dried artificially. Revista Brasileira de Sementes, 34, 70-77.

Merritt, D.J. & Dixon, K.W. (2011). Restoration seed banks - a matter of scale. Science, 332, 424-425.

Merritt, D.J., Martyn, A.J., Ainsley, P., Young, R.E., Seed, L.U., Thorpe, M., Hay, F.R., Commander, L.E., Shackelford, N., Offord, C.A., Dixon, K.W. & Probert, R.J. (2014). A continental-scale study of seed lifespan in experimental storage examining seed, plant, and environmental traits associated with longevity. Biodiversity and Conservation, 23, 1081-1104.

Meyer, D.J.L. (2005). Seed Development and Structure in Floral Crops. CABI Publishing, Oxfordshire, UK & Cambridge, USA.

Miller, A.L. (2005). Tetrazolium Testing for Flower Seeds. CABI Publishing, Oxfordshire, UK & Cambridge, USA.

Milosevic, M., Vujakovic, M. & Karagic, D. (2010). Vigour Tests as Indicators of Seed Viability. Genetika, 42, 103-118.

Mira, S., Gonzalez-Benito, M.E., Ibars, A.M. & Estrelles, E. (2011). Dormancy release and seed ageing in the endangered species *Silene diclinis*. Biodiversity and Conservation, 20, 345-358.

Mitlacher, K. (2009). Samenbankuntersuchung von Arten der Strandrasen-Gesellschaft (*Myosotis rehsteineri*, *Deschampsia littoralis*, *Ranunculus reptans* und *Littorella uniflora*) am Bodensee (Wasserburg) und Starnberger See (Allmanshausen). Bericht 2006. - Unveröffentlichtes Gutachten im Auftrag des Bayerischen Landesamtes für Umwelt

Moles, A.T. & Westoby, M. (2006). Seed size and plant strategy across the whole life cycle. Oikos, 113, 91-105.

Moles, A.T., Hodson, D.W. & Webb, C.J. (2000). Seed size and shape and persistence in the soil in the New Zealand flora. Oikos, 89, 541-545.

Moles, A.T., Warton, D.I. & Westoby, M. (2003). Do small-seeded species have higher survival through seed predation than large-seeded species? Ecology, 84, 3148-3161.

Mondoni, A., Probert, R.J., Rossi, G., Vegini, E. & Hay, F.R. (2011). Seeds of alpine plants are short lived: implications for long-term conservation. Annals of Botany, 107, 171-179.

Moore, R.P. (1973). Tetrazolium staining for assessing seed quality. Seed ecology (ed. by Heydecker, W.), pp. 347-366. Butterworths, London.

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

Muñoz Garmendia, F. & Navarro, C. (1999). Sanguisorba. Flora Iberica (ed. by Castroviejo, S.). Real Jardín Botánico, C.S.I.C., Madrid.

Nagel, M., Kranner, I., Neumann, K., Rolletschek, H., Seal, C.E., Colville, L., Fernández-Marín, B. & Börner, A. (2014). Genome-wide association mapping and biochemical markers reveal that seed ageing and longevity are intricately affected by genetic background and developmental and environmental conditions in barley. Plant, Cell & Environment, 38, 1011-1022.

Nathan, R., Schurr, F.M., Spiegel, O., Steinitz, O., Trakhtenbrot, A. & Tsoar, A. (2008) Mechanisms of long-distance seed dispersal. Trends in Ecology & Evolution, 23, 638-647.

Nei, M. (1972). Genetic distance between populations. American Naturalist, 106, 283-292.

Neumann Silva, V., Cicero, S.C. & Bennett, M. (2012). Relationship between eggplant seed morphology and germination. Revista Brasileira de Sementes, 34, 597 - 604.

Newton, R.F., Hay, F. & Probert, R. (2009). Protocol for comparative seed longevity testing. Technical Information Sheet_01. Available at: http://www.kew.org/sites/default/files/1_ppcont_014163_Protocol%20for%20comparative%20seed%20longevity%20testing_0.pdf (accessed 2014).

Nguyen, T.P., Keizer, P., van Eeuwijk, F., Smeekens, S. & Bentsink, L. (2012). Natural Variation for Seed Longevity and Seed Dormancy Are Negatively Correlated in Arabidopsis. Plant Physiology, 160, 2083-2092.

Niedzielski, M., Walters, C., Luczak, W., Hill, L.M., Wheeler, L.J. & Puchalski, J. (2009). Assessment of variation in seed longevity within rye, wheat and the intergeneric hybrid triticale. Seed Science Research, 19, 213-224.

Nikolaeva, M.G. (1967). [Physiology of deep dormancy in seeds.] Leningrad, Russia: Izdatel'stvo 'Nauka' (in Russian). [Translated from Russian by Z. Shapiro (1969), National Science Foundation, Washington, DC, 219.].

Nikolaeva, M.G. (1977). Factors controlling the seed dormancy pattern, North Holland, Amsterdam.

Nordborg, G. (1967). The genus *Sanguisorba* section Poterium. Experimental studies and taxonomy. Opera Bot, Lund.

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 of London B: Biological Sciences, rspb20102769.

Noronha, A., Andersson, L. & Milberg, P. (1997). Rate of change in dormancy level and light requirement in weed seeds during stratification. Annals of Botany, 80, 795-801.

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

Ooi, M.K.J., Auld, T.D. & Denham, A.J. (2009). Climate change and bet-hedging: interactions between increased soil temperatures and seed bank persistence. Global Change Biology, 15, 2375-2386.

Orme, D., Freckleton, R.P., Thomas, G., Petzoldt, T., Fritz, S. & Isaac, N. (2012). The caper package: comparative analysis of phylogenetics and evolution in R. R package, version 5(2)

Page, R.D.M. (1996). TREEVIEW: An application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences, 12, 357-358.

Pagel, M.D. (1992). A method for the analysis of comparative data. Journal of Theoretical Biology, 156, 431-442.

Pagel, M.D. (1999). Inferring the historical patterns of biological evolution. Nature, 401, 877-884.

Pakeman, R.J., Small, J.L. & Torvell, L. (2012). Edaphic factors influence the longevity of seeds in the soil. Plant Ecology, 213, 57-65.

Paun, O., Schönswetter, P., Winkler, M., IntraBioDiv-Consortium & Tribsch, A. (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.

Payne, R.W., Harding, S.A., Murray, D.A., Soutar, D.M., Baird, D.B., Glaser, A.I. & Channing, I.C. (2008). GenStat 11th Edition. Reference Manual

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.

Peco, B., Traba, J., Levassor, C., Sanchez, A.M. & Azcarate, F.M. (2003). Seed size, shape and persistence in dry Mediterranean grass and scrublands. Seed Science Research, 13, 87-95.

Perez-Garcia, F., Gonzalez-Benito, M.E. & Gomez-Campo, C. (2007). High viability recorded in ultra-dry seeds of 37 species of Brassicaceae after almost 40 years of storage. Seed Science and Technology, 35, 143-153.

Perry, G. & Garland, T. (2002). Lizard home ranges revisited: Effects of sex, body size, diet, habitat, and phylogeny. Ecology, 83, 1870-1885.

Petit, R.J., Aguinagalde, J., de Beaulieu, J.L., Bittkau, C., Brewer, S., Cheddadi, R., Ennos, R., Fineschi, S., Grivet, D. & Lascoux, M. (2003). Glacial refugia: hotspots but not melting pots of genetic diversity. Science, 300, 1563-1565.

Pfeifer, M., Schatz, B., Xavier Pico, F., Passalacqua, N.G., Fay, M.F., Carey, P.D. & Jeltsch, F. (2009). Phylogeography and genetic structure of the orchid *Himantoglossum* hircinum (L.) Spreng. across its European central - marginal gradient. Journal of Biogeography, 36, 2353-2365.

Piqueray, J., Bottin, G., Delescaille, L.-M., Bisteau, E., Colinet, G. & Mahy, G. (2011). Rapid restoration of a species-rich ecosystem assessed from soil and vegetation indicators: the case of calcareous grasslands restored from forest stands. Ecological Indicators, 11, 724-733.

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. (1993). Die Dauerhaftigkeit von Diasporenbanken in Böden und deren Bedeutung

für den Arten- und Biotopschutz am Beispiel von Kalkmagerrasenstandorten. Verhandlungen der GfÖ, 22, 229-240.

Poschlod, P. (2015). Geschichte der Kulturlandschaft: Entstehungsursachen und Steuerungsfaktoren der Entwicklung der Kulturlandschaft, Lebensraum-und Artenvielfalt in Mitteleuropa. Verlag Eugen Ulmer, Stuttgart.

Poschlod, P. & Jackel, A.K. (1993). The dynamics of the generative diaspore bank of calcareous grassland plants. 1. Seasonal dynamics of diaspore rain and diaspore bank in 2 calcareous grassland sites of the Suebian-alb. Flora, 188, 49-71.

Poschlod, P. & Bonn, S. (1998). Changing dispersal processes in the central European landscape since the last ice age: an explanation for the actual decrease 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., Deffner, A., Beier, B. & Grunicke, U. (1991). Untersuchungen zur Diasporenbank von Samenpflanzen auf beweideten, gemähten, brachgefallenen und aufgeforsteten Kalkmagerrasenstandorten. Verhandlungen der GfÖ, 20, 893-904.

Poschlod, P., Bakker, J.P., Bonn, S. & Fischer, S. (1996). Dispersal of Plants in fragmented landscapes. Pages 123-127 in Settele, J., Margules, C., Poschlod, P. & Henle. K., editors. Species Survival in Fragmented Landscapes. Kluwer, Dordrecht.

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, 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 & Phytotaxonomica, 38, 263-271.

Poschlod, P., Bakker, J.P. & Kahmen, S. (2005a). Changing land use and its impact on biodiversity. Basic and Applied Ecology, 6, 93-98.

Poschlod, P., Tackenberg, O. & Bonn, S. (2005b). Plant dispersal potential and its relation to species frequency and coexistence. Vegetation ecology (ed. by Van Der Maarel, E.), 147-171. Blackwell, Oxford.

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.CZ (ed. by Szabó, P. and Hédl, R.), pp. 130-143. Institute of Botany of the Czech Academy of Sciences, Brno.

Poschlod, P., Abedi, M., Bartelheimer, M., Drobnik, J., Rosbakh, S. & Saatkamp, A. (2013). Seed ecology and assembly rules in plant communities. Vegetation ecology (ed. by Van Der Mareel, E. & Franklin, J.). Wiley-Blackwell, Chichester.

Pott, R. (1996). Die Entwicklungsgeschichte und Verbreitung xerothermer Vegetationseinheiten in Mitteleuropa unter dem Einfluss des Menschen. Tuexenia, 16, 337-369.

Pritchard, H.W. & Dickie, J.B. (2003). Predicting Seed Longevity: the use and abuse of seed viability equations. Seed conservation: turning science into practice. (ed. by Smith, R.D., Dickie, J.D., Linington, S.H., Pritchard, H.W. and Probert, R.J.), pp. 653-721. Kew Publishing, Kew.

Pritchard, J., Wen, X. & Falush, D. (2009). STRUCTURE ver. 2.3. University of Chicago, Chicago.

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

Probert, R.J., Daws, M.I. & Hay, F.R. (2009). Ecological correlates of *ex situ* seed longevity: a comparative study on 195 species. Annals of Botany, 104, 57-69.

Purschke, O., Sykes, M.T., Reitalu, T., Poschlod, P. & Prentice, H.C. (2012). Linking landscape history and dispersal traits in grassland plant communities. Oecologia, 168, 773-783.

Quinger, B., Bräu, M. & Kornprobst, M. (1994). Lebensraumtyp Kalkmagerrasen. 2 Teilbände. Bayer. Staatsministerium für Landesentwicklung und Umweltfragen (StMLU) u. Bayer. Akademie für Naturschutz und Landschaftspflege (ANL), Munich.

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

Reisch, C. (2008). Glacial history of *Saxifraga paniculata* (Saxifragaceae): molecular biogeography of a disjunct arctic-alpine species from Europe and North America. Biological Journal of the Linnean Society, 93, 385-398.

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., Windmaißer, T., Vogler, F., Schuhwerk, F. & Meyer, N. (2015). Genetic structure of the rare and endangered *Hieracium wiesbaurianum* group (Asteraceae) in Bavaria. Botanical Journal of the Linnean Society, 177, 112-123.

Revell, L.J. (2012). Phytools: An R package for phylogenetic comparative biology (and other things). Methods in Ecology and Evolution, 3, 217-223.

Revell, L.J., Harmon, L.J. & Collar, D.C. (2008). Phylogenetic signal, evolutionary process, and rate. Systematic Biology, 57, 591-601.

Riecken, U., Finck, P., Raths, U., Schröder, E. & Ssymank, A. (2006). Rote Liste der gefährdeten Biotoptypen Deutschlands. Zweite fortgeschriebene Fassung. Naturschutz und Biologische Vielfalt, 34, 318.

Ritz, C. & Streibig, J.C. (2005). Bioassay analysis using R. Journal of Statistical Software, 12, 1-22.

Rogers, S.O. & Bendich, A.J. (1994). Extraction of total cellular DNA from plants, algae and fungi. Plant molecular biology manual (ed. by Gelvin, S.B. & Schilperoort, R.A.), 183-190. Springer, Netherlands.

Römermann, C., Tackenberg, O. & Poschlod, P. (2005) How to predict attachment potential of seeds to sheep and cattle coat from simple morphological seed traits. Oikos, 110, 219-230.

Ruddiman, W.F. & Thomson, J.S. (2001). The case for human causes of increased atmospheric CH4 over the last 5000 years. Quaternary Science Reviews, 20, 1769-1777.

Saatkamp, A., Affre, L., Dutoit, T. & Poschlod, P. (2009). The seed bank longevity index revisited: limited reliability evident from a burial experiment and database analyses. Annals of Botany, 104, 715-724.

Saatkamp, A., Affre, L., Dutoit, T. & Poschlod, P. (2011). Germination traits explain soil seed persistence across species: the case of Mediterranean annual plants in cereal fields. Annals of Botany, 107, 415-426.

Saatkamp, A., Poschlod, P. & Venable, D.L. (2014). The Functional Role of Soil Seed Banks in Natural Communities. Seeds: The Ecology of Regeneration in Plant Communities (ed. by Gallagher, R.S.), 263-295. CABI Publishing, Wallingford.

Sádlo, J. (2007). Diversity of vegetation of the Czech Republic, its determinants and history (in Czech). Vegetation of the Czech Republic. 1. Grassland and Heathland Vegetation (ed. by Chytrý, M.). Academia, Prague.

Sang, T. & Zhong, Y. (2000). Testing hybridization hypotheses based on incongruent gene trees. Systematic Biology, 49, 422-434.

Santiso, X., Lopez, L., Retuerto, R. & Barreiro, R. (2016). Phylogeography of a widespread species: pre-glacial vicariance, refugia, occasional blocking straits and long-distance migrations. AoB Plants, 8, plw003.

Schaal, B.A., Hayworth, D.A., Olsen, K.M., Rauscher, J.T. & Smith, W.A. (1998). Phylogeographic studies in plants: problems and prospects. Molecular Ecology, 7, 465-474.

Scheepens, J.F., Frei, E.S. & Stöcklin, J. (2013). Glacial history affected phenotypic differentiation in the alpine plant, *Campanula thyrsoides*. Plos One, 8, e73854.

Scheuerer, M. & Ahlmer, W. (2003). Rote Liste gefährdeter Gefäßpflanzen Bayerns mit regionalisierter Florenliste. Bay. Landesamt für Umweltschutz, Augsburg.

Schlueter, P.M. & Harris, S.A. (2006). Analysis of multilocus fingerprinting data sets containing missing data. Molecular Ecology Notes, 6, 569-572.

Schmidt, M., Fischer, P. & Becker, C. (2007). Zur Herkunft von Pflanzenarten anthropo-zoogener Kalk-Halbtrockenrasen Mitteleuropas-Überle-

gungen am Beispiel des nördlichen deutschen Hügel-und Berglandes. Hercynia N. F., 40, 257-267.

Schoeman, J., Buckley, Y.M., Cherry, H., Long, R.L. & Steadman, K.J. (2010). Inter-population variation in seed longevity for two invasive weeds: *Chrysanthemoides monilifera* ssp *monilifera* (boneseed) and ssp *rotundata* (bitou bush). Weed Research, 50, 67-75.

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

Schönswetter, P., Stehlik, I., Holderegger, R. & Tribsch, A. (2005). Molecular evidence for glacial refugia of mountain plants in the European Alps. Molecular Ecology, 14, 3547-3555.

Schwienbacher, E., Marcante, S. & Erschbamer, B. (2010). Alpine species seed longevity in the soil in relation to seed size and shape - A 5-year burial experiment in the Central Alps. Flora, 205, 19-25.

Sebasky, M.E., Keller, S.R. & Taylor, D.R. (2016). Investigating past range dynamics for a weed of cultivation, *Silene vulgaris*. Ecology and Evolution,

Simak, M. & Gustafsson, A. (1953). X-ray photography and sensitivity in forest tree species. Hereditas, 39, 458–468.

Smith, P., Dickie, J., Linington, S., Probert, R. & Way, M. (2011). Making the case for plant diversity. Seed Science Research, 21, 1-4.

Smith, R.D., Dickie, J.B., Linington, S.H., Pritchard, H.W. & Probert, R.J. (2003). Seed Conservation: turning science into practice. Kew Publishing, Kew.

Socolowski, F., Cicero, S.M. & Vieira, D.C.M. (2011). Seed weight of *Xylopia aromatica* (Annonaceae): quality evaluation from X-ray and seedling emergence. Scientia Agricola, 68, 643-646.

Soubani, E., Hedrén, M. & Widén, B. (2014). Phylogeography of the European rock rose *Helianthemum nummularium* (Cistaceae): incongruent patterns of differentiation in plastid DNA and morphology. Botanical Journal of the Linnean Society, 176, 311-331.

Stebbins, G.L. (1984). Polyploidy and the distribution of the arctic-alpine flora: new evidence and a new approach. Bot. Helvetica, 94, 1-13.

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

Stöcklin, J. & Fischer, M. (1999). Plants with longer-lived seeds have lower local extinction rates in grassland remnants 1950-1985. Oecologia, 120, 539-543.

Sturiao, W.P., Landgraf, P.R.C. & Rosa, T.P. (2012). X-ray test for the evaluation of the internal morphology of the seeds of seafortia palm trees. Semina-Ciencias Agrarias, 33, 3003-3008.

Sutkowska, A., Pasierbiński, A., Warzecha, T., Mandal, A. & Mitka, J. (2013). Refugial pattern of *Bromus erectus* in Central Europe based on Issr fingerprinting. Acta Biologica Cracoviensia Series Botanica, 55, 107-119c.

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

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.

Tackenberg, O., Poschlod, P. & Bonn, S. (2003). Assessment of wind dispersal potential in plant species. Ecological Monographs, 73, 191-205.

Tallavaara, M., Luotob, M., Korhonenc, N., Järvinend, H. & Seppäb, H. (2015). Human population dynamics in Europe over the Last Glacial Maximum. Proceedings of the National Academy of Sciences, 112, 8232-8237.

Tausch, S., Leipold, M., Reisch, C. & Poschlod, P. (2015). Genbank Bayern Arche - ein Beitrag zum dauerhaften Schutz gefährdeter Pflanzenarten in Bayern. ANLiegen Natur, 37, 82 - 91.

Telewski, F.W. & Zeevaart, J.A.D. (2002). The 120-yr period for Dr. Beal's seed viability experiment. American Journal of Botany, 89, 1285-1288.

Thompson, K. & Grime, J.P. (1979). Seasonal variation in the seed banks of herbaceous species in ten contrasting habitats. Journal of Ecology, 67, 893-921.

Thompson, K. & Grime, J.P. (1983). A Comparative-Study of Germination Responses to Diurnally-Fluctuating Temperatures. Journal of Applied Ecology, 20, 141-156.

Thompson, K., Band, S.R. & Hodgson, J.G. (1993). Seed size and shape predict persistence in soil. Functional Ecology, 7, 236-241.

Thompson, K., Bakker, J.P. & Bekker, R.M. (1997). The soil seed banks of North West Europe: methodology, density and longevity. University Press, Cambridge.

Thompson, K., Bakker, J.P., Bekker, R.M. & Hodgson, J.G. (1998). Ecological correlates of seed persistence in soil in the north-west European flora. Journal of Ecology, 86, 163-169.

Thompson, K., Ceriani, R.M., Bakker, J.P. & Bekker, R.M. (2003). Are seed dormancy and persistence in soil related? Seed Science Research, 13, 97-100.

Tomescu, A.M. (2000). Evaluation of Holocene pollen records from the Romanian Plain. Rev Palaeobot Palynol, 109, 219-233.

Toole, E.H. & Brown, E. (1946). Final results of the Duvel buried seed experiment. Journal of Agricultural Research, 72, 201-210.

Traba, J., Azcarate, F.M. & Peco, B. (2006). The fate of seeds in Mediterranean soil seed banks in relation to their traits. Journal of Vegetation Science, 17, 5-10.

Triponez, Y., Arrigo, N., Espíndola, A. & Alvarez, N. (2015). Decoupled post-glacial history in mutualistic plant-insect interactions: insights from the yellow loosestrife (*Lysimachia vulgaris*) and its associated oil-collecting bees (*Macropis europaea* and *M. fulvipes*). Journal of Biogeography, 42, 630-640.

Tuckett, R.E., Merritt, D.J., Hay, F.R., Hopper, S.D. & Dixon, K.W. (2010). Dormancy, germination and seed bank storage: a study in support of *ex situ* conservation of macrophytes of southwest Australian temporary pools. Freshwater Biology, 55, 1118-1129.

Tutin, T.G., Heywood, V.H., Burges, N.A., Moore, D.M., Valentine, D.H., Walters, S.M. & Webb, D.A. (1968). Flora Europaea, Rosaceae to Umbelliferae. University Press, Cambridge.

Tweddle, J.C., Dickie, J.B., Baskin, C.C. & Baskin, J.M. (2003). Ecological aspects of seed desiccation sensitivity. Journal of Ecology, 91, 294-304.

Twyford, A.D. & Ennos, R.A. (2012). Next-generation hybridization and introgression. Heredity, 108, 179-189.

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.

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.

Valko, O., Torok, P., Tothmeresz, B. & Matus, G. (2011). Restoration Potential in Seed Banks of Acidic Fen and Dry-Mesophilous Meadows: Can Restoration Be Based on Local Seed Banks? Restoration Ecology, 19, 9-15.

van der Burg, W.J., Aartse, J.W., Vanzwol, R.A., Jalink, H. & Bino, R.J. (1994). Predicting Tomato Seedling Morphology by X-Ray-Analysis of Seeds. Journal of the American Society for Horticultural Science, 119, 258-263.

Van Treuren, R., de Groot, E.C. & van Hintum, T.J. (2012). Preservation of seed viability during 25 years of storage under standard genebank conditions. Genetic Resources and Crop Evolution, 60, 1407-1421.

Venable, D.L. & Brown, J.S. (1988). The selective interactions of dispersal, dormancy, and seed size as adaptations for reducing risk in variable environments. American Naturalist, 131, 360-384.

von Blanckenhagen, B. & Poschlod, P. (2005). Restoration of calcareous grasslands: the role of the soil seed bank and seed dispersal for recolonisation processes. Biotechnologie, Agronomie, Société et Environnement, 9, 143–149.

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.

Walck, J.L., Hidayati, S.N., Dixon, K.W., Thompson, K. & Poschlod, P. (2011). Climate change and plant regeneration from seed. Global Change Biology, 17, 2145-2161.

Wäldchen, J., Pusch, J. & Luthardt, V. (2005). Zur Diasporen-Keimfähigkeit von Segetalpflanzen: Untersuchungen in Nord-Thüringen. Beiträge für Forstwirtschaft und Landschaftsökologie, 34, 145-156.

Walker, E.A., Hermann, J.M. & Kollmann, J. (2015). Grassland restoration by seeding: seed source and growth form matter more than density. Applied Vegetation Science, 18, 368-378.

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.

Walsh, D.G.F., Waldren, S. & Martin, J., R. (2003). Monitoring seed viability of fifteen species after storage in the Irish threatened plant genebank. Biology and endvironment: Proceedings of the Royal Irish Academy, 103B, 59-67.

Walters, C. (1998). Understanding the mechanisms and kinetics of seed aging. Seed Science Research, 8, 223-244.

Walters, C. (2015). Genebanking Seeds from Natural Populations. Natural Areas Journal, 35, 98-105.

Walters, C., Hill, L.M. & Wheeler, L.J. (2005a). Dying while dry: kinetics and mechanisms of deterioration in desiccated organisms. Integrative and Comparative Biology, 45, 751-758.

Walters, C., Wheeler, L.M. & Grotenhuis, J.M. (2005b). Longevity of seeds stored in a genebank: species characteristics. Seed Science Research, 15, 1-20.

Walters, C., Ballesteros, D. & Vertucci, V.A. (2010). Structural mechanics of seed deterioration: Standing the test of time. Plant Science, 179, 565-573.

Watson, L. & Dallwitz, M.J. (1992 onwards). The families of flowering plants: descriptions, illustrations, identification, and information retrieval. Version: 19th December 2012. URL http://delta-intkey.com.

Webb, C.O. & Donoghue, M.J. (2005). Phylomatic: tree assembly for applied phylogenetics. Molecular Ecology Notes, 5, 181-183.

Webb, C.O., Ackerly, D.D. & Kembel, S.W. (2008). Phylocom: software for the analysis of phylogenetic community structure and trait evolution. Bioinformatics, 24, 2098-2100.

Wendel, J.F. & Doyle, J.J. (1998). Phylogenetic incongruence: window into genome history and molecular evolution. Molecular systematics of plants II, pp. 265-296. Springer, US.

Wietsma, W.A., Deinum, D., Teunissen, H.A.S. & van den Berg, R.G. (2014). Phylogenetic relationships within *Fritillaria* section *Petilium* based on AFLP fingerprints. Plant Systematics and Evolution, 301, 1043-1054.

Wikstrom, N., Savolainen, V. & Chase, M.W. (2001). Evolution of the angiosperms: calibrating the family tree. Proceedings of the Royal Society B-Biological Sciences, 268, 2211-2220.

Windmaißer, T., Kattari, S., Heubl, G. & Reisch, C. (2016). Glacial refugia and postglacial expansion of the alpine-prealpine plant species *Polygala chamaebuxus*. Ecology and Evolution, 6, 7809-7819.

Witter, M.S. & Carr, G.D. (1988). Adaptive Radiation and Genetic Differentiation in the Hawaiian Silversword Alliance (Compositae, Madiinae). Evolution, 42, 1278-1287.

Yan, D., Duermeyer, L., Leoveanu, C. & Nambara, E. (2014). The functions of the endosperm during seed germination. Plant and Cell Physiology, pcu089.

Yeh, F.C., Yang, R. & Boyle, T. (1999). POPGENE Version 1.3.1 - Microsoft Window based Freeware for Population Genetic Analysis - Quick User Guide. University of Alberta, Alberta

Zhao, L.P., Wu, G.L. & Cheng, J.M. (2011). Seed mass and shape are related to persistence in a sandy soil in northern China. Seed Science Research, 21, 47-53.

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