Application of DNA marker systems to test for genetic imprints of habitat fragmentation in *Juniperus communis* L. on different spatial and temporal scales

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Integration of scientific knowledge into conservation measures

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften
(Dr. rer. nat.)

dem Fachbereich Biologie
der Philipps-Universität Marburg
vorgelegt von

Inga Maria Michalczyk
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Marburg/Lahn, 2008

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Einsatz von DNA-Marker-Systemen zur Überprüfung genetischer Effekte der Habitatfragmentierung auf *Juniperus communis* L. innerhalb unterschiedlicher räumlicher und zeitlicher Skalen

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Einbindung wissenschaftlicher Erkenntnisse in Naturschutzmaßnahmen

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The research pertaining to this thesis was carried out at the Department of Conservation Biology at the Philipps-University of Marburg from January 2005 to September 2008 under the supervision of Prof. Dr. Birgit Ziegenhagen.

Vom Fachbereich Biologie

der Philipps-Universität Marburg als Dissertation am 18. September 2008 angenommen.

Erstgutachterin: Prof. Dr. Birgit Ziegenhagen

Zweitgutachter: Prof. Dr. Gerhard Kost

Tag der mündlichen Prüfung am 11. November 2008

Neues wagen, Positives entdecken, jeden Tag.

This thesis is based on the following three publications and manuscripts. They will be referred to in the text by the term ‘paper’ and their roman numerals. They are reviewed in chapter 2 and 3 of this thesis. After a short general introduction into the main topics, the main results of the respective scientific analyses are given and interpreted. For detailed information about materials and methods as well as for further results and discussions see appended publications and manuscripts.

I **Genetic support for recurrent fragmentation and founder events of juniper populations in Central Europe**

Inga M. Michalczyk, Yvonne A. Lücke, Stefan Huck and Birgit Ziegenhagen

Manuscript submitted to *The Holocene*.

Reviews received with the comment ‘possibly acceptable after moderate revision’.

II **Characterization of highly polymorphic nuclear microsatellite loci in *Juniperus communis* L.**

Inga M. Michalczyk, Federico Sebastiani, Anna Buonamici, Eva Cremer, Christina Mengel, Birgit Ziegenhagen and Giovanni G. Vendramin

Molecular Ecology Notes (2006) 6, 346-348.

III **Reduction of population sizes has not yet affected genetic diversity of juniper, *Juniperus communis* L.**

Inga M. Michalczyk, Ronald Bialozyt, Frank Schlütz and Birgit Ziegenhagen

Manuscript submitted to *Biological Conservation.*

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Synthesis

German summary – Zusammenfassung

Juniperus communis L., im Folgenden auch Wacholder genannt, ist die am weitesten verbreitete Konifere der Holarktis. In Europa stellt er eine typische Art der halboffenen Kulturlandschafts-Ökosysteme dar. Dieser halbnatürliche Landschaftstypus wurde v. a. durch intensive Aufforstungen, aber auch durch die Intensivierung der Landwirtschaft und deren gleichzeitige Aufgabe in so genannten Ungunstlagen verkleinert und ist heute stark fragmentiert. Deshalb ist der ehemals landschaftsbestimmende Wacholder in vielen europäischen Ländern nur noch in kleinen, räumlich voneinander isolierten Reliktpopulationen anzutreffen. Viele dieser Populationen weisen ein Naturverjüngungsdefizit und eine einheitliche, überalterte Vertikalstruktur auf.

Um Wacholder als einen Vertreter der europäischen Kulturlandschaft und das durch ihn geprägte Landschaftsbild, die Wacholderheide, zu erhalten, erachte ich Restaurierungsmaßnahmen als unabdingbar. Hierin ist die Motivation der vorliegenden Doktorarbeit begründet.

Zunächst nutzte ich unterschiedliche DNA-Marker-Systeme, um verschiedene Wacholderpopulationen auf unterschiedlichen räumlichen und zeitlichen Skalen auf genetische Effekte der Habitatfragmentierung hin zu überprüfen. Die gewonnenen Ergebnisse sollten anschließend dazu dienen, die auf regionaler Ebene untersuchten Wacholderheiden naturschutzfachlich zu bewerten und einen wissenschaftlich begründeten Managementplan für die Restaurierung von Wacholderheiden zu erstellen. Dieser Plan sollte sich vornehmlich mit Nachbesserungen der Bestände durch Ausbringen von Saat- und Pflanzgut beschäftigen.

Auf europäischer Ebene setzte ich so genannte Amplified Fragment Length Polymorphism (AFLP-) Marker ein, um Aspekte der biogeographischen Geschichte des Wacholders zu rekonstruieren und mögliche genetische Abstammungslinien zu identifizieren. Es wird angenommen, dass derartige Abstammungslinien geographische Areale beschreiben, innerhalb derer Saat- und Pflanzgut ausgetauscht werden kann. Die genetische Analyse ließ keine Abstammungslinien erkennen. Vielmehr deuten die Ergebnisse auf ein Fehlen grundlegender phylogeographischer Signale in Europa hin. Aufgrund der AFLP-Daten und im Zusammenhang mit Kenntnissen aus anderen wissenschaftlichen Disziplinen vermute ich, dass der Wacholder während der letzten Eiszeit in Zentraleuropa in zahlreichen kleinen und

geeigneten Habitaten überdauerte. Möglicherweise waren diese Habitate diffus über Zentraleuropa verteilt und veränderten sich unter Umständen im Laufe der Zeit mehrmals in ihrer Größe und geographischen Lage. Für die Zeit zwischen der letzten maximalen Eisausdehnung (Last Glacial Maximum, LGM) bis heute sehe ich für Wacholder periodisch auftretende Fragmentierungs- und Gründerereignisse als außerordentlich wahrscheinlich an. Diese Vermutung liegt begründet in den erhaltenen AFLP-Ergebnissen, aber auch in den ökologischen Ansprüchen des Wacholders, in seinen „life-history traits“, in palynologischen Daten und in Kenntnissen über die historischen Einflüsse des Menschen auf die Natur.

Auf regionaler Ebene, im Rheinischen Schiefergebirge, einer Mittelgebirgsregion in Westdeutschland, setzte ich nukleäre Mikrosatelliten- (nuclear Simple Sequence Repeat, nSSR-) Marker ein, um Einblicke in die genetische Struktur und Diversität von acht reliktischen Wacholderpopulationen zu erhalten. Um die genetische Identität der Bestände durch Nachbesserungsmaßnahmen mit Saat- und Pflanzgut nicht zu verändern und deren genetische Diversität zu erhalten bzw. zu fördern, ist es notwendig, deren genetische Struktur und Diversität zu kennen. Gleichzeitig testete ich mittels der nSSR-Marker die Populationen auf mögliche genetische Effekte der derzeitigen Habitatfragmentierung. Die untersuchten Mikrosatellitenorte wurden im Rahmen der vorliegenden Doktorarbeit charakterisiert, und es erfolgte eine detaillierte Validierung. Zusätzlich zur genetischen Untersuchung der Altbestände analysierte ich in einer Fallstudie die genetische Diversität und Differenzierung unterschiedlicher Pollenwolken, die in der Folgegeneration (Embryonen) reproduktiv effektiv geworden sind. Eigens hierfür wurde im Rahmen der vorliegenden Doktorarbeit ein neues Computerprogramm entwickelt. Darüber hinaus wurden durch die Analyse von unterschiedlichen Moospolstern bzw. den darin abgelagerten Pollenkörnern physikalische Pollenflugdistanzen für Wacholder bestimmt. Für Nachbesserungsmaßnahmen bzw. die räumliche Organisation von Saat- und Pflanzgut im Zusammenhang mit den bereits vorhandenen Altbäumen könnten derartige Daten von gewisser Relevanz sein. Die beachtlich hohe genetische Diversität und das Fehlen genetischer Flaschenhalseffekte in allen untersuchten Wacholderbeständen des Rheinischen Schiefergebirges sowie das Fehlen eines „isolation-by-distance“-Effektes deuten darauf hin, dass sich die heutige Habitatfragmentierung noch nicht auf die genetische Struktur und Diversität der acht Wacholderpopulationen ausgewirkt hat. Stattdessen vermute ich, dass diese genetischen Parameter seit Beginn der Fragmentierung „festgefroren“ sind. Die genetische Diversität der Folgegenerationen ist im Vergleich zu den Altbeständen nicht reduziert, obwohl die palynologische Untersuchung auf äußerst lokal begrenzten Pollenflug hinweist.

Auf Grundlage der Ergebnisse aus den genetischen Analysen erfolgte eine naturschutzfachliche Bewertung der untersuchten Wacholderpopulationen im Rheinischen Schiefergebirge. Hierfür erstellte ich ein Leitbild, welches auf weit verbreiteten populationsökologischen und -genetischen Theorien basiert. Warum diese Bewertung nicht zufriedenstellend ausfiel, wird im Rahmen der vorliegenden Doktorarbeit detailliert diskutiert. Weiterhin beleuchtete ich im Hinblick auf die „life-history traits“ und die innerhalb dieser Arbeit postulierte biogeographische Geschichte des Wacholders die Kriterien und deren Qualitätsforderungen des aufgestellten Leitbildes kritisch.

Aufgrund der nun vorliegenden genetischen Ergebnisse sowie aufgrund des Naturverjüngungsdefizites in allen untersuchten Wacholderpopulationen bleibt es weiterhin ungewiss, ob sich die heutige Habitatfragmentierung im Rheinischen Schiefergebirge in Zukunft negativ auf deren genetische Struktur und Diversität auswirken wird. Allerdings erscheint es als sehr wahrscheinlich, dass sich Populationen, die sich dauerhaft nicht verjüngen, durch sukzessives Sterben seneszenter Individuen verkleinern und in der Folge komplett aussterben werden. Deshalb entwickelte ich im Rahmen der vorliegenden Doktorarbeit einen nachhaltigen, demographisch und genetisch begründeten Managementplan zur Restaurierung sich nicht verjüngender Wacholderpopulationen. Dieser Plan basiert auf den hier gewonnenen Ergebnissen und auf Expertenwissen. Er umfasst Leitlinien und Empfehlungen für die Sammelstrategie von Pflanzmaterial, für dessen Behandlung im Gewächshaus und für dessen Inverkehrbringen. Nach einem genetischen Screening mit den im Rahmen dieser Doktorarbeit veröffentlichten nSSR-Markern kann der Managementplan in vollem Umfang auch in gefährdeten Wacholderpopulationen anderer Regionen bzw. Länder Europas angewendet werden.

English summary

Juniperus communis L., in the following also referred to as *J. communis* and juniper, is the conifer taxon with the largest distribution area in the Holarctic. In Europe it represents a typical species of the cultural landscape. Mainly due to afforestation as well as due to the intensification of agriculture and its retreat from unfavourable sites, this characteristic semi-natural open landscape type has been decreased in size and is nowadays highly fragmented. Therefore, the formerly continuous and widespread juniper populations are currently divided into small and spatially isolated relics in numerous European countries. Additionally, many of these populations suffer from an absence of natural regeneration and consist predominantly of senescent individuals.

In order to maintain juniper as a valuable element of the cultural landscape in Europe, I considered a restoration management to be indispensable. The goal of the present thesis is based on this consideration.

Using different DNA marker systems, I firstly tested various juniper populations on different spatial and temporal scales for potential imprints of habitat fragmentation. Afterwards my intention was to evaluate the analysed populations on the regional scale in terms of nature conservation and to develop a scientifically based conservation management plan, which should focus on planting activities.

In a Europe-wide study I used an Amplified Fragment Length Polymorphism (AFLP) marker approach to reconstruct aspects of the biogeographic history of juniper and to detect potential distinct genetic lineages. Those lineages are supposed to delineate geographic regions within which plant material can be interchanged. The genetic analysis revealed no distinct genetic lineages. Hence it suggested an absence of underlying phylogeographic signals throughout the whole of Europe. Along with other scientific findings about juniper these results point to a glacial persistence of juniper in Central Europe. I suppose that during the last glacial period, this species managed to survive in several small and suitable habitats, which were probably diffusely scattered and permanently changing. Moreover, based on the genetic results and knowledge about the ecology of juniper, its life-history traits, palynological data as well as the history of human influences, I hypothesise that recurrent fragmentation and founder events since the last glacial maximum (LGM) up until today are highly likely to have occurred in this species.

On a regional scale, i.e. in the Rhenish Uplands, a mountainous region in West Germany, I used nuclear microsatellite markers (also called nuclear Simple Sequence Repeat or nSSR markers) to gain insights into the genetic structure and variation of eight relict juniper populations. Such knowledge is necessary for planting activities in order to prevent negative effects in the respective populations. At the same time, I tested the eight populations for genetic imprints of the recent habitat fragmentation. The investigated nSSR loci in juniper were characterised within the scope of this thesis. A detailed validation of the newly developed nSSR markers is presented. In addition to the analysis of adult juniper generations, I performed a case study by investigating the genetic diversity and differentiation of different pollen clouds, which have become reproductively effective in the filial generations (juniper embryos). For this purpose a specific computer software was developed as part of the present thesis. Next, a palynological study was conducted to determine physical pollen flow distances of juniper pollen grains. In terms of plantings I assume that such data is relevant for the spatial organisation of already existing juniper individuals and the respective plant material. Considerably high levels of genetic diversity and an absence of recent genetic bottlenecks in all populations as well as an absence of an isolation-by-distance effect led me to the assumption that the current habitat fragmentation has not yet affected the genetic diversity in the eight juniper populations in the Rhenish Uplands. Instead, I postulate that the genetic diversity and differentiation have been 'frozen' since the recent fragmentation started. The genetic diversity of the filial generation is not reduced in comparison to the adult generation, although the palynological study points to locally restricted pollen flow distances.

After defining a 'Leitbild' for viable juniper populations based on widely accepted population ecological and genetic theories, I used the genetic results to evaluate the analysed populations of the Rhenish Uplands in terms of nature conservation. The reasons why this evaluation was not satisfactory are discussed in detail. Further on, I commented critically on the evaluation criteria of the 'Leitbild' and their respective quality demands with regard to the life-history traits of juniper and its biogeographic history as presented within this thesis.

Based on the presented genetic results and on the apparent absence of natural regeneration in all populations it remains uncertain whether the current habitat fragmentation will affect the genetic diversity and structure of the eight populations in the Rhenish Uplands deleteriously in the future. However, if juniper will not start with natural regeneration again, this will certainly lead to an extinction of the respective populations because without substitution, senescent individuals will gradually die off. Thus, in the distant future juniper will probably

become extinct in areas where it does not regenerate naturally. Therefore, I developed a sustainable, demographically and genetically substantiated restoration management plan as a final outcome of this thesis. It is based on the genetic analysis presented here and on expert knowledge, and it includes guidelines and recommendations concerning the collection of plant material, its treatment in the greenhouse and plantings in the field. After a screen with the newly developed nSSR markers for juniper, the entire management plan can be transferred directly to other European regions where juniper populations are threatened also.

1 Introduction

Landscape or habitat fragmentation divides formerly continuous habitats into several smaller and spatially isolated remnants (Young et al., 1996). The theory that the reduction of population size and the loss of connectivity affect several demographic and genetic processes is widely accepted (Oostermeijer et al., 2003; Washitani et al., 2005). In plant ecology isolation of populations can often lead to a disruption of mating systems with an ensuing decrease of gene flow and an increase of genetic drift and inbreeding (Ellstrand and Elam, 1993). As a result, genetic diversity in terms of heterozygosity is lost and alleles become fixed (Templeton et al., 2001). Thereby, genetic differentiation arises and, as a long-term effect, isolation-by-distance effects occur (Young et al., 1996). Increased homozygosity in a population may be associated with reduced fitness and a loss of genetic variation may reduce the potential to adapt to new environmental conditions (Fisher, 1930; Templeton et al., 2001). However, some recent studies could not detect any negative genetic effects of habitat fragmentation in the analysed populations (e.g. O'Connell et al., 2006; Williams et al., 2007). Depending on different life-history traits of the species, the genetic consequences of habitat fragmentation seem to be more or less pronounced. Thus, short-lived species and/or species with less effective gene flow are often more severely affected by habitat fragmentation than e.g. long-lived species with highly effective gene flow (see e.g. review Lowe et al., 2005).

Within this thesis, I used different DNA marker based methods to test for possible genetic imprints of habitat fragmentation in *Juniperus communis* L. on different spatial and temporal scales.

J. communis, the Common Juniper (from now on also referred to as *J. communis* and juniper) represents a typical species of the cultural landscape in Europe. This landscape type is characterised by a mosaic of grasslands, cultivated fields, open shrublands and woodlands (Vos and Meekes, 1999). Many open landscape ecosystems developed due to prolonged human activities and human interaction with nature (Vos and Meekes, 1999). Often, these anthropogenically created areas are characterised by high diversity in plants and animals (Phillipps, 1995; Vos and Meekes, 1999; Haberl et al., 2004). Besides their ecological value, they offer a special cultural and aesthetical value as well (Phillipps, 1995; Vos and Meekes, 1999; Haberl et al., 2004). However, intensive afforestation activities, which started in the early 19th century, led to a decrease of many traditionally managed habitats (Hasel and Schwartz, 2002). In addition, both intensification of agriculture on the one hand and retreat

from unfavourable sites on the other hand have fragmented or actually replaced former open grasslands and open shrublands and with this their typical species assemblages (Hunziker and Kienast, 1999; Bender et al., 2005). On these accounts, cultural landscapes are nowadays often facing a high need for nature conservation since they offer habitats of rare and endangered species (e.g. De Klemm, 1985; Amanatidou, 2005).

Juniper stands constitute typical cultural landscape ecosystems. They exhibit a unique landscape character and are valuable components of the landscape in terms of culture, aesthetics and recreation. *J. communis,* the species of interest, has been of particular importance for humans for hundreds of years. Its wood has been used for making fire, to cure meat and as timber for arts and crafts. The fruits have been employed to flavour food and alcohols and the wood, the needles as well as the cones have been utilised in medicine (Hegi, 1935; Bolliger et al., 1983).

J. communis is an evergreen, dioecious gymnosperm (Cupressaceae). In the Holarctic, it represents the conifer taxon with the largest distribution area, ranging from North America to most of Europe (except the Azores, Balearic Islands and Crete), North Africa and North Asia up to the Himalaya (Thomas et al., 2007) (Fig. 1). It is known to have a broad ecological and physiological amplitude (Thomas et al., 2007). Being a pioneer species, juniper grows mainly as a coloniser on raw soils from the subarctic tundra down to semideserts on both limestone and acidic soils (Farjon, 1998). Its distribution reaches from sea level up to the timberline (Aas and Riedmiller, 1987). Light availability is the only restriction in terms of habitat demands (Ellenberg et al., 1991). Consequently, juniper has to compete with other woody plants, especially shade-casting trees, which results in succession and distribution turn-by-turn with the shade-casters (Iversen, 1954). Although it is wind-dispersed, juniper pollen is assumed to be more or less locally deposited (Huntley and Birks, 1983). For seed dispersal, birds are considered the most important dispersal agents (Hegi, 1935; Turcek, 1961; Livingston, 1972). However, seeds can also be dispersed by wind, gravity, hares and sheep (Vedel, 1961; Thomas et al., 2007). Estimating the age of juniper can be difficult since branches often break apart with age, building multi-stemmed bushes (Rodwell, 1991). Sometimes the decumbent branches can root, which leads to clonal-like structures (Ward, 1973; Clifton et al., 1997). Nevertheless, juniper is known as a long-lived species with an average age of 100 to 200 years (e.g. Clifton et al., 1997; Ward, 2007). Moreover, individuals with an age of approximately 1000 years are described (e.g. Hegi, 1935; Kallio et al., 1971).

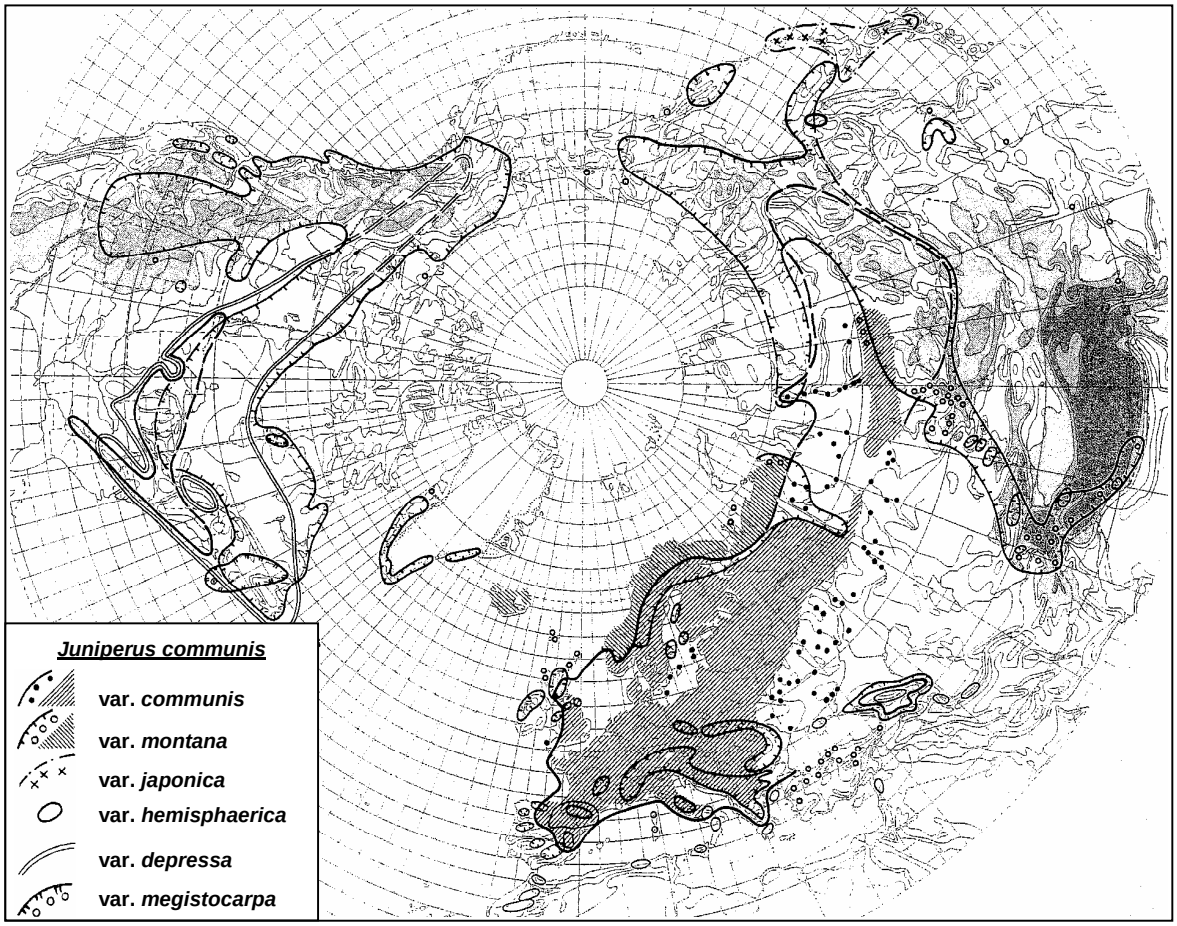

Fig. 1: Global distribution of *J. communis* (modified from Hultén and Fries, 1986).

Since 1992, *J. communis* communities are listed in Annex I of the EU-Habitat Directive (code 5130) in order to protect and conserve juniper stands as habitats with a Europe-wide specific relevance. In the meantime, *J. communis* is also recognised at the European Forest Genetic Resources Programme (EUFORGEN, see homepage http://www.bioversityinternational.com/networks/euforgen/). On a national scale, e.g. in Germany, juniper has already been protected by the Federal Nature Conservation Act since 1936. However, despite many efforts, juniper populations still decline in many parts of Europe (reviewed in Verheyen et al., submitted).

J. communis suffers from multifarious problems in Europe, especially in cases when it grows in semi-natural and therefore open landscapes created by traditional human land use practices. The following points are probably the most influential problems:

1. Formerly continuous and widespread juniper populations of the cultural landscape in Europe are nowadays highly fragmented and thus divided into several small and spatially isolated remnants (e.g. Pott and Hüppe, 1991; García et al., 1999; van der Merwe et al., 2000; Oostermeijer and de Knegt, 2004; Verheyen et al., 2005; Thomas et al., 2007; Provan et al., 2008; Verheyen et al., submitted).

2. In numerous European countries juniper is faced by a virtual lack of natural regeneration (e.g. Ward, 1982; Hüppe, 1995; García et al., 1999; van der Merwe et al., 2000; Oostermeijer and de Knegt, 2004; Verheyen et al., 2005; Provan et al., 2008; Verheyen et al., submitted).

3. A high number of seeds does not harbour viable embryos. Even more, many seed coats are empty in different populations all over Europe (e.g. García, 2000; Clifton et al., 1997; Falke, 2002; Verheyen et al., submitted).

Since the 1980s, the importance of genetic factors in conservation biology has been greatly emphasised (Frankham et al., 2002). At least since the United Nations adopted the Convention on Biological Diversity (CBD) at the Conference on Environment and Development in Rio de Janeiro (UNCED, 1992), genetic diversity, as one of the three fundamental levels of biodiversity, has played a major role for nature conservation purposes. Thus, the new scientific discipline ‘conservation genetics’ with its central dogma ‘genetic variability and hence genetic variation is beneficial’ has emerged (Pertoldi et al., 2007). Within this field, which is based on widely accepted population genetic theories (in the following also referred to as conservation genetics paradigm), researchers are looking at the effects of habitat fragmentation on the genetic diversity and structure of populations and at the dynamics of adaptation to new environmental conditions (Frankham et al., 2002; Freeland, 2005; Lowe et al., 2006; Ouborg et al., 2006; Pertoldi et al., 2007).

Within the present thesis I used different DNA marker based systems to gain insights into the genetic diversity and structure of various juniper populations on different spatial scales in Europe. Besides strengthening the knowledge about juniper with regard to habitat fragmentation, my aim was to use the scientific results in order to evaluate the analysed populations on the regional scale in terms of nature conservation purposes. Moreover, my intention was to develop a sustainable, demographically and genetically substantiated restoration management plan for juniper populations, which suffer from a lack of natural

regeneration. The compiled guidelines and recommendations should focus on planting activities and shall contribute to the maintenance of juniper populations as valuable components of the cultural landscape in Europe.

With regard to a conservation management for juniper and hence to planting activities, the detailed goals of my scientific research were as follows:

1. Using an AFLP (Amplified Fragment Length Polymorphism) marker approach on a large geographical scale, I tried to reconstruct aspects of the biogeographic history of juniper and to detect refugial gene pools and/or distinct genetic lineages (chapter 2 and paper I). For genetically based and species-specific plantings it is recommended to know about the large-scale geographical pattern of genetic lineages and with this the pattern of genetic variants, which result from historic processes. Regions containing the same genetic variants are deemed to form 'seed transfer zones' or regions within which plant material can be moved without negative consequences on population fitness (Gömöry et al., 1998; Hufford and Mazer, 2003).

2. In order to maintain the level of genetic diversity and to prevent inbreeding and/or outbreeding depression by planting activities, the genetic variation and structure of the respective populations have to be investigated (see e.g. Barrett and Kohn, 1991; Frankham et al., 2002; O'Brien et al., 2007). Therefore, I carried out a comprehensive nuclear microsatellite marker analysis (also called nuclear Simple Sequence Repeat marker or nSSR marker analysis) in eight relict juniper populations on a regional scale. The precise location of those populations is in the 'Rhenish Uplands', a mountainous region in West Germany (chapter 3.3 and paper III). Additionally, my aim was to investigate potential genetic effects of the recent habitat fragmentation within this region. For this analysis I developed specific nSSR markers for *J. communis* (chapter 3.1 and paper II) and I performed a detailed validation of these markers (chapter 3.2 and paper II, III) within this thesis.

3. I also carried out a case study by analysing the genetic diversity and differentiation of various single tree progenies (juniper embryos) in the Rhenish Uplands. Under the assumption of locally restricted pollen flow I expected a genetic bottleneck from the adult to the filial juniper generation (chapter 3.3 and paper III) and a high level of differentiation between different pollen clouds to be reproductively effective in the embryos (chapter 3.3

and paper III). To analyse different half-sib embryo families, a special computer software called Swap was developed within this thesis (paper III). The aim of the programme is to sample the paternal alleles from the embryo genotypes, particularly for cases where an embryo has the same heterozygote genotype as its mother. In addition to the genetic analysis, pollen grains deposited in moss cushions at different distances from a male donor were counted. On the one hand, the analyses concerning juniper pollen should once again give an indication of potential genetic effects of habitat fragmentation. On the other hand, they should give an idea about physical pollen flow distances. In terms of plantings these could be relevant to the spatial organisation of already existing juniper individuals and the respective plant material (chapter 3.3 and paper III).

2 Biogeographic history of *Juniperus communis* in Central Europe: historic fragmentation effects on a large spatial scale

Biogeographic analyses concentrate on the former and recent spatial distribution patterns of plant and animal taxa and try to relate these patterns with the history of the Earth. In addition, biogeography focuses on processes of populations, biocenoses and biomes. It overlaps and complements various scientific disciplines like e.g. geography, climatology, palaeontology, palynology, community ecology, systematics and evolutionary biology, which includes phylo- and/or population genetics. The climatic oscillations of the Quaternary (since ~ 2.4 myr) have been one of the main drivers for the recent distribution patterns of plant and animal taxa on a large geographic scale (Stebbins, 1950; Hewitt, 1996). The glacial periods led to repeated range contractions, mainly into suitable southern and eastern refugia (Bennett et al., 1991; Avise, 1992; Hewitt, 1996). Some argue that during the long-term isolation within these glacial refugia multiple genetic bottlenecks with further genetic drift and ensuing genetic differentiation resulted in distinct genetic lineages and even in speciation (Hewitt, 1996). Further on, it is supposed that during interglacial periods the refugial genetic lineages expanded and recolonised e.g. Central Europe via successive founder events, mostly from diverse directions (Hewitt, 1996; 2000; Petit et al., 2003). In circumstances where species did not migrate or withdraw to more suitable areas during glacial periods, they had to cope with drastic climatic and environmental changes. This was probably achieved by recruiting genes or gene combinations from the gene pool, which increased fitness under the new environmental conditions. If they did not succeed, they became extinct (Fisher, 1930; Hewitt, 1996; 2000; Templeton, 2001; Bettin et al., 2007).

Nowadays, different genetic marker systems like e.g. chloroplast (cp), mitochondrial (mt) and/or AFLP markers contribute to the reconstruction of species-specific scenarios during and after the last glacial period (e.g. Petit et al., 2002; Schönswetter et al., 2005; Magri et al., 2006; Cheddadi et al., 2006; Bettin et al., 2007). These DNA markers offer the possibility to reveal the developed range-wide distribution patterns of genetic variants or rather genetic lineages. These genetic lineages indicate the historic genetic processes described above during the time of survival in refugia and during the recolonisation.

In Europe, the bio- and/or phylogeographic histories of several tree species have been more or less reconstructed with the help of palaeobotanic and genetic analyses (e.g. Petit et al.,

2002; Magri et al., 2006; Cheddadi et al., 2006). The '*tabula rasa* hypothesis' is supposed to apply to these species during the Last Glacial Maximum (LGM) in Central Europe. This hypothesis suggests the complete absence of plant and animal taxa in Central Europe during the last glacial period and successive recolonisation from refugia outside the main glaciated areas (Nordal, 1987). Indeed, for e.g. white oaks as well as for Common Beech and Scots Pine it is shown that they contracted to different southern and eastern refugia (e.g. Petit et al., 2002; Magri et al., 2006; Cheddadi et al., 2006). Also for other woody species such as Heather, Goat Willow and Silver Birch the *tabula rasa* hypothesis may hold true, although charcoal remains and/or genetic analyses allow the assumption that these cold-tolerant species did not contract to the common southern refugia (Rendell and Ennos, 2002; Palmé et al., 2003a; b). Apart from the range contraction into diverse refugia, the range expansion also proceeded in a species-specific way. Thus, e.g. several oak species recolonised Central Europe from Mediterranean as well as from eastern refugia (Petit et al., 2002), whereas e.g. *Fagus sylvatica* and *Pinus sylvestris* recolonised Central Europe mainly from eastern refugia (Magri et al., 2006; Cheddadi et al., 2006).

Juniper displays a high ecological and physiological plasticity (Thomas et al., 2007). This is mainly reflected by its recent distribution pattern. Thus, juniper currently grows e.g. within Mediterranean regions but also within discontinuous and continuous permafrost areas (Fig. 1) (Hultén and Fries, 1986; Thomas et al., 2007). This could lead to the assumption that this species did not withdraw to the traditionally described southern and eastern refugia during the LGM. However, there are no fossil remains supporting this assumption. Therefore, I performed an AFLP marker approach to reconstruct the behaviour of juniper during and after the last glaciation and at the same time to test for potential refugial gene pools or rather distinct genetic lineages and for potential recolonisation routes through Europe (paper I).

On the European scale the following questions were addressed:

1. Did the cold-tolerant woody species *J. communis* contract to the traditionally described southern and eastern refugia during the LGM?
2. Did juniper recolonise the Rhenish Uplands from diverse refugia?

I analysed a total of 23 populations originating from six European countries (Spain, Germany, Italy, Lithuania, Poland, Slovakia) with an AFLP marker system using the primer-enzyme-

combination *Mse / Pst* (Vos et al., 1995; van der Merwe et al., 2000). The main focus of the populations investigated was on Central Europe with 15 populations (Fig. 2).

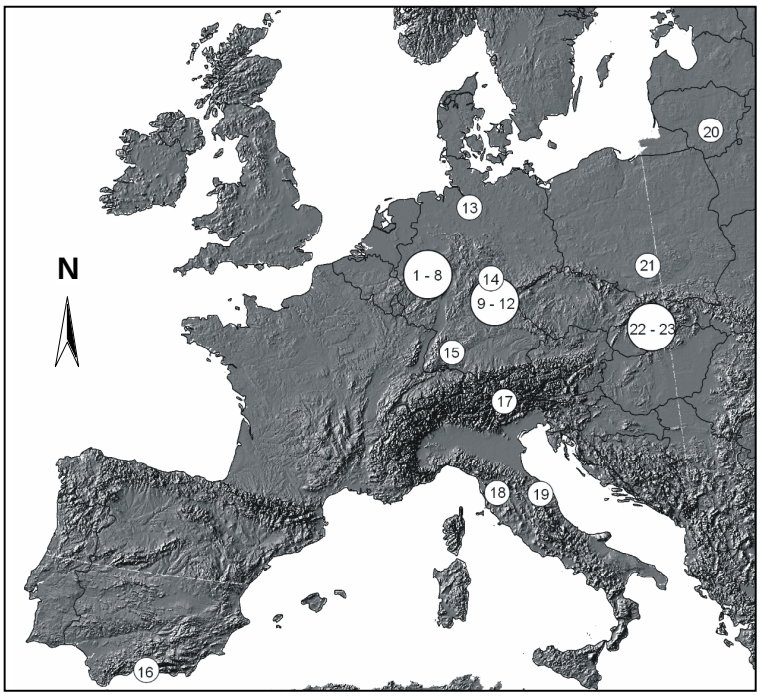

Fig. 2: Sampled juniper populations in Europe. The numbers within the circles indicate the population numbers. Populations 1 - 15 originate from Germany (1 - 8 Rhenish Uplands, 9 - 12 Franconian Alb, 13 Lower Saxony, 14 Thuringia, 15 Swabian Alb), 16 from Spain (Andalusia), 17 - 19 from Italy (17 South Tyrol, 18 Tuscany, 19 Marche), 20 from Lithuania (Kauno), 21 from Poland (Slaskie) and 22 - 23 from Slovakia (22 Liptov, 23 Zvolen).

Among all European populations I detected substantial genetic differentiation (Φ_{pt} = 0.413***). This phenomenon might give a hint for the contraction of juniper into southern and/or eastern refugia during the last glaciation (e.g. Hewitt, 1996). However, although each population clustered distinctively in a Principle Coordinate Analysis (PCoA), which explains the high Φ_{pt} - value, there was no consistency in the clusters with the geographical distribution of the populations (Fig. 3). For example, the German populations from the Rhenish Uplands (dark blue) and the Franconian Alb (red), respectively, did not cluster together, whereas the population from Spain (pink) clustered with the two populations from Slovakia (light blue) (Fig. 3). In addition, neither a Bayesian approach nor geostatistic procedures showed a meaningful coherence between the genetic pattern and the geographical origin of the populations. These results suggest a lack of underlying phylogeographic signals. Therefore, I neglect the *tabula rasa* hypothesis for juniper during the LGM. Consequently, no post-glacial recolonisation routes in Central Europe could be detected or rather described for this species.

Thus, the question concerning different recolonisation routes in the Rhenish Uplands becomes irrelevant.

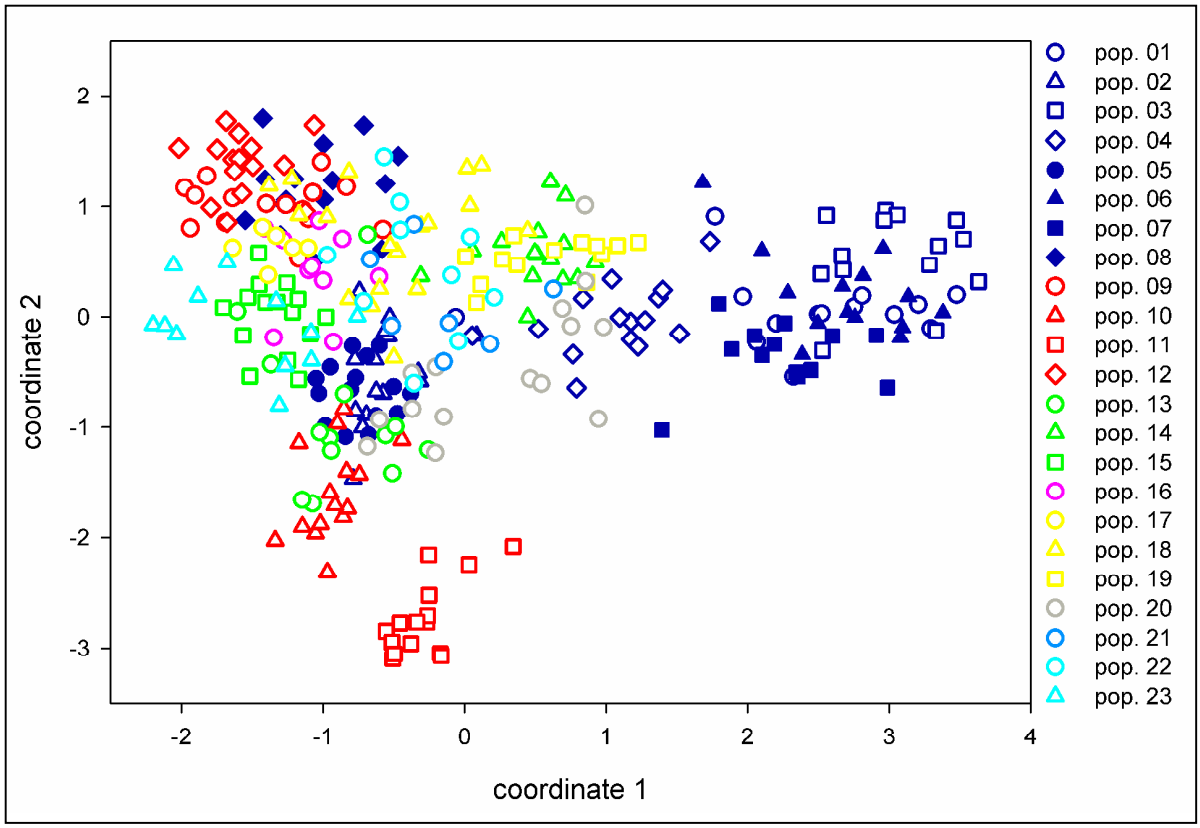

Fig. 3: Principal Coordinate Analysis (PCoA) of 23 juniper populations from Europe based on AFLP data. The first two axes are plotted. The first axis represents 33.9 % of the total variation and the second 16.1 %. The population numbers indicate the origin of the populations: 1 - 15 Germany (1 - 8 Rhenish Uplands, 9 - 12 Franconian Alb, 13 Lower Saxony, 14 Thuringia, 15 Swabian Alb), 16 Spain (Andalusia), 17 - 19 Italy (17 South Tyrol, 18 Tuscany, 19 Marche), 20 Lithuania (Kauno), 21 Poland (Slaskie) and 22 - 23 Slovakia (22 Liptov, 23 Zvolen).

Based on the present AFLP analysis and on knowledge about the species' ecology and its life-history traits (Thomas et al., 2007) as well as on palynological data (e.g. Godwin, 1975; O'Connell et al., 1999; van der Hammen, 1951; Bertsch, 1961), I rather assume an alternative scenario for juniper in Central Europe during the last glacial period: juniper could have survived throughout Central Europe in several small and suitable habitats which were probably scattered diffusely and varied in size and geographical area. These habitats were probably connected via effective gene flow. New populations were probably founded by a random sample of seeds, originating from the remnant populations. Based on fossil records, a similar scenario for glacial persistence has already been declared for various thermophilous tree species in Eastern Europe, Hungary and Moldova (Willis and van Andel, 2004).

Mantel tests between the pairwise genetic distance and the pairwise geographic distance matrices revealed only a marginal indication of isolation-by-distance on the European scale (r_{xy} = 0.108***). These results corroborate the assumption of the glacial persistence of juniper throughout Central Europe. If juniper recolonised Central Europe from isolated refugia, one would expect to see a large isolation-by-distance effect on the European scale (see e.g. Dumolin-Lapègue et al., 1997). In contrast to the European scale, I detected considerably strong isolation-by-distance effects for the populations from the Rhenish Uplands (r_{xy} = 0.249***) and the Franconian Alb (r_{xy} = 0.565***). This points to genetic drift and reduced gene flow, which was probably caused by recent habitat fragmentation. However, since AFLP markers are dominant markers, genetic drift due to a loss of heterozygosity is not measurable (Meudt and Clarke, 2007). Therefore, I applied different nSSR markers in an ensuing genetic analysis (see chapter 3.3 and paper III).

Besides the assumed scenario concerning the glacial persistence in diffuse scattered habitats, I suppose that the formerly coherent gene pool of the highly light-demanding juniper across Europe has been affected by recurrent fragmentation and founder events up until today. I assume that the possible drivers for recurrent fragmentation events were and still are not only the last glaciation but also the succession of shade-casting trees such as *Pinus*, *Picea*, *Quercus* and *Fagus* (Firbas, 1949; Lang, 1994) as well as human activities (Pott, 1996; Küster, 1999; Mertz, 2000; Aas, 2003). Juniper may have survived at exceptional sites such as rock ledges, clearings or as an understorey in parts of the forests that did not form completely closed canopies. These habitats could have harboured founder populations, which then regained a wide distribution in Central Europe during different periods of forest usage or even devastation by man during e.g. the Bronze, Iron and/or Middle Age (Firbas, 1949). These assumptions are in line with palynological data from e.g. the Rhenish Uplands where Speier (1994; 1999) identified various occurrence intensities of juniper pollen grains since the Young Dryas (10.000 BP).

3 Population genetic analysis in the Rhenish Uplands: recent fragmentation effects on a regional scale

For population genetic analyses nuclear microsatellites or nuclear simple sequence repeats (nSSRs) are the best marker of choice (Vendramin et al., 2004). However, no such markers were available at the beginning of this thesis – neither for the genus *Juniper* nor for the species *J. communis*. As genetic markers, only AFLP, RAPD and allozyme marker systems were applied to *Juniperus* species at that time (e.g. van der Merwe et al., 2000; Adams and Pandey, 2003; Oostermeijer and de Knegt, 2004). Therefore, I developed specific nSSR microsatellite markers for *J. communis* (paper II). Afterwards, I used these markers for a population genetic analysis on a regional scale (paper III).

3.1 Development of species-specific nuclear microsatellite (nSSR) markers

Nuclear microsatellite markers are powerful tools to analyse the genetic diversity within and the genetic differentiation among populations. Moreover, they are widely applied in studies concerning spatial family structures, pollen- and seed-mediated gene flow as well as paternities and/or parentages (Vendramin et al., 2004). For these purposes, the markers should be able to discriminate between individuals and therefore to distinguish genotypes and clones (genetic fingerprint) (Vendramin et al., 2004; Lefort et al., 2000; Gomez et al., 2001). nSSRs can be found all over the genome. They are characterised by multiple repetitions of specific basepair motifs (1 - 6 basepairs in series), their high variability and therefore their high number of alleles per locus. The different alleles per locus can vary in the number of repetitions and thus in length (fragment length analysis) from one individual to another (Tautz, 1989). In most cases nSSRs are species-specific DNA markers. However, sometimes they can be transferred from one species to another of the same genus (e.g. genus *Quercus* (Steinkellner et al., 1997), genus *Abies* (Cremer et al., 2006)). Only in exceptional cases they can be used between different genera (e.g. genus *Quercus* and *Castanea* (Steinkellner et al., 1997) or genus *Populus* and *Salix* (Hanley et al., 2002)).

In order to develop nSSR markers for juniper, a microsatellite library enriched for di- (GA, GT, AT, GC), tri- (CAA, ATT, GCC) and tetranucleotide (GATA, CATA, ATAG) repeats was constructed, following the method described by Edwards et al. (1996). Afterwards a total of 215 clones were randomly chosen from the library and sequenced in one direction. In 79 % of

these sequences, a microsatellite motif could be detected. However, I discarded many of these sequences immediately as they revealed undesirable properties like extremely long and compound-interrupted SSR stretches (20 %), SSR stretches too close to the vector (20 %) and stretches that were too short (4 %). After sequencing the reverse direction of the remaining candidate clones, 38 primer pairs were designed for specific polymerase chain reaction (PCR) amplification of the loci. Only five nSSR loci showed scorable polymorphic bands and were therefore published together with the respective primer pair sequences. In contrast, the remaining 33 loci showed no amplification, multi-banding patterns, stutter that was too pronounced and/or monomorphic bands.

3.2 Validation and application of the newly developed nSSR markers

The nSSR analysis of 28 juniper individuals sampled in Spain, Germany and Slovakia did not reveal any significant case of linkage disequilibrium and a very high rate of polymorphism for all five loci (paper II). Thus, at first sight, all newly developed nSSR markers seemed to be useful for population genetic analyses.

However, based on a case study with approximately 300 samples, I decided to use only four nSSR markers for the following population genetic analysis (Jc 31, Jc 32, Jc 35, Jc 37). The decisive factor was that the locus Jc 16 showed various 'three-allele-banding patterns' in several cases, even after repeating whole PCR procedures. Juniper is known to be diploid (Thomas et al., 2007) and the remaining loci supported this by showing one- and two-banding-patterns (homozygotic and heterozygotic genotypes). Therefore, for the respective juniper individuals, I assume additional primer binding sites for the primer pair Jc 16. These primer binding sites could have resulted from mutations which have not occurred in all individuals. To gain deeper insights into this subject, many sequencing reactions would have to be carried out. The sequences then would have to be analysed for the respective primer annealing sites and the microsatellite motif (GT-repeats).

For the validation of the four-loci marker system (Jc 31, Jc 32, Jc 35, Jc 37) (paper III), I performed a test of reproducibility with a subsample of 25 individuals. The genotyping error was 0 % when amplifying DNA templates twice. However, I did not obtain PCR products from all templates. After genotyping 889 adult juniper samples (sample size of the regional population genetic analysis, see chapter 3.3 and paper III) the proportion of missing data was 2.69 % in total and 9.57 %, 0.27 %, 0.13 % and 0.8 % at the four loci Jc 31, Jc 32, Jc 35 and

Jc 37, respectively. Null alleles, which are supposed to introduce a bias into population genetic analyses (Dakin and Avise, 2004; Chapius and Estoup, 2007), occurred at all loci. They arise from mutations in the primer annealing site, causing amplification failure and leading to falsely interpreted heterozygote deficiencies (Jarne and Lagoda, 1996). Vice versa, 'real' heterozygote deficiencies, which are caused by population genetic processes like e.g. genetic drift and inbreeding, can be misinterpreted as null alleles (Chakraborty et al., 1992). Since population geneticists assume that different loci behave more or less concordantly, this misinterpretation can be avoided by excluding the respective loci (Dakin and Avise, 2004). Given that the null allele frequencies of Jc 31, compared to the other three loci, were disproportionately high in all cases, I considered this locus to show 'real' null alleles. To explicitly explore the phenomenon of introducing a bias into population genetic analyses due to high null allele frequencies, I conducted all calculations of the following population genetic analysis on the regional scale with four-loci genotypes as well as with three-loci genotypes where Jc 31 was omitted (see chapter 3.3 and paper III). Interestingly, all diversity parameters (mean allele number per locus, allelic richness, expected heterozygosity) were similarly high for both marker combinations. This implies that the four-loci genotype was nearly as informative as the three-loci genotype. Heterozygote deficiencies and fixed alleles were also detected for both marker combinations. However, after excluding Jc 31 these values decreased considerably. It is difficult to establish whether the low heterozygote deficiencies at the remaining three loci were caused by low null allele frequencies calculated for these loci or by 'real' deviations from the Hardy-Weinberg equilibrium (HWE), mainly because the algorithm of calculating null allele frequencies partly includes comparisons between H_o and H_e (e.g. software Cervus 3.0.3, Kalinowski et al., 2007). Moreover, deviations from HWE and fixation of alleles can arise from a sample size effect (Hedrick, 2000). Indeed, respective calculations of observed vs. expected frequencies of single-locus genotypes were linked to such a sample size effect in the dataset. This effect is possibly caused by the high number of alleles per locus (see e.g. Gregorius, 1980).

Since diversity parameters were similarly informative for both marker combinations and the average probability of identity (PI_{ave}, Ayres and Overall, 2004) was low enough to distinguish individuals properly, I considered the three-loci genotypes to be reliable. Since the slight heterozygote deficiencies and the fixation of alleles at the remaining three loci were probably caused by a sample size effect as mentioned above, I decided not to interpret these parameters in terms of deleterious effects of habitat fragmentation (see chapter 3.3 and paper III).

3.3 Genetic diversity and differentiation of eight *J. communis* populations in the Rhenish Uplands

As already mentioned, habitat fragmentation affects several different genetic and demographic processes and its extent depends mainly on the life-history traits of the respective species (Oostermeijer et al., 2003; Washitani et al., 2005; Lowe et al., 2005). It is assumed that habitat fragmentation often leads to restricted gene flow and increased genetic drift. This results in a reduction of genetic diversity, with the loss of rare alleles initially and loss of heterozygosity later on. There is also an ongoing fixation of alleles (Young et al., 1996; Templeton et al., 2001). If different alleles become fixed in different populations these will differentiate genetically from each other. Populations with low genetic diversity, i.e. with a limited gene pool, may be influenced by reduced fitness and may only have a slight chance to cope with changing environments successfully (Young et al., 1996; Templeton et al., 2001).

So far, several investigations on tree species concerning habitat fragmentation and its potential genetic consequences have been carried out (see e.g. review Lowe et al., 2005). Particularly for tropical angiosperm trees it was demonstrated that the isolation of populations and thus the disruption of breeding systems led to decreased gene flow, increased genetic drift and/or reduction of reproductive fitness (see e.g. review Lowe et al., 2005; Farwig et al., 2008; Hanson et al., 2008). In contrast, gymnosperm trees and especially conifers are thought to be less vulnerable to landscape fragmentation due to their life-history strategies such as wind pollination, efficient seed dispersal via wind and/or animals and longevity (Hamrick and Godt, 1996; see e.g. O'Connell et al., 2006; Williams et al., 2007). However, even for this tree classification deleterious genetic effects of habitat fragmentation could be found (e.g. Wang et al., 2005; Robledo-Arnuncio et al., 2004; Kettele et al., 2007).

In the Rhenish Uplands, juniper stands are highly fragmented and harbour only small relict juniper populations. Many of these populations suffer from a lack of natural regeneration. They do not show different ontogenetic stages but many multi-stemmed individuals. Therefore, I assumed at the beginning of the study that they were even- and over-aged. In a previous study I conducted tests on the viability of juniper embryos in populations of the Rhenish Uplands. The results indicated almost a complete absence of viable seeds for all populations (0.7 - 2.3 % viable seeds). In the meantime, these results have been integrated in a Europe-wide study concerning various reproduction and regeneration problems of juniper along a latitudinal gradient (see Verheyen et al., submitted).

In order to investigate potential genetic effects of the recent habitat fragmentation on eight relict juniper populations in the Rhenish Uplands, I used three and four of the five newly developed nSSR markers, respectively (paper III; see chapter 3.2 and paper III for explanations concerning the different marker combinations). Furthermore, I analysed single tree progenies at the same nSSR loci (paper III). With regard to the future restoration management, a palynological study was conducted to gain insights into physical pollen flow distances (paper III).

On the regional scale, I worked mainly on the following questions:

1. Are genetic effects of habitat fragmentation detectable in the eight adult populations of the Rhenish Uplands?
2. Is the genetic diversity of single tree progenies reduced compared to that of the adult generations?
3. Is genetic differentiation detectable among different pollen clouds?
4. Is the physical distance of juniper pollen flow locally restricted?

I collected needles from adult junipers of eight relict populations in a random grid design (Fig. 4). This grid design allowed me to detect clonal and/or family structures and it was used to estimate densities of juniper populations. As a clone and genet, I considered all procumbent branches originating from one single individual (genotype), regardless of whether these branches and ramets had roots or not. I recorded all sampled junipers bearing cones as female and all those without cones as potentially male. In order to analyse the filial generation genetically, I selected ten mother trees from two of the juniper stands (five from each population, Haberg and Heidbuechel) and harvested 150 dark blue cones from each mother tree. Four females grew at the margins and one in the centre of each juniper stand. To determine the physical distance of juniper pollen flow, a set of moss cushions was collected on a transect situated inside and outside of one juniper stand (Alendorfer Kalktriften). The pollen grains deposited within these moss cushions were counted and juniper pollen grains as proportion of the pollen sum (arboreal and non-arboreal pollen grains) as well as the concentration of juniper pollen grains per gram (after adding a known number of *Lycopodium* spores) were calculated. In addition, the distances between the investigated moss cushions and the respective nearest male juniper were measured.

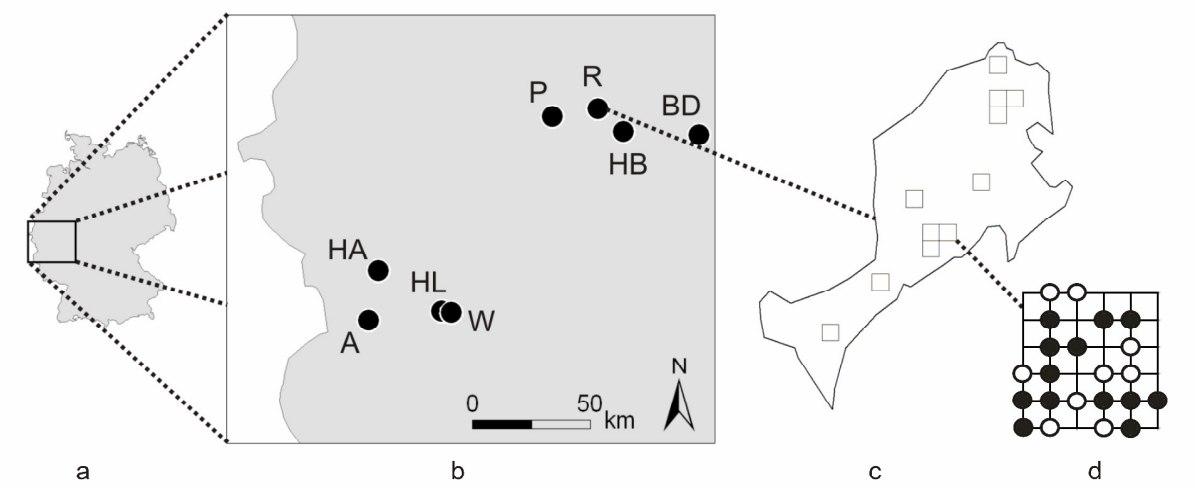

Fig. 4 (a - d): a: The location of the Rhenish Uplands in Germany. b: Analysed juniper populations in the Rhenish Uplands. The letters indicate the population names: A: Alendorfer Kalktriften, BD: Battenfelder Driescher, HA: Halsberg, HB: Haberg, HL: Heidbuechel, P: Piwittsmoor, R: Ruebenkamp, W: Waberner Heide. c: Position of 15 x 15 m plots (derived from the grid design) in the stand Ruebenkamp (R). d: One representative plot with 3 x 3 m grid point samples, where empty circles indicate female junipers and filled circles potential male junipers.

The average probability of identity calculated with the three- as well as with the four-loci genotypes was very low for all populations. Thus, clones could be properly identified. After clone identification and exclusion of all ramets of a genet except one, I was able to determine the real number of individuals sampled. Thus, I could calculate the number of juniper individuals per hectare and the sex ratio (female : male). The population density varied from 182 to 634 individuals per hectare in the populations Haberg and Piwittsmoor, respectively. In five populations the sex ratio had a male bias and ranged from 1 : 1.16 to 1 : 1.4 for the populations Halsberg and Ruebenkamp, respectively. In one population the sex ratio was balanced (1 : 1, Alendorfer Kalktriften) whereas in two populations there were more females than males (1 : 0.83 and 1 : 0.92 for the populations Battenfelder Driescher and Waberner Heide, respectively).

I detected a very low but significant global genetic differentiation value (F_{ST} = 0.014***) combined with high allelic richness (R_S over three loci varied from 18.67 (Haberg) to 22.08 (Piwittsmoor)) and a lack of recent genetic bottlenecks in all eight juniper populations. Based on these results, I hypothesise that the recent habitat fragmentation and therefore the reduction of habitat sizes, which started approximately in the early 19th century, has not yet affected the genetic diversity of all eight populations in a negative way. The results of the

conducted Mantel test rather point to a scenario where the detected genetic diversity and differentiation have been 'frozen' since the fragmentation started. This is probably the case because the detected absence of an isolation-by-distance effect and the relatively low and equal pairwise F_{ST} - values reflect a genetic structure, which usually appears shortly after a genetically homogeneous population has invaded a new area and where gene flow predominates genetic drift (Hutchison and Templeton, 1999). Thus, we may look back at a time when juniper regained the Rhenish Uplands by the invasion of individuals, originating from several small or one large founder population. Especially where habitat fragmentation occurred within a life-span of one generation, such 'freezing-effects' have already been reported for other tree species (e.g. Young et al., 1993; Aldrich et al., 1998; Lowe et al., 2005; Williams et al., 2007). For juniper, this phenomenon has also been suggested to occur in populations from England and from the Netherlands (van der Merwe et al., 2000; Oostermeijer and de Knegt, 2004).

I subjected the same eight juniper populations of the Rhenish Uplands to the AFLP marker approach (chapter 2 and paper I) as well as for the nSSR analysis. However, for the AFLP analysis I used only a subsample of each population. As stated earlier, I performed a Mantel test for both marker systems. Since the Mantel test of the AFLP data showed a significant isolation-by-distance effect for the Rhenish Uplands and the one of the nSSR data did not, these results seem to contradict each other at first sight. However, the contrary results are most likely based on the different characteristics of the marker systems I used. As opposed to nSSR markers, AFLP markers may include phylogenetic signals and loci that are relevant for selection as well (Meudt and Clarke, 2007).

Although habitat fragmentation has not yet produced genetically deleterious effects in the adult generations, this may still happen in future generations, especially if pollen deposition is locally restricted as declared by Huntley and Birks (1983). The palynological study included in this thesis indeed confirmed low physical pollen flow distances for juniper. A steep decline of juniper pollen grains as proportion of the pollen sum and as concentration of juniper pollen grains per gram with distance from a male donor was detected. At a distance of 5 m from the pollen source, there was a reduction of one third of the pollen grains (from 45 % to 15.2 % and from 33294 to 11275 pollen grains at 0.5 m and 5 m, respectively). After approximately 100 m only 1 % (759) of juniper pollen grains was found in the moss cushions.

However, the analysis of the reproductively effective pollen clouds of single tree progenies that were pooled did not corroborate the hypothesis of reduced genetic diversity in the filial generations. Allelic richness was similar in comparison to the respective adult generation. I could not detect any significant genetic differentiation among the adults and the respective filial generation. Indeed, I found significant genetic differentiation among the different pollen clouds in the Heidbuechel population. This was not the case in the Haberg population. Both populations showed similar densities but more and larger prostrate junipers occurred in the stand Heidbuechel, which is also where significant genetic differentiation emerged. Presumably, the genetic differentiation between different pollen clouds became reproductively effective through the overdominance of large pollen clouds produced by large male junipers in this population. This overdominance could possibly lead to the fixation of the respective father alleles and therefore to a reduction of genetic diversity in the filial generation.

Because of the low repetition number, the analysis concerning the single tree progenies and the palynological investigations represent only case studies. They can therefore not be used to generalise the results. Nevertheless, these analyses provide initial insights into the respective conditions and they offer valuable perspectives for ongoing hypotheses and studies (see chapter 5.2).

4 Integration of scientific knowledge into conservation measures

In the following, I use the scientific results of this thesis to evaluate the eight juniper populations of the Rhenish Uplands in terms of nature conservation purposes. Furthermore, I discuss the evaluation criteria and their quality demands with regard to the life-history traits and the hypothesis of the biogeographic history of juniper as presented in this thesis. Based on the results of my investigations, on what is known from the literature and on expert knowledge, I present guidelines and recommendations for a specific restoration management as a final outcome of this thesis.

4.1 Evaluation of *J. communis* populations for nature conservation purposes

To evaluate populations in terms of nature conservation a 'Leitbild' has to be defined first. Leitbild is a German word, which refers to a statement of a future desired state or situation. It was suggested to use this term also in the English scientific literature since there is no direct translation for both meanings of it, namely 'future desired condition' and 'vision' (Potschin and Haines-Young, 2003). A Leitbild should be composed of different parameters or criteria, which are highly valuable e.g. in terms of ecology and/or genetics. Furthermore, it should be possible to achieve the specified 'high quality' Leitbild in a specific area and in a conceivable time period (Uppenbrink and Knauer, 1987). The comparison between the predefined Leitbild with its given criteria and the actual state of the respective landscape, ecosystem or population, which results from data analyses, leads to the evaluation of the biological entity (Plachter, 1991).

4.1.1 Establishment of a Leitbild for *J. communis* populations

In the literature, no Leitbild has been defined for juniper populations so far. This is why I established one within this thesis. In order to develop a species-specific Leitbild and to evaluate the respective criteria of populations from a nature conservation perspective, it is necessary to know several reference values. The data required are mainly data from field studies, which are conducted in undisturbed populations and/or which assess the relationship between the respective criteria and fitness components, like e.g. reproduction success and/or survival rate (see e.g. Ouborg et al., 2006; Pertoldi et al., 2007). Since there are no such genetic data available for juniper yet, the presented Leitbild, or rather the respective criteria and their quality demands, follow the widely accepted conservation genetics paradigm (reviewed in Ouborg et al., 2006; Pertoldi et al., 2007). I selected the demographic criteria

and their respective quality demands following Ward (1982; 2007) and Dahr et al. (2008). While Ward worked on juniper and age-related criteria, Dahr and colleagues worked on different conservation strategies for *Taxus baccata.* This tree species has nearly the same ecophysiological characteristics and life-history traits as juniper and suffers from the same regeneration problems.

The Leitbild for J. communis populations is defined as follows:

Viable juniper populations with a chance of long-term survival under changing environmental conditions need to show different ontogenetic stages and especially natural regeneration. Their population size has to be adequate. The sex ratio should be more or less balanced. In order to evolve and adapt to changing environments, the amount of genetic variation has to be appropriately high. Consequently, a deviation from HWE, a fixation of alleles and an indication of a recent genetic bottleneck should not occur. There should be recent connectivity between the populations, at least via low rates of reproductively effective long-distance dispersal events (see also Mills and Allendorf, 1996; Bialozyt et al., 2006).

4.1.2 Evaluation of the analysed *J. communis* populations

Below, I evaluate the eight analysed juniper populations of the Rhenish Uplands in terms of nature conservation purposes. The actual state, which is based on the results of this thesis, is compared with the Leitbild that was defined above.

Different ontogenetic stages, natural regeneration and sex ratio

As mentioned earlier, I assumed that most of the juniper individuals of the eight analysed juniper populations at the beginning of the investigations were old, mainly because of their obviously senescent state. To determine the exact age of several juniper individuals, I took core samples and I used dendrochronological standard procedures (Schweingruber, 1983; Fritts, 2001). By tree ring counting, I dated most of the individuals to an age of approximately 30 to 50 years (data not shown in this thesis). However, I consider these results to be incorrect. I presume that in many cases, I did not investigate the main trunk because of the multi-stemmed morphology of the individuals and due to the fact that many procumbent branches and/or trunks were already partly rotted (see e.g. Rodwell, 1991). However, the genetic analysis pointed indirectly to a high population age. The occurrence of several juniper clones (multi-stemmed junipers) in the dataset and the male-biased sex ratio in five populations caused me to verify indirectly that the populations were old (see Falinski, 1980; Rodwell, 1991; Clifton et al., 1997). Moreover, in conjunction with the absence of natural

regeneration and the apparent absence of continuity in the ontogenetic stages, I consider them to be even- and over-aged. Suffering from a lack of natural regeneration and being over-aged can form an extinction risk in the distant future. This is because the dying-off of senescent individuals combined with a changing environment will probably lead to a loss of alleles and/or genotypes. As a result, fixation of alleles will occur and hence the ability to adapt to further environmental changes will be reduced more and more (Young et al., 1996; Templeton et al., 2001). Thus, at this time, I can not exclude a complete breakdown of over-aged clones and with this an extinction of the analysed juniper populations in the distant future. Therefore, I conclude that the survival of the analysed juniper populations based on the indications for being even- and over-aged (missing different ontogenetic stages, lack of natural regeneration, several multi-stemmed individuals, male-biased sex ratios) is uncertain, but it is most likely going to be threatened.

Genetic variation and recent genetic bottlenecks

To date, only one study has been conducted on the genetic diversity of *J. communis* by analysing nuclear microsatellite loci (Provan et al., 2008). Compared with that study and with population genetic analyses of related conifer species (e.g. O'Connell et al., 2006; Williams et al., 2007), I suppose that all genetic diversity parameters found within this thesis are considerably high. This, combined with the facts that there is no reduction of allelic richness in the filial generations, that there are no recent genetic bottlenecks in any of the eight populations and that there is no isolation-by-distance effect, led me to the assumption that the genetic diversity of all populations has so far not been reduced through the recent fragmentation events. However, I can not evaluate whether this situation ensures juniper's long-term survival, particularly due to the fact that none of the populations regenerate naturally. Therefore, analyses which correlate genetic variation and fitness parameters are indispensable (Ouborg et al., 2006; Pertoldi et al., 2007). Such analyses would possibly provide insights into a 'minimum amount' of genetic diversity responsible for successful reproduction and/or survival (see e.g. Hedrick and Kalinowski, 2000; Frankham et al., 2002; Frankham, 2005). However, with regard to the theory about an appropriate pool of genetic diversity to adapt to changing environments and with regard to the angle of the CBD and EUFORGEN, the detected high amount of genetic diversity must be considered to be highly valuable and highly worth protecting.

Deviation from HWE, fixation of alleles, connectivity via gene flow and population size

Since I decided not to interpret the parameters 'deviation from HWE' and 'fixation of alleles' due to the presence of a sample size effect and the presence of null alleles, which could have arisen from artificial problems, they can not contribute to the evaluation of the eight analysed juniper stands. Also the criterion 'connectivity via reproductively effective gene flow' can not be part of the evaluation because I did not analyse recent pollen- and/or seed-mediated gene flow among the populations directly via e.g. parentage analyses. Indeed, the regional genetic analysis in the Rhenish Uplands gave indications for gene flow among the populations. However, I considered this gene flow to be a historic imprint. An 'adequate population size' in terms of conservation genetics is characterised by a size, which allows for a 'minimum' amount of genetic diversity. It needs to be high enough to prevent inbreeding and/or inbreeding depression (e.g. Hedrick and Kalinowski, 2000; Frankham et al., 2002; Frankham, 2005). If population size falls below a certain threshold, genetic variation and with this the ability to adapt to changing environments will be lost inevitably (Young et al., 1996). For the Rhenish Uplands, I could not detect a significant relationship between the allelic richness and the size of the juniper stands (data not shown in this thesis). Moreover, there are currently no scientific studies available, which provide insights into a threshold of population size and amount of genetic diversity, since this would mean that juniper would be inbred, reduced in fitness and reduced in the ability to adapt to environmental changes. Therefore, it is not possible to use the criterion 'population size' for the current evaluation either.

Concluding summary

To sum up, the evaluation of the analysed eight juniper populations of the Rhenish Uplands based on the Leitbild established above is not satisfactory. At the moment, it is not possible to draw definitive conclusions about the long-term survival of juniper in the Rhenish Uplands. Several evaluation criteria could not be used due to the reasons mentioned. Only the criteria 'ontogenetic stages', 'natural regeneration' and 'genetic diversity' could be applied, although the detected amount of genet diversity could not be considered to be sufficient to ensure the survival of the populations. However, the evaluated populations are probably threatened by extinction and should therefore be protected and supported by nature conservation measures in order to ensure their survival.

4.2 Critical comments on the evaluation criteria with respect to the species *J. communis*

The criteria demanded above and their quality standards concerning the Leitbild and with this the evaluation of juniper populations from a nature conservation perspective are in accordance with widely accepted concepts of population ecology and population genetics (Frankham et al., 2002; Freeland, 2005; Lowe et al., 2006, Ouborg et al., 2006; Pertoldi et al., 2007). However, are these concepts really applicable to juniper? Moreover, is the achievement of the Leitbild criteria and their respective quality demands actually necessary for the survival of juniper? Maybe the quality demands are not valuable for this pioneer species because of its life-history traits and the biogeographic history that was postulated here.

Different ontogenetic stages, natural regeneration and sex ratio

At the moment, the sex ratio in five analysed juniper populations is male-biased. All eight populations seem to be even- and over-aged and do not show any natural regeneration. The question arises whether this situation *per se* forms an extinction risk or whether this is just a life-history strategy of juniper. Longevity is considered to be an alternative demographic strategy of juniper, next to sexual regeneration (García and Zamora, 2003). Maybe juniper is even using this strategy now to bridge a period of unfavourable conditions in the Rhenish Uplands (see García and Zamora, 2003)? Possibly, it will start with successful sexual regeneration again at some time in the future? If this should be the case, and if the genetic diversity will not have fallen below the above-mentioned threshold with all its negative consequences by then, the criteria 'different ontogenetic stages' and 'natural regeneration' are not useful for the evaluation. However, if juniper should not start with natural regeneration again, this would possibly present the most important extinction risk. This is particularly true in the case of severely changing environmental conditions, where a long-lasting absence of natural regeneration must be considered to be threatening (Young et al., 1996; Templeton et al., 2001).

Genetic variation, fixation of alleles, recent genetic bottlenecks and population size

And what about the genetic criteria and the criterion concerning population size? Is it really necessary for juniper to be genetically highly diverse? In fact, for long-term persistence, when adaptation will probably be necessary, a diverse gene pool is indispensable (Fisher, 1930; Templeton et al., 2001). However, what about the persistence for shorter periods and periods

in which the environment will not change dramatically? There are several examples where reduced diversity and resultant inbreeding as well as fixation of alleles were detected, but where inbreeding depression did not form a severe threat (e.g. Hoelzel, 1999; Russell et al., 2003; Picó et al., 2004). In fact, the opposite can even occur. Sometimes inbreeding leads to a positive effect by eliminating recessive alleles due to breeding with related individuals and ensuing natural selection (i.e. purging) (see review Byers and Waller, 1999). As mentioned before, I assume that juniper went through several genetic bottleneck situations due to recurrent fragmentation events in the past. Since it shows high genetic diversity and no recent genetic bottlenecks at present, I conclude the following: juniper has managed to cope with reduced population sizes and the accompanying reduction of genetic diversity for a while and it was also able to recoup from losses of genetic variation during expansion phases, probably due to new mutations (see Savolainen and Pyhäjärvi, 2007). Presumably, its life-history strategies 'longevity' and 'highly effective gene flow' via bird-dispersed seeds also contributed to the process of recovery (see e.g. Hamrick and Godt, 1996; Lowe et al., 2005; Thomas et al., 2007). Moreover, additional long-distance dispersal events may have occurred in the past when seeds were blown across the ground, especially in treeless, windy environments where the ground was covered by smooth frozen surfaces (Rendell and Ennos, 2002).

Deviation from HWE

Also, the deviation from HWE should not generally be regarded as a threat to the survival of populations. The fact that artificial circumstances, such as the presence of null alleles and/or sample size effects, can cause heterozygote deficiencies already reveals that this criterion is not appropriate for an evaluation (the same applies with fixed alleles) (Hedrick, 2000; Dakin and Avise, 2004; Chapius and Estoup, 2007). As mentioned before, I did not interpret and evaluate the detected deviations from HWE in terms of nature conservation. However, if there had been real heterozygote deficiencies for the analysed juniper populations, what assumptions could have been drawn from these findings? The concept of being in HWE is based on the conditions 'random mating', 'no natural selection', 'no mutation' and 'no immigration and emigration' (Hardy, 1908; Weinberg, 1908). Pertoldi and co-workers (2007) argue that population genetic models, which are based on equilibrium conditions, are not typically found in nature. I agree with this statement and postulate that the conditions for being in HWE are unrealistic or rather not necessary for juniper. As I assume that this species has contracted repeatedly to small fragmented habitats and has expanded again repeatedly from the last glaciation up until today, I consider a deviation from HWE to be quite normal for

juniper populations. Therefore, in my opinion, deviations from HWE do not *per se* mean an extinction risk for this species. Nevertheless, I can not exclude the possibility that juniper is able to come close to a new equilibrium some time after a fragmentation event and/or other genetic bottlenecks (e.g. changing environments, catastrophes), which have destabilised the former equilibrium (see e.g. Savolainen and Pyhäjärvi, 2007).

Connectivity via gene flow

The last evaluation criterion 'connectivity between populations via reproductively effective gene flow' should also be treated with caution. If the goal is to maintain a high level of genetic diversity, then gene flow among different populations is assumed to be of high value (Mills and Allendorf, 1996; Richards, 2000; Ingvarsson, 2001). However, if different populations are differently adapted in a heterogeneous landscape, then gene flow is not assumed to be valuable. It is even rather supposed to be detrimental because gene flow can disrupt the local adaptation and this results in a decrease in local average fitness (i.e. outbreeding depression) (see review Edmands, 2007). It has not been investigated so far whether juniper is locally adapted or whether it has the possibility to show a plastic response, which one might assume from its broad ecophysiological amplitude (see e.g. Pigliucci, 2005). Therefore, it is currently not possible to use the criterion 'connectivity via gene flow' for the evaluation in terms of nature conservation purposes.

4.3 Guidelines and recommendations for a specific restoration management

As stated earlier, it is uncertain at the moment whether the lack of natural regeneration in the Rhenish Uplands does indeed form a sort of extinction risk or whether juniper just uses it as a life-history strategy for now. Moreover, no one is able to predict whether juniper will start with natural regeneration before the above-mentioned complete breakdown of the remaining senescent individuals. Therefore, I currently consider a restoration management for the maintenance of juniper in the Rhenish Uplands to be indispensable. In terms of the conservation genetics paradigm, the CBD and the programme of EUFORGEN, this restoration management shall conserve juniper as a species in conjunction with all its genetic resources. Moreover, the conservation of the ecosystem 'juniper stand' as an important element of the cultural landscape in Europe shall be ensured with it.

The fact that old female juniper produce fewer viable seeds than younger females is already known (Ward, 1982). Thus, the first step of the restoration should be to rejuvenate the

populations by planting 'young' junipers. With this, I intend to rekindle the production of viable seeds. The second step should be the creation of promising germination sites to realise the reproduction events. It is not yet clear exactly how such germination sites should be created. However, several suggestions and ideas can be found in the literature (e.g. Oostermeijer and de Knegt, 2004; Verheyen et al., 2005; Thomas et al., 2007).

Here, only the first step of the restoration is considered. Thus, a detailed sustainable, demographically and genetically substantiated management plan concerning planting activities is listed below. All guidelines and recommendations are based on the results of this thesis and on expert knowledge. They can be transferred in entirety to other European regions where juniper is threatened also. However, a genetic screen with the nSSR markers that were presented here should be done first.

4.3.1 Guidelines and recommendations for the collection of plant material

a) I advise to use vegetative cuttings for plantings

Most juniper seeds in Europe are empty due to a variety of reasons (see e.g. García, 2000; Clifton et al., 1997; Falke, 2002; Verheyen et al., submitted) and the germination of juniper seeds is extremely difficult (Oostermeijer and de Knegt, 2004). Therefore, activities like harvesting seeds, sowing and germination of seeds would not be profitable. Thus, cuttings should be used.

b) Cuttings should be collected within specific regions

The Europe-wide AFLP analysis did not reveal any distinct historic genetic lineages. Thus, plant material can be collected and planted reciprocally throughout Europe. However, AFLP markers are considered to be neutral markers (Meudt and Clarke, 2007) and therefore do not account for local adaptation. In order to grant possible adaptation to e.g. edaphic conditions, aridity and/or solar radiation, I advise to harvest and interchange plant material only within regions with similar ecological and climatic conditions (Gömöry et al., 1998; Hufford and Mazer, 2003). These regions or seed transfer zones can be defined according to the conditions of provenance delimitations for tree species in Europe (for Germany see e.g. FoVHgV, 1994).

c) Cuttings should be collected gender-specifically

The nSSR marker analysis allowed the identification of clonal structures and therefore the identification of the real individual number sampled. Based on these results, male-biased sex

ratios were detected in five populations. Male-biased sex ratios in over-aged juniper populations are recorded several times (e.g. Clifton et al., 1997; Ward, 2007). However, the ultimate cause of differential mortality of female junipers is not yet fully understood (Ward, 2007). Nevertheless, I advise to balance the sex ratio by plantings. Therefore, cuttings should be collected gender-specifically and kept separately until planting. Due to the different flowering morphology, female junipers can be distinguished from males during the flowering period from April to June (Thomas et al., 2007). Afterwards, only females can be identified reliably since they bear cones.

d) Sampling in genetically depauperated populations and in clonal structures should be avoided

In order to prevent artificially induced genetic erosion or inbreeding and inbreeding depression via plantings, I advise to sample in genetically diverse populations (see e.g. Lynch, 1991; Hufford and Mazer, 2003). Moreover, I advise to avoid sampling in clonal structures. In doubtful situations sampling should be given up.

4.3.2 Guidelines and recommendations for the greenhouse

To achieve best rooting success, the following protocol should be used (Bolko Haase, personal communication). Root formation requires about one year. During this time conditions should be kept constant.

- The cuttings should be harvested from young junipers or young shoot, ideally 1 to 2 years old and 10 to 15 cm in length.
- Best harvesting time is from August until September.
- The cut surface should be treated with 5000 ppm 3-indolylbutyric acid solution for approximately 2 min.
- For sticking, the soil substrate should be a 50/50 mixture of peat and sand.
- The planting bed should be covered with a row cover (cloche) to guarantee high air humidity. However, a low powered sprinkler installation would be the best.

4.3.3 Guidelines and recommendations for plantings in the field

a) The cuttings collected within specific seed transfer zones should be planted within these zones again

To give consideration to a possibly adapted genomic background, the plant material collected within a specific region (or provenance) should be replanted within this region (Gömöry et al.,

1998; Hufford and Mazer, 2003). It is also possible to interchange cuttings from geographically different regions with similar ecological and climatic conditions.

b) The genetic diversity and structure of the target populations should not be falsified by cuttings, which offer completely different allele sizes

The allele sizes and the allele frequencies of the respective juniper populations are known due to the nSSR marker analysis. Since the genetic diversity of the analysed juniper populations in the Rhenish Uplands is considerably high, I advise to use cuttings, which offer already existing alleles to avoid potential outbreeding and/or outbreeding depression (Edmands, 2007). However, if the adult populations show very low amounts of genetic diversity and high frequencies of fixed alleles, it should be tried to balance this by cuttings, which show slightly different but not completely different allele sizes or rather moderate pairwise genetic distances compared to the adult junipers. If this procedure is adhered to and if there are reproduction and hence recombination events between old junipers and cuttings, the evolutionary potential of the populations could possibly be enriched.

c) It is advised to balance the sex ratio

For natural regeneration in the near future, young female junipers will be needed in the populations (Ward, 1982). Therefore, the sex ratio should be at least balanced by plantings. Actually, considering the relation between the age of females and the number of viable seeds (Ward, 1982) and the fact that only over-aged females are harboured in the analysed populations of the Rhenish Uplands, one could consider an increase of females through plantings.

d) Male junipers should be planted near females

The spatial organisation of juniper individuals inside the stands seems to play an important role for the successful reproduction and the genetic structure of the filial generation (Hopster and Greeve 1999; own results, see chapter 3.3 and paper III). Therefore, I advise to plant male junipers close to females. However, since the differentiation among the different pollen clouds in the population Heidbuechel may be due to an overdominance of single pollen clouds, which are probably descending from large, prostrate juniper individuals, a sufficient number of males should be planted in the neighbourhood of females.

5 Conclusions and perspectives

Below, I draw conclusions from the presented genetic analyses and the acquired knowledge with regard to the usability of the results for nature conservation purposes. Afterwards, I give several suggestions for further scientific analyses. Almost all of them aim at the transfer of scientific results to nature conservation measures. Since I consider the new era of 'eco-genomics' to be highly promising, this aspect is described in some more detail.

5.1 Imprints of habitat fragmentation in *J. communis* and the usability of the scientific results from a nature conservation perspective

The different DNA marker systems I used within this thesis only partially revealed genetic imprints of habitat fragmentation in *J. communis* on different spatial and temporal scales. On the one hand, along with other scientific knowledge about juniper, the Europe-wide AFLP analysis points to glacial persistence of this species in fragmented and small but suitable habitats throughout Central Europe and to an ensuing biogeographic history, which is most likely characterised by recurrent fragmentation events. On the other hand, although the populations are currently highly fragmented again, no genetic imprints of habitat fragmentation are detectable on the regional scale, neither in the adult nor in the filial generations. Since none of the populations that were analysed are able to achieve natural regeneration and due to the fact that no genetic reference data and no studies, which correlate genetic variation and fitness parameters, are available at the moment, the genetic results do not have the potential to contribute to predictions about juniper's long-term survival in the Rhenish Uplands. Thus, it still remains uncertain whether the analysed populations will be affected by the recent habitat fragmentation in the future and whether they will persist. Consequently, the same reasons account for the unsatisfactory evaluation concerning the analysed juniper populations and their chance for survival. However, as already discussed in detail, I question the usefulness of the compiled Leitbild criteria, which were based on the conservation genetics paradigm, for juniper in any case. In spite of everything, the results of the present thesis can now be used as reference data for further similar studies regarding the species *J. communis.*

Next to the scientific results and the newly developed computer programme Swap (available for free from http://www.uni-marburg.de/naturschutzbiologie/downloads/), another final outcome of this thesis is the restoration management plan I established. For the development

of this plan, all the results were invaluable and they might enhance the chances of survival of juniper if all the guidelines and recommendations were employed. After a screen with the newly developed nSSR markers, the restoration management plan can also be used for other European regions where juniper does not regenerate naturally and where its population size declines as well.

In conclusion, I would like to stress, that it is not the possibility of negative genetic effects caused by habitat fragmentation in the future but instead the current absence of natural regeneration and the already detectable even- and over-aged structures, which form the greatest extinction risk for juniper in the Rhenish Uplands as well as in other European countries. If juniper should not start with natural regeneration again, neither in the distant future nor after applying the management plan I presented, then the respective populations will become extinct because senescent individuals are gradually dying off without substitution.

5.2 Perspectives for further analyses

In order to verify the suggested glacial persistence of juniper in Central Europe, more fossil records are needed. Particularly records dating back to the LGM or near the LGM would be extremely useful. Genetic analyses of juniper populations originating from areas, which were definitely formerly glaciated, such as e.g. parts of Scandinavia and the northern British Isles, would also help to gain more detailed insights into the biogeographic history of juniper in Europe.

As mentioned earlier, the palynological analysis as well as the genetic analysis concerning the filial generations or rather the different pollen clouds were only case studies. To gain more insights into the respective topics, I recommend more and more comprehensive repetitions. Thereby, the hypothesis concerning the overdominance of large pollen clouds and with this the fixation of alleles in the filial generation could be tested as well. For this purpose, female junipers, their progenies and male junipers in the neighbourhood might be analysed at the nSSR loci that were characterised within this thesis. In addition, the exact geographical position of all analysed junipers and their dimensions or the dimensions of the respective clones might be measured. If there was an overdominance effect due to individual large male junipers, a mechanical reduction of clone sizes could possibly lead to a less pronounced dominance effect. Besides, this measure would lead to the creation of open space for new seedlings while maintaining all genotypes.

However, as also mentioned earlier, it is currently uncertain whether the fixation of alleles and additional detrimental processes, which are thought to be caused by habitat fragmentation and which are components of the conservation genetics paradigm, have deleterious effects on the fitness of juniper. Therefore, I suggest testing the theory about a potential relationship between respective genetic and fitness parameters. Populations from e.g. the Franconian Alb (Germany, own observations) and/or from Öland (Sweden, Rosén and Bakker, 2005) can be used as reference populations for studies concerning the regeneration success. If there is indeed a correlation between the amount of genetic diversity and the regeneration success detected, cuttings originating from these regions could be used for plantings. However, the question of potential local adaptation should also be considered here.

To evaluate the eight juniper populations of the Rhenish Uplands in terms of nature conservation, I used the results of an nSSR marker analysis. However, it is debatable whether this marker type is useful for detecting a loss of evolutionary potential (Ouborg et al., 2006). Neutral nSSR markers seem to be an excellent tool to study nonselective processes such as e.g. genetic drift and inbreeding. However, they are not necessarily suitable to quantify changes in selectively relevant DNA regions (Reed and Frankham, 2003). In the past, researchers considered selectively relevant loci to follow the same trend as selectively neutral loci. But, in the meantime it seems likely that the correlation between these two different DNA regions is actually low (Reed and Frankham, 2003). Indeed, Ouborg and co-workers (2006) warn against drawing a conclusion about the viability of a population and its potential to adapt to changing environments when it is exclusively based on observed levels of neutral marker variation. Nowadays, the analysis of selectively relevant gene variations is an alternative to the analysis of variation at neutral DNA markers (Hoffmann and Willi, 2008). This recently emerged field is called ecological genomics or eco-genomics and uses different genomic techniques (see e.g. Van Straalen and Roelofs, 2006; Ouborg and Vriezen, 2007). These include investigations of sequence variations in functional or candidate genes (mainly single nucleotide polymorphism (SNP) variations), variations in gene expressions e.g. by means of real-time PCR or microarrays (variations in cDNA, i.e. variations in expressed sequence tags - ESTs) and/or gene functions, which are coupled with phenotype (methods shortly reviewed in Ouborg and Vriezen, 2007). The aim is to analyse how the variation in functional genes and in gene expressions, subjected to selection, is correlated with population size and isolation as well as with quantitative traits (e.g. flowering time, growth form, frost hardiness, draught resistance, resistance against insects), mainly along environmental gradients (e.g. light availability, day length, temperature, draught, soil salinity,

solar radiation) (e.g. Stinchcombe et al., 2004; Ralph et al., 2006; Holliday et al., 2008). Such analyses can considerably further the knowledge about selection and about the potential to adapt to specific environmental conditions. The obtained knowledge can subsequently be used in the context of conservation genetics and restoration management (see e.g. Gonzáles-Martínez et al., 2006; Hoffmann and Willi, 2008). However, to analyse sequence variations in functional genes, the latter have to be identified species-specifically at first. The full and nearly full nuclear and organelle sequences of the model species (e.g. *Arabidopsis thaliana*, *Medicago truncatula, Lotus japonicus*, maize, rice and tomato; see e.g. http://mips.gsf.de/proj/plant/jsf/index.jsp) as well as of the tree species *Populus trichocarpa* (Tuskan et al., 2006) do indeed provide information about the position and constitution of such genes. For example, *Arabidopsis* shows high levels of sequence homology to the conifer species *Pinus taeda* (Kirst et al., 2003). However, a 'fine-tuning' has to be done for every new species of interest (Ouborg et al., 2006). So far, no candidate genes have been identified for juniper and there have been no studies about its gene expression. Therefore, I recommend working on these subjects. Detecting and locating variations in selectively relevant genes and/or gene expression along a latitudinal and/or longitudinal gradient may reveal whether the fragmented juniper populations in the Rhenish Uplands have the potential to adapt to global change and the predicted increase in temperature and eutrophication (see e.g. review Jump and Peñuelas, 2005). Moreover, combining eco-genomics and greenhouse as well as transplant experiments might make it possible to test juniper individuals for plastic response. If such response would exist, this would enrich the evolutionary potential of the respective populations (Pigliucci, 2005; Pertoldi et al., 2007). With regard to planting activities, seed transfer zones and their necessity could presumably be identified by eco-genomics. If an adaptation to e.g. temperature would actually occur, one might consider shifting cuttings from more Mediterranean regions to Central Europe.

After planting activities following the presented management plan, I recommend to set up an ecological and genetic long-term monitoring programme in the respective populations. This programme should ideally merge ecological field studies with investigations of neutral and selectively relevant genetic markers if available. Next to the control of success for the conducted conservation measures, this monitoring could help to detect unpredictable events and to respond to these quickly. Besides the monitoring of juniper populations in which cuttings have been planted, a monitoring should be arranged in control populations without planting activities. Within those control populations, one could analyse whether juniper turns

back to natural regeneration on its own and whether it has used longevity just as an alternative demographic strategy next to natural regeneration.

Finally, I would like to mention that, next to intrinsic reasons such as genetic factors, it is most likely that various extrinsic reasons are responsible for the threatening absence of natural regeneration, namely e.g. eutrophication, soil acidity, soil pathogens and/or missing mycorrhizae (see e.g. Oostermeijer and de Knegt, 2004; Thomas et al., 2007). In order to figure out what is causing this absence, genetic as well as ecological research should be continued and further specific conservation strategies should be generated. I consider the recommencement of the natural regeneration to be the only opportunity to ensure the survival of juniper in the long term.

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‘Ask not what genomics can do for ecology, but also what ecology can do for genomics’
(Martin Feder, personal communication, cited in Thomas and Klaper, 2004)

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http://mips.gsf.de/proj/plant/jsf/index.jsp (for different plant genome projects, state: 28. August 2008)

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http://www.uni-marburg.de/naturschutzbiologie/downloads/ (from this page the computer programme Swap can be downloaded for free, state: 22. August 2008)

7 Abbreviations

Φ_{pt}	F_{ST} - analogue to use for binary data, see F_{ST}
%	percentage
AFLP	Amplified Fragment Length Polymorphism
BP	Before Present
CBD	Convention on Biological Diversity
cDNA	complementary DNA
cm	centimetre
cp	chloroplast
DNA	desoxyribonucleidacid
e.g.	abbreviation of Latin '*exempli gratia*', for example
EST(s)	Expressed Sequence Tag(s)
et al.	abbreviation of Latin '*et allii*", and others
EU	European Union
EUFORGEN	European Forest Genetic Resources Programme
Fig.	Figure
FoVHgV	abbreviation of German 'Forstvermehrungsgut-Herkunftsgebietsverordnung', ordinance about the provenances for forest reproductive material
F_{ST}	proportion of the total genetic variance contained in a subpopulation relative to the total genetic variance (values range from 0 to 1, 1 implies total differentiation among populations)
H_e	expected proportion of heterozygotes
H_o	observed proportion of heterozygotes
HWE	Hard-Weinberg Equilibrium
i.e.	abbreviation of Latin '*id est*', it is
km(s)	kilometre(s)
L.	Linneaus
LGM	Last Glacial Maximum (~ 18.000 BP)
m	metre
min	minute
mt	mitochondrial
myr	million years
nSSR(s)	nuclear Simple Sequence Repeat(s)

PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PI_{ave}	Probability of Identity, i.e. an estimate of the average probability that two unrelated individuals, drawn from the same randomly mating population, will by chance have the same multi-locus genotype (calculated under the assumption of inbreeding)
pop.	population
ppm	parts per million
R_S	allelic richness
r_{xy}	correlation coefficient of the Mantel test (values range from 0 to 1, 0 implies no correlation)
SNP	Single Nucleotide Polymorphism
UNCED	United Nations Conference on Environment and Development
var.	abbreviation of Latin '*varietas*', variety
vs.	versus

Publications and Manuscripts

Paper I

Genetic support for recurrent fragmentation and founder events of juniper populations in Central Europe

Inga M. Michalczyk, Yvonne A. Lücke, Stefan Huck and Birgit Ziegenhagen

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Genetic support for recurrent fragmentation and founder events of juniper populations in Central Europe

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Abstract

In contrast to the thermophilous tree species, which did not survive in Central Europe during the Last Glacial Maximum (LGM) and therefore recolonized from various southern or eastern refugia, we put forward the hypothesis that *Juniperus communis*, an ecologically highly plastic species, could have survived in a cold-steppe like biome of the LGM. Lacking general fossil evidence from the LGM, we used an AFLP (Amplified Fragment Length Polymorphism) marker approach to elaborate on the assumption: if juniper survived in Central Europe and on the whole did not post-glacially recolonize the area from diverse refugia outside Central Europe, we would expect the formerly coherent gene pool to have been affected by long-term recurrent fragmentation and founder events, which are due to the natural and anthropogenic history of the taxon. Using AFLP markers in 23 juniper populations sampled throughout Europe and focussing mainly on Germany, we found a high level of genetic differentiation. The lack of underlying phylogeographic signals or other meaningful geographic structures, as well as the considerable isolation-by-distance effect on a regional scale are discussed against the background of the species' ecology, life-history traits and the history of human influence. Thereafter, recurrent fragmentation and founder events since the LGM have become highly likely. The strong fragmentation effect of recent land-use changes is confirmed by the positive correlation between population size and genetic diversity. Inferences for conservation issues are drawn.

Keywords: *Juniperus communis*, AFLPs, genetic differentiation, historical biogeography, Last Glacial Maximum

Introduction

In range-wide studies, different genetic markers such as chloroplast (cp), mitochondrial (mt) or Amplified Fragment Length Polymorphism (AFLP) markers assisted in reconstructing the bio- and phylogeographic history of various European plant species during and after the last glacial period. Whenever the taxa occurred in pollen diagrams, it was highly advantageous to consult data from fossil records as well (Lascoux *et al.*, 2004). In wind-pollinated woody species, therefore, both disciplines together, paleobotany and population genetics, came up with European large-scale syntheses (eg Petit *et al.*, 2002; Magri *et al.*, 2006; Cheddadi *et al.*, 2006). Also, benefiting from the profound palynological background on European tree species (Huntley and Birks, 1983; Bennett *et al.*, 1991), these syntheses shed light on the locations of refugia and migration routes and dated the arrival and/or the extinction of populations back to certain locations of the species' range. Moreover, range-wide distribution patterns of genetic variants visualized the geographical partitioning of refugial genetic lineages as well as the zones of contact, hybridisation and/or introgression of these lineages (Petit *et al.*, 2003).

In sum, more or less complete bio- and/or phylogeographic histories were obtained for those numerous woody thermophilous tree species recolonizing Central Europe from different southern or eastern refugia (eg Petit *et al.*, 2002; Magri *et al.*, 2006; Cheddadi *et al.*, 2006). Hence, with an increasing number of studies, the *tabula rasa* hypothesis (Nordal, 1987) for Central Europe was strengthened for thermophilous woody taxa. From charcoal and fossil records of the late Weichselian, however, debates arose as to whether the woody thermophilous species could have survived in Central and Eastern Europe at the borderlines of permafrost during the last glaciation (Willis *et al.*, 2000; Willis and van Andel, 2004). The proof of survival is still missing, particularly during the Last Glacial Maximum (LGM: 18,000 BP), due to a lack of fossil signals in this period in Central Europe. In the east, in Hungary and Moldova, refugia cannot be excluded (Willis and van Andel, 2004).

Although cold-tolerant woody species such as willow and birch survived outside the common southern refugia (Palmé *et al.*, 2003a; 2003b), also for these taxa, the Central Europe *tabula rasa* hypothesis may hold true as well. What, however, about the cold and drought-tolerant woody taxa of our present-day heathlands such as juniper?

Juniperus communis L. (Cupressaceae) is an important bush or tree species of the cultural landscape in Central Europe. Nowadays, its populations are highly fragmented and reduced in size (van der Merwe *et al.*, 2000; Oostermeijer and de Knegt, 2004; Verheyen *et al.*, 2005). From a holarctic perspective, juniper represents the conifer taxon with the largest distribution area, ranging from North America, to Europe in its entirety, and Asia. It is the most-wide spread taxon world-wide (Hegi, 1935; Farjon, 1998), and known to have a broad ecological

amplitude. Juniper grows mainly as a colonizer of raw soils (pioneer) from the subarctic tundra down to semideserts on both acidic and chalky soils (Farjon, 1998). Its distribution reaches from sea level up to the timberlines (Aas and Riedmiller, 1987). Light availability is its sole claim to the habitat (Ellenberg *et al.*, 1991). This forces the shrub to compete with other woody plants, especially shade-casters, which results in succession and distribution turn-by-turn with the shade-casting trees (Iversen, 1954).

While the woody species studied so far mainly follow the *tabula rasa* hypothesis, present-day distribution (Hultén and Fries, 1986) and the ecological plasticity of juniper could be indications for its survival in suitable scattered habitats throughout Central Europe during the LGM. So far, this hypothesis could not be proven, because of the lack of fossil records for this period in Central Europe.

We therefore attempted to reconstruct aspects of the biogeographic history of juniper with the help of genetic markers. The approach is based on the following assumption: if juniper had survived in Central Europe and mainly did not post-glacially recolonize the area from diverse refugia outside of Central Europe, we would expect the formerly coherent gene pool to have been affected by long-term recurrent fragmentation and founder events, due to the natural and anthropogenic history of the taxon (Firbas, 1949; Lang, 1994). To elaborate on this assumption, we applied AFLP markers (Vos *et al.*, 1995; van der Merwe *et al.*, 2000) in a range-wide study. Genetic differentiation and genetic diversity parameters were estimated and explored in a geographic context. Furthermore, the patterns of genetic variation and differentiation were discussed with regard to conservation issues.

Materials and Methods

Plant material

A total of 309 individuals were collected in 23 populations from six European countries (Figure 1, Table I). The individual number per population ranged from six in South Tyrol (population 17) to 18 in Tuscany (population 18), in most cases, however, 15 samples were collected. The main focus of the study was on Central Europe with 15 populations. Eight populations originated from the Rhenish Uplands, a region in West Germany, and four from the Franconian Alb in Southern Germany. The remaining three German populations were sampled in different regions known for juniper heathlands. While two to three populations were sampled in Italy and Slovakia, just one each was sampled in Spain, Lithuania and Poland. In order to avoid sampling in clonal or family structures, plant material was taken from junipers separated by more than 40 m. After that, the needles collected were shock frozen with liquid nitrogen and stored at -80 °C until extraction.

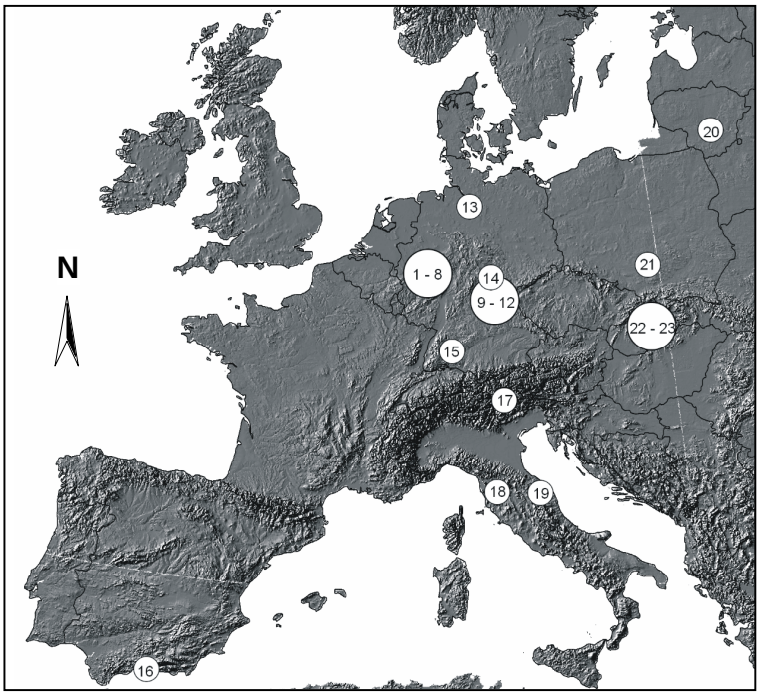

Figure 1: Sampled juniper populations in Europe. The numbers in the circles indicate the population numbers shown in Table I.

DNA extraction

Genomic DNA was extracted from the frozen needles (approx. 100 mg) following the protocol by Dumolin *et al.* (1995). As a slight modification, the protocol included an additional and final treatment with 0.5 µg RNase at 37 ℃ for 30 min.

AFLP procedure

AFLP analysis was performed as described by Vos *et al.* (1995) and van der Merwe *et al.* (2000). The enzyme combination *Mse*I and *Pst*I (Fermentas, Leon-Rot, Germany) was used for digestion of 2000 ng genomic DNA. Afterwards *Mse*I and *Pst*I adapters were ligated to the restriction fragments. For the pre-amplification, we used the respective primer pair *Mse*I+A and *Pst*I+A.

In a pilot study, we used two different selective primer-enzyme-combinations (PECs), namely *Mse*I.2/*Pst*I1.3 and *Mse*I.3/*Pst*I.1, as described by van der Merwe *et al.* (2000). In both cases, *Ms*e primers were fluorescence labelled with Cy5. The estimates of diversity parameters and results of a Principal Coordinate Analysis (PCoA) did not differ when the systems were used either separately or combined. Thus, we decided to use just one PEC (*Mse*I2/*Pst*I.1.3) for the whole dataset. This PEC created more polymorphic loci than the other.

The pre-amplification was carried out in a total PCR volume of 50 µl, with 5 µl of restriction-digested and ligated DNA, 10 x PCR buffer (Invitrogen, Karlsruhe, Germany), 0.5 mM of each dNTP, 0.2 U *Taq* polymerase (Invitrogen), 1.5 mM $MgCl_2$ and 0.5 mM of each primer. The PCR profile of the pre-amplification was: denaturation at 94 °C for 5 minutes (min), followed by 25 cycles of 94 °C for 30 seconds (s), 60 °C for 30 s, 72 °C for 60 s and a final extension of 72 °C for 10 min. The final amplification volume of 20 µl contained 5 µl of 1 : 5 dilution of pre-amplification product(s), 10 x PCR buffer (Invitrogen), 0.25 mM of each dNTP, 1.5 mM $MgCl_2$, 0.1 U of *Taq* polymerase (Invitrogen), 0.5 mM of Primer *Pst*I.1.3 and 0.1 mM of labelled Primer *Mse*I.2. The PCR profile of the final amplification was: denaturation at 94 °C for 5 min, 1 cycle of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 60 s followed by 12 cycles of 94 °C for 30 s, 64 °C for 30 s, 72 °C for 60 s and 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s with a final extension of 72 °C for 10 min.

Data analysis

Data scoring

The products of the AFLP final amplification were analysed by means of the Automatic Laser Fluorescence express II (ALFexpress II, GE Healthcare, Munich, Germany) and 'high resolution' polyacrylamid gels. Bands were analysed by ALFwin™ Instrument Control version 2.0 and were scored by ALFwin™ Fragment Analyser 1.03.01 as present (1) or absent (0). Only fragments between 50 and 300 bps, with a relative height above 5 %, were scored. Bands with slight migration differences, a common phenomenon in gel electrophoresis, were assigned to the same band position (Ziegenhagen *et al.*, 2003). In a next step, the mistyping error was estimated (Bonin *et al.*, 2004) and before the genetic data analysis, all monomorphic loci were discarded.

Genetic diversity

To estimate the genetic diversity of each population, we calculated the percentage of polymorphic loci (PLP). For this purpose, we used the programme AFLPDiv at the level of 1 % confidence (first cited in Coart *et al.*, 2005). Due to the unequal numbers of individuals per population, we applied rarefaction with six individuals, as this was the lowest sample size in the dataset. A regression analysis was carried out with 'R' version 2.5.0 (Ihaka and Gentleman, 1996) between the degree of polymorphism and the size of the German populations. Since we had no complete counts of individuals, we regarded the area size of the juniper stands as an estimator of population size. This analysis was carried out to test for the possibility of genetic drift as a consequence of fragmentation.

Genetic differentiation and geostatistics

Population differentiation occurs when a large proportion of the total genetic variation is found among populations. To detect and test for such a differentiation, we used an AMOVA (Analysis of Molecular Variance) with 9999 random permutations (Excoffier *et al.*, 1992; Peakall *et al.*, 1995; Michalakis and Excoffier, 1996). This was run for the whole data set and separately on a regional scale, for the populations of the German regions Rhenish Uplands and Franconian Alb. The AMOVA was based on the squared pairwise genetic distance matrix according to Huff *et al.* (1993) and Peakall *et al.* (1995).

As an estimate of genetic differentiation, we calculated Φ_{pt} via AMOVA (Peakall *et al.* 1995). This is analogue to Wright's F_{ST} (Wright, 1951) and should be used for binary data (Peakall and Smouse, 2005).

The conversion of the squared pairwise genetic distance matrix into a covariance matrix was used as input into a Principle Coordinate Analysis (PCoA). This multivariate technique is based on an algorithm by Orloci (1978). We used this method to visualise the variability of the individuals' pairwise genetic distances along two axes.

To study the underlying genetic structure in more detail, we used the Bayesian multilocus assignment method by Corander *et al.* (2003), as implemented in the software BAPS version 3.2. The programme clusters data (genetic mixture analysis) at either a group level or at an individual level. When clustering at the individual level, the programme needs an *a priori* expectation for the maximum number of groups (K). The programme allows multiple inputs of K. For each K - value, BAPS tries to determine the optimal partitions, stores these internally, and, after all the runs have been processed, it merges the stored results according to the log-likelihood values. In our analysis, values of K ranging from 1 to 23 (the number of studied populations) were explored. The optimisation algorithm of BAPS is stochastic and consequently, different results can be obtained for the same value of K. Hence, we used 20 replicates for each value of K.

Furthermore, to detect maximal genetic differences between groups of populations in a geographic context, we carried out Spatial Analysis of Molecular Variance (SAMOVA) with the software SAMOVA version 1.0 (Dupanloup *et al.*, 2002). SAMOVA was undertaken assuming two to 20 groups (maximum programme setting) with a simulated annealing process of 100 repeats.

Finally, we tested the correlation between the pairwise linear genetic distance matrix and the pairwise geographic distance matrix using a Mantel test (Smouse *et al.*, 1986; Smouse and Long, 1992). The correlation coefficient r_{xy} was calculated and tested for significance. The procedure was carried out at different spatial scales ranging from a European scale down to a

regional scale. Afterwards a test for significant differences between the r_{xy} - values of the different spatial scales was conducted with 'R'. With the Mantel test, we aimed at analysing the relationships between genetic and geographical distances for isolation-by-distance phenomena.

AMOVA, PCoA and Mantel tests were calculated using GenAlEx version 6 (Peakall and Smouse, 2005).

Results

To test the reproducibility of the AFLP marker system, we repeated the whole AFLP procedure twice with 30 individuals. The mistyping error was moderate with a value of 2.65 %.

Genetic diversity

Diversity statistics are summarized in Table I. Using the AFLP method, a total of 216 scorable fragments was generated. A high proportion of these was polymorphic (208 fragments, 96 %) and was used for all calculations. The total number of polymorphic loci ranged from 35 (population 11) to 104 (population 20). The percentages of polymorphic loci varied from 16.6 % (population 11) to 49.3 % (population 20).

There was a significant correlation between the percentage of polymorphic loci (PLP 1 %) and the size of juniper stands in Germany (Figure 2), indicating genetic drift through fragmentation.

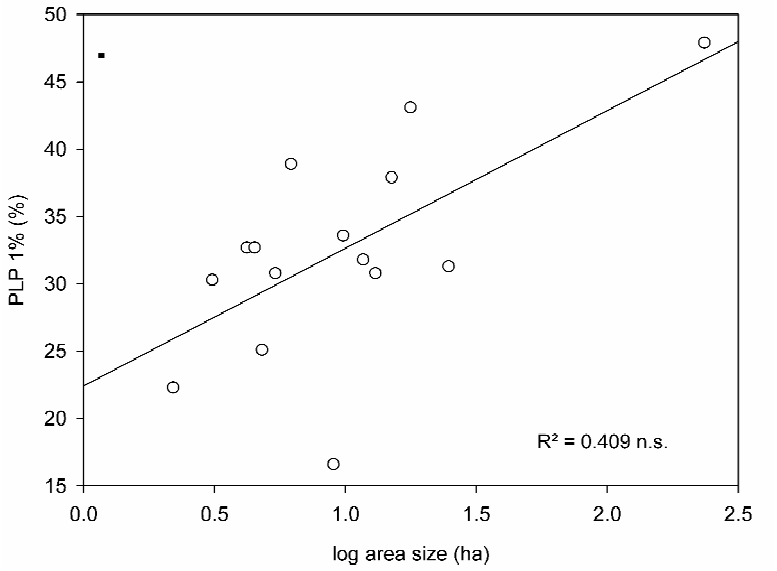

Figure 2: Regression analyses between log area size of the German juniper populations and the percentages of polymorphic loci (PLP 1 %).

Table I: List of the juniper populations analysed and their origin. Also given are the sample sizes, the number of polymorphic loci and the percentages of polymorphic loci (PLP 1 %).

Population	Region or province	Country	Latitude	Longitude	Sample size	Number of polymorphic loci	Percentages of polymorphic loci (PLP 1 %)
1	Rhenish Uplands	Germany	50.3646	6.6421	14	91	43.1
2	Rhenish Uplands	Germany	51.0415	8.6398	15	66	31.3
3	Rhenish Uplands	Germany	50.5516	6.7037	14	65	30.8
4	Rhenish Uplands	Germany	51.0638	8.1844	15	71	33.6
5	Rhenish Uplands	Germany	50.3960	7.0742	15	80	37.9
6	Rhenish Uplands	Germany	51.1286	7.7602	14	67	31.8
7	Rhenish Uplands	Germany	51.1528	8.0356	13	64	30.3
8	Rhenish Uplands	Germany	50.3905	7.1334	15	81	38.9
9	Franconian Alb	Germany	49.8131	11.3546	15	69	32.7
10	Franconian Alb	Germany	50.0214	11.0852	15	53	25.1
11	Franconian Alb	Germany	50.0452	11.2097	15	35	16.6
12	Franconian Alb	Germany	49.9732	11.3066	15	65	30.8
13	Lower Saxony	Germany	53.1658	9.9605	13	101	47.9
14	Thuringia	Germany	50.7265	11.0582	15	69	32.7
15	Baden-Wuerttemberg	Germany	48.2449	8.9780	15	47	22.3
16	Andalusia	Spain	36.7473	-3.8827	10	51	24.2
17	South Tyrol	Italy	46.5483	11.5581	6	40	19.0
18	Tuscany	Italy	43.4534	11.0917	18	62	29.4
19	Marche	Italy	43.3660	13.1005	13	44	20.9
20	Kauno	Lithuania	54.8167	24.2000	15	104	49.3
21	Slaskie	Poland	51.9838	18.9356	7	59	28.0
22	Liptov	Slovakia	49.0408	19.7752	10	96	44.5
23	Zvolen	Slovakia	48.3514	19.0427	12	93	44.1

Genetic differentiation and geostatistics

The AMOVA revealed that there was a comparably large amount proportion of variation found among populations. The variation among populations ranged from 41 % at the European level to 39 % and 28 % on the regional scale for the Rhenish Uplands and the Franconian Alb, respectively (Table II). Accordingly, the value of Φ_{pt} (Table II) was highly significant with a value of 0.413 (P = 0.001) for the European dataset and lower for the Rhenish Uplands (Φ_{pt} = 0.393, P < 0.001) and the Franconian Alb (Φ_{pt} = 0.276, P < 0.001) (Table II).

To resolve possible patterns of pairwise genetic distances, we carried out a PCoA (Figure 3). The first axis explained 33.9 % and the second 16.1 % of the total variation. Each population clustered distinctively, there was, however, no overall consistency in the clusters with the geographical distribution of populations. For example, the German populations from the Rhenish Uplands (dark blue) and the Franconian Alb (red), respectively did not cluster together. However, the population from Spain (pink) clustered with the two populations from Slovakia (light blue).

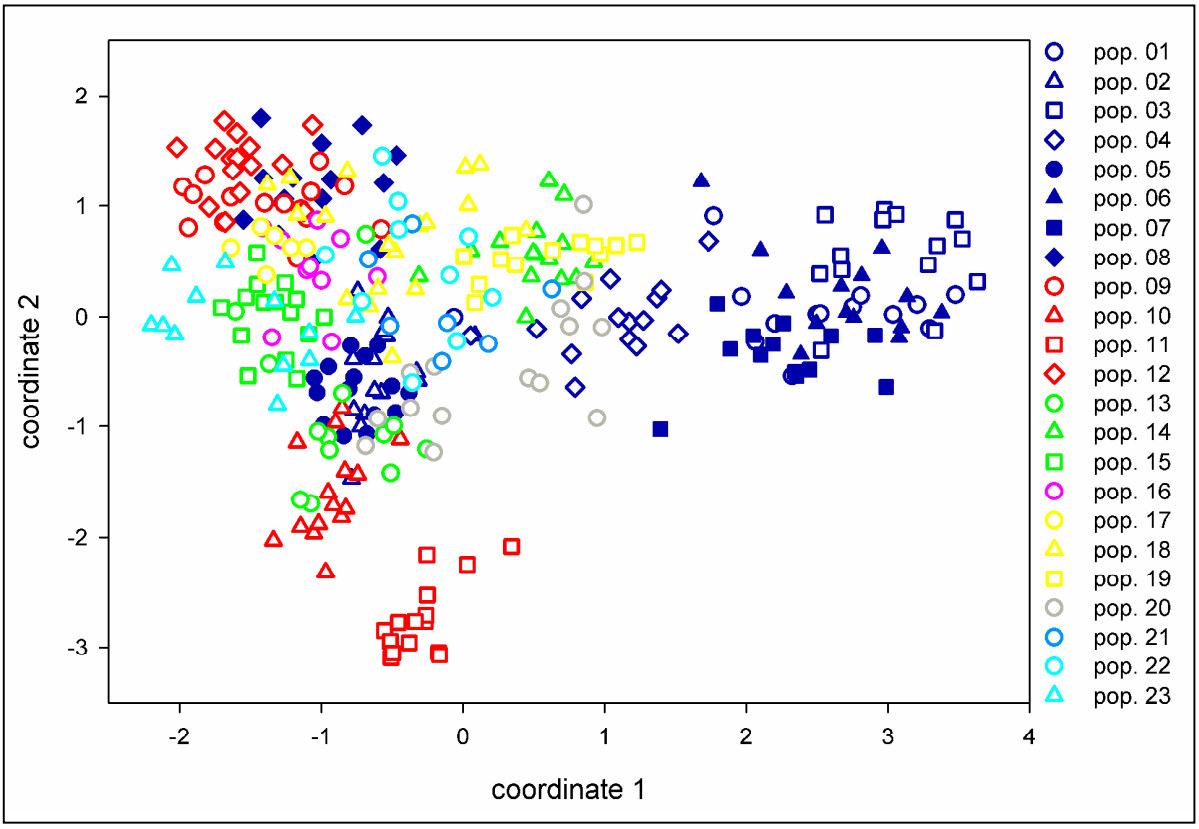

Figure 3: Two-dimensional plot of the Principle Coordinate Analysis of the European AFLP dataset. The first two axes are plotted. The first axis represents 33.9 % of the total variation, the second 16.1 %. Samples/populations originating from the same region are marked with the same colour: dark blue: Rhenish Uplands, red: Franconian Alb, light green: remaining German populations, pink: Spain, yellow: Italy, grey: Lithuania, medium blue: Poland, light blue: Slovakia.

The analysis of the underlying genetic structure according to the Bayesian model came up with numerous group and individual based clusters. With both procedures, at the group level as well as at the individual level, highest *a posteriori* probabilities (-17259.4 and -17157.3, respectively) were obtained for K = 10. The analysis at group level revealed similar results as at the individual level. Although there were clusters indicating genetic relatedness, the Bayesian approach did not yield homologous results between the geographic and the genetic structure (data not shown). We therefore omitted further analyses such as the neighbourjoining procedure, as a phylogeographic signal was obviously missing.

Similarly, SAMOVA did not recover geographically meaningful groups with significant structure. The F_{CT} - values fluctuated between 0.186 (10 groups) and 0.234 (20 groups) with $P < 0.001$ for both values (detailed data not shown).

The overall r_{xy} - values of the Mantel tests were significant for all three cases and they showed all significant differences between each other at the 99 %-confidence level (Table II, Figure 4). However, only a weak correlation could be found between the geographic and the genetic distance matrix with a comparably low value of 0.108 when all populations were included. The strongest correlation was obtained with the populations of the Franconian Alb ($r_{xy} = 0.565$, $P < 0.001$) (Table II, Figure 4). In contrast to the European scale, a comparably strong isolation-by-distance has become effective on the regional scale.

Table II: Results of the AMOVAs and Mantel tests on different geographic levels.

Regions	Genetic variation within populations [%]	Genetic variation among populations [%]	Φ_{pt} [1]	r_{xy}
Europe	59	41	0.413***	0.108***
Rhenish Uplands	61	39	0.393***	0.249***
Franconian Alb	72	28	0.276***	0.565***

[1] estimate of genetic differentiation, calculated via AMOVA
[2] correlation coefficient of the Mantel tests

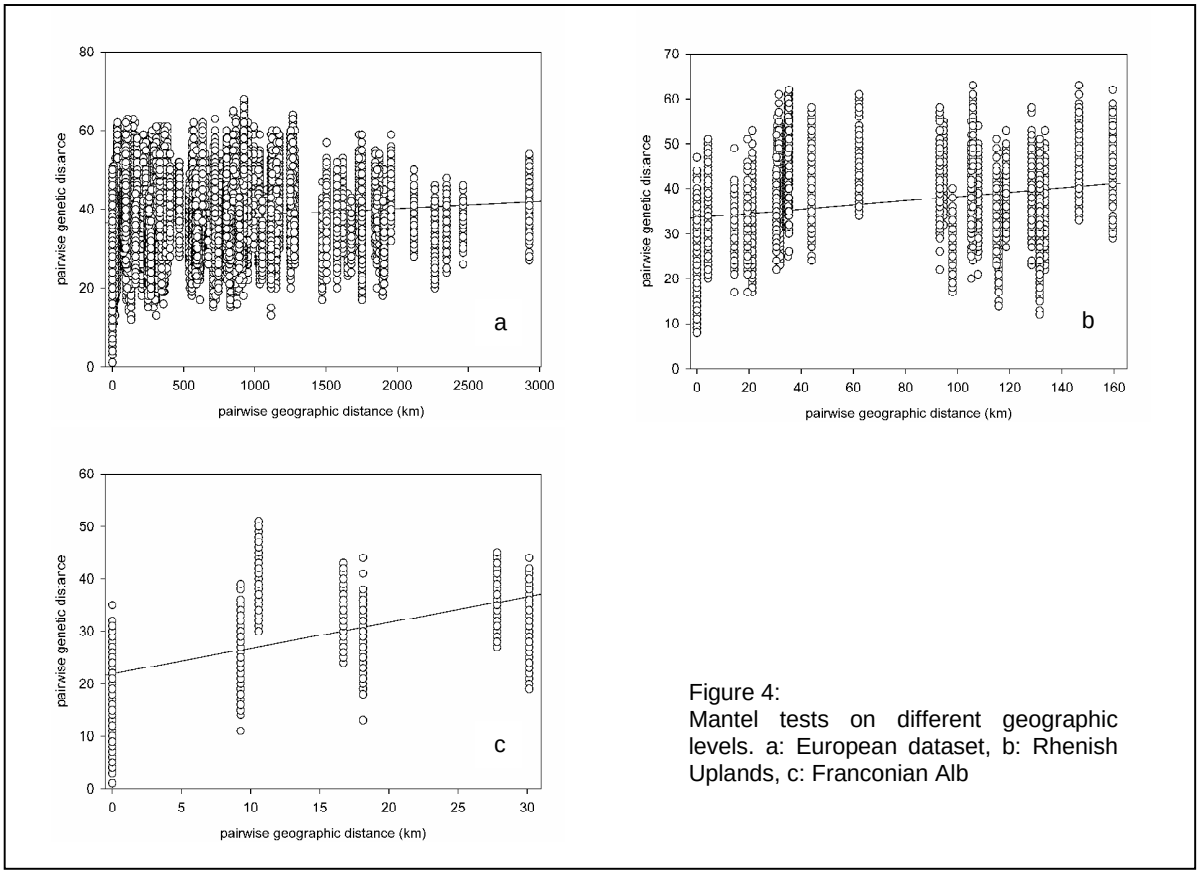

Figure 4:
Mantel tests on different geographic levels. a: European dataset, b: Rhenish Uplands, c: Franconian Alb

Discussion

AFLP markers for reconstructing the biogeographic history of juniper

Lacking fossil evidence for tracing the fate of juniper during the LGM in Central Europe, we opted for a genetic marker-assisted approach. In cases where genetic markers were used in order either to confirm or complement palaeobotanic findings in a bio- or phylogeographic context, organelle (cp and mt) DNA markers were frequently consulted (review in: Petit and Vendramin, 2007). Due to the fact that suitable cp and mt DNA markers for juniper have not yet been designed and the transfer of available *Pinaceae* cp primers (Vendramin *et al.*, 1996) was not successful, we chose AFLP markers. These have been recently re-evaluated as useful tools for the reconstruction of historical events of populations (review in: Meudt and Clarke, 2007 and used, for example, in: van der Merwe *et al.*, 2000; Schönswetter *et al.*, 2005). In fact, because of the considerable number of loci involved, these markers may out-compete even nuclear microsatellite markers when used, as in this case, for discrimination and differentiation purposes among populations (Woodhead *et al.*, 2005; own unpublished nuclear microsatellite data on juniper). Since AFLPs target numerous polymorphic loci with

different mutation rates, spatial genetic patterns of differentiation derived from AFLPs may be shaped by phylogenetic and/or population genetic processes (Meudt and Clarke, 2007). Indeed, we did find a high level of differentiation among the European juniper populations, which can result from an imprint of recolonization routes and/or by isolation events followed by genetic drift. Consequently, to elaborate on the two possible historic scenarios, the survival of juniper in the LGM or not, we performed a comprehensive analysis of genetic distances using ordination methods, a Bayesian approach and geostatic procedures. Furthermore, we analysed the relationship between population size and genetic diversity to unravel possible effects of fragmentation.

Which of the scenarios can be supported genetically in the light of what is known about junipers' history since the end of the Pleistocene, as shaped by ecology, life-history traits and human influence?

Biogeography of *J. communis* in Central Europe – a history of fragmentation and founder events since the LGM

Though not available for the LGM, fossil records of *J. communis* dating from a period between 15,000 and 11,000 BP in Ireland (Godwin, 1975; O'Connell *et al.*, 1999), in the Netherlands (van der Hammen, 1951), in Brandenburg (North-East Germany) (Brande and Wolters, 1997), and at Lake Constance (south Germany) (Bertsch, 1961) support the assumption that this species could have survived in Central Europe, even in close proximity to the ice-sheets. In particular, some very early records were reported from areas, which were covered by ice during the Quaternary cooling (Godwin, 1975; O'Connell *et al.*, 1999; van der Hammen, 1951; Bertsch, 1961). At present, the cold-tolerant species has a circumpolar distribution and is able to grow in discontinuous and continuous permafrost areas such as the North Scandian and North-Alaska (Hultén and Fries, 1986; Schultz, 2000). Juniper, therefore, seems to be able to grow along the edges of the glaciated areas, possibly in a biome such as a cold forest steppe (Willis and van Andel, 2004). Moreover, the potential to grow in altitudes over 1,600 m above sea-level (Aas and Riedmiller, 1987), suggests the survival of juniper on so-called nunataks. Using genetic markers, Stehlik *et al.*, (2001), Stehlik *et al.*, (2002) and Stehlik (2002) described such behaviour for *Eritrichium nanum* and *Rumex nivalis* in the Alps.

In the present study, we were able to find substantial genetic differentiation among the different European juniper populations. Of course, the phenomenon as such is still in accordance with a *tabula rasa* hypothesis, as was claimed for other species. Here, long-term isolation of populations in refugia outside Central Europe led to distinct genetic lineages, which could be traced back to southern refugia (eg Hewitt, 1996). In our case, however, as

shown by the PCoA and the Bayesian approach as well as by geostatistics (SAMOVA), the genetic differentiation could not be attributed to geographical patterns favouring a postglacial recolonization of juniper into Central Europe. We therefore propose an alternative scenario. Juniper could have survived throughout Central Europe in suitable scattered and diffuse, i.e. fragmented and probably permanently-altering habitats. A similar scenario is described for various thermophilous tree species in Central and Eastern Europe (Willis and van Andel, 2004). The populations in these habitats were possibly connected through effective gene flow and new populations were presumably founded by a random sample of seeds originating from small founder populations encompassing a broad geographical area. Support for such a scenario comes from studies of seed dispersal. Juniper seeds are mainly dispersed by birds and small mammals (Hegi, 1935; Turcek, 1961; Livingston, 1972). Moreover, long-distance events may have occurred when seeds were blown across the ground, especially in treeless, windy environments, where the ground is covered by smooth frozen surfaces (Rendell and Ennos, 2002).

Since juniper is a pioneer and light-demanding shrub (Ellenberg *et al.*, 1991; Lang, 1994), we should consider a later and further fragmentation in the Holocene, when shade-tolerant tree species or highly-competitive shade-casters such as oak and/or beech trees appeared (Firbas, 1949). Since juniper is known to become suppressed in forests and especially in closed canopies (Iversen, 1954), the populations gradually diminished or decreased in size until about 2,500 years ago, when beech had completely recolonized Central Europe. However, it can be assumed that juniper was able to survive at exceptional sites such as rock ledges, clearings or as an understorey in parts of the forests not forming completely closed canopies. These habitats could have harboured founder populations, which then regained a large distribution in Central Europe, followed by different periods of forest usage or even devastation by man. Human influence was apparent already in the Neolithic, continuing during the Bronze and Iron Ages and had its historical climax in the Middle Ages (Firbas, 1949). At that time, juniper became a dominant shrub in heathlands and grasslands. Palaeobotanic findings in the Rhenish Uplands, Germany, strengthen this assumption (Speier, 1994, 1999). Finally, we assume that fragmentation of the large mediaeval juniper populations took place again with intensive re-afforestation activities starting in the early 19th century (Hasel and Schwartz, 2002).

To test whether the scenario of survival in the LGM in Central Europe holds true, we explored our data for isolation-by-distance effects at differing spatial scales. When all the populations were included in the Mantel test, we found only a marginal indication of isolation-by-distance. In contrast, we would expect a significant and large effect, in case that Central Europe had

been recolonized from isolated and/or fragmented refugia, as demonstrated for white oaks by Dumolin-Lapègue *et al.* (1997). Thus, we may conclude that there is an underlying, more or less coherent gene pool, where the observed differentiation is not caused by phylogenetic signals. Interestingly, there is less differentiation but a strong isolation-by-distance effect on the regional scale. Changes in silvi- and agricultural practices throughout the last centuries fragmented and reduced the formerly widespread juniper populations in Central Europe again (van der Merwe *et al.*, 2000; Oostermeijer and de Knegt, 2004; Verheyen *et al.*, 2005). Fragmentation affects mainly small populations through genetic drift and inbreeding depression. As a result, genetic differentiation occurs and, if gene flow is low, isolation-by-distance effects become measurable. In the end, decreasing population sizes are strongly correlated with a decreasing level of genetic diversity (Young *et al.*, 1996). Those decreasing levels of genetic differentiation and the increasing effects of isolation-by-distance detected throughout the different scales in this study could be due to restricted gene flow (also see Hamrick *et al.*, 1992). This leads to the question of the effectiveness of gene flow in this species, given the life-history traits 'wind-pollination' and 'seed dispersal by birds'. Indeed, though wind-dispersed, juniper pollen is known to travel relatively short distances (Huntley and Birks, 1983; Schlütz, personal communication). Concerning seed dispersal carried out by birds, we assume a less effective gene flow than seed dispersal by wind in the treeless cold steppes of the Pleistocene. From genetic studies of juniper in Great Britain, van der Merwe *et al.* (2000) also consider the effective gene flow via birds to be relatively low. They argue that the differentiation detected has become 'fossilized' through a recent loss of long-distance dispersal events. In the present study, the positive correlation between population size and degree of polymorphism in Germany finally postulates genetic drift as an overall fragmentation effect of Central European juniper.

Conclusion and lesson for conservation issues

Our AFLP study supports recurrent fragmentation and founder events for juniper populations in Central Europe since the LGM. We assume that juniper was able to survive in fragmented suitable habitats during the last ice age and the period of widespread Holocene forests. We postulate that after the ice retreated and the forests declined, especially in the Middle Ages, juniper stands were founded by seeds originating from source populations at exceptional sites. Given restricted pollen dispersal and a recent lack of long-distance seed dispersal, recent isolation-by-distance is strongly imprinted when we scale down to regions in Germany. To further test for genetic drift and loss of heterozygosity, we are applying nuclear microsatellite markers in ongoing studies.

Phylogeographic studies of thermophilous woody species lent themselves to the assignment of range-wide gene zones (Bucci *et al.* 2007). For forest requests or environmental conservation purposes, it is recommended to obtain reproductive material from those respective gene zones where the measures are taken. In some Central European regions, juniper populations are deficient in natural regeneration (eg Verheyen *et al.* 2005; Ward 1973; Fitter and Jennings 1975; Gilbert 1980; Pott and Hüppe 1991; García *et al.* 1999). As there is no evidence for distinct historic genetic lineages, it is feasible to collect plant material from any European juniper population for restoration purposes. However, we advise on the introduction of material harvested from genetically diverse populations. Moreover, source populations should originate from localities with similar climatic and ecological conditions in order to give consideration to the thereto adapted genomic background (Gömöry *et al.*, 1998).

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Paper II

Characterization of highly polymorphic nuclear microsatellite loci in *Juniperus communis* L.

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PRIMER NOTE

Characterization of highly polymorphic nuclear microsatellite loci in *Juniperus communis* L.

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Abstract

We developed five nuclear microsatellite markers in *Juniperus communis* L. using an enriched library method. Samples from 28 juniper individuals were collected in Spain, Germany and Slovakia and were analysed at the five loci. A high level of allelic diversity with values ranging from nine to 23 alleles was found. These highly polymorphic markers will be used in ongoing population genetic studies to evaluate the genetic resources and to contribute to the maintenance of genetic diversity of juniper in Middle Europe.

Keywords: juniper, simple sequence repeat

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Juniperus communis L. (Cupressaceae) is the conifer taxon with the widest distribution area in the world (Farjon 1998). Nevertheless, juniper heaths are one of the threatened elements of the recent cultural landscape in Europe, and heathlands, as such, currently belong to the most highly fragmented seminatural ecosystems in Western Europe (Bakker & Berendse 1999). A further problem is that *J. communis* L. is often suffering from regeneration deficiencies (Clifton *et al*. 1997). Programmes for maintaining its genetic resources and genetic diversity have been started. Therefore, knowledge is needed about the spatial organization of juniper individuals (clones) and the spatial organization of its genetic diversity as well as about gene flow as dependent from fragmentation and/or isolation. So far, population genetic studies in juniper have been carried out using amplified fragment length polymorphism (AFLP), random-amplified polymorphic DNA (RAPD) and allozyme markers (e.g. van der Merwe *et al*. 2000; Adams & Pandey 2003; Oostermeijer & de Knegt 2004; Michalczyk, unpublished). Since nuclear microsatellites are the markers of choice for studying small-scale genetic diversity and the mating system, we decided to develop such markers for ongoing population genetic research in juniper.

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A microsatellite library enriched for di- (GA, GT, AT, GC), tri- (CAA, ATT, GCC) and tetranucleotide (GATA, CATA, ATAG) repeats was constructed following the method described by Edwards *et al*. (1996). The total genomic DNA was extracted from needles of one *J. communis* individual following the protocol by Dumolin *et al*. (1995). A total of 215 clones randomly chosen from the library were sequenced in one direction. For sequencing, primers flanking the polycloning site of the pBlueScriptII vector (Stratagene) were designed external to the M13 universal primers and used for sequencing. The sequences are (5′–3′): for-CGATTAAGTTGGGTAACG; rev-GCTTTACACTTTATGCTTCC. Sequencing reactions were performed using the DYEnamic ET Terminators Sequencing Kit (Amersham Bioscience) and run on a capillary automatic sequencer (MEGABACE 1000, Amersham Bioscience). In 79% of these sequences, a microsatellite motif was detected. However, many of them were omitted immediately since they revealed nondesired properties like extremely too long and compound-interrupted simple sequence repeat (SSR) stretches (20%), SSR stretches too close to the vector (20%) and too short stretches (4%), respectively. After sequencing the reverse direction of the remaining candidate clones, 38 primer pairs were designed for specific polymerase chain reaction (PCR) amplification of the loci using the computer program PRIMER 3 (Rozen & Skaletsky 2000). Following extensive prescreening for the quality of the

amplified SSRs, which was performed on a subset of six individuals run on an automatic capillary sequencer (MEGABACE 1000, Amersham Bioscience), only five single loci SSRs were found to have scorable polymorphic bands. The other 33 loci showed no amplification, multibanding patterns, too pronounced stutter and/or monomorphic bands, respectively.

To characterize the five confirmed loci, a total of 28 individuals were sampled from six different populations (two from Germany, six individuals each; two further from Germany, one from Spain and one from Slovakia, four individuals each). The DNA was isolated from needles following the protocol by Dumolin *et al*. (1995). PCRs were performed in 25 μL containing 30 ng of template DNA, 1 × PCR buffer (Promega), 5 mM of each dNTPs, 1 U *Taq* polymerase (Go*Taq*, Promega), 1.5 mM and 2.5 mM $MgCl_2$, respectively (Table 1), 0.5% BSA and 2 μM of each primer. Two different PCR profiles were used to amplify the microsatellite loci: (a) denaturation at 94 °C for 4 min followed by 35 cycles at 94 °C 30 s, 50 °C 30 s, 72 °C 30 s and final extension at 72 °C for 7 min; and (b) denaturation at 94 °C for 5 min, 10 touchdown cycles at 94 °C 30 s, 60 °C 30 s (–1 °C/cycle), 72 °C 40 s, 25 cycles at 94 °C 30 s, 50 °C 50 s, 72 °C 40 s, and final extension at 72 °C 7 min (Table 1). PCRs were performed using a Biometra T1 thermo cycler (whatman Biometra, Goettingen, Germany). The amplification products were separated by capillary electrophoresis using MEGABACE 1000 (Amersham Bioscience) automatic sequencer. Alleles were sized using the size standard MegaBACE ET400-R (Amersham Bioscience) and the MEGABACE Fragment Profiler version 1.2 software (Amersham Bioscience). Primer labels are reported in Table 1.

Genetic diversity parameters were estimated using the software package GENALEX version 5 (Peakall & Smouse 2001) and the frequency of null alleles determined with the software package CERVUS version 2.0 (Marshall *et al*. 1998). To test all loci for linkage disequilibrium, we ran the Fisher's test with GENEPOP version 3.4 (Raymond & Rousset 1995).

The number of alleles observed for the five loci ranged from nine to 23. The observed heterozygosity and the expected heterozygosity ranged from 0.292 to 0.692 and 0.693 to 0.948, respectively. Nonsignificant deviation from Hardy–Weinberg (H–W) expectations was observed for Jc 035. The deviation from the H–W expectations ($P < 0.01$) observed at the other loci might be caused by the presence of null alleles (Table 1). The Fisher's test did not reveal any significant case of linkage disequilibrium (data not shown).

It has to be annotated that due to the fact that the samples tested with the new primer pairs do not derive from one single outcrossing population, the values of expected heterozygosity and the frequency of null alleles have to be treated with reserve. The high number of alleles detected at the five SSR loci promises a sufficient power of the multilocus genotypes. Preliminary calculations of exclusion

Table 1 Characteristics of *Juniperus communis* microsatellite markers

Locus name (Accession no.)	Primer sequence 5′–3′	Repeat motif	Clone size (bp) (allele size range)	T_a (°C)	$MgCl_2$ (mM)	PCR profile	No. of samples	No. of alleles	H_O	H_E	Null allele freq.
Jc 016 (DQ192499)	F: HEX-CAAAATGATGCTTATGATGA R: TGAAAATCATTGTTGTTTTCTT	$(GT)_{24}$	160 (118–154)	50	2.5	a	24	9	0.292	0.693*	0.41
Jc 031 (DQ192495)	F: FAM-CCTAATGTTGTAATCACGTATATCT R: TGACCTTGGGCGTATAGATT	$(CA)_{15}$	192 (174–242)	50	2.5	a	24	16	0.667	0.850*	0.12
Jc 032 (DQ192496)	F: FAM-ACATTGCAAATATGGGGTAA R: TTGATGAGTTGTTGAGTTATTAAG	$(AC)_{14}(ATC)_8$	203 (158–224)	50	1.5	b	24	16	0.583	0.922*	0.22
Jc 035 (DQ192497)	F: FAM-TGTGTTTATTCTCCCCATCT R: CCCCCAGTTATTCTAAACATT	$(CA)_{20}$	155 (131–167)	50	1.5	b	27	11	0.593	0.783^{NS}	0.13
Jc 037 (DQ192498)	F: FAM-GGCAATTAGTAAGGCACAAG R: TAAGGTGGATATCACCAAGG	$(TG)_{10}(AG)_{20}$	171 (176–222)	50	1.5	b	26	23	0.692	0.948*	0.16

F, forward primer; R, reverse primer T_a, annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity; Null allele freq., null allele frequency; *deviation from H–W expectation ($P < 0.01$); NS, not significant.

percentages reached values of more than 99% (data not shown).

In the nearest future, the described microsatellite markers will be used to analyse the genetic structure and diversity of juniper as affected by landscape fragmentation in Germany. Research of effective gene flow and spatial analyses will be accomplished.

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Paper III

Reduction of population sizes has not yet affected genetic diversity of juniper, *Juniperus communis L.*

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Reduction of population sizes has not yet affected genetic diversity of juniper, *Juniperus communis* L.

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Abstract

Nowadays many *Juniperus communis* populations represent only small relics in Europe, mainly due to habitat fragmentation and a virtual lack of natural regeneration. Using four nuclear microsatellite makers, we tested eight relict juniper populations in the Rhenish Uplands, Germany, for genetic and demographic effects of habitat fragmentation. Since one microsatellite locus showed high levels of null allele frequencies and it is presumed that null alleles introduce a bias into analyses of genetic diversity and differentiation, we explicitly studied this phenomenon by using different marker combinations. In addition to studies of the adult generations, we conducted a case study by analysing single tree progenies. With the help of a newly developed computer software, we analysed the genetic variation and differentiation of the reproductively effective pollen cloud. Next, we conducted a palynological study by counting contemporary pollen grains to gain insights into physical pollen flow distances. High allelic richness combined with an absence of recent bottlenecks and of an isolation-by-distance effect revealed that habitat fragmentation has not yet affected genetic diversity in all eight juniper populations. More likely, genetic diversity and differentiation have been 'frozen' since the last habitat fragmentation. Analyses of the single tree progenies did not indicate genetic drift, although the palynological study displayed locally restricted pollen flow distances. Based on our genetic analysis and on related studies, we present a catalogue of guidelines on how to restore juniper populations, which lack natural regeneration.

Keywords habitat fragmentation, pollen-mediated gene flow, clonal structure, null alleles, conservation management

1. Introduction

Cultural landscapes in Europe are characterised by fragmentation, which dissects continuous habitats into several smaller and spatially isolated remnants (Jongman, 2002; Young et al., 1996). Reduction of population size and loss of connectivity are supposed to have demographic and genetic consequences, both of which endanger species survival (Oostermeijer et al., 2003; Washitani et al., 2005). The isolation of populations and the ensuing disruption of mating systems, leads to decreased gene flow followed by genetic drift. Often, these situations end up in a decrease of genetic variation and inbreeding (Ellstrand and Elam, 1993). As a result, genetic diversity in terms of heterozygosity is lost and alleles can become fixed (Templeton et al., 2001). Genetic differentiation arises and, as a long-term effect of fragmentation, isolation-by-distance effects occur (Young et al., 1996). Dependent on different life history traits of the species e.g., short-lived vs. long-lived, less effective vs. highly effective gene flow, the genetic effects of fragmentation are more or less pronounced (Lowe et al., 2005).

The present study targets the potential genetic consequences of landscape fragmentation on relict *Juniperus communis* L. populations in the 'Rhenish Uplands', a mountainous region in West Germany. *J. communis* (from now on also referred to as juniper) is an evergreen dioecious gymnosperm. In Europe it represents an important but nowadays threatened element of the cultural landscape. The historical biogeography of this species is assumed to have been affected by long-term recurrent fragmentation and founder events. Presumably, the highly light-depending pioneer shrub contracted to exceptional sites in all over Europe during glacial periods and did so as well in times when trees were dominant. Whenever possible, the remaining populations acted as founder populations and regained a large distribution (Michalczyk et al., submitted), particularly in the period from the Middle Ages to the 19th century when heathlands, as a consequence of forest devastation, occupied large areas of the landscape. Nowadays, the formerly widespread juniper stands are highly fragmented again and represent only small relict populations (Küster, 1999). This phenomenon is not only known for the Rhenish Uplands but also in Northern Germany (Pott and Hüppe, 1991) and in southern parts of Germany, e.g. in the Franconian and Swabian Alb. Moreover, heavily fragmented juniper stands are recorded for e.g. Spain (García et al., 1999), Belgium (Verheyen et al., 2005), the Netherlands (Oostermeijer and de Knegt, 2004), Ireland (Provan et al., 2008) and England (review by Thomas et al., 2007). In Germany, juniper has already been protected by the Federal Nature Conservation Act since 1936. Since 1992 *J. communis*-communities are listed in Annex I of the EU-Habitat Directive (code 5130). In the meantime, *J. communis* is also admitted to the European Forest Genetic Resources

Programme (EUFORGEN). However, despite many efforts, juniper populations still decline in many parts of Europe, mainly because of habitat destruction, habitat degradation and a quasi lack of sexual reproduction (Verheyen et al., submitted).

Many juniper populations in the Rhenish Uplands do not show any natural regeneration and they seem to be even- and over-aged. An indication of over-aging is the shrub-like growth form of juniper individuals. The primary column-like growth form often changes with age as inner branches die from self-shading. Particularly in response to environmental conditions, like e.g. heavy wet snow, dead branches break out of the column and old bushes are prone to break apart (Rodwell, 1991). Sometimes procumbent, living branches can root (Ward, 1973; Clifton et al., 1997). Thus clonal-like structures emerge.

In a previous Europe-wide study, which was devoted to the Holocene biogeography of *J. communis* in Central Europe, we found a strong isolation-by-distance effect on regional scales (Michalczyk et al., submitted). However, since we used dominant AFLP markers, we were not able to test for Hardy-Weinberg equilibria (HWE) and therefore we could not definitely detect losses of heterozygosity through drift and/or inbreeding. This fact and conflicting results of genetic effects of fragmentation in tree species (see e.g. review Lowe et al., 2005) made us to perform a comprehensive study on highly fragmented juniper populations in the Rhenish Uplands. Using codominant nuclear microsatellite markers (nSSRs), we analysed adult individuals from eight relict juniper populations of small sizes varying from 3 to 25 ha for losses of genetic variation and diversity as a possible consequence of drift.

Next, we conducted a small case study. From two of the populations we analysed each five single tree progenies for allelic richness and genetic differentiation of the pollen clouds, which had become reproductively effective in the embryos. If juniper pollen was to realise a more or less strictly locally deposition (Huntley and Birks, 1983) without mediating effective gene flow, we would expect a further genetic bottleneck-effect, this time from the adult to the next generation. Also, we would expect a high level of genetic differentiation between the single pollen clouds. Since the underlying assumption of locally restricted pollen deposition was obtained with Himalayan juniper species (Miehe et al., 2006), we conducted a study on *J. communis* in one of the heathlands in the Rhenish Uplands. We counted pollen grains deposited in moss cushions at different distances from a male donor. Based on our results, on what is known from literature and on expert know how we compiled a catalogue of genetically based guidelines on how to restore juniper stands, which lack natural regeneration.

2. Material and Methods

2.1 Sampling of needle material

In the years 2003 to 2005, we collected a total of 889 samples from adult junipers in eight populations of the Rhenish Uplands (Fig. 1, Table 1). All eight juniper stands differed in area size. Their size varied between 3.1 (Ruebenkamp, R) and 24.8 ha (Battenfelder Driescher, BD) (Table 1). The smallest distance between two stands was 4.24 km (Heidbuechel, HL to Waberner Heide, W) and the largest was 159.55 km (Alendorfer Kalktriften, A to BD) (Table 3). Samples were collected in a random grid design. For this purpose a 15 x 15 m grid was placed on ortho-photos from the juniper stands using a geographic information system (ArcMap™ 9.2). In each juniper stand, eleven 15 x 15 m plots were randomly chosen for sampling needles (Fig. 1c). These plots had to be located entirely within the juniper stands and not to be dissected by any paths. In each of the plots an additional, smaller 3 x 3 m grid was placed and whenever an individual or a branch hit a grid point needle samples were taken (Fig. 1d). This small grid design allowed us to detect clonal and/or family structures and was used to estimate densities of juniper individuals. As a clone and genet we regarded all procumbent branches originating from one single individual (genotype) irrespectively of the fact whether this branch and ramet had roots or not. The sample size ranged from 55 (Halsberg, HA) to 162 (Piwittsmoor, P) adults (Table 1). Needles were shock frozen and stored at -80 °C until extraction. During collecti on, the sex of every sampled juniper was monitored and its spatial position geo-referenced with a Global Positioning System (Leica GS50, Munich, Germany). Junipers with cones were determined as females, junipers without cones as potential males (Fig. 1d).

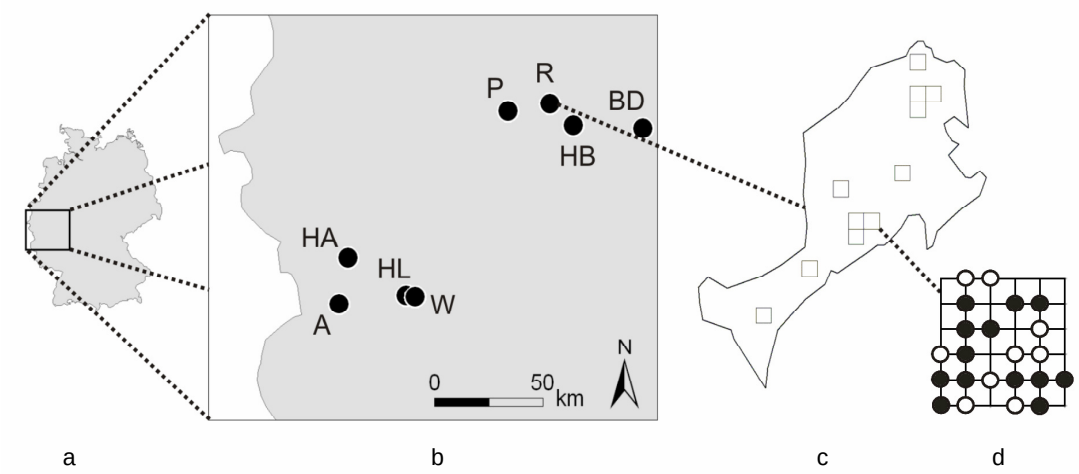

Fig. 1 (a - d) – a: The location of the Rhenish Uplands in Germany. b: Analysed juniper populations in the Rhenish Uplands. A: Alendorfer Kalktriften, BD: Battenfelder Driescher, HA: Halsberg, HB: Haberg, HL: Heidbuechel, P: Piwittsmoor, R: Ruebenkamp, W: Waberner Heide. c: Position of the 15 x 15 m plots in the stand Ruebenkamp (R). d: One representative plot with 3 x 3 m grid point samples, where empty circles indicate female junipers and filled circles indicate potential male junipers.

2.2 Sampling of seeds

From two populations (HB and HL), ten females were selected (five from each population) and 150 dark blue cones were harvested from each mother tree in 2005. We chose four females at the margins in the north, south, west and east and one in the centre of each juniper stand. The distances between them were approximately equal. After incubation in distilled water for 24 h, all seeds were cut. The embryos were taken out and stored at -80 °C until extraction. Due to seed predation and abortion (García et al., 2000), we did not find embryos in all seed coats. Therefore, the embryo numbers ranged from 41 to 185 in the population Haberg (HB) and from 35 to 122 in the population Heidbuechel (HL).

2.3 Molecular methods

Genomic DNA was extracted from frozen needles (approximately 100 mg) following the protocol by Dumolin et al. (1995). As a slight modification, the protocol included an additional and final treatment with 0.5 µg RNase at 37 °C for 30 min. Genomic DNA from the embryos was extracted with the extraction kit Nucleon Phyto Pure (GE Healthcare, Uppsala, Sweden). We genotyped all samples at four nuclear microsatellite markers developed in a previous study (Michalczyk et al., 2006). The amplification was carried out in a total PCR volume of 12.5 µl with 30 ng of template DNA, 5 x PCR reaction buffer (Promega, Mannheim, Germany), 5 mM of each dNTP, 0.5 U *Taq* polymerase (GoTaq, Promega), 0.2 µM of each primer (forward primer fluorescence labelled), 0.8 % BSA, and 1.5 mM, 2 mM or 2.5 mM $MgCl_2$, depending on the locus to be amplified. The PCR profiles were optimised as follows: For the primer Jc 31 (FAM-label), the protocol was best with denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C 30 s, 52 °C 50 s, 72 °C 80 s and a final extension of 72 °C for 7 min, using 2.5 mM $MgCl_2$. For the primer Jc 32 (FAM-label), optimal products were obtained with denaturation at 94 °C for 5 min foll owed by 35 cycles of 94 °C 30 s, 56 °C 50 s, 72 °C 90 s and a final extension of 72 °C f or 7 min, using 2.0 mM $MgCl_2$. For the primers Jc 35 (HEX-label) and Jc 37 (TMR-label), a touch-down procedure was successful with denaturation at 94 °C for 5 min, 10 touchdown cycles (-1 °C/cycle) of 94 °C 30 s, 60 °C 50 s (-1 °C/cycle), 72 °C 80 s, 25 cycles of 94 °C 30 s, 50 °C 50 s, 72 °C 40 s and a final extension at 72 °C 7 min, using 1.5 mM $MgCl_2$ each. For the amplification of the nSSR loci from embryo DNA we used 38 cycles for the primer pairs Jc 31 and Jc 32. For the primer pairs Jc 35 and Jc 37 we used 10 touchdown cycles and 28 additional cycles. In a test run, inconsistent results were obtained when the same embryo DNA was repeatedly amplified. This could be due to phytochemicals, especially essential oils, which can act as PCR inhibitors. Also, PCR can produce erroneous results when template DNA is available only in

small amounts (Taberlet and Luikart, 1999). Therefore, we repeated all amplifications twice. Only when the results were identical did we regard them as valid. Finally, only 8 to 40 (HB) and 7 to 14 (HL) embryos per mother tree could be used for data analysis.

2.4 Data scoring and validation of the marker system

The amplification products were separated by capillary electrophoresis using MegaBACE 1000 (GE Healthcare) automatic sequencer. Alleles were sized using the size standard MegaBACE ET400-R (GE Healthcare) and the MegaBACE Fragment Profiler 1.2 software (GE Healthcare). The reproducibility of the nSSR marker system was estimated according to Bonin et al. (2004). Accordingly, we repeated the amplification of a sub-set of 25 samples twice for each locus. The software MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004) was used to test for unexpected allele sizes. The resulting non-fitting allele sizes were corrected. Moreover, the number of missing data was calculated for each locus and over all loci. The probability of the presence of null alleles was estimated using the computer programme Cervus 3.0.3 (Kalinowski et al., 2007). To test the power of the marker system for an unambiguous individual identification or to properly address ramets of a genet, we calculated the average probability of identity (PI_{ave}) using the computer programme API-CALC 1.0 (Ayres and Overall, 2004). Clones and ramets, respectively, were identified by matching identical multi-locus genotypes (Cervus, Kalinowski et al., 2007). Before calculating the population genetic parameters all ramets of a genet except one were excluded from the whole data set.

2.5 Demographic data analysis

The numbers of genets were counted for all populations. Based on these numbers, which indicate the real individual number genotyped, we calculated the sex ratio (female : male). Based on the same numbers, we also calculated the number of juniper individuals per hectare as an extrapolation from the sample area of 11 x 225 m².

2.6 Population genetic analysis of the adult junipers

The computer software FSTAT 2.9.3.2 (Goudet, 2001) and Cervus 3.0.3 (Kalinowski et al., 2007) were used to calculate within and between population genetic parameters. For each population, the number of alleles per locus and the mean number of alleles were calculated (N_a). To correct for variation in sample size, we calculated the allelic richness (R_s) per locus and population using the rarefaction method described by El Mousadik and Petit (1996). Rarefaction size was 40 since this was the minimum sample size when all the data from all

loci were complete. Furthermore, the observed (H_o) and expected heterozygosity (H_e) (Nei, 1987) as well as the fixation index (F_{IS}) according to Weir and Cockerham (1984) for each locus and population were estimated. Deviations from HWE and respective levels of significance were assessed. To elaborate on the question whether the fragmentation of the formerly widespread juniper has caused recent genetic bottlenecks, we analysed the eight populations with the computer software BOTTLENECK 1.2.02 (Cornuet and Luikart, 1996). We did this under two different assumptions, first under the assumption of an infinite allele model (IAM) according to Kimura and Crow (1964), and second under the assumption of a stepwise mutation model (SMM) (Shriver et al., 1993; Valdes et al., 1993). Significance between the observed and expected allelic diversity and heterozygosity across loci was also tested (Wilcoxon sign-rank test). In order to detect genetic differentiation among the eight juniper stands, global F_{ST} and pairwise F_{ST} calculations were carried out following Weir and Cockerham (1984). Since some loci showed deviations from HWE throughout all populations, we tested the population differentiation (global F_{ST} and pairwise F_{ST}) as well as the heterozygote deficit within populations (F_{IS}) by permuting genotypes among samples, instead of permuting alleles, with the log-likelihood statistic G (Goudet et al., 1996). To test for an effect of isolation-by-distance, we performed a Mantel test using the computer programme GenAlEx 6.1 (Peakall and Smouse, 2005), while the pairwise F_{ST} - matrix derived from the calculations with FSTAT 2.9.3.2 (Goudet, 2001). The results were visualised by plotting the pairwise F_{ST} - values against the geographic distances.

2.7 Genetic variation and differentiation of the reproductively effective pollen clouds

In order to analyse the genetic variation and differentiation of the pollen clouds, which had become reproductively effective within the ten analysed half-sib embryo families, we developed the computer programme Swap. It is available for free from http://www.uni-marburg.de/naturschutzbiologie/downloads/. This programme was written to sample the paternal alleles from the embryo genotypes, particularly for cases where an embryo had the same heterozygote genotype as its mother. In such ambiguous cases, Swap permutes the relevant alleles (gametes) of the embryos via a jackknifing-procedure. Next, the programme simulates rarefaction using re-sampling without replacement on the basis of the smallest sample size. Thus, it calculates allelic richness after rarefaction (R_s). In addition, it uses the algorithms of Pons and Petit (1995) to estimate the within population diversity H_S and the total diversity H_T in order to finally estimate the genetic differentiation parameter G_{ST}. Via a second

jackknifing-procedure, the confidence limits and thereby the levels of significance are finally provided.

Two questions were addressed by the analysis of the pollen clouds with the help of Swap. On the one hand, we wanted to ascertain whether the adult populations and the next gamete populations, represented within the offspring, differ in terms of genetic variation and/or differentiation. All pollen clouds of a stand were pooled for this purpose. For comparison with the adults or parental generation, we had to transform the diploid genotypes of the adults into haplotypic data sets, so that the individual number was *quasi* doubled. This approach is permitted since it does not affect the allele frequencies. Another question was whether the single pollen clouds are differentiated among each other. Each pollen cloud that had been descending from a single female was deemed to be a single gametic population.

2.8 Palynological analysis

We collected a set of moss cushions on a transect situated inside and outside of the stand Alendorfer Kalktriften in August 2005. Inside the population, we measured the distances between a given sample and the nearest male juniper by using a measuring tape. To avoid influences through neighbouring shrubs, the samples were taken at sparsely juniper-covered areas. The distances between outside samples to the edge of the juniper population were calculated by GPS-data and digital maps using the computer programme Fugawi Global Navigator 4.5. The preparation of the pollen samples followed standard procedures using KOH, HF and acetolysis. Afterwards the pollen suspension was sieved in an ultrasonic bath (mesh max. 1.5 µm) and stored in glycerine (Erdtman, 1960; Moore et al., 1999). To calculate the concentration of juniper pollen grains per gram in every single moss cushion, the dried cushion was weighed and a known number of exotic markers (*Lycopodium* spores) was added (Benninghoff, 1962; Stockmarr, 1971). Seven samples were analysed until 200 arboreal pollen grains (excluding juniper) were found. The calculation of the proportion of juniper pollen grains is based on the pollen sum of all arboreal and non-arboreal pollen grains. The proportions and concentrations were plotted against the distances and best fit model was tested applying a linear, a quadratic and a power function model. Models were compared using an ANOVA. All analyses were conducted in ‘R’ version 2.5.0 (Ihaka and Gentleman, 1996).

3. Results

3.1 Validation of the marker system and clone identification

The test on reproducibility revealed that the genotyping error was 0 % for all four loci when DNA templates were amplified twice. On the other hand we had not obtained PCR products from all templates. In the adult junipers, the proportion of missing data was 2.69 % in total, and 9.57%, 0.27 %, 0.13 % and 0.8 % at the four loci Jc 31, Jc 32, Jc 35 and Jc 37. With the more difficult tissue of the embryos, all figures were higher with a total proportion of 12.17, and 31.98 %, 1.74 %, 0.41 % and 14.53 % at the four loci (in same order as above). Null alleles occurred at all loci. The frequencies varied between 0.344 and 0.631 (Jc 31), 0.011 and 0.071 (Jc 32), 0.014 and 0.159 (Jc 35) and 0.038 and 0.099 (Jc 37) (Table 2). However, following the ranking by Chapius and Estoup (2007), who analysed the prevalence and distribution of null allele frequencies at nSSR loci, the frequencies at the loci Jc 32, Jc 35 and Jc 37 were 'moderate' to 'negligible'. Only the null allele frequencies at locus Jc 31 were 'large' and are expected to introduce a bias into population genetic analyses. As there were no further nSSR markers available for *J. communis*, and in order to explicitly evaluate the effect of the high null allele frequencies at locus Jc 31, we calculated all population genetic parameters with and without this locus. Consequently, both results at three and four loci are given while the results obtained at four loci including Jc 31 are given in brackets. If there are no brackets given, the respective results were identical. To test for the power of individual identification or differentiation, we calculated PI_{ave}, which ranged from 7.91×10^{-6} (HL) (2.69×10^{-8}, P) to 1.76×10^{-5} (HB) (9.6×10^{-7}, HB). Even with three loci only, PI_{ave} was low enough to properly address juniper individuals and ramets of a genet.

3.2 Demographic data

The number of repeatedly sampled junipers, which equals the number of prostrate bushes bigger than 3 x 3 m in diameter, varied extremely among the eight populations, from 0 (HA) to 32 (HL) (Table 1). Sometimes one genet was composed of more than two ramets (see our previous definition). After clone identification and exclusion of all ramets of a genet except one, the original number of samples and therefore the number of individuals was drastically reduced (Table 1). The resulting number of 'true' individuals was used to calculate the density parameter 'individuals per hectare'. Here, the main difference between the stands became obvious with highly variable numbers of individuals spanning from 182 (HB) to 634 (P) per hectare (Table 1). In five populations, the sex ratio (female : male) was male-biased (HA, HB, HL, P, R). There were more females than males in two populations (BD, W) while the sex ratio was balanced in one population (A) (Table 1).

Table 1 – Characteristics of the investigated juniper populations, sample sizes and demographic parameters.

Population	Code	Area size (ha)	Latitude	Longitude	Sample size (N)	Number of prostrate junipers sampled repeatedly	Number of individuals corrected by clones	Number of juniper individuals per ha	Sex ratio (female : male)
Alendorfer Kalktriften	A	17.7	50.3646	6.6421	126	2	124	501	1 : 1
Battenfelder Driescher	BD	24.8	51.0415	8.6398	118	13	99	400	1 : 0.83
Halsberg	HA	5.4	50.5516	6.7037	55	0	55	222	1 : 1.16
Haberg	HB	9.8	51.0638	8.1844	70	13	45	182	1 : 1.37
Heidbuechel	HL	15	50.3960	7.0742	116	32	61	246	1 : 1.18
Piwittsmoor	P	11.4	51.1286	7.7602	162	5	157	634	1 : 1.28
Ruebenkamp	R	3.1	51.1528	8.0356	127	9	113	456	1 : 1.4
Waberner Heide	W	6.2	50.3905	7.1334	115	12	98	396	1 : 0.92

3.3 Genetic diversity of the adult populations

Diversity statistics are summarized in Table 2 for each locus separately and averaged over all four loci. The means for three loci without Jc 31 are given as well. In the case were we applied rarefaction, the rarefaction size increased from 40 (4 loci) to 45 (3 loci).

The analysed nSSR loci were highly polymorphic with a total number of alleles varying from 17 (Jc 35) to 51 (Jc 32). The mean number of alleles per population ranged from 18.67 (HB) (17.25, HB) to 29 (P) (27.5, P). The mean allelic richness per population varied from 18.67 (HB) (16.78, HB) to 22.08 (P) (20.638, P). Observed heterozygosity (H_o) ranged from 0.719 (HA) (0.605, HA) to 0.792 (HB) (0.697, P), and expected heterozygosity (H_e) from 0.856 (R) (0.857, R) to 0.885 (BD) (0.893, P). Locus Jc 31 deviated significantly from HWE in all populations. At the other loci significant deviations occurred as well but not throughout all populations. Operating with four loci, the fixation index was very high in all populations with mean values between 0.204 (R, $P < 0.001$) and 0.313 (HA, $P < 0.001$). This was different when the locus Jc 31 was excluded. In this case, mean F_{IS} ranged from 0.091 (HB, $P < 0.01$) to 0.193 (HA, $P < 0.001$).

Table 2 – Levels of genetic diversity and fixation within eight juniper populations for four nSSR loci and averaged over four and three loci. Additionally, the null allele frequencies are given.

Locus	N_a	R_s	H_o	H_e	HWE	F_{IS}	Null allele frequency
Alendorfer Kalktriften (A)							
Jc 31	25	19.11	0.236	0.879	**	0.732***	0.575
Jc 32	35	25.041	0.839	0.944	**	0.112***	0.059
Jc 35	9	7.47	0.565	0.725	**	0.222***	0.122
Jc 37	30	24.8	0.879	0.952	NS	0.077**	0.038
Over all	24.75	19.105	0.63	0.875	-	0.286***	0.2
Over 3 loci	24.67	19.784	0.761	0.873	-	0.137***	0.073
Battenfelder Driescher (BD)							
Jc 31	18	16.243	0.323	0.895	**	0.641***	0.47
Jc 32	35	27.769	0.859	0.957	NS	0.103***	0.052
Jc 35	10	7.645	0.616	0.754	**	0.184***	0.103
Jc 37	29	22.872	0.828	0.943	NS	0.123***	0.063
Over all	23	18.632	0.657	0.887	-	0.263***	0.172
Over 3 loci	24.67	20.137	0.768	0.885	-	0.137***	0.073
Halsberg (HA)							
Jc 31	10	9.634	0.264	0.799	**	0.671***	0.502
Jc 32	29	25.85	0.818	0.951	NA	0.141***	0.071
Jc 35	10	9.206	0.556	0.759	**	0.27***	0.159
Jc 37	25	23.448	0.782	0.939	NS	0.169***	0.086
Over all	18.5	17.035	0.605	0.862	-	0.313***	0.204
Over 3 loci	21.33	20.197	0.719	0.883	-	0.193***	0.105
Haberg (HB)							
Jc 31	13	13	0.2	0.891	**	0.778***	0.631
Jc 32	19	18.418	0.8	0.916	NS	0.128 **	0.061
Jc 35	10	9.553	0.733	0.756	NS	0.03 NS	0.014
Jc 37	27	26.149	0.844	0.953	NA	0.115**	0.055
Over all	17.25	16.78	0.644	0.879	-	0.263***	0.19
Over 3 loci	18.67	18.67	0.792	0.875	-	0.091**	0.043
Heidbuechel (HL)							
Jc 31	18	16.423	0.368	0.878	**	0.583***	0.411
Jc 32	33	27.97	0.869	0.95	NA	0.086**	0.042
Jc 35	8	7.495	0.574	0.687	NS	0.166*	0.087
Jc 37	25	22.474	0.869	0.943	NA	0.079*	0.038
Over all	21	18.591	0.67	0.865	-	0.229***	0.144
Over 3 loci	22	20.094	0.771	0.86	-	0.11***	0.056

Table 2 – continued

Locus	N_a	R_s	H_o	H_e	HWE	F_{IS}	Null allele frequency
Piwittsmoor (P)							
Jc 31	23	18.596	0.416	0.923	**	0.55***	0.378
Jc 32	43	29.36	0.865	0.957	**	0.096***	0.049
Jc 35	9	7.561	0.624	0.734	**	0.15***	0.084
Jc 37	35	27.036	0.882	0.958	NS	0.08***	0.04
Over all	27.5	20.638	0.697	0.893	-	0.219***	0.138
Over 3 loci	29	22.082	0.79	0.883	-	0.109***	0.058
Ruebenkamp (R)							
Jc 31	20	16.098	0.419	0.86	**	0.514***	0.344
Jc 32	38	28.135	0.867	0.95	**	0.087***	0.045
Jc 35	10	8.972	0.619	0.676	NS	0.084 NS	0.047
Jc 37	25	21.205	0.821	0.943	NS	0.129***	0.068
Over all	23.25	18.603	0.682	0.857	-	0.204***	0.126
Over 3 loci	24.34	20.105	0.769	0.856	-	0.1***	0.053
Waberner Heide (W)							
Jc 31	24	18.35	0.412	0.875	**	0.531***	0.359
Jc 32	30	25.61	0.928	0.956	NS	0.029 NS	0.011
Jc 35	11	8.699	0.592	0.696	NS	0.15*	0.085
Jc 37	29	24.183	0.776	0.949	**	0.183***	0.099
Over all	23.5	19.211	0.677	0.869	-	0.223***	0.139
Over 3 loci	23.33	20.084	0.765	0.867	-	0.121***	0.065

N_a = number of observed alleles; R_s = number of alleles after rarefaction with 40 and 45 individuals, respectively; H_o = observed heterozygosity; H_e = expected heterozygosity; HWE = significance of deviation from Hardy-Weinberg equilibrium; F_{IS} = fixation index. Significance levels: NS = not significant, * = significant at the 5 % level, ** = significant at the 1 % level, *** = significant at the 0.1 % level, all significance levels include Bonferroni corrections ; NA = not assessed

Neither under the IAM nor under the SMM did we find any evidence for recent bottlenecks in the fragmented eight juniper stands of the Rhenish Uplands (P > 0.05, one-tailed Wilcoxon sign-rank tests).

3.4 Genetic differentiation among adult populations

In order to measure genetic differentiation, we calculated global F_{ST} resulting in a highly significant value of 0.014 (0.014) (P < 0.001 for both marker combinations). Pairwise F_{ST} - values ranged from 0.0006 (HL and W) (0.0009, HL and W) to 0.037 (R and HL) (0.031, HB and R) and were significant in most cases (Table 3b). The overall r_{xy} - value of the Mantel test

was 0.035 (0.087). For three as well as for four loci there was no significant relationship between the two matrices (Fig. 2, for three loci only).

Table 3 (a - b) – a: Pairwise geographic distance matrix (km) and b: pairwise genetic differentiation matrices (F_{ST}) with levels of significance, upper triangle 3 loci, lower triangle 4 loci.

a	A	BD	HA	HB	HL	P	R	W
A	0							
BD	159.548	0						
HA	21.246	146.569	0					
HB	133.548	31.928	118.609	0				
HL	30.837	131.525	31.414	107.793	0			
P	115.780	62.197	98.079	30.486	94.677	0		
R	131.484	43.969	114.937	14.348	107.941	19.401	0	
W	34.958	128.402	35.294	105.249	4.241	93.164	105.876	0

b	A	BD	HA	HB	HL	P	R	W
A	0	0.0039***	0.0066***	0.0063**	0.0038*	0.006***	0.0305***	0.0073***
BD	0.0063***	0	0.0084**	0.0126***	0.0089***	0.0078***	0.0336***	0.0087***
HA	0.0085***	0.0119***	0	0.0123***	0.0071*	0.0049*	0.0219***	0.0061 NS
HB	0.0059**	0.0145***	0.0165***	0	0.0047 NS	0.0117***	0.0319***	0.0161***
HL	0.0027 NS	0.0079**	0.0058*	0.0063*	0	0.0078***	0.037***	0.0006 NS
P	0.0094***	0.0072***	0.0122***	0.0111***	0.0094***	0	0.0174***	0.0077***
R	0.027***	0.0267***	0.0204***	0.0312***	0.0295***	0.0188***	0	0.0356***
W	0.0049***	0.0093*	0.0073***	0.0161***	0.0009 NS	0.0113***	0.0284***	0

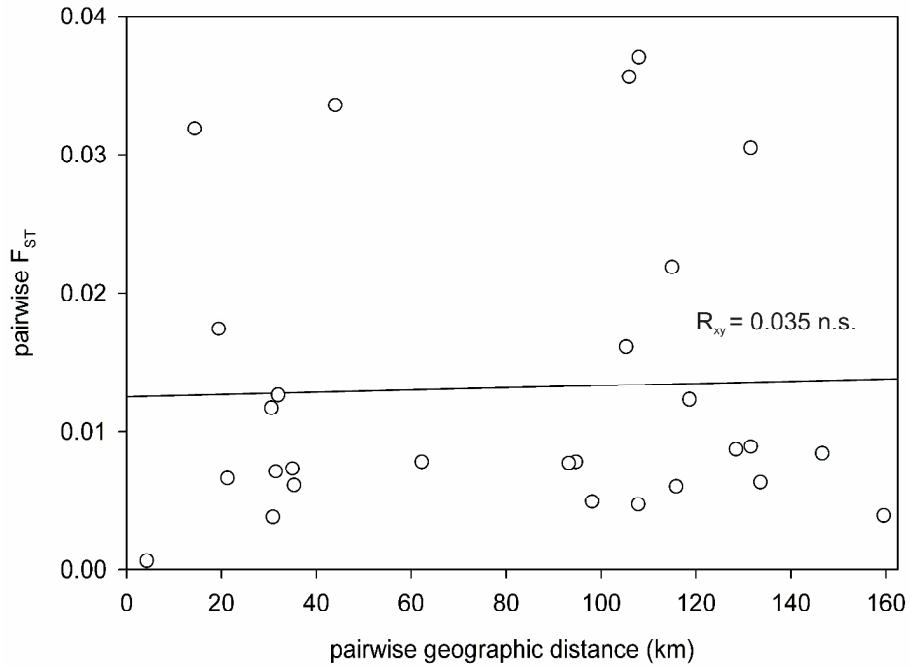

Fig. 2 – Relationship of pairwise F_{ST} - values and pairwise geographic distances (km) (3 loci only).

3.5 Genetic variation and differentiation of the reproductively effective pollen clouds

As a case study in two of the eight populations, we compared the allelic richness of the pollen clouds with the allelic richness of the adults after haploidising the diploid genotypes. Furthermore, we analysed the genetic differentiation among these two subsets in each of the two populations (Table 4).

Table 4 – Allelic richness of the adult junipers and the pooled pollen clouds in each of the two juniper populations. G_{ST} is given as an estimate of genetic differentiation between the two subsets. SD = standard deviation.

Population	R_s (3 loci)	SD (R_s)	R_s (4 loci)	SD (R_s)	G_{ST} (3 loci)	SD (G_{ST})	G_{ST} (4 loci)	SD (G_{ST})
HB adults	20	0.745	19.73	0.541				
HB embryos	19.9	0.448	19.35	0.7	-0.006 n.s.	0.001	-0.007 n.s.	0.001
HL adults	16.22	1.01	16.06	0.876				
HL embryos	15.67	0.685	16.05	0.86	-0.009 n.s.	0.002	-0.009 n.s.	0.002

For both combinations (three and four loci), the adult generation of the population Haberg (HB) showed a higher mean allelic richness than the one in the population Heidbuechel (HL). Interestingly, in each of the two populations, the adults and the paternal contribution to the embryos showed the same mean allelic richness. Also, in both populations the two subsets were not genetically differentiated for both marker combinations (Table 4). Next, we analysed the pollen clouds separately for genetic differentiation among them. If pollen-mediated gene flow was low, we would expect genetic differentiation to occur. In this aspect, the Haberg and the Heidbuechel population behaved differently. In the Haberg population we were not able to detect differentiation among the pollen clouds (G_{ST} = 0.0076 n.s., SD = 0.02 for three loci and G_{ST} = 0.0003 n.s., SD = 0.013 for four loci), whereas in the Heidbuechel population the pollen clouds were differentiated with G_{ST} = 0.0473* (SD = 0.019) for three loci and G_{ST} = 0.05** (SD = 0.02) for four loci.

3.6 Distances of pollen deposition

With increasing distance from the pollen source, the percentages and concentrations of juniper pollen grains declined steeply (Fig. 3). The power function $y = a \cdot x^{b}$ was found to giving the best fit with high values of R^2 (R^2 = 0.91 and 0.96 for percentages and concentrations, respectively), demonstrating the strong correlation of decreasing pollen availability with increasing distance over short distances. The relative and absolute abundances of juniper pollen were reduced to a third over a distance of less than 5 m between a male donor and a respective moss cushion (Table 5). This means a reduction from

45 % in 0.5 m distance to 15.2 % in 5 m distance and a reduction from a concentration of 33 294 juniper pollen grains per gram moss cushion to 11 275 grains per gram.

Table 5 – Moss cushions of the transect with samples 1 - 3 from inside the juniper population and samples 4 - 7 from outside.

Sample	Distance between moss cushion and next male juniper (m)	Percentages of juniper pollen grains	Concentration of juniper pollen grains per g moss cushion	Pollen sum of aboreal and non-aboreal pollen grains
1	0.5	45	33294	802
2	1	18.1	13373	448
3	5	15.2	11275	490
4	13	3	2185	440
5	110	1	759	680
6	140	0.4	282	524
7	220	0.4	325	680

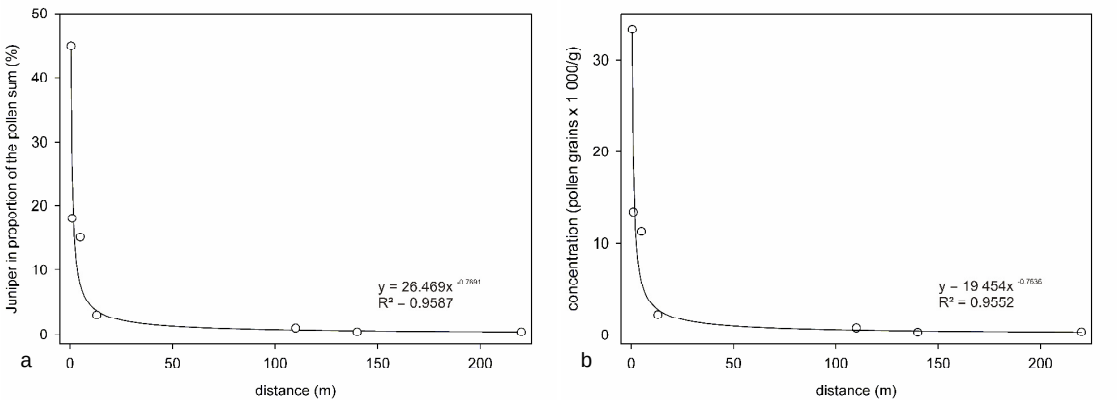

Fig. 3 (a - b) – Relation of the amount of juniper pollen grains with increasing distance from the pollen donor. a: proportion of juniper pollen grains and b: concentrations of juniper pollen grains per gram moss cushion, with lines of best fit, their formulas and coefficients of determination R^2.

4. Discussion

Nowadays, juniper habitats in many parts of Europe are highly fragmented and the populations are reduced in size. We used a nuclear microsatellite marker approach to gain insights into possible genetic consequences of habitat reduction and isolation of eight relict juniper populations in the Rhenish Uplands in Germany. Finally, a compact guideline for a genetic management of juniper populations is provided.

4.1 Validation of the marker system

Since nearly three decades, nSSR markers as powerful Mendelian markers are widely used for population genetic analyses including parentage analyses (Dakin and Avise, 2004). However, they entail a potential drawback, which is the occurrence of null alleles (Pemberton et al., 1995). Null alleles can occur through mutations in the primer annealing sites, thus amplification fails (Jarne and Lagoda, 1996). In addition to technical problems, null alleles can also result from a misinterpretation of a heterozygote deficiency reflecting deviations from HWE. The latter in turn can be caused by population genetic processes such as inbreeding, genetic drift or Wahlund effect (Chakraborty et al., 1992). By thorough multilocus analysis, these misinterpretations can be circumvented due to the fact that the mentioned factors should behave more or less concordant across all loci analysed (Dakin and Avise, 2004). Since the null allele frequencies at the locus Jc 31 were always considerably higher than those at the other three loci, we assumed this locus to exhibit 'real' null alleles. This assumption is corroborated by the large amount of missing PCR products at this locus that represents a status of quasi homozygosity of null alleles. It is generally supposed that loci with assured null alleles introduce a bias into analyses of genetic diversity and differentiation (Dakin and Avise, 2004; Chapius and Estoup, 2007). Interestingly, when we explicitly explored this phenomenon by keeping the locus Jc 31 within four-loci genotypes as well as by omitting it (three-loci genotypes), we came up with similar high values for all diversity parameters (N_a, R_s, H_e). What was even more interesting was the fact that in most cases the mean observed number of alleles was slightly higher for three than for four loci. Also, a heterozygote deficiency and fixation of alleles were detected for both combinations. However, these values substantially decreased after omission of locus Jc 31. The software Cervus (Kalinowski et al., 2007) provided low frequencies of null alleles at the loci (Jc 32, 35 and 37). This gave reason to ask whether the low but still present heterozygote deficiency was caused by the low frequencies of the null alleles detected or whether we were confronted with 'true' deviations from HWE. The question is hard to answer since the algorithm of calculating null allele frequencies partly includes comparisons between H_o and H_e. Or could there be another reason for deviations from HWE and fixed alleles in our case, e.g. such as a sample size effect, which contributes to the false positive results of a heterozygote deficiency (Hedrick, 2000)? Indeed, the large number of alleles detected at the four-loci genotypes should have demanded for larger sample sizes than ours (e.g. Gregorius, 1980). Actually, we got a strong signature of such a sample size effect, when we compared the observed with the expected frequencies of single-locus genotypes. Our careful evaluation finally allows us to rely on a three-loci genotype approach, which is as informative as working with four loci regarding

diversity parameters. Furthermore, when working with three-loci genotypes, the slight deviation from HWE and the positive fixation indices have most likely derived from the sample size effects. Therefore, we decided not to interpret them in terms of deleterious genetic effects of fragmentation.

4.2 Status of genetic diversity

All diversity parameters (N_a, R_S, H_e) were comparatively high in all eight juniper populations. This is not unexpected for a tree species, which pursues life-history strategies such as outbreeding, wind-pollination, efficient seed dispersal via wind and/or animals and longevity (Hamrick and Godt, 1996). High levels of genetic diversity were also found for juniper in other European countries (van der Merwe et al., 2000; Oostermeijer and de Knegt, 2004; Michalczyk et al., submitted). However, bearing in mind that the analysed juniper populations are highly fragmented (and therefore constitute isolated relict habitats) and that physical dispersal of pollen was short-distanced, the high values of genetic diversity are astonishing. We suggest that the fragmentation of the formerly widespread juniper stands in the Rhenish Uplands so far has had only little to no effect on the genetic diversity of the remnant populations. This is in line with the high levels of genetic diversity of juniper in fragmented heathlands in the Netherlands (Oostermeijer and de Knegt, 2004). Moreover, in the present study we had to reject the hypothesis of recent bottlenecks. Fragmentation of the formerly widespread juniper stands, which started approximately 200 years ago, has obviously not yet led to genetic erosion. Instead, a ‘freezing-effect’ of the formerly genetic diversity in all remnant populations has occurred. Being old and lacking natural regeneration, the populations present themselves as genetically diverse fragments of the old non-fragmented heathlands. Therefore, if ever there will be a genetic effect of the recent fragmentation, we postulate a time-lag in genetic response of juniper. ‘Freezing effects’ of genetic diversity are reported for other long-lived tree species as well, especially in cases where the fragmentation event is within the life-span of one generation (e.g. Young et al., 1993; Aldrich et al., 1998; Lowe et al., 2005; Williams et al., 2007). Moreover, this phenomenon is also assumed for long-lived juniper in England and the Netherlands (van der Merwe et al., 2000; Oostermeijer and de Knegt, 2004).

4.3 Past processes of gene flow

Accepting that the ‘frozen’ remnants do not exhibit current gene flow within and among stands, the question arises about past gene flow, which can be estimated by the degree of genetic differentiation. The mean value of genetic differentiation (G_{ST}) reported for biparental

inheritance in gymnosperms is approximately 0.116 (Petit et al., 2005). Compared to that, we found a smaller significant value of global differentiation (F_{ST} = 0.014). Our finding corresponds to that found for juniper in the Netherlands and Ireland (F_{ST} = 0.026, Oostermeijer and de Knegt, 2004; $\Phi_{ST(N)}$ = 0.096, Provan et al., 2008). As a result of our Mantel test, we clearly had to reject the hypothesis of an isolation-by-distance effect. At first sight, this seems to conflicts with the isolation-by-distance effect on the regional scale in Central Europe found with AFLP markers in our previous study (Michalczyk et al., submitted). However, the contrary results are most likely based on the different marker categories we used. As opposed to nSSR markers, AFLP markers may include phylogenetic and selectively relevant signals as well (Meudt and Clarke, 2007). The scatterplot of the pairwise genetic and geographic distances, which shows relatively low and equal pairwise F_{ST} - values, matches exactly with case II of the model by Hutchison and Templeton (1999). This scenario II is likely to reflect the genetic structure shortly after a homogeneous source population has invaded a new habitat. Here, we find additional evidence for the hypothesised 'frozen' diversity. This scenario might offer us a look back at a time when juniper had founded populations or had re-expanded in the Rhenish Uplands from existing populations. Palynological data confirm that juniper has been continuously existent for several centuries in this region (Speier, 1994, 1999). The scatterplot allows an additional explanation regarding gene flow and drift. It supports the model in which gene flow predominates genetic drift. Thus panmixia prevails (Hutchison and Templeton, 1999). Against the background of the 'frozen' situation we therefore have to consider that in former times gene flow could have been stronger than genetic drift. Van der Merwe et al. (2000) discuss long-distance seed dispersal by birds, which has been more prevalent in former times than today. This leads us to assume that in the future, further losses of over-aged individuals and a lack of previous and future sexual recombinations could lead to a genetically deleterious effect of fragmentation. This seems to be different in Ireland where recent bottlenecks and therefore fragmentation effects could not be excluded (Provan et al., 2008).

4.4 Current pollen-mediated gene flow

If fragmentation has not yet affected genetic diversity in the long-lived juniper, could we postulate a time-lag? And consequently, could we postulate that fragmentation will affect the next generations, and also affects them to an even larger extent under the assumption of locally restricted gene flow via pollen? Our study on physical distances of pollen dispersal revealed evidence for short-distance deposition. Concerning reproductively effective pollen dispersal, there is evidence for spatially restricted pollen dispersal from the literature. A

significant negative relationship between the distance to the nearest male juniper and the seed production in Dutch juniper populations indicates short pollen flow distances (Hopster and Greeve, 1999). Our case study on genetic variation and differentiation of reproductively effective pollen clouds is the first which uses nSSRs markers on DNA from juniper embryos. The results reveal that the pooled reproductively effective pollen clouds of single tree embryos exhibit similar values of allelic richness compared to the respective adult stand. Obviously, when collecting paternal alleles from five half-sib embryo families from the corners and the centre of a habitat, there was no genetic drift from the adult to the filial generation. This is reflected by the absence of differentiation among the adults and the embryos. However, when we considered each pollen cloud separately, the two populations (HB and HL) behaved different. One of it (HL) exhibited significant genetic differentiation among the pollen clouds. The present case study cannot be used to generalise our results but can give valuable perspectives for ongoing hypotheses and studies. The juniper population Heidbuechel, which showed significantly differentiated pollen clouds, harbours similar low numbers of individuals per hectare as does the Haberg population. This means both populations show the same density of individuals. But the pollen clouds were genetically differentiated in only one of them. The latter population is characterised by a great number of large prostrate bushes or spatially expansive clonal structures (32 cases). We cannot exclude the fact that such large individuals produce large pollen clouds, which prevail in the progeny of neighboured females and introduce fixation of alleles. That means that low population density is not necessarily an indicator of future genetic drift and fixation of alleles. Systematic investigations concerning the impact of the spatial organisation of old juniper individuals on the mating system should be carried out in further studies.

4.5 Conclusions

Our genetic analysis supports a ‘freezing effect’ of high genetic diversity in and low genetic differentiation among eight juniper populations in the Rhenish Uplands since the times when the recent habitat fragmentation started approximately 200 years ago. The analysis of single tree progenies from two populations did not show a decrease of genetic diversity and therefore no negative effect of habitat fragmentation in the filial generation. However, since all analysed juniper populations suffer from a lack of natural regeneration and therefore are over-aged, the survival of juniper in the Rhenish Uplands remains uncertain. Most likely it is threatened with extinction even if it would persist as a species with a sole asexual reproduction for a while. Thus, juniper heathlands may retain a certain vitality until a complete breakdown of over-aged clones. Since this kind of juniper die-off may be predicted also in

other European countries (Verheyen et al., submitted and references therein), we elaborated on a detailed management plan for over-aged juniper populations lacking regeneration.

4.6 Guidelines for a genetic management

It is already known that old female junipers produce fewer viable seeds than younger females (Ward, 1982). Thus, the first step should be to rejuvenate the populations by planting 'young' junipers. With this we hope to rekindle the production of viable seeds. Second, we should create promising germination sites to realise the reproduction events. Based on our genetic analyses, on what is known about juniper and on expert know how, the first step will be described in detail below. It is not yet clear exactly how promising germination sites should be created. However, several suggestions and ideas can be found in the literature (e.g. Oostermeijer and de Knegt, 2004; Verheyen et al., 2005; Thomas et al., 2007).

Which plant material shall we use for plantings, from where shall we take it and where shall we apply it?

1. Since most of the seeds of juniper in Europe are empty due to a variety of reasons (see Verheyen et al., submitted and references therein) and since the germination of juniper seeds is extremely difficult (Oostermeijer and de Knegt, 2004), we advise the use of vegetative cuttings for the process of rejuvenation.
2. Plant material can be collected from any European juniper population since a Europe-wide AFLP analysis did not reveal any distinct historic genetic lineages (Michalczyk et al., submitted). However, we advise harvesting this material from genetically diverse populations and in localities with similar climatic and ecological conditions compared to the target locations (Michalczyk et al., submitted).
3. To prevent genetic erosion due to plantings, collecting plant material in clonal structures should be avoided.
4. Male-biased sex ratios in over-aged juniper stands have been recorded several times (e.g. Clifton et al., 1997; Ward, 2007; the present study). Although the ultimate cause of differential mortality of females is not yet fully understood (Ward, 2007), we advise to collect the cuttings gender-specifically and at least to balance the sex ratio through plantings. In fact, considering the correlation between age of females and number of viable seeds (Ward, 1982), one could think about an exceeding increase of females through plantings.
5. The spatial organisation of juniper individuals inside the stands seems to play an important role for the successful reproduction and the genetic structure of the filial generation (Hopster and Greeve, 1999; the present study). Therefore male junipers should be planted near

females. However, there should be a sufficient number of males in the neighbourhood of females avoiding an overdominance of a single pollen cloud.

6. To achieve best rooting success, the following protocol should be used (B. Haase, personal communication): The cuttings shall be sampled from young junipers or young shoots (1 - 2 years old, 10 - 15 cm in length) ideally from August until September. The cut surface should be treated with 5000 ppm 3-indolylbutyric acid solution for approximately 2 min. For sticking, the soil substrate shall be a 50/50 mixture of peat and sand. The planting bed should be covered with a row cover (cloche) to guarantee high air humidity. Root formation requires about one year. During this time conditions should be kept constant.

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Declaration

I hereby declare that the dissertation entitled “Application of DNA marker systems to test for genetic imprints of habitat fragmentation in *Juniperus communis* L. on different spatial and temporal scales – Integration of scientific knowledge into conservation measures” submitted to the Department of Conservation Biology at the Philipps-University of Marburg is the original and independent work carried out by me under the guidance of the PhD committee. The dissertation has not been submitted previously to receive any Degree, Diploma or other similar titles.

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