Genetic diversity and population structure of the relict forest trees *Zelkova carpinifolia* (Ulmaceae) and *Pterocarya fraxinifolia* (Juglandaceae) in the South Caucasus

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Because the living environment is what really sustain us

E.O. Wilson (2008)

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Summary

The dissertation presents an analysis of the genetic diversity and population structure of two mesophytic trees *Zelkova carpinifolia* (Ulmaceae) and *Pterocarya fraxinifolia* (Juglandaceae) in the South Caucasus. The Caucasus represents one of the global biodiversity hotspots. In part, its enormous biological diversity is constituted by a type of mesophytic forests which once covered larger areas in Eurasia. The current range of these forests is considerably reduced due to the major climate and tectonic changes from the Miocene up to climate oscillations in the Quaternary. Therefore, these forests and many of their species (being paleoendemics) have a relict status. There are two major climate refugia in the southern Caucasus region: the Colchis at the eastern coast of the Black Sea and the Hyrcan at the southern coast of the Caspian Sea. The Caucasian elm, *Zelkova carpinifolia*, and the Caucasian wingnut, *Pterocarya fraxinifolia*, are very characteristic trees of the Caucasian relict forests, for both of which the fossil record indicates a much wider distribution in the past. The current distribution area covers the Colchis and the Hyrcan, and some populations are found in southeastern Anatolia and the Zagros Mountains. *P. fraxinifolia* also occurs at the foothills of the Greater Caucasus and adjacent valley, whereas *Z. carpinifolia* grows in the Lesser Caucasus in Garabagh. Despite their similar distribution area, their ecological preferences differ. *Zelkova carpinifolia* grows on both moist and dry stony soils, whereas *Pterocarya fraxinifolia* requires moist soils and humid air.

In the present work, a representative sampling of natural populations of *Z. carpinifolia* and *P. fraxinifolia* across their distribution area was carried out. Thirteen nuclear microsatellite markers were developed for *Z. carpinifolia* using next-generation sequencing (454 pyrosequencing). The genetic diversity and structure in both tree species was evaluated using nuclear and chloroplast loci. A specific objective was to test in how far Colchic and Hyrcanian populations of the trees are genetically separated. A further objective was to locate potential climate refugia and areas of high genetic diversity that deserve attention for conservation.

Extensive sampling of populations of *Z. carpinifolia* and *P. fraxinifolia* across their main distribution ranges in the South Caucasus and northern Iran was carried out with fieldwork between 2010 and 2013. Thirty populations of *Z. carpinifolia* and of *P. fraxinifolia* were included for microsatellite analysis, respectively, while an incremented number of 40 populations were studied for chloroplast haplotypes in *P. fraxinifolia*.

In *Z. carpinifolia* and *P. fraxinifolia*, the number of clonal individuals, detected as individuals with identical multilocus genotypes, varied substantially among sampling localities. In general, a low total number of alleles (2.35-3.63), a medium heterozygosity (0.425-0.604) and a low number of private alleles (0.01-0.14) were detected in *Z. carpinifolia*. Although relatively low numbers of alleles (2.8-4.9) and medium levels of heterozygosity (0.482-0.711) were also revealed in *P. fraxinifolia*, they were significantly higher than in *Z. carpinifolia*, especially in Hyrcan. The high number of private alleles (0.01-0.28) was also detected for populations of *P. fraxinifolia* from Hyrcan.

The analysis of molecular variance (AMOVA) of nuclear microsatellite loci indicated low differentiation among *Z. carpinifolia* populations. No significant differentiation among the Colchic and Hyrcanian populations of *Z. carpinifolia* was found. Bayesian analysis, which is based on the assignment of individuals to different clusters using individual membership coefficients inferred from allele frequencies, clustered the populations in two groups (Colchic and Hyrcanian) but with a wide gradient. The lack of a strong genetic structure and of patterns of isolation-by-distance suggests that gene flow through pollen is extensive in *Z. carpinifolia*. Such extensive gene flow can, therefore, reduce inbreeding and genetic drift. The natural populations of *Z. carpinifolia* are, however, highly geographically structured at chloroplast DNA loci, indicating limited seed dispersal in the species in contrast to the extensive pollen dispersal.

Although *P. fraxinifolia* is wind-pollinated, significant differentiation between Colchis and Hyrcan were revealed at the nuclear microsatellite loci. The two regions also differ at chloroplast DNA haplotype diversity. Eleven chloroplast haplotypes of *P. fraxinifolia* were found in Hyrcan, whereas only two were found in Colchis and the Greater Caucasus. In *P. fraxinifolia*, population structure is significant at both nuclear and chloroplast DNA loci, indicating more limited gene flow by both pollen and seeds.

The higher genetic diversity in the Hyrcan, in addition to the high number of private alleles and chloroplast haplotypes in this region, possibly indicates the existence of multiple past climate refugia in the region for *P. fraxinifolia*. This potentially also applies to *Z. carpinifolia*, although our sampling in the Alborz Mountains (N-Iran) is more sparse in this species. The existence of past refugia for *P. fraxinifolia* in Colchis cannot be ruled out based on the results of the present study.

The results of this doctoral thesis present a first comprehensive study of the genetic diversity and structure of natural populations of two relict tree species in the Caucasus. *Z.*

carpinifolia and *P. fraxinifolia* are both wind-pollinated trees, but the patterns of gene flow inferred using nuclear microsatellite markers are different in two species. The low production of pollen in *Z. carpinifolia* does not limit the high gene flow among existing populations, whereas abundantly produced pollen in *P. fraxinifolia* is also widely dispersed among populations, but restricted in dispersal among geographic regions. The ecological preferences of the species might explain these patterns of gene flow by pollen, so that pollen is dispersed over the large distances in the dry air, whereas in the humid air pollen mainly disperses locally. The phylogeographical patterns revealed at chloroplast loci suggested past diversification of *Z. carpinifolia* populations (chloroplast data were provided by collaborators) in both potential refugia and diversification of *P. fraxinifolia* populations in Hyrcan. So, this study also represents one of the first attempts to localizing the past climate refugia for mesophytic trees and shrubs in the Caucasus using genetic data.

To confirm the existence of past climate refugia in Colchis and Hyrcan, comparative analyses of the population genetics and phylogeography of other tree and shrub species in the region with different patterns of dispersal and reproductive systems should be performed. In future studies, more extensive sampling should be carried out, especially in Turkey and Iran, to obtain a complete picture of the genetic structure of both species throughout their distribution range.

The information obtained is useful in the planning of future conservation management strategies, and for *ex situ* and *in situ* protection of relict Caucasian tree species. The following implications for conservation should be considered in the future: (i) forest fragmentation which may cause the inbreeding and genetic drift in populations has yet no effect on the genetic diversity of the natural populations due to the high gene flow among and within populations; (ii) some sites with higher genetic diversity at nuclear and/or chloroplast loci (high number of alleles and haplotypes private to populations) in both Colchis and Hyrcan should be protected; in particular the populations from Hyrcan deserve particular attention for conservation. (iii) plant material used in the reforestation practices should consider the provenances, so that the seeds should be produced from the local populations in order to maintain the unique genetic variation of each site.

Zusammenfassung

In der vorgelegten Dissertation wird die genetische Diversität und Populationsstruktur der mesophytischen Reliktbäume *Zelkova carpinifolia* (Ulmaceae) und *Pterocarya fraxinifolia* (Juglandaceae) im Südkaukasus untersucht. Der Kaukasus gehört zu den globalen Biodiversitäts-Hotspots. Diese enorme biologische Vielfalt wird zum Teil durch einen Typ mesophytischer Wälder konstituiert, der einst größere Gebiete in Eurasien bedeckte. Bedingt durch die klimatischen und tektonischen Veränderungen seit dem Miozän, bis hin zu den Klimaoszillationen im Quartär, sind diese Wälder heute auf kleine Areale reduziert. Diese Wälder und ein Großteil ihrer Arten (sie sind Paläoendemiten) haben daher Reliktstatus. Es gibt zwei große klimatische Refugialräume in der südlichen Kaukasus-Region: Die Kolchis an der Ostküste des Schwarzen Meeres und der Hyrkan an der Südküste des Kaspischen Meeres. Die kaukasische Zelkove (*Zelkova carpinifolia*) und die Kaukasische Flügelnuss (*Pterocarya fraxinifolia*) sind charakteristische Elemente der kaukasischen Reliktwälder und für beide Arten zeigt der Fossilbeleg eine einst deutlich weitere Verbreitung als heute. Das aktuelle Areal umfasst die Kolchis und den Hyrkan, und einige Populationen komen in Südostanatolien und dem Zagros Gebirge vor. *Pterocarya fraxinifolia* gedeiht außerdem am Fuß des Großen Kaukasus und benachbarten Tälern, und *Z. carpinifolia* weist Populationen im Kleinen Kaukasus, in Garabagh, auf. Trotz ihrer ähnlichen Verbreitungsgebiete unterscheiden sich die ökologischen Präferenzen der beiden Arten. *Zelkova carpinifolia* wächst sowohl auf feuchten als auch trockenen steinigen Böden, wohingegen *Pterocarya fraxinifolia* feuchte Böden und feuchte Luft benötigt.

Im Rahmen der vorliegenden Arbeit wurden die natürlichen Populationen von *Z. carpinifolia* und *P. fraxinifolia* repräsentativ in ihrem Gesamtareal beprobt. Für *Zelkova* wurden mit Hilfe eines Next-Generation-Sequencing Ansatzes (454 Pyrosequenzierung) selbst Kern-Mikrosatelliten-Marker entwickelt. Dies ergab 13 polymorphe Kern-Mikrosatelliten-Marker für *Z. carpinifolia*. Die genetische Diversität und Struktur in beiden Baumarten wurde dann durch Verwendung von Kern- und Chloroplasten-Loci analysiert. Eine zentrale Fragestellung war dabei zu verstehen, in wie weit die kolchischen und die hyrkanischen Populationen voneinander genetisch isoliert sind. Ein weiteres Ziel war die Örtlichkeiten möglicher klimatischer Refugien und Gebiete mit hoher genetischer Diversität festzustellen, die besondere Aufmerksamkeit für Naturschutz-Maßnahmen erfordern.

In den Jahren 2010 bis 2013 wurden Feldarbeiten durchgeführt, um Proben von *Z. carpinifolia* und *P. fraxinifolia* über ihr Hauptverbreitungsgebiet im Südkaukasus und im nördlichen Iran hinweg zu sammeln. Dreißig Populationen von *Z. carpinifolia* und 40 Populationen von *P. fraxinifolia* wurden dafür beprobt, wobei lediglilch 30 Populationen von *P. fraxinifolia* in die Kern-Mikrosatelliten-Analysen einbezogen wurden.

Bei *Z. carpinifolia* und *P. fraxinifolia* variiert die Anzahl der klonalen Individuen (identifiziert als Individuen mit identischen Multilocus-Genotypen) erheblich zwischen den einzelnen Fundorten. Im Allgemeinen wurde eine geringe Gesamtanzahl von Allelen (2.35-3.63), eine mittlere Heterozygotie (0.425-0.604) und eine geringe Anzahl an Privaten Allelen (0.01-0.14) in *Z. carpinifolia* nachgewiesen. Obwohl auch eine relativ geringe Anzahl an Allelen (2.8-4.9) und ein mittleres Level an Heterozygotie (0.482-0.711) bei *P. fraxinifolia* gefunden wurde, war es signifikant höher als bei *Z. carpinifolia*, insbesondere im Hyrcan. Eine große Zahl privater Allele (0.01-0.28) wurde für die hyrkanischen Populationen von *P. fraxinifolia* ermittelt.

Die Analyse der molekularen Varianz (AMOVA) der Kern-Mikrosatelliten zeigte eine nur geringe Differenzierung zwischen den Populationen von *Z. carpinifolia*. Es wurde keine signifikante Differenzierung zwischen den Populationen der Kolchis und des Hyrkan gefunden. Allerdings zeigte die Bayesianische Analyse, die auf der Zuordnung von Individuen zu unterschiedlichen Clustern basierend auf individuellen Zugehörigkeits-Koeffizienten, berechnet ausgehend von den realen Allelfrequenzen, beruht, zwei Cluster von Populationen (Kolchis und Hyrkanien). Beide Cluster sind allerdings durch einen weiten Gradienten miteinander verbunden. Das Fehlen einer starken genetischen Struktur, sowie das Fehlen von Mustern der räumlichen Isolierung, weisen darauf hin, dass der Genfluss bei *Z. carpinifolia* durch Pollen offenbar sehr umfangreich ist. Es liegt daher nahe anzunehmen, dass Inzucht und Genetische Drift bei dieser Art verringert werden. Im Gegensatz dazu sind die natürlichen Populationen von *Z. carpinifolia* in den Chloroplasten-DNA-Loci hochgradig geographisch strukturiert, was auf begrenzte Samenausbreitung in der Art, im Gegensatz zur umfangreichen Pollenausbreitung, hindeutet.

Obwohl auch *Pterocarya fraxinifolia* windbestäubt ist, zeigen die Daten der Kern-Mikrosatelliten deutliche Unterschiede zwischen den Populationen in der Kolchis und dem Hyrkan. Die beiden Regionen unterscheiden sich auch sehr stark in der Diversität der Chloroplasten-DNA-Haplotypen. Während 11 Haplotypen wurden im Hyrkan gefunden wurden, sind es nur zwei in der Kolchis und im Großen Kaukasus. Sowohl Kern- als auch

Chloroplasten-DNA-Loci weisen bei *P. fraxinifolia* eine signifikante Populationsstruktur auf, was auf limitierten Genfluss durch Pollen und Samen rückschließen lässt.

Die höhere genetische Diversität im Hyrkan, zusätzlich zu der hohen Anzahl an privaten Allelen und Chloroplasten-Haplotypen in dieser Region, deutet möglicherweise auf die Existenz von mehreren Refugien für *Z. carpinifolia* und *P. fraxinifolia* gerade in den Gebirgen südlich des Kaspischen Meeres hin. Daher verdienen die Populationen des Hyrkan besondere Beachtung für den Naturschutz. Allerdings kann die Existenz von Refugien von *P. fraxinifolia* auch in der Colchis, basierend auf den Ergebnissen der vorliegenden Arbeit, nicht ganz ausgeschlossen werden.

Die Ergebnisse dieser Doktorarbeit stellen die erste umfassende Studie der genetischen Diversität und Struktur der natürlichen Populationen zweier Reliktbaumarten im Kaukasus dar. Dennoch sollten vergleichende Analysen der Populationsgenetik und der Phylogeographie von anderen Bäumen und Sträuchern der Region durchgeführt werden, um die Existenz vergangener Klima-Refugien in der Kolchis und im Hyrkan zu bestätigen. In weiterführenden Studien sollte außerdem ein umfangreicheres Sampling vor allem in der Türkei und im Iran durchgeführt werden, um ein noch besseres Bild der genetischen Struktur beider Arten im gesamten Verbreitungsgebiet zu erhalten.

Die Ergebnisse dieser Arbeit sind für die Planung von Naturschutz-Strategien und für den *ex situ* und *in situ* Schutz von reliktischen kaukasischen Baumarten nützlich. Die folgenden Empfehlungen für den Naturschutz lassen sich in diesem Zusammenhang ableiten: (i) Fragmentierung der mesophytischen Wälder, die grundsätzlich Inzucht und genetische Drift in den Populationen verursachen kann, hat wahrscheinlich noch keinen starken Effekt auf die genetische Diversität der natürlichen Populationen aufgrund eines hohen Pollen-bedingten Genflusses; (ii) einige Gebiete mit höherer genetischer Diversität sowohl im Kern- als auch Plastidengenom (große Zahl von privaten Allelen und Haplotypen in einzelnen Populationen) sowohl in der Kolchis als auch im Hyrkan sollten geschützt werden; die Populationen des Hyrkan sollten dabei besonders hohe Aufmerksamkeit bekommen; (iii) bei der Produktion von Pflanzenmaterial, das für Wiederaufforstungsmaßnahmen verwendet wird, sollten Provenienzen berücksichtigt werden, denn nur durch die Verwendung von Saatgut aus benachbarten, lokalen Populationen kann die einzigartige genetische Variabilität der unterschiedlichen Waldgebiete erhalten werden.

Contents

CHAPTER 1

General introduction

1.1. Project overview and aims of the study

This doctoral study was carried out in the framework of the project "Developing Tools for Conserving the Plant Diversity of the Transcaucasus" supported by the Volkswagen Foundation in the programme "Between Europe and the Orient". The project is based on the "Caucasus Plant Biodiversity Initiative" that has been established between the Botanical Garden and Botanical Museum Berlin-Dahlem (BGBM) of the Freie Universität Berlin (Germany) and various botanical institutes from countries of the South Caucasus, including the Institute of Botany of the Azerbaijan National Academy of Sciences (Azerbaijan).

As one of the most important biodiversity hotspots in the Northern Hemisphere (Myers *et al.*, 2000), with more than 2000 endemic plant species, the Caucasus currently attracts the attention of conservation scientists from around the world. There are two potential past climate refugia in the Caucasus, where different plant taxa persisted through major climate changes during the Quaternary: the Colchis at the eastern coast of the Black Sea and the Hyrcan at the southern coast of the Caspian Sea. They are valuable due to their relict mesophytic forests that once covered larger area in Eurasia but largely disappeared due to climate change. However, long-lasting human impact on forests in the Caucasus, especially in the first half of the 20th century when lowland mesophytic forests were cleared for agricultural purposes, resulted in a significant reduction of the forest area and loss of suitable habitats for relict trees.

The main goal of the doctoral study was to evaluate and compare the levels of genetic variation and the genetic structure of natural populations of two Caucasian relict tree species, *Zelkova carpinifolia* and *Pterocarya fraxinifolia*, that have similar geographic distributions but different ecologies. The Caucasian elm, *Zelkova carpinifolia*, is a deciduous tree that covered large lowland areas in the past but now has a patchy distribution due to forest clearing for agricultural purposes and logging for its highly valuable timber. It is evaluated as "Near Threatened" by the International Union for Conservation of Nature (IUCN) and deserves particular attention from a conservation point of view. The Caucasian wingnut, *Pterocarya fraxinifolia*, is a deciduous relict tree that once also was widespread in the lowland

riparian communities but now remains in just a few areas that have not been transformed by humans. It is considered as "Lower Risk/Least Concern" by the IUCN. Both species are the only representatives of their respective genera in continental western Eurasia and their closest relatives occur in East Asia. *Zelkova carpinifolia* grows in mesophytic forests, but is more adapted to a drier Mediterranean-type climate. *Pterocarya fraxinifolia* is adapted to a humid warm temperate climate and grows in the vicinity of watercourses. Both species mainly occur in Colchis and Hyrcan, but some isolated populations are found in southeastern Anatolia and the Zagros Mountains. *Zelkova carpinifolia* also grows in the dry forests of the Lesser Caucasus and *Pterocarya fraxinifolia* in the moist forests at the foothills of the Greater Caucasus. *Z. carpinifolia* and *P. fraxinifolia* are abundantly represented in the fossil spectra and were used as indicators of past climate changes. However, no previous investigations concerning the genetic diversity of these two species in particular nor in any other relict tree species from the Caucasus had been carried out before this doctoral project.

Studying the genetic structure of natural populations of relict trees is necessary in order to obtain information for future conservation planning and management. Therefore, the goals of the presented doctoral study were: (1) to perform representative population-level sampling of *Z. carpinifolia* and *P. fraxinifolia* in the South Caucasus and adjacent areas; (2) to develop nuclear microsatellite markers for *Z. carpinifolia* using high-throughput next-generation sequencing technology; (3) to evaluate the genetic diversity and population structure of *Z. carpinifolia* using the newly developed nuclear microsatellite markers; (4) to evaluate the genetic diversity of *P. fraxinifolia* using nuclear microsatellite markers previously developed in closely related species; (5) to perform a phylogeographical analysis of chloroplast haplotypes of *P. fraxinifolia*; (6) to compare the population genetic structure based on both nuclear and chloroplast markers between the two tree species, including chloroplast data of *Z. carpinifolia* available from the collaborators in the course of the study; and (7) to infer the existence of potential climate refugia in the Caucasus using the obtained genetic data.

My dissertation follows the cumulative format and contains the following chapters: a general introduction (Chapter 1), three research manuscripts (Chapters 2, 3 and 4) and a general discussion (Chapter 5). Chapters 2, 3 and 4 are structured as journal articles and contain their own introduction, material and methods, results, discussion, figures and tables. Chapter 2 and 3 are published respectively in the *Applications of Plant Sciences* and the *American Journal of Botany*, and Chapter 3 is under review in the *Journal of Biogeography*. All references are provided after Chapter 5. Appendices to the chapters are given at the end of the thesis.

1.2. Population genetics and phylogeography of trees

1.2.1. Population genetics and phylogeography

Population genetics is an essential part of evolutionary biology that is dedicated to the study of the genetic structure of populations of a single species and the change of this genetic structure over space and time. It is based on the development of mathematical models of change in allele or gene frequency and their test on empirical data in order to draw conclusions about the evolutionary processes shaping the genetic structure in populations. The knowledge of species population genetics also provides tools for conservation of biodiversity by determining the priority units and for development of the appropriate management programs. The emergence of the field of phylogeography that links population genetics with phylogenetics provided new insights into understanding of the historical processes that influenced species distributions (Avise, 2000, 2009). Together these two disciplines allowed to reveal the populations and areas that served in the past as sources of genetic diversity and those that were recolonized from the source populations (Petit *et al.*, 2003; de Lafontaine *et al.*, 2013).

Genetic structure is defined in terms of the genetic variation (variation in allele and genotype frequencies) distributed within and among populations and individuals and this variation is influenced by microevolutionary forces such as mutation, genetic drift, natural selection and gene flow (Hedrick, 2011). The Hardy-Weinberg model (Hardy, 1908; Weinberg, 1908) describes populations consisting of infinite numbers of individuals that are subject to random mating in the absence of these microevolutionary forces. By comparing the observed genetic diversity of natural populations with the expected diversity under Hardy-Weinberg equilibrium (HWE) conditions, which result in stable allele and genotype frequencies over generations, one can infer the impact of microevolutionary forces on the studied populations.

Mutation acts as the ultimate source of genetic variation and increases the level of variation within populations. Most of the mutations are thereby selectively neutral or deleterious. Genetic drift, in turn, is a random process of allele loss or fixation due to the finite size of natural populations and reduces genetic variation within populations. Genetic drift potentially has strong effects on the genetic diversity in bottlenecked populations and populations established through founder events. Genetic bottlenecks occur when populations experience a sharp reduction in census size and cause a reduction of the effective population

size, regardless of potential increases in census size in the following generations, and a loss of genetic diversity. The establishment of populations by a limited number of individuals, a process known as founder event, reduces the level of genetic variation within the "sink" population compared to the source population.

Natural selection is the process in which the environment influences the relative chances of the different genotypes in a population to transfer their alleles to the next generation. As a consequence of natural selection, adaptive evolution, during which allele frequencies change and the average fitness of a population increases, may occur. However, natural selection may also prevent evolution to occur and maintain the genetic variation present in a population.

The genetic exchange among populations or gene flow has an important influence on the genetic diversity and is crucial for the maintenance of genetic diversity in small populations (Ellstrand, 1992). Gene flow can overcome the effects of genetic drift and keep allele frequencies similar across populations while increasing genetic diversity within populations (Hedrick, 2011). Extensive gene flow among populations that are adapted to different environment may, however, counteract local adaptation processes (Ellstrand, 1992).

The distribution of genetic variation among individuals of a population is affected by the mating system. Populations may experience nonrandom mating, such as inbreeding or outbreeding, which affects the genotype frequencies in the population. Inbred populations experience an increase of homozygosity and possibly inbreeding depression, whereas outbreeding causes a heterozygosity excess and possibly outbreeding depression (Ellstrand, 1992). In small isolated populations the chance for inbreeding is higher than in large populations and simultaneous effect of inbreeding and genetic drift facilitates extinction of small populations.

1.2.2. Genetic marker technology

Since the establishment of population genetics at the beginning of the last century, theoretical knowledge in the field developed fast, whereas bursts in the generation of empirical data occurred much later following the development of molecular markers and techniques used in genetic analyses (Hedrick, 2011). Among the first molecular markers widely used in population genetics were allozymes and their analysis is based on the differential electrophoretic mobility of enzyme variants (Hamrick *et al.*, 1992; Schlotterer, 2004). Later, direct measures of DNA variability were developed: restriction fragment length

polymorphisms (RFLP) (Botstein *et al.*, 1980) and DNA fingerprinting based on minisatellites (Gill *et al.*, 1985). However both methods were largely abandoned since the development of polymerase chain reaction (PCR)-based DNA markers. Among them, microsatellites, randomly amplified polymorphic DNAs (RAPD), amplified fragment length polymorphisms (AFLP) and single nucleotide polymorphisms (SNPs) were successfully used in population genetic studies in addition to direct sequencing of target organellar or nuclear DNA (Schlotterer, 2004). The recent development of next-generation sequencing techniques provided cost-effective and fast tools to gain large amounts of DNA sequence data (Zhou *et al.*, 2010).

1.2.2.1. Microsatellite markers

Microsatellites, simple sequence repeats (SSR) or short tandem repeats (STR) represent the frequently occurring tandem repeats of motifs of 1-6 nucleotides in the genome. Microsatellites are one of the most variable genetic markers and polymorphisms are detected as length differences of PCR amplified fragments, not as actual DNA sequences (Fig. 1.1; Ellegren, 2004). Dinucleotide, trinucleotide and tetranucleotide repeat markers are most commonly used, whereas mononucleotide repeats are difficult to amplify and repeats with more nucleotides are less abundant (Selkoe & Toonen, 2006).

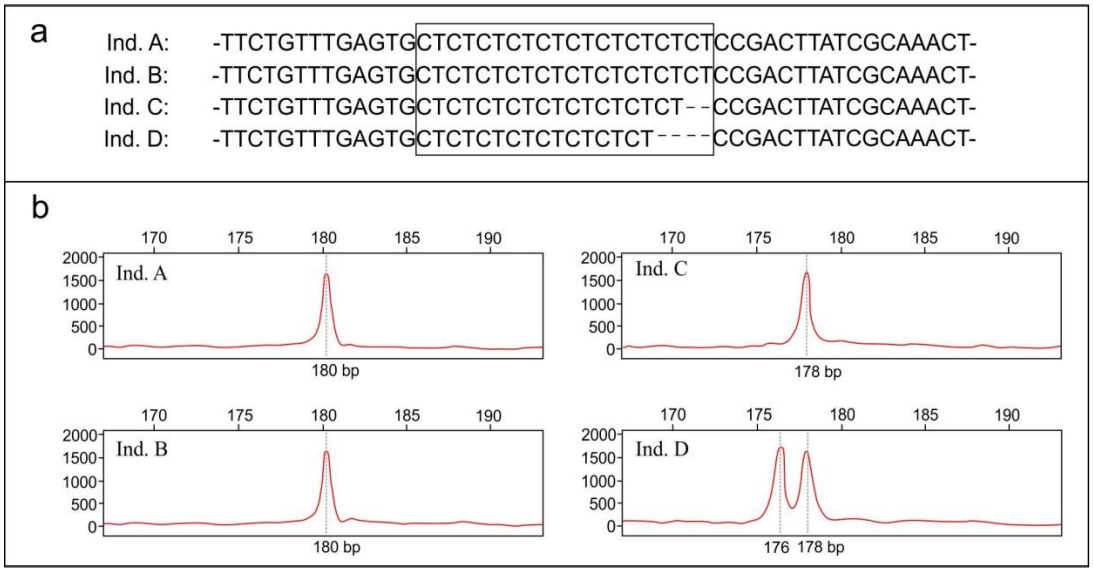

Fig. 1.1. Hypothetical example of a partial DNA sequence of the locus containing microsatellite repeat $(CT)_n$ in four individuals (panel **a**): Ind. A and Ind. B contain the same number of repeats, $(CT)_{10}$; Ind. C and Ind. D contain respectively 9 and 8 repeat motifs, and respective locus is shorter. The polymorphism in microsatellite loci of diploid organisms is detected as length differences of PCR products (panel **b**): Ind. A and B are homozygous and both loci are 180 bp long; Ind. C is also homozygous (178 bp long); Ind. D is heterozygous and contains two alleles of different size (loci 176 and 178 bp long).

Microsatellites are usually found in noncoding sequences, including both introns and spacers and their size may reach 40 bp or more. Polymorphisms are caused by strand slippage during DNA replication and two main models are applied for microsatellite evolution: the infinite allele model (IAM) in which new alleles appear independently of the ancestral allele during every mutation event and the stepwise mutation model (SMM) in which a new allele is created by the stepwise addition or removal of a repeat unit to the ancestral allele (Jarne & Lagoda, 1996). Microsatellites became the marker of choice in many population genetic studies because they are selectively neutral, species-specific, co-dominant and highly variable (Selkoe & Toonen, 2006). However, the main disadvantage of microsatellite markers is homoplasy: alleles that are not identical by descend, and which could even have different sequence motifs, may still have the same size (Estoup *et al.*, 2002).

1.2.2.2. Chloroplast DNA markers

Interest in the use of chloroplast DNA sequences in phylogenetic analyses was promoted through the *rbc*L study of Chase *et al.* (1993) and similar studies using genes such as *mat*K, *ndh*F and *atp*B. Later it was shown that noncoding cpDNA is more variable due to its higher mutation rate and more useful for inferring phylogenies and phylogeographical histories (Gielly & Taberlet, 1994; Shaw *et al.*, 2005, 2007; Borsch & Quandt, 2009; Dong *et al.*, 2012). Chloroplast DNA in angiosperms is usually maternally inherited and plant migration or establishment in a new environment depends on seed dispersal, so that cpDNA markers are widely used to track recolonization routes of plants after the Pleistocene glaciations (Bai *et al.*, 2010; Ohtani *et al.*, 2013). Among many other noncoding cpDNA markers, the following were used in the present study: the *trn*L intron and *trn*L-*trn*F intergenic spacer, the *trn*S-*trn*G intergenic spacer and *trn*G intron and the *rpl*32-*trn*L intergenic spacer. These represent some of the most variable noncoding cpDNA markers in plants (Fig. 1.2; Shaw *et al*., 2005, 2007; Dong *et al*., 2012).

Taberlet *et al.* (1991) developed primers to sequence the whole *trn*T-*trn*L-*trn*F cpDNA region, including the group I intron in the *trn*L gene and two intergenic spacers. This marker became very popular (Shaw *et al*., 2005), but difficulties in the sequencing of the *trn*T-*trn*L spacer caused the increasing use of just the *trn*L intron and *trn*L-*trn*F spacer (Borsch & Quandt, 2009). The region is easily amplified in many plants using universal primers (Taberlet *et al*., 1991) and the intron ranges from approximately 250 to 1400 bp while the spacer ranges from approximately 100 to 500 bp (Shaw *et al*., 2005).

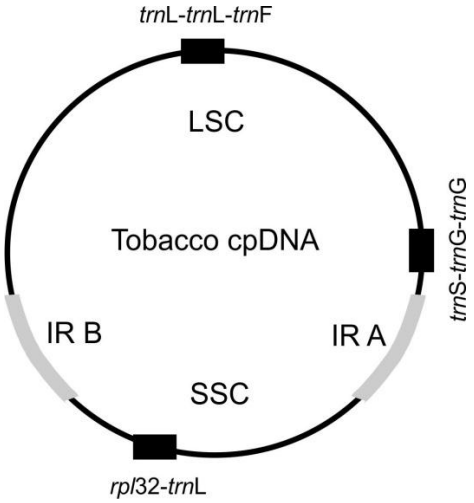

Fig. 1.2. Schematic representation of the location of chloroplast DNA regions sequenced in this study (black boxes) in the circular chloroplast DNA of *Nicotiana tabacum*. *trn*L-*trn*L-*trn*F and *trn*S-*trn*G-*trn*G are situated in the large single copy (LSC), whereas *rpl*32-*trn*L is situated in the small single copy (SSC) regions. The inverted repeats (IR A and IR B) are shown in grey.

The *trn*S-*trn*G-*trn*G cpDNA region is very variable across plant species and populations, and more variable than *trn*L-*trn*L-*trn*F (Shaw *et al*., 2005; Dong *et al*., 2012). In addition, many primers for the amplification of the region were developed (Hamilton, 1999; Shaw *et al*., 2005, 2007; Tesfaye *et al*., 2007; Murdock, 2008). The *trn*G intron (group II intron; Borsch & Quandt, 2009) ranges from approximately 697 to 1008 bp and the *trn*S-*trn*G intergenic spacer ranges from 619 to 1035 bp in different plant groups. However, some plant groups lack the spacer (Shaw *et al*., 2005).

The spacer *rpl*32-*trn*L was shown to be the most variable intergenic region among studied noncoding cpDNA sequences (Shaw *et al*., 2007; Dong *et al*., 2012). However, it may be absent in some plant groups (Dong *et al*., 2012). It is situated in the small single-copy (SSC) of cpDNA and ranges from 543 to 1417 bp due to the large insertions and deletions found in different plant taxa (Shaw *et al*., 2007).

1.2.3. Population genetics of tree species - state of the art

According to Niklas (1997), "any perennial plant with a permanent, woody, self-supporting main stem or trunk, ordinarily growing to a considerable height, and usually developing branches at some distance above the ground" is considered a tree. Extant tree species constitute 15-25% of the total number of vascular plants (Scotland & Wortley, 2003; Petit & Hampe, 2006) and make up the forests that currently cover 31% of the Earth's land surface (Keenan *et al*., 2015). The development of the tree growth habit is an evolutionary successful adaptation that is due to the process of secondary growth, which produces woody stems in both dicot forest trees and herbs (Groover, 2005).

Trees are characterized by a variety of features that allow their long-term persistence or longevity: a large size, a widely branching root system and a wide crown. These features allow the reception of sufficient sunlight, and the efficient absorption of nutrients and water from the soil. Trees are resistant to short-lasting abiotic stress factors, such as drought, flooding, fires and strong winds, and can overcome damage to and loss of the vital parts through epicormic branching and stump sprouting, replacement of roots and clone formation. They can develop an adaptive growth in response to disturbances by leaning the stems and sweeping the branches upwards (Lanner, 2002). Moreover, the long lifetime of woody species increases the chance of successful reproduction. Although unfavorable environmental conditions could affect flowering and decrease seed production, long-lived woody species have multiple chances for reproduction. An outcrossing mating system is predominant in trees due to their large size in addition to the presence of mechanisms that prevent self-fertilization, such as inbreeding depression, self-incompatibility and dioecy (Petit & Hampe, 2006). However, all tree features have their drawbacks: e.g. a large size requires high maintenance costs, a long lifetime causes delayed maturity, a long generation time reduces not only the extinction risk but also the speciation rate, and extensive seed production usually meets high recruit mortality due to unsuitable microhabitats, high competition among dense seedlings or suppression by the understory herbs (Clark *et al.*, 1999; Petit & Hampe, 2006).

The genetic diversity of woody species is different from the diversity of herbaceous plants, but varies extensively among the different tree species. The main features that shape the genetic diversity of woody species are: lifespan, the mating system, the seed dispersal mechanism and species distribution (Hamrick *et al.*, 1992). The lifespan of trees and their generation time are long and many seeds are produced, but very few individuals reach maturity so that the effective population sizes of trees tend to be closer to the census population sizes than is the case for herbal species (Petit & Hampe, 2006).

Despite the presence of an outcrossing mating system in most of the woody species (Petit & Hampe, 2006), genetic bottlenecks and inbreeding have been detected in small and isolated tree populations. E.g. evidence for the occurrence of bottlenecks in the sweet chestnut, *Castanea sativa*, from eastern Anatolia, in addition to high numbers of private alleles, suggested that these populations are isolated from west European populations and might represent a past climate refugium for the species (Mattioni *et al.*, 2013). Tree species with both sexual and asexual reproduction maintain higher levels of gene diversity, as clonal reproduction during less favorable conditions could preserve genetic diversity until the conditions are again suitable for sexual reproduction (Lanner, 2002). One of the most

prominent examples is the quaking aspen, *Populus tremuloides* (Callahan *et al.*, 2013), a species that is reproducing both sexually and by root sprouting, and of which a clonal population in the Rocky Mountains (USA) has presumably persisted since the ice ages (Lanner & Stetter, 1984). The extensive vegetative reproduction in some relict species with low genetic diversity, such as the white elm, *Ulmus leavis* (Venturas *et al.*, 2013), may also have played an important role in the survival of relict species through changing environments.

The large size of trees and their extensive production of pollen and/or seeds under suitable environmental conditions, multiple reproduction cycles, outcrossing mating system and wind-pollination usually result in extensive gene flow among populations (Petit & Hampe, 2006). As a consequence, genetic differentiation is high within and low among populations (Hamrick *et al.*, 1992), and species-level genetic diversity is preserved (Lowe *et al.*, 2005).

Gene flow in plants occurs due to pollen- and seed-dispersal, and different mechanisms of dispersal influence the levels of genetic diversity. Gene flow through pollen may be high both in wind-pollinated and animal-pollinated woody species (Hamrick *et al*., 1992), although the distances over which pollen is dispersed by wind may prevail those of animal-mediated dispersal which depend on the behavior of the pollinator (Starr *et al.*, 2013). Species with animal seed dispersal, on the other hand, have higher genetic diversities than species with gravity and wind-dispersed seeds (Hamrick *et al*., 1992).

The spatial structure of populations may differ between closely related tree species. For example, differentiation among populations of the endangered East Asian species of ash, *Fraxinus mandshurica*, is very low due to high levels of gene flow among populations (Hu *et al.*, 2008). In *Fraxinus excelsior* and *Fraxinus angustifolia* from Europe, however, populations were shown to be highly structured and this structure is explained by the existence of several climate refugia that preserved plant taxa in the past (Heuertz *et al.*, 2004; Temunovic *et al.*, 2013).

Limited or absent gene flow among tree populations is usually due to the existence or emergence of natural barriers (mountains, water basins, etc.). For example, in the relict tree *Euptelea pleiospermum* from East Asia mountain ridges were shown to serve as barriers and river valleys as corridors for gene flow (Wei *et al.*, 2013a). But, human impact on forests also affects the gene flow among and within populations. Fragmentation and degradation of forests should cause a loss of genetic variation and increase in differentiation among populations as gene flow within and among populations reduces, and genetic drift increases (Lowe *et al.*, 2005). Inbreeding usually impacts tree populations immediately after disturbance and genetic

diversity is slowly lost in subsequent generations (Lowe *et al*., 2005). The habitat loss in species with limited seed-dispersal leads to an early decline in genetic diversity, as in the evergreen broad-leaved tree *Castanopsis sclerophylla* (Zhang *et al.*, 2012). Nevertheless, observed effects of fragmentation may be limited as trees are long-lived and too few generations may have elapsed since the fragmentation (Andrianoelina *et al.*, 2009).

1.3. The Caucasus – a biodiversity hotspot

Thirty five biodiversity hotspots are known across the world as areas with high biodiversity, a high number of endemic species and undergoing habitat loss (Myers *et al.*, 2000; Mittermeier *et al.*, 2004, 2011). The Caucasus is one of the significant biodiversity hotspots in West Asia. Only 10% of its primary vegetation cover remains from the 500,000 km^2 it once occupied (Myers *et al.*, 2000). Two thousand and ninety-one endemic species of plants are known for the Caucasus ecoregion, and 1,752 of them were evaluated for the Red List of The Caucasus (Solomon *et al.*, 2014).

1.3.1. The geography and geological history of the Caucasus

The Caucasus region is mainly mountainous and lies between the Black and the Caspian Seas on the border between Europe and Asia (Fig. 1.3). The major mountain ranges are the Greater Caucasus (or Caucasus Major) and the Lesser Caucasus (or Caucasus Minor). The area north to the Greater Caucasus, known as Ciscaucasia or the North Caucasus, includes part of the Southern Federal District of the Russian Federation (the republic of Adygea, Krasnodar krai and part of Rostov oblast) and the North Caucasian Federal District (Stavropol krai and the autonomous republics of Chechna, Dagestan, Ingushetia, Kabardino-Balkaria, Karachay-Cherkessia and North Osetia-Alania). The area to the south of the Greater Caucasus is known as Transcaucasia or the South Caucasus and includes Armenia, Azerbaijan and Georgia, extending further to northeastern Turkey and northwestern Iran. The Rioni-Kura (or Transcaucasian) intermountain depression separates the Greater Caucasus from the Lesser Caucasus and the Armenian and the Javakheti volcanic plateaus, whereas the Surami Range (or Likhi Ridge) connects the Greater and the Lesser Caucasus and divides the Rioni-Kura depression into the western (Rioni or Colchic) and the eastern (Kura or Kura-Aras) lowlands (Volodicheva, 2003). The Lesser Caucasus in western Georgia links to the eastern Pontic Mountains of northeastern Turkey, and the Talysh Mountains of southeastern Azerbaijan link

to the Alborz (or Elburz) mountain range of northern Iran. The Lankaran (or Lenkoran) and the Caspian lowlands that spread from the foothills of the Talysh and Alborz Mountains to the Caspian Sea also belong to the South Caucasus.

The Caucasus is a central part of the Alpine-Himalayan orogenic belt and its complex structure and landscape of high mountain ranges, intermountain depressions and volcanic highlands is a result of the continuous convergence of the Eurasian and African-Arabian plates (Adamia *et al.*, 2011). The rate of the convergence is estimated at approximately 15–30 mm per year (Demets *et al.*, 1990; Reilinger *et al.*, 2006). The region is affected by tectonic shortening and orogenesis due to the lack or thinning of a lithosphere beneath the collision zone (Koulakov *et al.*, 2012).

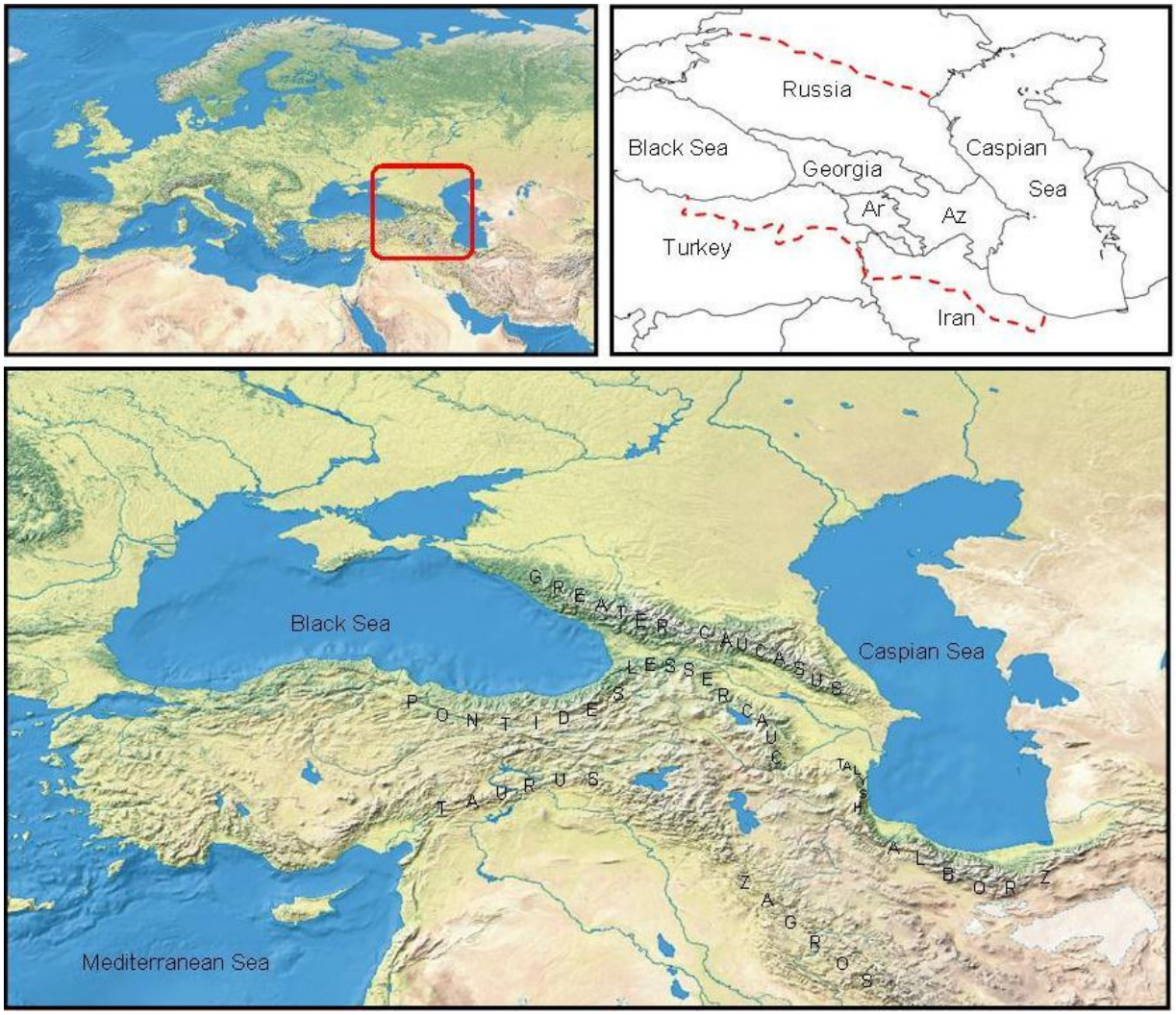

Fig. 1.3. Location of the Caucasus on the topographical map of western Eurasia (upper left map); country map of the Caucasus (upper right map), where the Caucasus ecoregion is enclosed between red dashed lines as designated by World Wildlife Fund (WWF); and topographical map of the Caucasus and adjacent areas with main mountain ranges (lower map). Abbreviations: Ar – Armenia, Az – Azerbaijan.

During the Late Proterozoic-Cenozoic, the region belonged to the ancient oceans and its Eurasian and African-Arabian margins. Small terrains (the South Caucasus) detached from Gondwana (the African-Arabian plate) during the Early Paleozoic (400-500 Ma) and moved northwards to the Eurasian plate, closing the Prototethys ocean and opening the Palaeotethys ocean. Their movement was completed approximately 350 Ma (Adamia *et al.*, 2011). Movement of the African-Arabian platform continued together with subsequent replacement of the Tethys ocean by the Neotethys and Paratethys oceans. Initial uplift, folding and erosion of the Sevan-Akera suture zone of the Lesser Caucasus occurred in the Paleocene (66-56 Ma) and continued until the Miocene (23 Ma). Deformations and subsequent uplift took place due to erosion throughout the Miocene (23-5.3 Ma) and due to volcanic activity up to the present day (Sosson *et al.*, 2010). The northward moving African-Arabian plate pushed the Lesser Caucasus into the Eurasian continental margin initiating the formation of the Greater Caucasus (Philip *et al.*, 1989). The uplifts of the western and central Greater Caucasus occurred respectively in the Oligocene (33.9-23 Ma; Vincent *et al.*, 2007) and the Pliocene (5.3-2.6 Ma; Avdeev & Niemi, 2011). The Talysh Mountains were mainly formed during the Eocene (56-33.9 Ma), whereas erosional deformation took place in the Oligocene and deposition of volcanic material in the Quaternary age (2.2-0.2 Ma; Vincent *et al.*, 2005). By the late Miocene, the region mainly represented a dry land, except for some lowland areas with shallow sea water. The Rioni and Lankaran lowlands are young and were uncovered from seawater during the Quaternary period (Adamia *et al*., 2011).

During the Late Pleistocene (115-10 ka), the largest glaciers in the Caucasus measured up to 50-70 km and only two of them descended to 600-750 m a.s.l. in the Central Greater Caucasus, whereas the others descended to only 1600-1200 m a.s.l. However, they have never reached the foothills or the lowlands. Glaciers on the Lesser Caucasus and the Armenian highland were insignificant (Milanovsky, 2000) and the lowest altitudes reached by glaciers in the Lesser Caucasus were about 2500-2700 m a.s.l. (Gobejishvili *et al.*, 2011). In Alborz, the snowline was about 600-1100 m lower than the present level (Ferringo, 1988) and no traces of Pleistocene glaciers have been found in the Talysh Mountains (Maruashvili & Khazaradze, 1982).

1.3.2. The climate of the Caucasus

A complex landscape provides heterogeneous climates in the Caucasus region. The Greater Caucasus range prevents cold air masses to reach the South Caucasus. Winters are colder and summers are drier in the eastern Caucasus in comparison to the western Caucasus. In the Russian part of the Caucasus, the climate ranges from a dry continental climate in the east to a warm-temperate climate in the west with annual precipitation ranging from 200 to 800 mm. The climate in western Georgia along the Black sea coast is warm-temperate and humid, with 4500 mm of annual precipitation, whereas to the east it becomes drier with 200-300 mm of annual precipitation (Mediterranean type temperate climate). In northeastern Turkey such a change is observed in the north-south direction from the Black Sea coast to the interior part of the country. The climate in Armenia is mainly dry continental with annual precipitation of ca. 300 mm in the lowlands and 800-1000 mm in the mountainous regions. The climate in Azerbaijan is also changing from humid and warm to dry and hot in a west-east direction: in the Kura-Aras lowland, the Absheron Peninsula and the Zuvand highland (Diabar), the climate is mainly semi-arid with annual precipitation of about 200 mm; precipitation reaches 300-900 mm in the mountain foothills, up to 1300 mm on the southern slopes of the Greater Caucasus and up to 1400 mm in the Lankaran lowland and the eastern slopes of the Talysh Mountains. In Iran, the climate also ranges from warm-temperate humid on the southwest coast of the Caspian Sea along the Talysh and Alborz mountains to a Mediterranean temperate type in the northwestern part of the country, with 280-330 mm of annual precipitation (Solomon *et al.*, 2014).

There are 1,521 glaciers in the Caucasus (Panov *et al.*, 2008) and the biggest ones develop in the Elbrus and Kazbegi mountains of the Greater Caucasus. The north-facing slopes of the Greater Caucasus develop more glaciers than the southern slopes (Volodicheva, 2003). However, the surface area of many glaciers in the northern Greater Caucasus has decreased over the last 200 years (Bushuyeva, 2013). A higher number of glaciers is found on the northern slopes of the western Alborz Mountains than on the eastern Alborz Mountains (Ferringo, 1988). In the Lesser Caucasus, glaciers are only found on high mountain peaks.

1.3.3. A short overview of the botanical explorations in the Caucasus

The first botanical studies in the Caucasus started in the 17th century. Among naturalists and botanists that carried out numerous expeditions to explore the flora of the

region were J. P. de Tournefort, J. C. Buxbaum, S. Gmelin, I. Güldenstädt, F. Hohenaker, C. A. Meyer, P. Pallas, C. Steven, G. Radde, F. Ruprecht, P. Trautvetter, K. Koch, N. Busch, N. Kuznetsov, G. Woronov and many others. The first publication on the flora of the region was written by M. von Bieberstein in *Flora Taurico-Caucasica* (1808) and described about 2,000 Caucasian plant species. During the 19th century, Caucasian species were included in *Prodromus* of de Candolle (1824-1873), *Flora Rossica* of Ledebour (1841-1853; about 3,000 species), *Flora Orientalis* of Boissier (1867-1884) and *Flora of the Caucasus* of V. I. Lipskiy (1899; about 4,500 species). In 1901, N. Kuznetsov, N. Bush and A. Fomin began to publish *Flora Caucasica Critica*, but publication was interrupted during World War I and the Russian Revolution. A great contribution to the flora of the region was made by A. A. Grossheim, who published, among other titles, the comprehensive *Flora of the Caucasus* (1928-1967), *The analysis of Caucasian Flora* (1936), *Manual of the Caucasian Plants* (1949) and the first *Flora of Azerbaijan* (1934-1936). The next major work describing Caucasian species was *Flora of USSR* (ed. V. L. Komarov, 1934-1960). Publication of the first *Flora of Georgia* was initiated in 1941 by N. N. Ketskhoveli and D. I. Sosnovsky, and of the first *Flora of Armenia* in 1954 by A. L. Takhtajan. *Flora of North Caucasus* was published by A. I. Galushko in the period 1978-1981. During the 20th century, many works on flora of different regions of the Caucasus were published. By the 21st century, old publications were out-of-date and a new *Caucasian Flora Conspectus* was edited by A. L. Takhtajan (2003-2008). Unfortunately, none of the floras published included northeastern Turkey and northwestern Iran. First data on the endemic species of all the regions belonging to the Caucasus ecoregion was a *Red list of the Endemic Plants of the Caucasus* (Solomon *et al.*, 2014).

1.3.4. The flora and vegetation of the Caucasus

Although debatable, the total number of plant species found in the Caucasus ranges from 5,000 to 6,350 depending on the source (Grossheim, 1936; Gagnidze *et al.*, 2002; Takhtajan, 2003-2008). The highest number of species is calculated for Azerbaijan (4,500), Georgia (4,100) and northeastern Turkey (4,000), with the highest number of Caucasian endemics found in Georgia (1,320) and the Russian Caucasus (1,300) (Solomon *et al.*, 2014). The most abundant families in numbers of species include Apiaceae, Asteraceae, Brassicaceae, Campanulaceae, Caryophyllaceae, Fabaceae, Poaceae, and Rosaceae with more than 100 species represented in the Caucasus (Solomon *et al.*, 2014). The richness of the

Caucasian flora is caused by the influence of three floristic regions: Euro-Siberian (or Circumboreal; Takhtajan, 1986), Mediterranean and Irano-Turanian.

Vegetation types include coniferous and deciduous forests, alpine and subalpine meadows, steppes and semi-deserts. Coniferous forests consist of pines (*Pinus* sp.), fir (*Abies nordmanniana* Spach.), spruce (*Picea orientalis* (L.) Peterm.) and junipers (*Juniperus* sp.). Swamp broad-leaved forests are dominated by *Alnus glutinosa* subsp. *barbata* (C. A. Mey) Yalt. and common lowland forests on the damp soils by *Quercus hartwissiana* Steven with *A. glutinosa* subsp. *barbata* and *Carpinus betulus* L. in the west, *Q. robur* subsp. *imeretina* (Steven ex Woronow) Menitsky in the central Caucasus and *Q. castaneifolia* C. A. Mey together with *Parrotia persica* C. A. Mey and *Zelkova carpinifolia* (Pall.) K. Koch in the southeast. Abundant lianas such as *Smilax excelsa* L. and *Periploca graeca* L. are found in humid lowland forests. On the riverbanks, gallery forests develop with *Populus* sp. in more arid areas and *Pterocarya fraxinifolia* (Poir.) Spach. together with *Alnus glutinosa* subsp. *barbata* and/or *A. subcordata* C. A. Mey in humid areas. *Pistacia atlantica* Desf. forms open pistachio forests in *Artemisia* and *Salsola* semi-deserts. The lower and middle mountain zone is covered with forests of beech *Fagus orientalis* Lipsky and oak *Quercus petraea* subsp. *iberica* (Steven ex M. Bieb.) Krassiln., the latter mixed with *Carpinus* sp., *Acer* sp., *Tilia* sp., *Ulmus* sp. and *Fraxinus excelsior* L. The upper mountain zone is covered by park like forests with *Betula* sp., *Acer* sp. and *Quercus macranthera* Fish. & C.A. Mey et Hohen. or *Q. pontica* K. Koch. (Grossheim, 1930).

A high diversity of species and endemics is found in the alpine and subalpine vegetation belts with *Bromus variegata* (M. Bieb.) Holub., *Campanula* L., *Carex* L., *Colchicum speciosum* Steven, *Festuca* L., *Poa* L., *Ranunculus caucasicus* M. Bieb., *Rhododendron* L., *Stachys* L., *Taraxacum* F.H. Wigg., *Trifolium ambiguum* M. Bieb. and many other genera and species. Subalpine meadows with tall herbaceous plants occur between 1,800 and 2,200 m in the Greater Caucasus and between 2,700 and 2,800 in the Lesser Caucasus; alpine meadows with short grasses usually occur between 2400 and 3000 m or higher (Solomon *et al.*, 2014).

Steppes are covered by *Bothriochloa ischaemum* (L.) Keng, feather-grass *Stipa tirsa* Steven as well as different Poaceae species and xerophytes. Semi-deserts are covered by *Artemisia* sp. and *Salsola* sp. *Halocnemum strobilaceum* (Pall.) M. Bieb., *Halostachys belangeriana* (Moq.) Botsch. and *Kalidium capsicum* (L.) Ung.-Sternb. grow on semi-deserts with saline soils (solonchaks). Xerophytic vegetation is represented by *Acantholimon* Boiss.,

Ajuga L., *Astragalus* L., *Campanula* L., *Daphne* L., *Paliurus spina-christi* Mill., *Prunus* L., *Psephellus* Cass., *Salvia* L., *Silene* L., *Ziziphora* L. and others (Grossheim, 1930).

1.4. The climate refugia of the South Caucasus

1.4.1. The role of climate refugia in the worldwide preservation of biodiversity

Global cooling of earth began in the Paleogene (65 Ma) and resulted in the formation and growth of the Antarctic ice cap since the Oligocene (35 Ma) and the Arctic ice cap which was established 2.4 Ma. Quaternary ice sheets advanced and receded with 41 ka cycles until 0.9 Ma, after which the cycles' duration increased to 100 ka when glaciations became pronounced (Hewitt, 2000; Milne & Abbott, 2002). The warm-temperate and subtropical (or even tropical) taxa of the Northern Hemisphere started to retreat southwards as temperatures dropped and aridity increased. They found refugia in the areas where wet and warm climates were preserved (Milne, 2006). The term "refugium" in this context was used for the first time by Heusser (1955). Bennett *et al.* (1991) stated that the term is used "to refer to the area of any size in which a taxon persisted at any population density during a cold stage". Later, Bennett & Provan (2008) suggested that refugia represent population bottlenecks. According to Keppel *et al.* (2012), "refugia are habitats that components of biodiversity retreat to, persist in and can potentially expand from under changing environmental conditions". Consequently, only locations that provide long-term preservation of the habitats with favorable climatic conditions where populations could have persisted up to the present day represent climate refugia (Stewart *et al.*, 2010; Tzedakis *et al.*, 2013; Gavin *et al.*, 2014). Refugia have been classified as glacial and interglacial, mesic and arid, macro and micro (Keppel *et al.*, 2012). The most studied refugia for the temperate taxa are from Europe and North America (Bennett *et al.*, 1991; Hewitt, 2000; Petit *et al.*, 2003), and, more recently, from East Asia (Bai *et al.*, 2010; Shi *et al.*, 2014). The described refugia are usually located at the southern latitudes in areas with a heterogeneous landscape. In Europe, refugia have been suggested to exist in Iberia, Italy, the Balkans, and around the Black and Caspian Seas (Hewitt, 1999; Leroy & Arpe, 2007). During the warm stages, taxa spread out of their refugia and recolonized areas to the north. However, it was shown that the cryptic refugia for the temperate taxa might have existed at more northern latitudes within the large area covered by permafrost, e.g. in Carpathian and Beringia, where taxa could have persisted at low densities (Provan & Bennett, 2008).

The inference of refugia was previously based on palaeoecological sources (Huntley & Birks, 1983) that provide detailed information about the past distributions of organisms (Bennett & Provan, 2008). Currently, however, the identification of refugia increasingly relies on genetic data (e.g. phylogeography and population genetics). Due to the long demographic histories of the populations preserved at refugia, their genetic diversities should be high, whereas populations outside the refugia usually possess lower levels of intraspecific variation as a consequence of successive founder events during postglacial recolonization (Petit *et al.*, 2003). For example, most of northern Europe was recolonized from the southern refugia, so it is genetically less diverse (Hewitt, 2000). However, this concept of "southern richness and northern purity" might be misleading, because high genetic diversity may be found in contact zones where genetic lineages from different refugia meet (Bennett & Provan, 2008) as it was shown for European tree populations at median latitudes (Petit *et al.*, 2003).

Recently, species distribution modelling became the next important tool used to locate refugia using information on both present and past species distributions (Keppel *et al.*, 2012). Although the above methods each have their own limitations, together they can provide crucial insights necessary to understand the processes that lead to climate refugia preserving taxa in the past (Gavin *et al*., 2014), aid in the management of an ongoing anthropogenic climate change and forecast future refugia (Keppel *et al*., 2012). One of the predictions is that the climate refugia of the past may counter the effects of ongoing and future climate changes as they provide long-term persistence of species. However, past refugia provided taxa with a shelter to persist through climate cooling whereas the ongoing climate warming could cause the extirpation of populations in the southern refugia and force populations to migrate towards more northern latitudes to establish new refugia (Gavin *et al.*, 2014).

Climate refugia, where different forest trees and shrubs survived during the Quaternary climatic oscillations, existed in a few regions of West Asia: in western Anatolia, western Taurus Mt., the upper reaches of the Tigris river, Levant, Colchis and Hyrcan (Tarkhnishvili *et al.*, 2012). Among these refugia, Colchis at the eastern coast of the Black Sea and Hyrcan at the southern coast of the Caspian Sea have played significant roles in the preservation of relict mesophytic trees and shrubs in the Caucasus (Fig. 1.4). Both regions are considered to belong to the Euxino-Hyrcanian floristic province of the Euro-Siberian floristic region and share many floristic elements (Table 1.1; Zohary, 1973; Browicz, 1989). Some of these species are more abundant in either Colchis or Hyrcan and some are distributed beyond those regions. Despite the abovementioned similarities, both regions have a different floral composition and, to a large extent, developed independently as the uplift of the mountain ranges between them

predates the establishment of the Colchic and Hyrcan climate refugia (Grossheim, 1926; Kolakovsky, 1961; Sosson *et al.*, 2010).

Table 1.1. The list of species shared between Colchis (Euxinian province) and Hyrcan according to Zohary (1973) and Browicz (1989).

Acer campestre L.	*Jasminum officinale* L.
A. cappadocicum Gled.	*Juglans regia* L.
A. platanoides L.	*Juniperus communis* L.
Alnus glutinosa subsp. *barbata* (C. A. Mey) Yalt.	*Leptopus chinensis* (Bunge) Pojark.
Betula pendula Roth.	*Prunus cerasifera* Ehrh.
Carpinus orientalis Mill.	*P. laurocerasus* L.
Carpinus betulus L.	*Pterocarya fraxinifolia* (Poir.) Spach.
Castanea sativa Mill.	*Punica granatum* L.
Celtis planchoniana K. I. Chr.	*Ribes biebersteinii* DC.
Crataegus germanica (L.) Kuntze	*Smilax excelsa* L.
C. microphylla K. Koch	*Sorbus subfusca* (Nordm.) Boiss.
C. pentagyna Waldst. & Kit ex Willd.	*Taxus baccata* L.
Daphne pontica L.	*Tilia cordata* Mill.
Diospyros lotus L.	*Ulmus laevis* Pall.
Fagus orientalis Lipsky	*Vaccinium arctostaphylos* L.
Fraxinus excelsior L.	*Zelkova carpinifolia* (Pall.) K. Koch
Hypericum androsaemum L.	etc.

1.4.2. Palaeoecological evidence for the preservation of the relict taxa in the Caucasus

The modern flora of the Caucasus and adjacent regions is composed of the elements of different floral types, particularly temperate ("Arcto-Tertiary" or "Turgay") and tropical or subtropical ("Indo-Malesian" or "Poltava") floras (Kryshtofovich, 1929; Zohary, 1973). At the early Paleogene, the flora around the Tethys Ocean was presumably tropical and extended to the high latitudes in the Northern Hemisphere (e.g. London Clay flora; Reid & Chandler, 1933; Zohary, 1973). The tropical elements began to retreat southwards since the Oligocene, but co-existed with temperate taxa throughout the Eocene-Miocene before being majorly replaced (Abuzjarova, 1962; Pantic & Nikolic, 1963; Ivanov *et al.*, 2011; Shatilova *et al.*,

2011; Alizade *et al.*, 2014). By the end of the Miocene-Pliocene, the subtropical/tropical elements disappeared from Europe (Schekina, 1962; Ivanov *et al.*, 2011) and found shelter in the Euxino-Hyrcanian province. Relicts of this flora are *Diospyros lotus* L., *Albizia julibrissin* Durazz., *Buxus sempervirens* L., *Dioscorea caucasica* Lipsky, *Ficus carica* L., etc., and its xero-tropical derivatives are *Ceratonia* L., *Laurus* L., *Smilax* L., *Ruscus* L., etc.

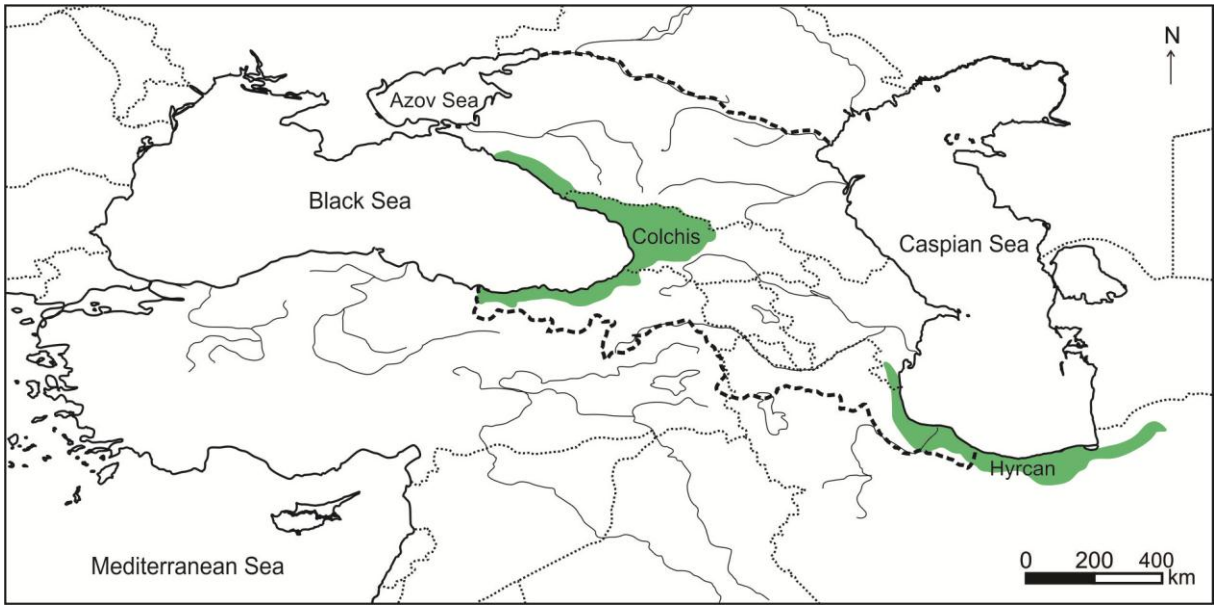

Fig. 1.4. The past climate refugia in the Caucasus: Colchis at the Black seacoast and Hyrcan at the Caspian seacoast (in green). The Caucasus ecoregion is enclosed between dashed lines as designated by WWF.

Temperate taxa were widespread in the Northern Hemisphere throughout the Miocene-Pliocene and extended southwards as far as the central Sahara Mountains in Africa and the Lower Jordan valley in the Middle East (Zohary, 1973). Climate change towards lower temperatures and higher aridity caused the extinction of many temperate taxa (e.g. *Carya* Nut., *Pterocarya* Kunth, *Parrotia* C. A. Mey, *Tsuga* Carriere and *Zelkova* Spach.) in Europe and North America at the end of the Pliocene and throughout the Pleistocene (Chaney, 1947; Lona & Riccardi, 1961; Beucher, 1963; Van Campo, 1964; Zohary, 1973; Kovar-Eder *et al.*, 2006). Some relict species persisted throughout the Pleistocene in the Caucasus and adjacent areas (Joannin *et al.*, 2010; Shatilova *et al.*, 2011; Tagieva *et al.*, 2013), but aridification of the region resulted in extirpation of many of the thermophilous trees and shrubs across the Caucasus (Ramezani *et al.*, 2008; Shatilova *et al.*, 2011). Amongst the tropical and temperate elements that underwent extinction in the Caucasus are *Acacia* Mill., *Carya* Nut., *Castanopsis* Spach., *Cinnamomum* Schaef., *Cupressus* L., *Dicksonia* L'Her., *Engelhardia* Blume, *Ginkgo*

L., *Litsea* (Lam.), *Magnolia* L., *Myrica* L., *Sequioa* Endl., *Thuja* L., *Tsuga* Carriere, palms, etc. (Zohary, 1973; Shatilova *et al.*, 2011; Alizade *et al.*, 2014).

1.4.3. Colchis as a climate refugium of the South Caucasus

The Euxinian floristic province spreads along the Black Sea coast from southeastern Bulgaria (Ropotamo valley) over the mountains of European Turkey (Istranca Daglari), Northern Anatolia and western Georgia to Krasnodar Krai in Russia (Browicz, 1989). Colchis represents the eastern part of the Euxinian province from the Melet valley near Ordu in Northern Anatolia (Davis, 1971) to Meskhiyskiy pereval (Kolakovsky, 1961) or Tuapse (Takhtajan, 1986) in Krasnodar Krai in Russia. In the soviet literature, the southern border is marked along the Coruh river in Batumi (Georgia; Kolakovsky, 1961). The exceptional climatic conditions of the Colchic triangle with 4,500 mm of annual precipitation, bounded to the Black Sea coast by the eastern Pontic Mountains, the western Lesser Caucasus and the western Greater Caucasus, created the environments suitable for many mesophytic taxa to survive during the past climate changes. The ever-humid climate of western Colchis changes to a drier temperate one in eastern Georgia (Denk *et al.*, 2001). The total area of the Colchic forests constitutes approximately three million hectares (Nakhutsrishvili *et al.*, 2011); however, the lowland vegetation of Colchis has largely been destroyed by human impact and large areas have been transformed for agricultural purposes.

Colchis is dominated by both mixed mesophytic broad-leaved forests with oriental beech (*Fagus orientalis* Lipsky), tanniniferous oak (*Quercus hartwissiana* Steven) and sweet chestnut (*Castanea sativa* Mill.) with a dense understory of evergreen shrubs such as *Rhododendron ponticum* L., cherry laurel (*Prunus laurocerasus* L.), holly (*Ilex colchica* Pojark.), *Daphne pontica* L., ivy (*Hedera helix* L. and *H. colchica* (K. Koch) K. Koch), box (*Buxus sempervirens* L. (= *B. colchica* Pojark.)), *Ruscus hypophyllum* L., *R. aculeatus* L. (= *R. ponticus* Woronow), or *Q. robur* subsp. *imeretina* (Steven ex Woronow) Menitsky with *Zelkova carpinifolia* (Pall.) K. Koch (Fig. 1.5) and coniferous forests with fir (*Abies nordmanniana* Spach.) and spruce (*Picea orientalis* (L.) Peterm.). Colchic forests are characterized by many endemic species, among which *Epigaea gaulterioides* (Boiss. & Balansa) Takht., *Osmanthus decorus* (Boiss. & Balansa) Kasapligil, *Quercus pontica* K. Koch, *Rhododendron ungerni* Trautv., *R. smirnowii* Trautv. and *Rhodothamnus sessilifolius* P. H. Davis are considered as localized Colchic endemics; *Abies nordmanniana* Spach., *Betula medwediewii* Regel, *Buxus sempervirens* L., *Daphne glomerata* Lam., *Hedera*

colchica K. Koch, *Hypericum xylosteifolium* (Spach) N. Robson, *Philadelphus coronaries* L., *Rhamnus imeretia* Dippel., *R. microcarpa* Boiss., *Rhododendron caucasicum* Pall., *Sorbus colchica* Zinserl., *Staphylea colchica* Steven and *Viburnum orientale* Pall. are endemics primary associated with the Colchic sector but extending beyond it. The Euxinian endemics are *Quercus hartwissiana* Steven and *Acer heldreichii* subsp. *trautvetteri* (Medw.) A. E. Murray, whereas some species such as *Rhododendron ponticum* L., *Ilex colchica* Pojark., *Pyracantha coccinea* M. Roem., *Rhododendron luteum* Sweet extend beyond the Euxinian province (Grossheim, 1930; Browicz, 1989).

Fig. 1.5. *Quercus robur* subsp. *imeretina* - *Zelkova carpinifolia* forest in Ajameti Nature Reserve, central Colchis, Georgia. Photo: Gerald Parolly, Botanical Garden and Botanical Museum Berlin-Dahlem (BGBM), Germany.

Quercus hartwissiana Steven, *Acer campestre* L., *Carpinus betulus* L. are main components of lowland forests with *Ficus carica* L., *Fraxinus excelsior* L. and *Ulmus glabra* Huds. as secondary species, whereas wetland lowland forests of Colchis are co-dominated by *Carpinus betulus* L., *Pterocarya fraxinifolia (Poir.) Spach.* and *Alnus glutinosa* subsp. *barbata* (C. A. Mey) Yalt. or dominated by the latter (Denk *et al.*, 2001). *Fagus* L., *Castanea* Mill., *Alnus* L., *Corylus avellana* L. and *Quercus* L. cover the middle mountain zone together with *Taxus baccata* L., *Tilia begoniifolia* Steven, *Ulmus glabra* Hudson, etc. *Fagus orientalis* Lipsky dominates the higher mountain zone. Higher in mountains *Picea orientalis* (L.)

Peterm. plays a supreme role in forest mountain vegetation occurring with other trees such as beech and oaks. Other wide mountain communities consist of *Pinus sylvestris* L. and *Abies nordmanniana* Spach. (Zohary, 1973).

1.4.4. Hyrcan as a climate refugium of the South Caucasus

The Hyrcan represents a narrow forest belt along the southern Caspian seashore from the Talysh Mountains and the Lankaran lowland of southeastern Azerbaijan (Lankaran District) over the northern slopes of the Alborz Mountains and Caspian lowland through Iran (Gilan, Mazandaran and Golestan Provinces) up to the Khorasan province and extending into the Kopet Dag Mountains in Turkmenistan (Browicz, 1989). The total estimated area of the Hyrcanian forests is approximately 1.96 million hectares (Nakhutsrishvili *et al.*, 2011). Hyrcan is less humid than Colchis, with about 1,400 mm of annual precipitation in the Talysh Mountains and Lankaran lowland, and up to 2,000 mm of annual precipitation in western Hyrcan in Iran. The amount of precipitation decreases towards the east (Khalili, 1973; Akhani *et al.*, 2010) and summer droughts occur in the Lankaran lowlands (Grossheim, 1926).

Hyrcan is covered by mixed mesophytic broad-leaved forests and the main differences in vegetation from Colchic forests are the absence of spruce, fir and rhododendron species, and the sparser evergreen understory. Nevertheless, the total number of woody species is higher in Hyrcan than in Colchis (Nakhutsrishvili *et al.*, 2011). Evergreen shrubs are *Buxus sempervirens* subsp. *hyrcana* (Pojark.) Takht. (= *B. hyrcana* Pojark.), *Danae racemosa* (L.) Moench, *Daphne pontica* L., *Ilex aquifolium* L., *I. spinigera* (Loes.) Loes., *Hedera pastuchovii* Woronow, *Prunus laurocerasus* L. and *Ruscus hyrcanus* Woronow (Grossheim, 1930). Local Hyrcanian endemics are *Alnus subcordata* C. A. Mey, *Frangula grandifolia* (Fisch. & C. A. Mey) Grubov, *Gleditsia caspia* Desf. (= *G. caspica* Desf.), *Ilex spinigera* (Loes.) Loes., *Parrotia persica* C. A. Mey, *Populus caspica* Bornm., *Quercus castaneifolia* C.A. Mey and *Ruscus hyrcanus* Woronow. However, the latter two species have also been found at the foothills of the eastern Greater Caucasus in Azerbaijan. Characteristic Hyrcanian species that extend in adjacent areas as well are *Acer velutinum* Boiss., *Albizia julibrissin* Durazz., *Buxus sempervirens* subsp. *hyrcana* (Pojark.) Takht., *Danae racemosa* (L.) Moench, *Euonymus velutinus* (E. Mey) Fisch. & C. A. Mey., *Hedera pastuchovii* Woronow and *Pyrus boissieriana* Buhse (Browicz, 1989).

Lowland forests are co-dominated by *Alnus subcordata* C. A. Mey, *A. glutinosa* subsp. *barbata* (C. A. Mey) Yalt. and *Pterocarya fraxinifolia* (Poir.) Spach. on alluvial soils (Fig.

1.6). These alluvial forests and flooded riverbanks also contain *Salix* spp., *Fraxinus excelsior* L., *Ficus carica* L., *Buxus sempervirens* subsp. *hyrcana* (Pojark.) Takht., *Albizia julibrissin* Durazz., *Gleditsia caspia* Desf., *Morus nigra* L., *Celtis australis* L., *Acer velutinum* Boiss., *Prunus cerasifera* Ehrh., *P. laurocerasus* L., *Crataegus germanica* (L.) Kuntze, *Paliurus spina-christi* Mill., *Populus caspica* Bornm., *Punica granatum* L., etc., in addition to lianas. The foothills and lower mountain zones are covered by *Quercus castaneifolia* C. A. Mey, *Parrotia persica* C. A. Mey, *Carpinus* sp., *Zelkova carpinifolia* (Pall.) K. Koch, *Diospyros lotus* L., *Albizia julibrissin* Durazz., *Alnus* sp. and *Pterocarya fraxinifolia* (Poir.) Spach. Other communities are *Quercus-Castanea* forests in the west, and *Quercus-Acer cappadocicum* Gled. as well as *A. velutinum* Boiss. in the east. Highly mesic beech forests develop at higher altitudes. Xeric *Quercus macranthera* Fish. & C. A. Mey et Hohen. develops at the highest mountain zones reaching the timberline (Zohary, 1973; Scharnweber *et al.*, 2007; Akhani *et al.*, 2010).

Fig. 1.6. Mixed deciduous Hyrcanian forest with *Pterocarya fraxinifolia* along Istisu river, Azerbaijan. Photo: Elmira Maharramova.

Among the main threats for forest trees in Hyrcan are silvopasture, lopping or pollarding, selective logging as different timber is used for construction and as fuel-wood (Scharnweber *et al*., 2007), urbanization, industrialization, development of tourism, agriculture and fires (Akhani *et al*., 2010).

1.5. The relict trees of the South Caucasus as study objects

Relicts, the "survivors from the past", represent an essential component of biodiversity (Habel *et al.*, 2010) and can be classified as taxonomic or biogeographic relicts. Taxonomic relicts are the remnant species of taxa that were taxonomically diverse in the past and biogeographic relicts are narrowly distributed species that descended from taxa that were geographically widespread in the past (Lomolino *et al.*, 2006; Habel *et al.*, 2010). Some of the relict trees and shrubs in the South Caucasus such as

Acer cappadocicum Gled.
Acer velutinum Boiss.
Albizia julibrissin Durazz.
Alnus subcordata C. A. Mey
Betula medwediewii Regel
Diospyros lotus L.
Epigaea gaultherioides (Boiss. & Balansa) Takht.
Gleditsia caspia Desf.
Hedera pastuchovii Woronow
Jasminum officinale L.
Pterocarya fraxinifolia (Poir.) Spach.
Pyrus boissieriana Buhse
Quercus castaneifolia C. A. Mey
Zelkova carpinifolia (Pall.) K. Koch

have distributions that are disjunct from the ones of closely related taxa (biogeographic relicts), whereas *Danae racemosa* (L.) Moench, *Osmanthus decorus* (Boiss. & Balansa) Kasapligil and *Parrotia persica* C. A. Mey are the only representatives of their respective genera (taxonomic relicts) (Browicz, 1989).

1.5.1. The Caucasian elm, *Zelkova carpinifolia*

1.5.1.1. The genus *Zelkova* (Ulmaceae)

The elm family, Ulmaceae Mirbel., consists of eight known genera: *Ampelocera* Klotzsch., *Chaetachme* Planch., *Hemiptelea* Planch., *Holoptelea* Planch., *Phyllostylon* Capan. ex Benth. & Hook, *Planera* J. F. Gmel., *Ulmus* L. and *Zelkova* Spach. The genus *Zelkova* is represented by six extant species of deciduous trees and shrubs that have disjunct distributions

across Eurasia: two species, *Z. sicula* Di Pasq., Garfi & Quezel and *Z. abelicea* (Lam.) Boiss., are found in the Mediterranean basin (Sicily and Crete), one species, *Z. carpinifolia*, in South Caucasus and adjacent areas, and three species in East Asia: *Z. schneideriana* Handel-Mazzetti and *Z. sinica* C. K. Schneider in China, and *Z. serrata* (Thunberg) Makino in eastern China, Japan, Korea, Taiwan and the Kuril islands. The genus once was wider distributed in the Northern Hemisphere (Northern America and Eurasia; van der Hammen *et al.*, 1971; Tanai & Wolfe, 1977; Burnham, 1986; Follieri *et al.*, 1986; Denk & Grimm, 2005 and references therein), but went extinct in North America (Chaney, 1947), North Africa (Zohary, 1973) and mainland Europe. In mainland Europe, it occurred for the last time near Rome (Italy) in the Pleistocene (about 31 ka; Follieri *et al*., 1986).

Regarding their conservation status, *Z. sicula* is evaluated by the IUCN Red List as "Critically Endangered" (Garfi, 2006) since only two small populations in Sicily are left (Garfì *et al.*, 2011), *Z. abelicea* as "Endangered" (Kozlowski *et al.*, 2012a) and *Z. carpinifolia* as "Lower risk/Near Threatened" (Güner & Zielinski, 1998). *Z. hyrcana* A. Grossh et A. Jarm. was originally described as a different species in Hyrcan, but was later shown to be an ecological or age form of *Z. carpinifolia* (Sokolov, 1951; Sosnovsky, 1952).

1.5.1.2. Current knowledge of the phylogeny and population genetics of *Zelkova*

Simple leaves with craspedodromous venation and simple tooth margins unify the species in the genus *Zelkova*, whereas leaf architecture (epidermal features) was assumed to distinguish among the different *Zelkova* species (Wang *et al.*, 2001). However later it was shown that leaf variability is too high and its use in distinguishing fossil species is very problematic (Denk & Grimm, 2005). A phylogenetic study of the Ulmaceae based on few morphological characters was not able to resolve a clade with *Zelkova*, *Ulmus*, *Planera*, *Hemiptelea* and *Chaetoptelea* (Zavada & Kim, 1996), but a later study by Wiegrefe *et al.* (1998) using cpDNA restriction site mapping showed that *Hemiptelea* is a sister group to the low-supported clade of *Zelkova*, *Ulmus* and *Planera*. Fineschi *et al.* (2002) used the chloroplast marker *trn*L and the nuclear internal transcribed spacer (ITS2) to assess the differentiation among the western Eurasian species of *Zelkova*, and chloroplast microsatellites and RFLPs to assess the genetic diversity within populations of Mediterranean species. They showed that the Mediterranean species *Z. sicula* and *Z. abelicea* differ from *Z. carpinifolia* both in *trn*L and ITS2, and differ from each other in *trn*L sequences; however no intraspecific variation in Mediterranean species was found. Denk & Grimm (2005) evaluated the

phylogeny within the genus *Zelkova* and found out that Caucasian *Z. carpinifolia* is placed between two East Asian species (*Z. schneideriana* and *Z. serrata*) and a Japanese-Mediterranean clade (*Z. serrata*, *Z. abelicea* and *Z. sicula*) using ITS (Fig. 1.7 a) or it is placed together with Japanese *Z. serrata* in a sister clade of the Mediterranean clade using morphological characters.

The phylogeography of western Eurasian species of *Zelkova* was recently evaluated by Christe *et al*. (2014) using two ITS and two chloroplast loci (Fig. 1.7 b). They revealed high numbers of chloroplast haplotypes in Caucasian *Z. carpinifolia* (15 haplotypes) and Mediterranean *Z. abelicea* (13 haplotypes), whereas each of two extant populations of *Z. sicula* possessed only one chloroplast haplotype. The strong phylogeographical structure in *Z. carpinifolia* suggested ancient diversification among populations from Colchis and Hyrcan. A high number of ribotypes was also found for *Z. carpinifolia* and *Z. abelicea* (13 and 7 ribotypes) based on ITS variability, but low or no variation within populations in *Z. carpinifolia*. They also suggested a hybrid origin of *Z. sicula*, which is clonal and triploid and its chloroplast haplotypes were located in the haplotype network between *Z. abelicea* and *Z. carpinifolia*, some ITS ribotypes and leaf morphology were shown to be close to *Z. abelicea* or *Z. carpinifolia*.

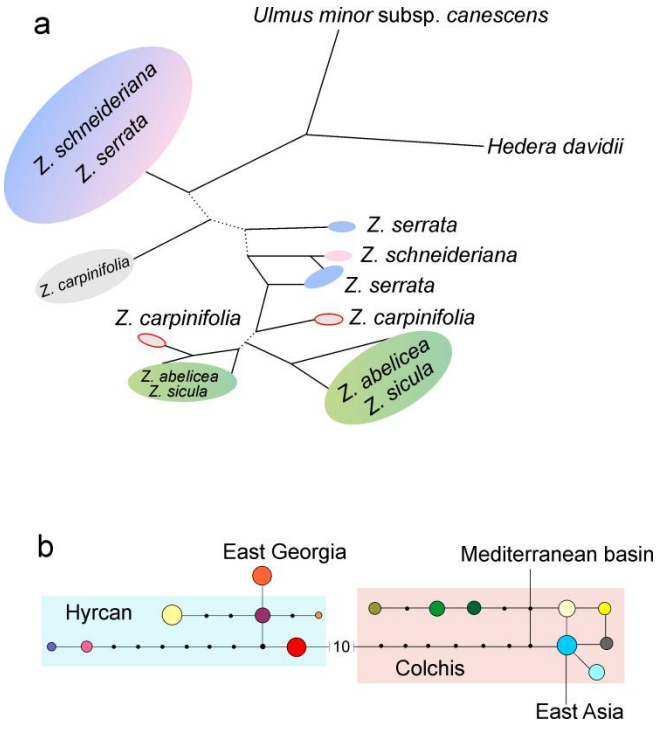

Fig. 1.7. Schematic representation of the phylogenetic relationships in genus *Zelkova* (panel **a**) adapted from Denk & Grimm (2005): unrooted Maximum Likelihood phylogram inferred from ITS markers. *Z. carpinifolia* position in the phylogram has a low support (<50%, dotted lines). Specimens of *Z. carpinifolia* sampled from Georgia (grey) and from St. Petersburg Botanical Garden (grey with red outline) did not group together.
Partial chloroplast DNA haplotype network of *Z. carpinifolia* adapted from Christe *et al.* (2014) (panel **b**): Colchic and Hyrcanian haplotypes differ by 19 mutational steps. One population from eastern Georgia belongs to the Hyrcanian group of haplotypes.

Fig. 1.8. Growth habit and bark variation in *Zelkova carpinifolia*: **a** – tall trees of *Z. carpinifolia* (left) and *Quercus castaneifolia* (right) with high crown in Hyrcanian forests, Azerbaijan; **b** - *Z. carpinifolia* with a short, wide trunk and wide crown in an open stand in village in Lankaran, Azerbaijan; **c** – bark of *Z. carpinifolia* in Astara, Azerbaijan; **d**, **e** – bark of *Z. carpinifolia* trees in Ajameti Nature Reserve, Georgia. Photo: a - Evelyne Kozlowski, Natural History Museum of Fribourg (NHMF), Switzerland; b, c – Elmira Maharramova; d, e – Gerald Parolly, BGBM, Germany.

1.5.1.3. Morphology of *Zelkova carpinifolia*

Z. carpinifolia is a large tree of about 20-35 m (up to 40 m) high, and 1-2 m (up to 4 m) in diameter. The bark is greenish-grey in young trees and brownish-grey in older trees, exfoliating leaving pink gaps (Fig. 1.8). The wood is white in young trees and with reddish-brownish heartwood in adults. The sapwood is slightly yellow. The year rings can be clearly seen from cuts.

Fig. 1.9. Leaves, flowers and fruits of *Zelkova carpinifolia*: a – a twig with male and female flowers, Lyon Botanical Garden, France; b – drupes at the leaf axils of the fruiting twig, Lankaran, Azerbaijan; c – leaves and buds of *Z. carpinifolia* in Lerik, Azerbaijan. Photo: a – Evelyne Kozlowski, NHMF, Switzerland, b – Emanuel Gerber, NHMF, Switzerland, c – Gerald Parolly, BGBM, Germany.

Leaves are elliptical or elongated-elliptical, acuminate with rounded or a slightly cordate unequal base, 4-8 cm (up to 10 cm) long and 2-4 cm (up to 6 cm) wide with 4-8 (up to 12) pairs of veins (Fig. 1.9). Leaves are smaller on fruiting twigs and larger on vegetative young shoots. Leaves are crenate with teeth that are usually blunted, the leaf lamina is dark green on the upper surface, slightly pubescent in younger age, and the underside is light green, rough and pubescent at veins, the petiole is short (1-2 mm long). The leaves open in

April (March) and fall in November, when they become colored yellow or brown (Sokolov, 1951; Kozlowski & Gratzfeld, 2013).

The twigs are reddish-brown and pubescent. Buds are elongate-conical, bulging, 2-3 mm long, reddish-brown or brown, fimbriated with white pubescence scales.

The tree is monoecious, but there is some confusion in the literature if the female flowers are actually bisexual (Sokolov, 1951; Tutayuk, 1975) or not (Penkovsky, 1901; Sosnovsky, 1952; Gulisashvili, 1961). From two to five male flowers with 4-5 stamens each are clustered in glomeruli sitting in the lower, more or less leafless part of one-year old twigs at the leaf axils (Fig. 1.9). The female (or bisexual) flowers are sitting solitary at the leaf axils in the upper part of the same twig (Fig. 1.9). They are greenish-white or brownish with a bell-shaped, 4-5 toothed naked or slightly pubescent perianth. The pistil has a unilocular ovary and two pubescent stigmas on two short columns (Tutayuk, 1975). Pollen grains are brownish with 4 or 5 oval pores and pollen is distributed by the wind. Flowering takes place simultaneously with leaf opening in April-May (sometimes March).

The fruit is a greenish, angular drupe with netlike venation, naked or with short pubescence, and measures up to 3-4 (7) mm in diameter (Fig. 1.9). The seeds are dark brown. Fruits mature in August-September (or October). Dispersal occurs mainly by gravity and wind, although zoochory is mentioned (Sokolov, 1951). Fruiting twig with 2-5 (7) drupes become separated from the mother tree together and are potentially transported by the wind (Gulisashvili, 1961). However, similar twigs of *Z. serrata* are not carried over very large distances (Hoshino, 1991). Thirty to eighty percent of seeds are usually undeveloped and the corresponding drupes are empty. Fruiting might not take place every year or the intensity of seed production may differ from year to year (Sokolov, 1951; Sosnovsky, 1952; Gulisashvili, 1961).

The root system is extensive and consists of 2-3 or more levels of side roots. Side roots of the first level are usually 10-15 m long (Penkovsky, 1901). *Z. carpinifolia* reproduces both sexually and by root and stump sprouting, but vegetative reproduction is preferred. Root sprouting may continue up to 5-6 years after a tree has been felled.

1.5.1.4. Ecology and distribution of *Zelkova carpinifolia*

Zelkova carpinifolia is a long-lived, deciduous, light-demanding but shadow-tolerant, wind-resistant tree, sensitive to the spring and winter frosts and gets damaged by temperatures below –20 ºC (Sokolov *et al.*, 1977). *Z. carpinifolia* trees live 300 years or longer, grow fast

until they are 60-80 years old and at the age of 30-50 years reach 15-20 m high. It is a mesophytic tree that prefers moist clay or loamy soils, but does not resist swamping, and grows extensively in humid ravines with humus-rich soils. At the same time, however, it is mesoxerophytic and can grow on dry stony soils (Fig. 1.10 & 1.12; Sokolov, 1951; Denk *et al*., 2001).

Z. carpinifolia occurs mainly in Hyrcan (southeastern Azerbaijan and northern Iran), Colchis (western Georgia and northeastern Turkey) and the Lesser Caucasus (western Azerbaijan). In addition, more isolated populations are found in the Zagros Mountains in Iran, in eastern Georgia, eastern Anatolia (Turkey) (Fig. 1.11; Gulisashvili, 1961; Browicz & Zielinski, 1982; Davis, 1982; Denk *et al*., 2001; Akhani *et al*., 2010) and presumably in Armenia near the village of Udjanis (Mulkidajnan, 1969).

Fig. 1.10. *Zelkova carpinifolia* park-like forest in Lankaran, Azerbaijan. Photo: Elmira Maharramova.

Z. carpinifolia grows in lowlands and lower to middle mountain ranges, but can be found at altitudes higher than 1500 m a.s.l. in dry habitats. It grows in Colchis (Georgia) between the Kvirila and Rioni rivers at the foothills of the Surami and Rachin mountain ranges at altitudes of 100-600 m a.s.l., together with *Quercus robur* and *Carpinus orientalis*,

on dry calcareous soils. In Kakheti-Zagatala (eastern Georgia, northwestern Azerbaijan) single trees were found at altitudes of 400-750 m a.s.l. in sympatry with *Quercus robur*, *Carpinus betulus*, *Acer* sp. and *Juglans regea* (Sokolov *et al*., 1977).

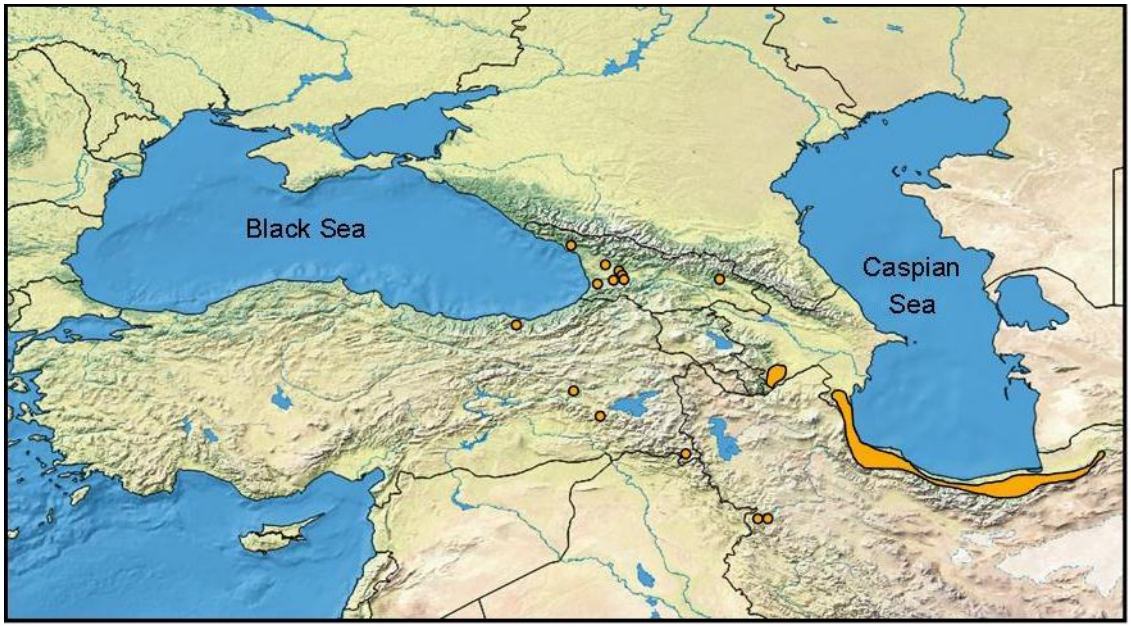

Fig. 1.11. Distribution of *Zelkova carpinifolia* in western Asia according to Browicz & Zielinski (1982).

Two-hundred and eight ha of a total of 770 ha of the Babaneuri Nature Reserve (eastern Georgia) at the left bank of the Alazani River is covered by *Z. carpinifolia* forest (Stuchlik & Kvavadze, 1993). In Garabagh (the Lesser Caucasus), it forms the small shrubs (although trees up to 15 m high do occur) and co-occurs with *Quercus petraea* subsp. *iberica* and *Crataegus* sp. In Hyrcan, it can grow at altitudes up to 1200-1500 m a.s.l. and occupies the first story of the forest together with *Quercus castaneifolia*, *Fraxinus excelsior* and *Acer* sp., whereas the second story is occupied by *Parrotia persica*, *Carpinus betulus*, *Pterocarya fraxinifolia* and *Buxus sempervirens* subsp. *hyrcana* (Sokolov *et al*., 1977). Close to the Zuvand highland (Talysh, Azerbaijan), where summers are hot and the climate is dry, *Z. carpinifolia* forms shrubs that are exposed to bovine grazing (Fig. 1.12; Gulisashvili, 1961).

Due to the decorative vase-shaped or cylindrical crown with a round top, *Z. carpinifolia* is used as an ornamental tree in Botanical Gardens worldwide (Kozlowski & Gratzfeld, 2013) and was cultivated in Crimea and the Caucasus (Gulisashvili, 1961). It was also proposed to be used to fortify the soils due to its deep and wide root system. But the exclusive value of *Zelkova carpinifolia* is given by its timber: dense, resilient, light and stable wood. The density exceeds that of oak, hornbeam, beech and ash trees. The wood gains a

brownish color in the water, is rot-resistant and obtains an attractive pattern after polishing. It was widely used in construction and joinery, shipbuilding, construction of bridges, water mills and underwater piles. Quality charcoal is produced from the wood of *Z. carpinifolia* (Sosnovsky, 1952).

Fig. 1.12. Talysh Mountains close to Zuvand highland, Azerbaijan, covered with shrubby *Zelkova carpinifolia* trees. Photo: Elmira Maharramova.

1.5.1.5. Economic value, main threats and conservation

The agricultural transformation of landscapes in the warm temperate zones of the South Caucasus and adjacent areas, through felling of forests and establishing tea, citrus, nut and rice plantations, became one of the major threats of the 20th century for the relict forests in the Caucasus. However, this process still continues in northern Iran. Other threats include urbanization, construction of roads and expansion of touristic attractions and, in particular for *Z. carpinifolia*, the past extensive logging for its valuable timber along with lopping, pollarding and bovine grazing (Fig. 1.13; Scharnweber *et al.*, 2007).

The logging of *Z. carpinifolia* trees is now prohibited and protection of *Zelkova carpinifolia*, along with other relict forest trees and shrubs, was established in few protected areas: the Ajameti Managed Reserve and the Babaneuri Nature Reserve (Georgia), the Hirkan National Park (Azerbaijan) and few protected areas in Iran (e.g. Golestan National Park).

Fig. 1.13. Lopped tree of *Zelkova carpinifolia* (a) and felled tree of *Pterocarya fraxinifolia* (b). *Z. carpinifolia* trees develop unusual form after lopping (c, d). Lerik, Azerbaijan. Photo: Elmira Maharramova.

Despite the popularity in the Botanical Gardens of the world, it is scarcely represented in the countries of its origin. At the same time, only 3% of its worldwide collections are of known origin (Kozlowski *et al.*, 2012b). An international project "*Zelkova*" was launched in 2010 to establish a new framework for the conservation of these relict trees (Kozlowski & Gratzfeld, 2013).

1.5.2. The Caucasian wingnut, *Pterocarya fraxinifolia*

1.5.2.1. The genus *Pterocarya* (Juglandaceae)

The walnut family, Juglandaceae A. Rich. ex Kunth. (Fagales), includes the genera *Alfaroa* Standl., *Carya* Nutt., *Cyclocarya* Iljinsk., *Engelhardia* Lesch. ex Blume, *Juglans* L., *Oreomunnea* Oerst., *Platycarya* Siebold. & Zucc., *Pterocarya* Kunth. and *Rhoiptelea* Diels & Hand.-Mazz. The diversification of these extant genera took place in the Paleogene (Xiang *et al.*, 2014) and the Juglandaceae family is extensively represented in the fossil record by fruits, leaves, inflorescences, wood and pollen (Zohary, 1973; Manchester, 1989; Ivanov *et al.*, 2011; Shatilova *et al.*, 2011; Alizade *et al.*, 2014). Most of the extant species of the wingnut genus *Pterocarya* (*P. hupehensis* Skan., *P. macroptera* Batalin, *P. stenoptera* C. DC., *P. tonkinensis* Dode and *P. rhoifolia* Siebold & Zucc.) are distributed in East Asia, whereas only one species, *P. fraxinifolia* (= *P. pterocarpa* (Michx.) Kunth. ex Iljinsk.), occurs in West Asia. The oldest fossil fruits of *Pterocarya* date back to the Eocene-Oligocene in North America and Eurasia, but most date back to the Neogene (Manchester & Dilcher, 1982). In western Eurasia (Germany (Koutsodendris et al., 2010), Poland (Bińka *et al.*, 2003), Lithuania (Kondratienė & Šeirienė, 2003), Ukraine (Gerasimenko & Rousseau, 2008), France (Andrieu *et al.*, 1997; Guiter *et al.*, 2008), Italy (Ravazzi *et al.*, 2005; Corrado & Magri, 2011)) as well as in Anatolia (Biltekin *et al*., 2015), the Caucasus (Joannin *et al.*, 2010; Shatilova *et al*., 2011; Tagieva *et al.*, 2013) and Iran (Ramezani *et al.*, 2008), *Pterocarya* fossils dating back to the Pleistocene interglacial periods have been found. However, wingnuts went extinct in western Eurasia, except in the Euxino-Hyrcanian Province and its small southern outposts.

1.5.2.2. The phylogenetic relationships within the Juglandaceae

According to Manning (1978), the family Juglandaceae is divided into two subfamilies, four tribes and seven genera based on detailed morphological features such as floral envelopes, leaf architecture and fruits. The subfamily Platycaryoideae contained one genus, *Platycarya*, of the tribe Platycaryeae, whereas the subfamily Juglandoideae consisted of three tribes. The tribe Juglandeae included two genera, *Juglans* and *Pterocarya*, with *Pterocarya* being divided into two subgenera, *Pterocarya* and *Cyclocarya*. The tribe Engelhardieae included the genera *Engelhardia*, *Oreomunnea* and *Alfaroa*, and the tribe Hicorieae consisted of the single genus *Carya* (Manning, 1978).

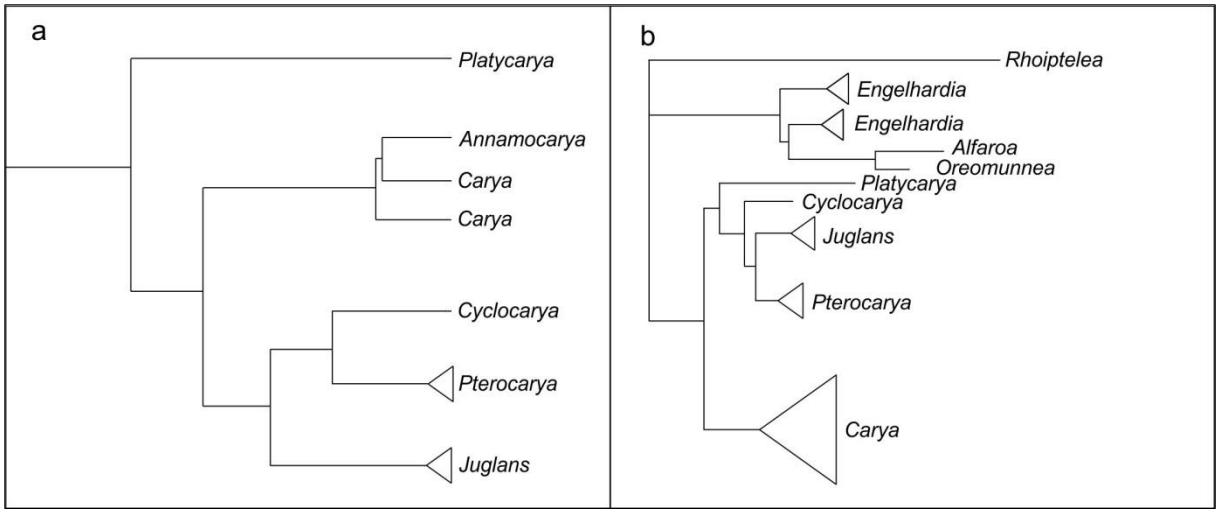

Fig. 1.14. Different phylogenetic relationships within the tribe Juglandoideae (*Platycarya*, *Carya*, *Cyclocarya*, *Juglans* and *Pterocarya*) of Juglandaceae: a – adapted from Manos & Stone (2001) and based on combined data from nuclear and chloroplast DNA, morphological and chemical datasets; b – adapted from Zhang *et al.* (2013) and based on nuclear and chloroplast DNA markers.

Incorporation of molecular genetic markers into the phylogenetic analyses by Manos & Stone (2001), however, suggested the existence of two subfamilies, Juglandoideae and Engelhardioideae, and two tribes within the Jugandoideae: Platycaryeae, containing the genus *Platycarya*, and Juglandeae, with the genera *Pterocarya*, *Juglans*, *Cyclocarya* and *Carya*. *Pterocarya* was shown to form a clade with *Cyclocarya*, which is sister to *Juglans* (Manos & Stone, 2001; Manos *et al.*, 2007), although some studies suggested reverse relationships for *Juglans* and *Cyclocarya* (Fig. 1.14; Fjellstrom & Parfitt, 1995; Zhang *et al.*, 2013; Xiang *et al.*, 2014).

Evolutionary studies, including phylogenetics, biogeography and population genetics, have been carried out within the genera *Juglans* (Stanford *et al.*, 2000; Aradhya *et al.*, 2007) and *Carya* (Zhang *et al*., 2013), but, so far, no such studies have been performed for *Pterocarya*.

1.5.2.3. The morphology of *Pterocarya fraxinifolia*

The Caucasian wingnut, *P. fraxinifolia*, is a deciduous, summer-green and mesophytic tree that grows 20-30 m (up to 40 m) tall and 1-1.5 m (up to 2 m) in trunk diameter. It has a wide cylindrical crown with a rounded top and a straight trunk. The bark is smooth, greyish and about 2 cm thick in young trees, and dark grey, vertically furrowed and 3-5 cm thick in older trees (Fig. 1.15).

Fig. 1.15. Differential growth habit of *Pterocarya fraxinifolia* in the forest (a) and in the open floodplain (c), Zagatala, Azerbaijan; b - the bark of *P. fraxinifolia*, Moscow forest, Lankaran, Azerbaijan. Photo: a, c – Elmira Maharramova, b – Gerald Parolly, BGBM, Germany.

The bark of the branches is also grey and smooth, whereas young shoots are olive-green with a brownish scale-like pubescence. The buds are naked or have 2-4 scales that fall apart after the first winter frost, 10-15 mm (up to 20 mm) long and dark brown with short

dense pubescence. The leaves are usually odd-pinnate, 30-35 cm (up to 40 cm) long and 18-20 cm (up to 25 cm) wide, and have 5-9 or 11-23 (up to 25) leaflets (Fig. 1.16). The leaflets are 8-12 cm (up to 15 cm) long and 4-5 cm (up to 6 cm) wide, have short petioles or are sessile, have elongated and serrated margins, have an underside that is pubescent at the vein axils and are unequal or narrowly wedge-shaped at the base. The number of veins varies between 8 and 15, and the veins do not reach the marginal teeth. The leaves of the young sprouts are larger, 60-75 cm long and 40-45 cm wide. The vegetation period lasts for 210-230 days (Sokolov, 1951; Iljinskaya, 1953; Gulisashvili, 1961; Manning, 1978).

The wingnut is a monoecious tree with many-flowered male (staminate) and female (pistillate) aments (catkins) (Fig. 1.16). The staminate aments, which are 3-4 times more abundant than the female ones, are 4-7 cm long, solitary in the axils of the side leafs or bud scars on the lower part of the twig (the old growth). The male flowers have a perianth with 1-4 (or 3-6) lobes and 6-18 stamens. The pistillate aments are located at the twig apex (the new growth) and contain 40 or more sessile female flowers (Iljinskaya, 1951; Gulisashvili, 1961). The perianth of the female flowers is tubular, has four lobes and the bract and bracteoles, which develop into the wings of the fruit, are both fused with the ovary (Manning, 1978). The pistil has two stigmas and a unilocular ovary.

The pollen grains have 5-6 (up to 8) pores (Manning, 1978), are produced in great abundance and are distributed by the wind (Gulisashvili, 1961; Kvavadze, 2001). After the blooming, the male aments fall apart in 3-5 days, whereas the female aments grow fast and mature in 2-3 weeks. The aments with fruits grow about 20-40 cm (up to 70 cm) long. The aments that produce normal fruits may also develop on the old trunks and surface roots (Gulisashvili, 1961). The number of fruits per ament varies between 30 and 80.

The fruit is a two-winged drupe, is irregularly turbinate, and measures up to 3.5-4.5 cm wide and 1-1.5 cm long with wings (Fig. 1.16). The wings are wider than the drupe, coriaceous, rounded and naked. *P. fraxinifolia* blooms in mid-April until mid-May and fruits in September – October (or earlier in the summer). The drupes fall apart as the seeds develop, mainly during the windy and rainy days of November. They are dispersed by wind up to 25-30 m away from the mother tree, but can be dispersed further away by the watercourses (Gulisashvili, 1961). The root system is extensive with numerous side roots. Vegetative reproduction through sprouting of the roots and stumps occurs often and is the preferred means of reproduction under natural conditions (Iljinskaya, 1951; Gulisashvili, 1961). Trees that grow from seeds start to produce seeds from the age of 18-25 years, whereas vegetatively produced trees start to reproduce sexually from the age of 10-12 years (Gulisashvili, 1961).

Fig. 1.16. Morphology of *Pterocarya fraxinifolia*: a – the branch of *Pterocarya fraxinifolia* with the leaves, Ismayilli, Azerbaijan; b – the male and female aments on the flowering twig, Lyon Botanical Garden, France; c & d – the catkins with winged drupes, Astara, Azerbaijan. Photo: a, b – Evelyne Kozlowski, NHMF, Switzerland, c, d – Emanuel Gerber, NHMF, Switzerland and Gerald Parolly, BGBM, Germany.

1.5.2.4. The ecology and distribution of *Pterocarya fraxinifolia*

Pterocarya fraxinifolia is a fast growing tree that reaches a height of 9-12 m in about 10 years and 30 m in about 30 years, with trunks that grow up to 80-100 cm wide in 60-80 years. One-year old sprouts are 1.5-2 m high and 6-year old sprouts are 3.5-4 m tall with a

diameter of about 4 cm. It lives up to 250 years and then becomes dry, hollow and rots from the root up. The tree is light-demanding and mesophytic, and gets damaged by temperatures under -30 °C. It is wind-resistant, but branches may break under the weight of snow (Gulisashvili, 1961). *P. fraxinifolia* is a moist-loving tree that grows along the river banks and in the floodplains on semi-deep and moist soils (sandy loam, loam, silt loam and clay; Sheykholislami & Ahmadi, 2009), and in ravines on skeletal humus-rich soils. Its growth and development are influenced both by air and soil humidity, but it develops only poorly in swamps (Gulisashvili, 1961).

Currently, *P. fraxinifolia* occurs in western Anatolia (Turkey), in Colchis (northeastern Turkey and western Georgia), on the southern slopes of the Greater Caucasus and the Alazan-Agrichay valley (eastern Georgia and northwestern Azerbaijan) and in Hyrcan (southeastern Azerbaijan and northern Iran). Isolated populations can be found in southeastern Anatolia (Turkey) and the Zagros Mountains of Iran (Fig. 1.17; Gulisashvili, 1961; Browicz & Zielinski, 1982; Denk *et al*., 2001; Akhani & Salimian, 2003; Biltekin *et al*., 2015).

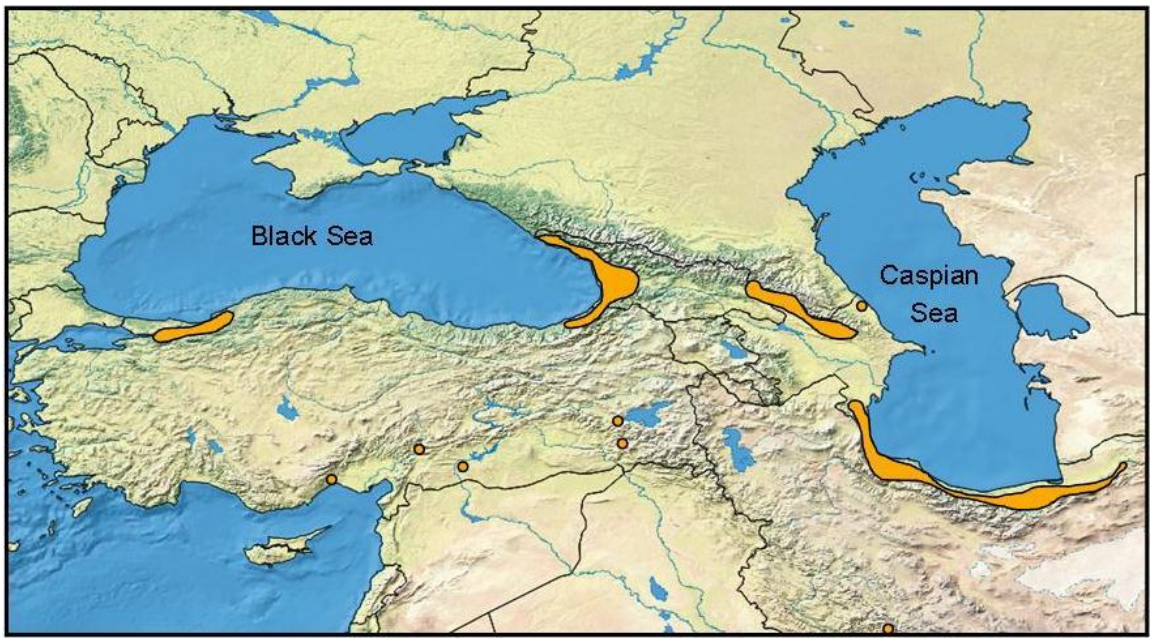

Fig. 1.17. Distribution of *Pterocarya fraxinifolia* in western Asia according to Browicz & Zielinski (1982) and Akhani & Salimian (2003).

It grows in the lowlands and in the low and middle mountain zone up to 400-500 m in Colchis, up to 1000-1100 m a.s.l. in the Greater Caucasus and up to 800-1200 m a.s.l. in Hyrcan. In the Zagros Mountains, one population was found at 1700 m a.s.l. (Akhani & Salimian, 2003).

It forms pure stands or, more often, occurs together with *Alnus glutinosa* subsp. *barbata*, *Carpinus betulus*, *Ulmus glabra*, *Acer platanoides*, *A. campestre*, *Fagus orientalis*, *Juglans regea* and/or *Populus canescens* (Fig. 1.18). In Hyrcan, it grows together with *Alnus subcordata* and *Acer velutinum*, and may be found together with *Quercus castaneifolia*, *Carpinus betulus*, *Parrotia persica* and *Zelkova carpinifolia*. *P. fraxinifolia* trees are scattered in *Betula-Carpinus* and *Betula-Quercus* moist forests in Khachmaz (eastern Greater Caucasus, Azerbaijan). The understory of the moist *Pterocarya-Alnus* forests is usually represented by *Cornus australis*, *Sambucus ebulus*, *Corylus avellana*, *Prunus sp.*, *Ligustrum vulgare*, *Crataegus germanica*, etc. In degraded forests, lianas such as *Smilax excelsa*, *Hedera* sp., *Periploca graeca*, *Vitis* vinifera, Lonicera *caprifolium* and *Humulus lupulus* develop extensively on *P. fraxinifolia* and may form the impassable thickets (Sokolov *et al.*, 1977).

Fig. 1.18. *Pterocarya fraxinifolia* forest in Zagatala, the Greater Caucasus, Azerbaijan. Photo: Elmira Maharramova.

1.5.2.5. The economic value, main threats and conservation

The wingnut tree is characterized by many valuable traits. Its wood is soft, light, resilient and white with a reddish tint. In dry conditions, it is durable and worm-resistant, but it rots fast in humid conditions. The timber is used for construction of the house-building materials and barns for drying of tobacco leaves, as well as for making troughs, vats, bowls, tare, etc. The cork is used for tying grapevines and for wickerwork. The bark, leaves and staminate aments can be used for coloring of silk in grey and wool in brown colors. The leaves and fruits contain vitamin C, the antibiotic juglone, some poisonous substances and have diaphoretic properties. The leaves are used for fishing. The strong and branched root system provides a wind-resistance that exceeds that of *Alnus* and *Populus*, and the trees are useful for the fortification of river banks. It is also used as an ornamental in parks in Europe, North America, Central Asia and, to a lesser degree, the Caucasus (Iljinskaya, 1951; Gulisashvili, 1961).

Agricultural development and deforestation resulted in a change of the lowland water regime, such that the environmental conditions are not suitable anymore for wingnut growth. Under the name of *P. pterocarpa*, *P. fraxinifolia* is listed in the IUCN Red List as "Lower Risk/Least Concern" (Firsov, 1998). Together with other relict trees, the protection of *P. fraxinifolia* is realized in protected areas and nature reserves in Azerbaijan, Georgia and Iran. However, tree felling continues outside the protected areas for road and electric lane expansions, as well as for bridge construction.

CHAPTER 2

Development of nuclear microsatellites for the Arcto-Tertiary tree *Zelkova carpinifolia* (Ulmaceae) using 454 pyrosequencing

2.1. Introduction

Zelkova carpinifolia (Pall.) K. Koch. (Ulmaceae) is a representative species of the Arcto-Tertiary relict flora (Wang *et al*., 2001; Milne & Abbot, 2002; Kozlowski *et al*., 2012b). It occurs naturally in Hyrcan and Colchic forests in Iran, Azerbaijan, Georgia, and Turkey (Browicz & Zielinski, 1982; Denk & Grimm, 2005; Kvavadze & Connor, 2005; Akhani *et al*., 2010). Its valuable timber has led to uncontrolled logging, which has resulted in a general population decline. Therefore, *Z. carpinifolia* was listed under the International Union for Conservation of Nature (IUCN) category "Near Threatened" (Güner & Zielinski, 1998). To plan conservation strategies for *Z. carpinifolia*, investigation of its genetic diversity is of great importance (Kozlowski *et al*., 2012b). The phylogeography of the species needs to be understood as there may be specific refugia within the range of the species that harbor relatively high genetic diversities and regionally adapted genotypes may occur.

The first studies of genetic diversity in *Zelkova* Spach. employed inter-simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) markers (Fineschi *et al*., 2004). Primers targeting microsatellite loci in *Z. serrata* (Thunb.) Makino (Fukatsu *et al*., 2005) did not amplify well in *Z. carpinifolia* and revealed only limited polymorphism (unpublished data). The current study therefore aimed to develop polymorphic microsatellite markers for *Z. carpinifolia* using 454 pyrosequencing.

2.2. Methods and Results

2.2.1. Plant material and DNA extraction

Zelkova carpinifolia samples were collected from six populations (10–20 individuals per population) in Azerbaijan and Iran (see Table A1 in Appendix A). Genomic DNA was extracted from silica gel-dried leaves using either the NucleoSpin Plant II kit (Macherey

Nagel, Düren, Germany) following the manufacturer's protocol or a three-fraction cetyltrimethylammonium bromide (CTAB) method (Borsch *et al*., 2003).

2.2.2. Microsatellite marker development

Construction of the enriched microsatellite libraries was carried out according to the protocol of Glenn & Schable (2005) with modifications. Genomic DNA was digested in 25-μL reactions containing 1 × NEBuffer 3.1, 0.1 mg/mL bovine serum albumin (BSA), 0.05 M NaCl, 20 U *Eco*RI, 10 U *Mse*I restriction enzymes (New England Biolabs, Frankfurt, Germany), and 2.4 μg genomic DNA during a 2-h incubation at 37°C. Digested DNA was run on a 2% agarose gel, and 200–400-bp fragments were extracted using the Gel/PCR DNA Fragment Extraction Kit (Avegene, Zollikofen, Switzerland) following the manufacturer's protocol.

To prepare double-stranded adapters, forward (*Eco*RI: 5′-CTCGTAGACTGCGTACC, *Mse*I: 5′-GACGATGAGTCCTGAG) and reverse (*Eco*RI: 5′-AATTGGTACGCAGTCTACGAG, *Mse*I: 5′-TACTCAGGACTCATCGTC) adapters (Metabion, Martinsried, Germany) were mixed in equimolar amounts (10 μM), incubated at 95°C for 5 min, and allowed to slowly cool down to room temperature. Adapters were ligated to the restriction fragments in 40-μL reactions containing 0.8 μM of each of the double-stranded *Eco*RI and *Mse*I adapters, 1 × T4 DNA ligase buffer, and 600 U T4 DNA ligase (New England Biolabs). Reactions were performed at 16-°C for 12 h and successful ligation was tested by PCR amplification in 50-μL reactions containing 4 μL of the adapter-ligated DNA, 1 × Taq DNA polymerase buffer, 1 × PCR enhancer solution, 150 μM of each dNTP, 2 U Taq DNA polymerase (PeqLab, Erlangen, Germany), 35 ng/μL BSA, 0.5 μM *Eco*RI primer (5′-CTCGTAGACTGCGTACCAATTC), and 0.5 μM *Mse*I primer (5′-GACGATGAGTCCTGAGTAA) (Metabion) using the following temperature profile: 95°C for 2 min; 20 cycles of 95°C for 20 s, 60°C for 20 s, 72°C for 1.5 min; and 72°C for 10 min. Two mixtures of 3′-biotinylated oligonucleotides [1: $(AG)_{12}$, $(TG)_{12}$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$ and 2: $(ACAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(ACTG)_6$] were used to generate two separate libraries of adapter-ligated genomic DNA enriched for repetitive motifs. Hybridization of biotinylated oligonucleotides and adapter-ligated genomic DNA, followed by the capturing of hybridized DNA on Dynabeads (Life Technologies, Darmstadt, Germany) and subsequent washing and resuspension, followed the protocol of Glenn & Schable (2005). Recovery PCR was performed with 2 μL of enriched DNA in the same reaction mixture and the temperature profile as described above, but with 25 cycles.

The enrichment procedure was repeated and the second recovery PCR was carried out with an *Eco*RI oligonucleotide primer extended with a Roche 454 pyrosequencing adapter (5′-GCCTCCCTCGCGCCATCAG-3′) and a specimen-specific barcode sequence and an *Mse*I oligonucleotide primer extended with a Roche 454 B sequencing adapter (5′-GCCTTGCCAGCCCGCTCAG-3′; see Table A2 in Appendix A).

Two enrichment libraries were prepared for each of six *Z. carpinifolia* specimens (population ZE053_IR, see Table A1 in Appendix A). Enrichment libraries were purified using the Amplicon Library Preparation Protocol (Roche, Branford, Connecticut, USA) according to the manufacturer's instructions. Lib-L Kits and the GS FLX Titanium Sequencing Kit XLR70 (Roche) were used for the library sequencing on the Roche GS FLX System. The resulting DNA sequences were analyzed using the R package SeqinR (Charif & Lobry, 2007). The 454 pyrosequencing yielded 86,058 total reads ranging from 40 to 577 bp with an average length of 138 bp.

2.2.3. Microsatellite screening

Screening for microsatellite loci and primer design was performed using QDD software version 1 (Meglécz *et al*., 2010), which identified 136 microsatellite loci containing di-, tri-, and tetranucleotide repeat motifs. A total of 80 microsatellite loci with 5–45 repeats, including three loci with tetranucleotide repeats, 17 loci with trinucleotide repeats, and 60 loci with dinucleotide repeats, were selected randomly, and oligonucleotide primer combinations with a GC content of 35–60% and melting temperature (T_m) ranging between 57 °C and 60 °C were tested for proper PCR amplification on genomic DNA extracted from three specimens (Table 2.1). A gradient PCR was carried out for each primer pair in a 25-μL reaction volume containing 20–40 ng DNA, 0.4 μM each of forward and reverse primer (Eurofins MWG Operon, Ebersberg, Germany), 1 × TaqBuffer S, 1.5 mM $MgCl_2$, 250 μM of each dNTP, and 0.75 U Hot Taq polymerase (PeqLab). The temperature profile was as follows: 96°C for 2 min, 54 ± 8°C for 30 s, 72°C for 30 s; followed by 34 cycles of 95°C for 30 s, 54 ± 8°C for 30 s, 72°C for 30 s; and 72°C for 15 min. PCR performance was assessed by gel electrophoresis. Of the initial 80 loci tested, 40 primer pairs failed to amplify a product. The reactions with the other 40 primer pairs were repeated in 50-μL reaction volumes.

Table 2.1. Characteristics of 13 polymorphic and five monomorphic nuclear microsatellite loci for *Zelkova carpinifolia*.

Locus	Forward primer sequence (5'—3')	Reverse primer sequence (5'—3')	Fluorescent label	Repeat motif	*T*a (°C)	Allele size range (bp)	GenBank/ENA accession no.
ZMS_1	AAGGTTCATCCCATGAGCAC*	GTCCAGTCCGGCATCTTTT	6-FAM	$(CTT)_7$	60	238-259	KF408341
ZMS_2	CAAGCTGCCAATACTCCACA*	GGTTTGGTTTCTGCTTGGAA	VIC	$(AGA)_7$	60	181-213	KF408342
ZMS_3	CCCAACAGCACTTGAAGCTA*	TTTGTGTAGTCTTTGGCCCC	6-FAM	$(CA)_8$	60	136-138	KF408343
ZMS_4	ACCGTTGGAAACAAAACTCG*	AGGGCAGTAGAGCTTGATCG	NED	$(AG)_9$	52	105-131	KF408344
ZMS_5	TTTTGTTCGTTGTTGCATGTC*	AGGCTTAGGTTGGGTGGTTT	PET	$(GAA)_6$	60	99-114	KF408345
ZMS_6	TTTGGTGCTTAGCATGTGGA*	TCTTTTGTTTGCGATAAGTCG	6-FAM	$(CT)_8$	58	101-120	KF408346
ZMS_7	TCTCTGTTATGATCACGTCTCCA	GAGCTAAACCCCATTTCAAATATAA*	NED	$(AC)_9$	60	120-128	KF408347
ZMS_8	ACGAACAAGATCGACAGAGG*	ATTGGATGGCACACGTAATTC	PET	$(TC)_{12}$	60	188-204	KF408348
ZMS_9	CAGGTGATTGGAATTGGAAAA*	CCTCACAGAGTACGAGGGATG	6-FAM	$(AC)_7$	62	171-196	KF408350
ZMS_10	CTGCGAGGGCAGTAGGTAAG*	TCCTAGATCCTTTGATATCTTTCTCA	VIC	$(GA)_8$	62	175-189	KF408351
ZMS_11	AGTAGGCTTGGAAGCAAATGA	TTGGAACTGCAATCGCTTT*	VIC	$(GT)_9$	58	96-115	KF408352
ZMS_12	CTTCCAATCATTCCGAGAGC*	TGACATCTCAAAGGTTGCCA	PET	$(CA)_7$	58	100-104	KF408353
ZMS_13	AACAAAGAATCTTCATCCCC*	GCATTTGGTTTTACCTAAAGTTACAG	NED	$(TG)_9$	58	110-116	KF408354
ZMS_14	GCGGTTGAGAGGAGAAAGAA	ACCAAACCCATCAACCATTG*	6-FAM	$(AGG)_{10}$	57	105†	HG737351
ZMS_15	GCTCCTCTTCAGCCAAACAC*	CTTCATCCTCGTTTGCATCA	6-FAM	$(AGG)_{12}$	60	128 †	HG737352
ZMS_16	GCCGGTACTGATTCCTTCAA*	GGACCATCTCACCAACATCC	6-FAM	$(AAG)_5$	60	233 †	HG737353
ZMS_17	AGCCCGTGCTTTTGAATATG	GGATAAGGCTATTTTGGCCC*	6-FAM	$(GA)_8$	55	167 †	HG737354
ZMS_18	GCCACGTAAACAGAGTAAGGG*	GCATGTTGGTGCTTTGAGAA	6-FAM	$(TG)_7$	58	103 †	HG737355

Note: ENA = European Nucleotide Archive; Ta = optimal annealing temperature. *Labeled primer, †Expected size.

PCR products were run on 1.5% agarose gel and extracted using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) following the manufacturer's protocol. PCR products were sequenced at Macrogen (Seoul, Korea) using standard Sanger sequencing to confirm locus-specificity of the oligonucleotide primers. Only the 20 primer pairs that specifically amplified the targeted microsatellite loci were retained for further analyses. Polymorphism assessment used genomic DNA extracted from 25 *Z. carpinifolia* specimens (five specimens per population; Appendix A Table A1) and fluorescently labeled forward or reverse primers (Applied Biosystems, Warrington, United Kingdom), and the same reaction conditions as in the previous step. Two primer pairs did not amplify a product in all individuals and were excluded from further analysis (Table 2.1). The rest of the PCR products were sent to Macrogen for fragment analysis.

2.2.4. Microsatellite marker data analysis

Electropherograms from the fragment analysis were analyzed using GENEMARKER software version 1.95 (SoftGenetics, State College, Pennsylvania, USA). Analyzed amplification products with unscorable peaks were not considered to be useful in the studies of variability due to the possible misinterpretation of data. Thirteen primer pairs produced polymorphic and heterozygous loci (accession numbers KF408341–KF408348, KF408350–KF408354, Table 2.1). The Excel Microsatellite Toolkit (Park, 2001) was used for the calculation of the number of alleles, observed (H_O) and expected (H_E) heterozygosities, and polymorphism information content (PIC) values. Allele sizes ranged from two to eight alleles per locus across 25 individuals from five different populations, with H_O and H_E varying between 0.12 and 0.84, and 0.25 and 0.81, respectively (Table 2.2).

2.3. Conclusions

In the current study, we used 454 pyrosequencing to obtain sequence reads for microsatellite primer development in *Z. carpinifolia*. After thorough evaluation, we present 13 primer pairs for amplifying polymorphic microsatellite loci in *Z. carpinifolia*. These 13 loci are proposed to be used for the microsatellite screening of naturally distributed *Z. carpinifolia* populations, but they may also cross-amplify in closely related species. Analysis of the obtained allele frequencies and knowledge of the individual genotypes will help us to

understand the population structure and relationships between and within different populations of *Zelkova* species.

Table 2.2. Results of initial primer screening on specimens from five populations of *Zelkova carpinifolia.*

	ZE001_AZ (*N*=5)			ZE007_AZ (*N*=5)			ZE013_AZ (*N*=5)			ZE017_AZ (*N*=5)			ZE021_AZ (*N*=5)			Total (*N*=25)			
Locus	*A*	H_O	H_E	*A*	H_O	H_E	*A*	H_O	H_E	*A*	H_O	H_E	*A*	H_E	H_E	*A*	H_O	H_E	PIC
ZMS_1	2	0.6	0.47	4	1	0.78	2	0.8	0.53	4	1	0.71	4	0.6	0.64	5	0.8	0.6	0.55
ZMS_2	3	1	0.71	4	1	0.73	4	0.6	0.53	4	0.8	0.73	3	0.8	0.64	8	0.84	0.7	0.64
ZMS_3	2	1	0.56	2	0.8	0.53	2	0.8	0.53	2	0.2	0.56	2	0.2	0.47	2	0.6	0.5	0.37
ZMS_4	3	0.8	0.6	4	0.8	0.8	4	0.8	0.8	5	1	0.84	5	0.4	0.8	8	0.76	0.8	0.78
ZMS_5	3	0.4	0.38	2	0.4	0.36	1	0	0	1	0	0	3	0.6	0.51	5	0.28	0.3	0.25
ZMS_6	2	0	0.36	2	0	0.36	3	0	0.62	5	0.4	0.87	5	0.2	0.82	8	0.12	0.8	0.76
ZMS_7	3	1	0.64	4	1	0.78	4	0.6	0.64	2	0.8	0.53	3	0.8	0.6	4	0.84	0.7	0.64
ZMS_8	5	1	0.82	4	1	0.73	4	1	0.73	3	0.8	0.6	4	0.2	0.64	7	0.8	0.8	0.74
ZMS_9	3	0.4	0.62	3	0	0.71	4	0.6	0.73	3	0.6	0.51	3	0.2	0.51	5	0.36	0.7	0.65
ZMS_10	2	0.2	0.56	4	0.6	0.73	3	0.4	0.6	2	0.2	0.47	4	0.8	0.78	5	0.44	0.7	0.61
ZMS_11	1	0	0	3	0.4	0.71	2	0	0.36	5	0.4	0.82	2	0	0.36	6	0.16	0.5	0.45
ZMS_12	2	0.6	0.47	2	0.4	0.36	2	0.2	0.2	2	0.2	0.2	1	0	0	3	0.28	0.3	0.23
ZMS_13	3	0.6	0.64	3	1	0.64	2	0.8	0.53	2	0.2	0.2	2	0.2	0.2	4	0.56	0.5	0.44

Note: A = number of alleles; H_E = expected heterozygosity (Nei's unbiased gene diversity); H_O = observed heterozygosity; *N* = sample size; PIC = polymorphism information content.

CHAPTER 3

Analysis of nuclear microsatellites reveals limited differentiation between Colchic and Hyrcanian populations of the wind-pollinated relict tree *Zelkova carpinifolia* (Ulmaceae)

3.1. Introduction

Conservation of relict plants that were able to survive through the Quaternary climatic oscillations and persist today in isolated refugial areas is crucial for maintaining the global diversity of plants, especially considering the current climate trend of increasing temperatures and water stress (Petit *et al*., 2005; Kozlowski *et al*., 2012b). The Arcto-Tertiary relict flora, that covered large parts of the northern hemisphere during the Eocene but since the Miocene has moved southward due to a shift toward a dryer and cooler climate (Chaney, 1947; Milne & Abbott, 2002; Milne, 2006), is represented today by many temperate tree genera, among which *Zelkova* gained a particular interest in the recent years (Fineschi *et al*., 2002 ; Denk & Grimm, 2005; Kvavadze & Connor, 2005; Søndergaard & Egli, 2006; Garfi *et al*., 2011; Kozlowski *et al*., 2012b; Christe *et al*., 2014). The fossil record supports the past wide distribution of the genus (Wang *et al*., 2001; Denk & Grimm, 2005) and its extinction in North America, North Africa, and Europe as a result of climatic shifts in the Quaternary period (Chaney, 1947; Kvavadze & Connor, 2005). The last appearances of *Zelkova* in mainland Europe were in Rome (Italy) and date back to 31 thousand years ago (ka) (Follieri *et al*., 1986).

Zelkova carpinifolia (Pall.) C. Koch. is the only species of the genus *Zelkova* that occurs in western Asia. The remaining *Zelkova* species are restricted to Mediterranean islands or have a wider distribution in East Asia. It has been shown that six regions in western Asia could have served as climate refugia for forest vegetation during the Last Glacial Maximum (LGM): Colchis, western Anatolia, western Taurus, upper reaches of the Tigris River, Levant, and the southern Caspian basin (Tarkhnishvili *et al*., 2012). There are two relict forest regions in the Caucasus and adjacent areas: the Hyrcanian forest on the southern coast of the Caspian Sea, bounded by the Talysh and Alborz mountain ranges, and the Colchic forest on the eastern coast of the Black Sea, bounded by the western Greater and Lesser Caucasus mountains and the Likhi Ridge (Nakhutsrishvili *et al*., 2011). *Zelkova carpinifolia* occurs naturally in the

Hyrcan region of Azerbaijan and Iran and the Colchis of western Georgia, but also grows in the south Lesser Caucasus (Garabagh, Azerbaijan), eastern Georgia (Babaneuri Strict Nature Reserve), Zagros Mountains (Iran), and the eastern part of Turkey (Fig. 3.1; Gulisashvili, 1961; Davis, 1982; Kvavadze & Connor, 2005; Akhani *et al*., 2010). The upheaval of the Greater Caucasus and the Lesser Caucasus mountain ranges began, respectively, at the end of the Miocene-Pliocene era (Avdeev & Niemi, 2011) and the Pliocene (Sosson *et al*., 2010), whereas Colchic and Hyrcanian forests are believed to have emerged during the Upper Pliocene (Kolakovsky, 1961). These forests are now completely separated from each other and consist of distinctive plant communities, with several common relict plant species (Nakhutsrishvili *et al*., 2011). Climatic oscillations during the Holocene influenced the distribution of *Z. carpinifolia* as it was shown for Georgia (Kvavadze & Connor, 2005) and Iran (Djamali *et al*., 2008; Ramezani *et al*., 2008).

Fig. 3.1. Geographical distribution of *Zelkova carpinifolia* in the Caucasus and Iran. Gray circles represent the distribution according to Browicz & Zielinski (1982); black circles represent sites sampled in this study. Red dotted lines enclose Caucasus ecoregion as designated by the World Wildlife Fund (WWF). Map was created in ArcGIS Desktop version 10.2.2 (ESRI, 2014). Country borders, streams, water bodies and terrain shapefiles were taken from ArcGIS Online (ESRI, 2014).

The phylogenetic relationships in the genus *Zelkova* were studied by Denk & Grimm (2005) using morphological characters and ribosomal internal transcribed spacer (ITS). Fineschi *et al*. (2002) addressed the genetic variation in natural populations of two

Mediterranean species (*Z. sicula* and *Z. abelicea*) and their comparison to *Z. carpinifolia* using different molecular techniques, but no variation within the Mediterranean species was found. Christe *et al*. (2014) addressed the phylogeographical patterns in three western Eurasian species: *Z. sicula*, *Z. abelicea*, and *Z. carpinifolia* using two chloroplast (*trn*H-*psb*A and *trn*L) and nuclear (ITS1 and ITS2) regions and detected high levels of genetic variation and strong phylogeographical structure using chloroplast markers. Currently, *Z. carpinifolia* mainly occurs in lowlands and middle mountain ranges, where, more recently, tree populations have become fragmented due to anthropogenic activities such as selective logging for timber, silvopasture, tree lopping and cutting for fuel, agricultural developments, construction of roads, tourism developments, fires, and extensive urbanization and industrialization (Gulisashvili, 1961; Scharnweber *et al*., 2007; Ramezani *et al*., 2008; Akhani *et al*., 2010; Kozlowski & Gratzfeld, 2013). As a consequence, *Z. carpinifolia* is listed as "Near threatened" (Güner & Zielinski, 1998) according to the criteria of the International Union for Conservation of Nature (IUCN).

The main aims of the current study were to (1) assess the nuclear genetic diversity across natural populations of *Z. carpinifolia*, (2) evaluate the differentiation between Hyrcanian and Colchic populations at nuclear loci, and (3) identify possible refugial areas as areas of high conservation value.

3.2. Materials and Methods

3.2.1. Study species and sampling sites

Zelkova carpinifolia is a mesophytic deciduous tree species that occurs mainly in mixed lowland, riverside, and ravine forests with trees such as *Quercus* sp., *Carpinus* sp., *Acer* sp., *Ulmus* sp., *Parrotia persica*, *Fraxinus excelsior*, *Gleditsia caspia*, *Albizia julibrissin*, *Diospyros lotus*, and *Pterocarya fraxinifolia.* Some individuals grow to 40 m tall and 2–3 m in diameter and have an expected lifespan of more than 300 yr. At high elevations, up to 1500 m a.s.l., the species occurs in the form of small shrubs. *Zelkova carpinifolia* is andromonoecious with male flowers clustering together at the basis of the current year's twigs, while bisexual flowers are sitting alone in the leaf axils. Flowering takes place in March and April, and pollination is by wind. The fruit is a greenish, angular, rugous drupe that is usually dispersed with part of the twig by wind.

Between 2010 and 2012, leaf samples were collected from 495 individuals of *Z. carpinifolia* among 30 geographical sites (10–20 individuals per site): 19 sites from Azerbaijan, 6 sites from Georgia, 4 sites from Iran, and 1 site from Turkey (Fig. 3.1, see Table B1 in Appendix B). Specimens were collected from both protected and unprotected areas, more or less untouched forests, forests degraded by human activities in the past and naturally regenerating forests, and park-like forest stands and remnant tree stands in villages. The distances among sites varied from 1 to 1000 km. The minimum distance between sampled trees was approximately 10 m, except for small sites where minimum distance was lower, but sampling of neighboring trees was prevented. Per site, one voucher specimen was collected, and voucher specimens were deposited in the herbaria of the Botanical Garden and Botanical Museum Berlin-Dahlem (Germany) and the Natural History Museum in Fribourg (Switzerland). Collection numbers were not assigned for specimens from Iran, Georgia, and Turkey, and herbarium vouchers were not collected for sites ZE053_IR and ZE069_AZ. Collected leaves were dried and stored in silica gel until DNA extraction.

3.2.2. DNA extraction and microsatellite genotyping

Total genomic DNA was extracted from silica-gel-dried leaves using the NucleoSpin Plant II kit (Macherey Nagel, Düren, Germany) following the manufacturer's protocol and individuals were initially genotyped at 10 microsatellite loci described previously for *Z. carpinifolia* (Maharramova *et al*., 2014). Except for loci ZMS_4 and ZMS_9, microsatellite markers were amplified by multiplex polymerase chain reaction (PCR) using three mixes of oligonucleotide primers: mix 1 amplified microsatellite loci ZMS_12 and ZMS_13; mix 2 amplified loci ZMS_1, ZMS_2, ZMS_5 and ZMS_7; and mix 3 amplified loci ZMS_3 and ZMS_8. PCRs were performed in 25-μL reaction volumes containing 20–40 ng template DNA, 0.4 μM of each forward and reverse primer (Eurofins MWG Operon, Ebersberg, Germany), 1 × TaqBuffer S (PeqLab, Erlangen, Germany), 1.5 mM $MgCl_2$, 250 μM of each dNTP, 0.2 mg/μL bovine serum albumin (BSA), and 0.75 U Hot *Taq* polymerase (PeqLab). Either the forward or reverse primer of each primer combination was labeled with a fluorescent dye (6FAM, VIC, NED or PET; Applied Biosystems, Warrington, UK; see Table B2 in Appendix B). The PCRs were carried out using the following temperature profile: initial denaturation at 96°C for 2 min, annealing at 57°C (mix 1), 60°C (mixes 2 and 3), 62°C (for ZMS_9) or 52°C (for ZMS_4) for 1 min, and primer extension at 72°C for 1 min; 30 cycles of denaturation at 95°C for 30 s, annealing at the aforementioned temperatures for 30 s,

and primer extension at 72°C for 30 s; final extension at 72°C for 15 min. Proper PCR amplification was checked by agarose gel electrophoresis and PCR products were cleaned up using the Gel/PCR DNA Fragment Extraction Kit (Avegene Life Sciences, Taipeh, Taiwan). Fragment analysis was performed by Macrogen (Seoul, Korea) using GeneScan 500 LIZ as internal size standard.

3.2.3. Statistical analysis

Genotypes were scored using GENEMARKER version 1.95 (SoftGenetics, State College, Pennsylvania, USA) and manually adjusted where necessary. Because *Z. carpinifolia* is actively reproducing by sprouting, identity analysis as implemented in the program CERVUS version 3.0.3 (Kalinowski *et al*., 2007) was performed to identify clones. Multilocus genotype diversity was estimated as a modification of the Simpson index (Pielou, 1969; Berg & Hamrick, 1994): $D_G = 1 - \{\Sigma[n_i(n_i - 1)]/[N(N - 1)]\}$, where n_i is the number of individuals of genotype i and N is the total number of individuals. The clone size was calculated as the ratio N_r/N_g and the frequency of clones per site as $1 - N_g/N_r$, where N_r is the total number of individuals sampled per site (ramets) and N_g is the number of different genotypes per site (genets) (McClintock & Waterway, 1993; Chung & Epperson, 2000). Genetic diversity parameters are usually measured with an exclusion of clonal individuals (Setsuko *et al*., 2004; Wei *et al*., 2013b). However, it was shown that clonal individuals do not affect the levels of genetic diversity significantly (Chung *et al*., 2005), but rather affect the spatial genetic structure of populations (Berg & Hamrick, 1994; Setsuko *et al*., 2004; Chung *et al*., 2005; Schueler *et al*., 2006). Except for the analysis of null alleles, all further analyses were performed using both the data set with all ramets and the data set with genets only to assess whether the measured parameters are affected by the inclusion of clonal individuals. Failed PCR amplifications that could be caused by the presence of null alleles or other technical issues were treated as missing data, and the percentage of missing data were calculated manually. However, null alleles can also occur at heterozygous loci and cause a homozygote excess. The program MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al*., 2004) was used to identify null alleles and to calculate null allele frequencies for all loci.

Genetic diversity was estimated in the program ARLEQUIN version 3.5 (Excoffier & Lischer, 2010) as allelic richness (A), observed heterozygosity (H_O) and expected heterozygosity (H_E) under the assumption of Hardy–Weinberg (HW) genotypic proportions. Polymorphism information content (PIC), commonly used in linkage analysis as a measure of

polymorphism for a marker locus (Botstein *et al*., 1980), was calculated in CERVUS. The occurrence of alleles private to a single site was assessed. To correct for differences in sample size between the geographical sites, we calculated allelic richness and private allelic richness using rarefied subsamples of 10 individuals (excluding ZE061_TUR with only 8 individuals) with HP-RARE version 1.1 (Kalinowski, 2005).

ARLEQUIN was used to assess deviations from HW equilibrium using a locus-by-locus exact test (1 000 000 Markov chain steps and 100 000 dememorization steps), to calculate Wright's fixation index F_{IS} (Weir & Cockerham, 1984) and to assess pairwise linkage disequilibrium (LD) between loci using a likelihood ratio test with 10 000 permutations to calculate the significance of the observed likelihood ratios.

An exact test of population differentiation based on genotype frequencies was performed with ARLEQUIN using 100 000 Markov chain steps and 10 000 dememorization steps. For visualizing the genetic structure of sampled sites, multidimensional scaling was performed using the cmdscale routine in the program R version 3.0.2 (R Core Team, 2013) with a matrix of pairwise R_{ST} distances between geographic sites calculated in ARLEQUIN.

Population genetic structure was investigated using the program STRUCTURE version 2.3.4 (Pritchard *et al*., 2000) with a model that allows mixed ancestry of individuals and assumes correlated allele frequencies within populations. Five independent runs were performed with the number of clusters (K) varying from 2 to 25, a burn-in period of 100 000 iterations and a data collection period of 1 000 000 iterations. Bayesian clustering is based on the assignment of individuals to K clusters (populations) by estimating the membership coefficients for each individual in each cluster and indicating the maximal number of clusters using the posterior probability of the data for a given K [Ln $P(D)$]. However, Evanno *et al*. (2005) showed that the maximum value of Ln $P(D)$ does not always indicate the "true" number of clusters, and instead ΔK, the rate of change in the log probability of data between successive K values, should be used. The program STRUCUTREHARVESTER web version 0.6.93 (Earl & vonHoldt, 2012) was used to analyze the clustering results, to calculate ΔK and to produce input files for the program CLUMPP version 1.1.2 (Jakobsson & Rosenberg, 2007), which permutes the results of different clustering runs and produces a single table with individual membership coefficients. The results were visualized using the program DISTRUCT version 1.1 (Rosenberg, 2004).

For partitioning the total genetic variation among groups, among sites within groups, and within sampling sites, analysis of molecular variance (AMOVA) across all loci was performed in ARLEQUIN using pairwise genetic distances defined as R_{ST} and 10 000

permutations to assess the significance of the variance components. For this purpose, the sampling sites were initially grouped together based on their geographical distribution into a Colchic group with samples from West Georgia, Turkey, and East Georgia (ZE054_GEO is situated outside the Colchis, but placed here due to its close proximity) and a Hyrcanian group with the samples from Azerbaijan and Iran. A second AMOVA was performed using only non-admixed populations as suggested by the STRUCTURE results. Pairwise genetic distances between sampling sites, estimated as R_{ST} (Slatkin, 1995) and F_{ST} (Wright, 1949), were obtained in ARLEQUIN, and significances were assessed using a permutation test with 10 000 permutations. The adjustment of *P* values for multiple comparisons were implemented in R (R Core Team, 2013) using the method controlling the false discovery rate (FDR; Benjamini & Hochberg, 1995). Correlation between R_{ST} and F_{ST} was assessed in R (R Core Team, 2013) using a Mantel test (Mantel, 1967) implemented in the "vegan" package (Legendre and Legendre, 1998). To evaluate the relationship between genetic and geographic distances, we performed a Mantel test in ARLEQUIN (significance was assessed using 100 000 permutations) using pairwise R_{ST} as genetic distances and pairwise geographic distances obtained using the GEOGRAPHIC DISTANCE MATRIX GENERATOR 1.2.3 (Ersts, 2013).

3.3. Results

3.3.1. Genetic diversity in *Zelkova carpinifolia*

Identical multilocus genotypes were found in 28 sampling sites and the multilocus genotypic diversity (D_G) ranged between 0 (all multilocus genotypes identical) for ZE061_TUR and 1 (no identical multilocus genotypes) for ZE072_AZ and ZE054_GEO (average value of D_G = 0.922; Table 3.1). Excluding ZE061_TUR, the lowest value of D_G (0.767) and the largest clone size (3.2) were observed for site ZE031_AZ. The individuals (ramets) with identical multilocus genotypes were considered to belong to the same genet. From the 495 ramets that were screened, 379 genets were detected. The frequency of clones varied from 0 (ZE072_AZ and ZE054_GEO) over 0.688 (ZE031_AZ) to 0.875 (ZE061_TUR). Disregarding clonal individuals, the highest estimated null allele frequencies (NAF) were detected for loci ZMS_5 (0.037), ZMS_7 (0.054), ZMS_4 (0.184), and ZMS_9 (0.235). Loci ZMS_4 and ZMS_9 were not amplified in, respectively, 1.6% and 15% of the individuals, whereas ZMS_5 and ZMS_7 were amplified in almost all individuals.

Table 3.1. Genetic diversity parameters calculated for 30 geographical sites of *Zelkova carpinifolia*.

No	Sampling site	Region	N_r	N_g	A	A_R	P_{AR}	$H_{O(r)}$	$H_{E(r)}$	$H_{O(g)}$	$H_{E(g)}$	$F_{IS(r)}$	$F_{IS(g)}$	D_G	N_r/N_g	$Freq_c$
1	ZE001_AZ	H	20	14	3.63	3.22	-	0.719	0.555	0.714	0.567	-0.455	-0.359	0.963	1.4	0.3
2	ZE007_AZ	H	20	12	3.88	3.54	-	0.769	0.567	0.75	0.58	-0.255	-0.175	0.947	1.7	0.4
3	ZE013_AZ	H	18	13	3.13	2.81	-	0.575	0.49	0.567	0.52	0.07	0.045	0.948	1.4	0.278
4	ZE017_AZ	H	20	16	3.38	3.16	-	0.56	0.534	0.558	0.531	0.012	-0.024	0.974	1.3	0.2
5	ZE019_AZ	H	20	18	4	3.49	0.02	0.469	0.474	0.479	0.487	0.027	0.028	0.984	1.1	0.1
6	ZE021_AZ	H	20	16	4.13	3.42	0.14	0.431	0.453	0.452	0.473	0.17	0.189	0.974	1.3	0.2
7	ZE027_AZ	H	20	14	3.38	2.89	-	0.493	0.446	0.486	0.452	-0.195	-0.227	0.953	1.4	0.3
8	ZE031_AZ	H	17	5	2.5	2.35	-	0.518	0.455	0.6	0.559	-0.257	-0.139	0.767	3.2	0.688
9	ZE034_AZ	H	18	14	3.75	3.42	0.05	0.569	0.527	0.545	0.514	0.03	0.033	0.967	1.3	0.222
10	ZE036_AZ	H	15	9	3.5	3.29	-	0.629	0.552	0.603	0.566	0.071	0.157	0.924	1.7	0.4
11	ZE043_AZ	H	18	16	3.25	2.94	0.03	0.556	0.493	0.563	0.508	-0.049	-0.033	0.987	1.1	0.111
12	ZE044_AZ	H	20	14	3.63	3.24	0.01	0.53	0.527	0.534	0.547	-0.03	-0.013	0.968	1.4	0.3
13	ZE051_AZ	H	10	8	3.63	3.63	-	0.6	0.56	0.589	0.574	-0.146	-0.068	0.956	1.3	0.2
14	ZE062_AZ	H	10	8	2.75	2.75	-	0.514	0.468	0.505	0.45	-0.257	-0.191	0.956	1.3	0.2
15	ZE067_AZ	H	20	17	3.75	3.29	0.02	0.619	0.559	0.61	0.562	-0.127	-0.034	0.979	1.2	0.15
16	ZE069_AZ	H	13	12	3.63	3.46	0.1	0.505	0.457	0.505	0.464	-0.122	-0.11	0.987	1.1	0.077
17	ZE072_AZ	H	13	13	3.75	3.59	0.12	0.602	0.604	0.602	0.604	-0.12	-0.12	1	-	-
18	ZE074_AZ	H	20	16	3.5	3.05	-	0.436	0.438	0.455	0.45	-0.036	-0.086	0.974	1.3	0.2
19	ZE075_AZ	H	20	18	3.5	3.1	0.01	0.524	0.518	0.512	0.525	-0.011	0.077	0.984	1.1	0.1
20	ZE053_IR	H	21	14	3.5	3.05	-	0.524	0.485	0.503	0.475	-0.14	0.055	0.942	1.3	0.25
21	ZE076_IR	H	12	10	3.75	3.57	-	0.413	0.518	0.41	0.542	-0.081	-0.111	0.955	1.2	0.167
22	ZE077_IR	H	13	7	3.5	3.33	0.06	0.549	0.476	0.551	0.502	0.135	0.182	0.872	1.9	0.462
23	ZE079_IR	H	11	5	3	2.94	-	0.511	0.417	0.525	0.467	0.198	0.386	0.818	2.2	0.545
24	ZE054_GEO	EG	20	20	3	2.78	-	0.478	0.425	0.478	0.425	-0.03	-0.03	1	-	-
25	ZE055_GEO	C	13	11	3.25	3.17	-	0.567	0.544	0.545	0.537	-0.023	-0.016	0.974	1.2	0.154
26	ZE057_GEO	C	18	14	3.5	3.18	-	0.59	0.506	0.583	0.538	-0.122	-0.074	0.947	1.4	0.3
27	ZE058_GEO	C	10	9	3.25	3.25	-	0.588	0.567	0.583	0.577	-0.277	-0.21	0.978	1.1	0.1
28	ZE059_GEO	C	20	18	3.75	3.3	0.06	0.459	0.494	0.47	0.505	0.055	0.087	0.989	1.1	0.1
29	ZE060_GEO	C	20	17	3.75	3.38	-	0.446	0.522	0.462	0.537	0.196	0.153	0.988	1.1	0.105
30	ZE061_TUR	C	8	1	1.25	-	-	-	-	-	-	-1	0	0	8	0.875

Notes: Region corresponds to Hyrcan (H), Colchis (C) or East Georgia (EG). N = number of individuals, A = allelic richness, A_R = rarefied allelic richness, P_{AR} = private allelic richness, H_O = observed heterozygosity, H_E = expected heterozygosity, F_{IS} = fixation index ($P > 0.05$, non-significant), D_G = multilocus genotypic diversity, N_r/N_g = clone size, $Freq_c$ = frequency of clones per site, r = ramets, g = genets. The mean values are presented.

Locus ZMS_2, which showed 5% of missing data, did not show any evidence for the presence of null alleles, indicating that missing data are not caused by the presence of null alleles alone. General homozygote excess caused by the presence of null alleles was observed in nine sites at locus ZMS_9 and in 13 sites at locus ZMS_4. Loci ZMS_4 and ZMS_9 were excluded from all subsequent analyses.

In total, 51 alleles were observed for eight microsatellite loci. The number of alleles per locus (*A*) ranged from three alleles at locus ZMS_3 to 10 alleles at loci ZMS_2 and ZMS_8 (overall mean = 6.38 alleles). The total number of di- and trinucleotide repeats (*R*) ranged between 2 and 11 repeats per locus with an average of 6.25 repeats across all loci. The highest gene diversity (H_E) and/or polymorphism information content (PIC) were observed for loci ZMS_2 (respectively, 0.693 and 0.726) and ZMS_8 (respectively, 0.677 and 0.731), and the lowest for locus ZMS_5 (respectively, 0.114 and 0.123). Average gene diversity across all loci was 0.460 (see Table 3.2).

Table 3.2. Characteristics of eight microsatellite loci for *Zelkova carpinifolia*.

Locus	*A* (total)	*A* (mean ± SD)	*R* (total)	*R* (mean ± SD)	H_E (total)	H_E (mean ± SD)	PIC
ZMS_1	7	4.03 ± 1.098	7	5.03 ± 1.450	0.684	0.606 ± 0.151	0.634
ZMS_2	10	5.50 ± 1.526	11	7.10 ± 2.578	0.752	0.693 ± 0.104	0.726
ZMS_3	3	2.07 ± 0.365	2	1.07 ± 0.365	0.477	0.425 ± 0.118	0.366
ZMS_5	5	1.53 ± 0.629	4	0.60 ± 0.770	0.127	0.114 ± 0.156	0.123
ZMS_7	6	3.77 ± 0.971	5	3.57 ± 0.971	0.660	0.568 ± 0.159	0.621
ZMS_8	10	5.03 ± 1.189	10	6.43 ± 0.935	0.765	0.677 ± 0.095	0.731
ZMS_12	4	2.60 ± 0.724	3	2.23 ± 0.935	0.258	0.247 ± 0.161	0.247
ZMS_13	6	2.70 ± 0.794	8	2.27 ± 1.617	0.397	0.352 ± 0.154	0.355
mean	6.38	3.40 ± 0.539	6.25	3.87 ± 0.596	0.515	0.460 ± 0.080	0.475

Notes: *A* = number of alleles, *R* = allelic range (difference between min and max number of repeats), H_E = expected heterozygosity, PIC = mean polymorphism information content.

Genetic diversity parameters per sampling site are shown in Table 3.1. The average number of alleles across loci per site (*A*) ranged between 1.25 (ZE061_TUR) and 4.13 (ZE021_AZ), and the rarefied allelic richness (A_R) per site (calculated excluding ZE061_TUR) between 2.35 (ZE031_AZ) and 3.63 (ZE051_AZ). Average private allelic richness was also low (0.01–0.14), and private alleles were detected in 11 sampled sites (for up to three loci). The mean observed heterozygosity (H_O) per site varied between 0.410 (ZE076_IR) and 0.750 (ZE007_AZ), whereas the mean expected heterozygosity (H_E) varied

between 0.425 (ZE054_GEO) and 0.604 (ZE072_AZ). ZE061_TUR was not considered due to its clonal structure. Twelve sampling sites significantly deviated from HWE for one to three loci. The mean fixation index F_{IS} over all sites was slightly negative (−0.092), with F_{IS} ranging between −0.455 (ZE001_AZ) and 0.198 (ZE079_IR), but deviations of F_{IS} from zero were not significant ($P > 0.05$). Among a total of 840 tests for pairwise linkage disequilibrium among the eight loci, only 55 were significant ($P < 0.05$). Significant linkage disequilibria were detected in 21 sites for at least one locus pair, with two pairs of loci (ZMS_2 and ZMS_8; ZMS_2 and ZMS_7) linked in five different sites. Therefore, we assumed no physical linkage between loci. As shown in Table 3.1, the inclusion of clonal individuals did not change the estimates of genetic diversity much, although it did affect locus by locus significance of HWE and LD (data not shown).

3.3.2. Genetic structure of *Zelkova carpinifolia* populations

Among 435 tests for pairwise differentiation among 30 geographical sites with clonal individuals, only 73 were non-significant ($P > 0.05$). However, all pairwise population (site) comparisons were non-significant when clonal individuals were excluded from the analysis. No significant difference was found between matrices of F_{ST} and R_{ST} (Mantel test, 1000 permutations, $r = 0.519$, $P < 0.05$) showing the independence of our results from the applied microsatellite mutation model. Among a total of 435 R_{ST} pairwise comparisons, 146 were not significantly different from zero when clonal individuals were included in the analysis, and 251 were not significantly different from zero when clones were excluded (see Table B3a in Appendix B). Including clonal individuals, the lowest significant pairwise R_{ST} was between ZE001_AZ and ZE043_AZ (0.027, $P < 0.05$), and the highest was between ZE027_AZ and ZE053_IR (0.410, $P < 0.05$). When clones were excluded, the lowest pairwise R_{ST} was observed between ZE043_AZ and ZE067_AZ (0.054, $P < 0.05$), and the highest was between ZE027_AZ and ZE053_IR (0.489, $P < 0.05$; Table B3b in Appendix B). In general, ZE027_AZ and ZE053_IR were not only more distant from each other, but also from all other sampled sites. It can also be seen in Fig. 3.2, which presents the results of a multidimensional scaling. When clones were removed, the R_{ST} distances for ZE067_AZ, ZE062_AZ and ZE079_IR were not significantly different from zero (see Fig. B1 in Appendix B).

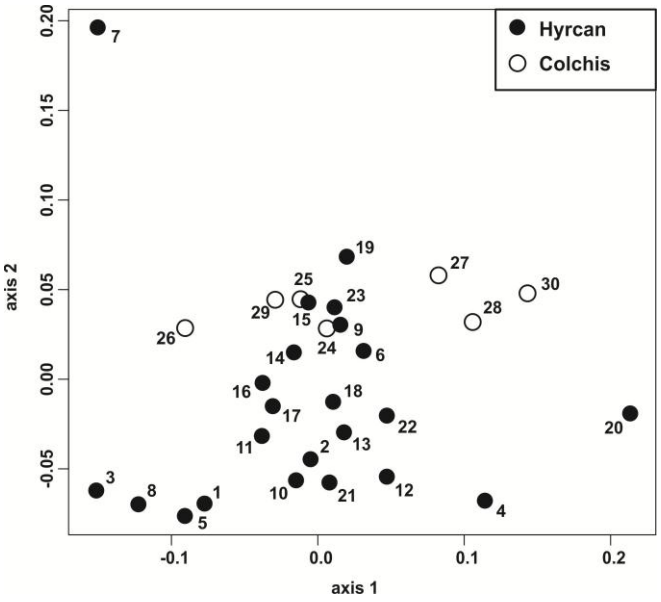

Fig. 3.2. Multidimensional scaling of 30 sites of *Zelkova carpinifolia* using pairwise R_{ST} as genetic distance (including clonal individuals). Circles represent sampling sites. Numbers correspond to the sites as shown in Table 3.1.

Additionally, Mantel test revealed no significant correlation between genetic and geographic distances in both datasets (r = 0.093, P > 0.05). Hierarchical AMOVA revealed low differentiation between the Colchic and Hyrcanian groups and among the sampled sites within the groups (respectively, 0.71% and 10.55%), with a non-significant fixation index for among group variation. The differences among individuals within sampling sites exhibited the major part of the total variation (88.74%; Table 3.3). Exclusion of the clonal individuals changed the results only slightly, preserving the same tendency for higher within-site differentiation.

Table 3.3. Results of hierarchical AMOVA for relict tree *Zelkova carpinifolia*.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation, %	Fixation indices
Among groups	1	111.424	0.11488	0.71	F_{CT}=0.00708
Among sites within groups	28	1979.74	1.71169	10.55	F_{SC}=0.10625*
Within sites	960	13822.982	14.39894	88.74	F_{ST}=0.11257*
Total	989	15914.145	16.2255		

Notes: *P < 0.05.

Analysis of the results of the Bayesian clustering as implemented in STRUCTURE revealed that the posterior probability of the data for a given K [Ln P(D)] increases with an increasing number of clusters (K) and that it reaches a maximum for K = 5. However, the optimal number of clusters suggested by the ΔK method corresponded to K = 3 (Fig. 3.3). A visual representation of individuals' membership coefficients also shows that three clusters capture the major structure in the data (Fig. 3.4). The STRUCTURE analysis separated Colchic

(blue cluster) and Hyrcanian (yellow cluster) groups with the third cluster (pink) inside the Hyrcanian group. Nevertheless, many sites are admixed with individuals sharing their memberships in two or all three clusters. The second AMOVA, using only non-admixed populations as suggested by the STRUCTURE results, showed 25.9% differentiation among sites ZE001_AZ, ZE027_AZ, and ZE058_GEO.

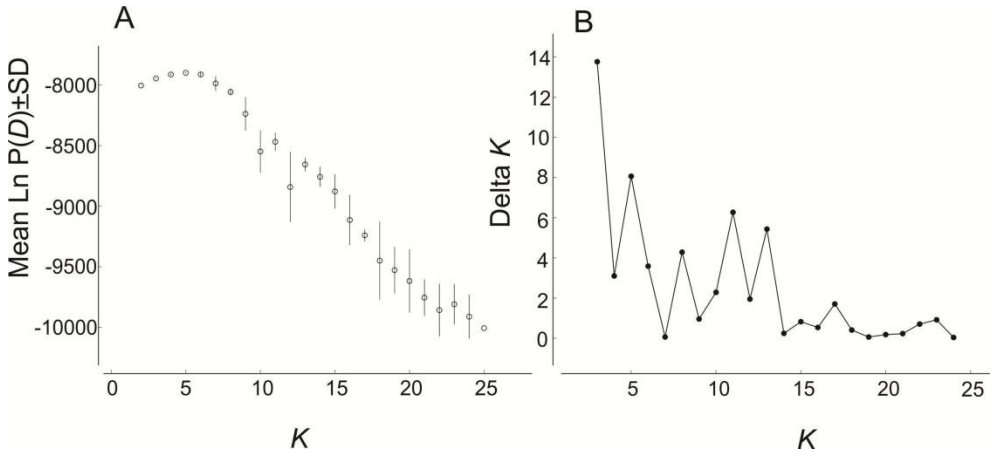

Fig. 3.3. Bayesian inference of the number of clusters (*K*) over five replicates for each *K*, using software STRUCTURE, based on 495 individuals of *Zelkova carpinifolia* collected from 30 geographical sites in the Caucasus. (A) Mean posterior probability of data for a given *K* [Ln *P*(D)]. (B) Rate of change in the log probability of data between successive *K* values (Δ*K*).

3.4. Discussion

3.4.1. Genetic diversity at nuclear microsatellite loci and frequency of clonal reproduction

The present study reports low to intermediate levels of genetic diversity in 30 sites sampled throughout the range of the relict tree *Zelkova carpinifolia* based on eight nuclear microsatellite loci. The lack of similar comprehensive studies of other trees from the Caucasus region prevents the recognition of general patterns of genetic diversity in its relict forests. Higher values of genetic diversity parameters (H_E=0.6–0.7, *A*=4–4.6) and high among population differentiation were shown for *Juglans regia* (Ibrahimov *et al*., 2010; Karimi *et al*., 2010), albeit that population sampling in both studies was biased toward low-distance scales. Usually long-lived woody species are likely to possess higher levels of genetic diversity than other life forms as they possess a higher proportion of polymorphic loci and more alleles per locus within their populations (Hamrick *et al*., 1992). However, similar levels

of genetic diversity at neutral loci (H_E = 0.48, A = 3.4) were described for *Ulmus leavis*, a close relative of *Zelkova*, from glacial refugia of the Iberian Peninsula (Spain) (Venturas *et al*., 2013).

Vegetative reproduction could delay the time among generations in disturbed environments and, therefore, buffer against the effect of fragmentation on the genetic diversity (Wei & Jiang, 2012). Root sprouting and stump shooting are also characteristic for *Zelkova* species (Gulisashvili, 1961; Nakagawa *et al*., 1998; Søndergaard & Egli, 2006), and almost all studied sites of *Z. carpinifolia* contained clonal individuals with the large clone size for ZE031_AZ severely affected by past logging and lopping. The site ZE061_TUR from Trabzon (Turkey) described previously as *Z. carpinifolia* subsp. *yomraensis* (Anşin & Gercek, 1991) seems to be established by only one individual, since all eight individuals represent the same multilocus genotype.

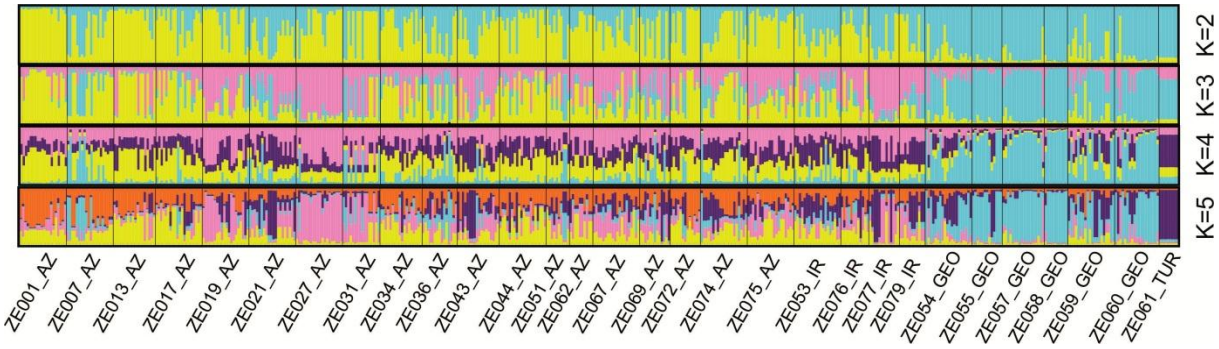

Fig. 3.4. Results of Bayesian clustering of 495 specimens of relict tree *Zelkova carpinifolia* for *K*=2-5, where the most probable number of clusters is *K*=3 with blue cluster representing Colchic group of populations and yellow and pink clusters representing the Hyrcanian group.

3.4.2. Gene flow between Hyrcanian and Colchic populations as a result of wind pollination

The current distribution of common shared relicts of Colchic and Hyrcanian forests such as *Zelkova carpinifolia* and *Pterocarya fraxinifolia* outside the climatic refugia could be a result of expansion processes during interglacials. However, the occurrence of their fossils in different parts of Georgia since the Miocene (Stuchlik & Kvavadze, 1998; Kvavadze & Connor, 2005) suggests that they also covered the region before the uplift of topographical barriers and formation of Colchic and Hyrcanian forests in the Upper Pliocene (Kolakovsky, 1961). Climatic oscillations during the Holocene and recent anthropogenic disturbance influenced the current distribution of *Z. carpinifolia* in the Caucasus. Isolation as well as

human-induced fragmentation is expected to cause a reduction of the gene flow among populations and an increase of inbreeding and random genetic drift in populations, resulting in strong genetic structure among regions and isolated populations (Young *et al*., 1996; Lowe *et al*., 2005). Such effects could also occur in *Zelkova* populations in the Caucasus. However, the obtained results indicate the same levels of genetic diversity, absence of inbreeding, and low differentiation between the two regions, with some private alleles detected for Hyrcan. Many temperate tree species are characterized by high diversity within populations and low differentiation among populations (Hamrick & Godt, 1996; Young *et al*., 1996; Sun *et al*., 2011; Lesser *et al*., 2013; Wei *et al*., 2013) due to the long lifespan, woody life form, outcrossing mating system, and wind pollination (Loveless & Hamrick, 1984; Heuertz *et al*., 2004). Wind pollination allows gene flow among populations that could override inbreeding and genetic drift and cause low among population differentiation and elimination of geographical structure (Lesser *et al*., 2013).

However, we found about 26% of variation among three non-admixed populations each representing one of the three clusters revealed by STRUCTURE analysis: ZE001_AZ of shrubs found at a high altitude (1205 m a.s.l.) near Zuvand highland (Lerik, Azerbaijan); ZE027_AZ, a small but dense tree stand surrounding the cemetery in Tengerud (Astara, Azerbaijan); and ZE058_GEO, a very small tree stand in the village of Rokiti (Baghdati, West Georgia) close to the Ajameti Nature Reserve. The observed admixture of the rest of the sampling sites could also be explained by pollen-mediated gene flow among populations within and between Colchis and Hyrcan. Even if *Z. carpinifolia* is a long-lived woody species and our sampling included overlapping generations of trees that could lead to an underestimation of the effects of the recent human-induced fragmentation as shown for black walnut (Victory *et al*., 2006), fragmentation caused by climatic changes and long-lasting isolation of the two regions would have been obvious from the detected patterns of genetic differentiation. Pollen of *Z. carpinifolia* was shown to be scarcely represented in sediments of Georgia (Stuchlik & Kvavadze, 1993), which was explained by the low yield of pollen production or poor pollen preservation in sediments. The limited pollen content (5–10%) was also described from fossil spectra of the Middle Miocene and Pliocene-Pleistocene from Georgia (Stuchlik & Kvavadze, 1993). Long-distance dispersal of pollen of *Z. carpinifolia* up to 100 km was shown by Kvavadze & Connor (2005). However, our findings indicate the possibility for pollen dispersal over larger distances (200 km or more). So far, no other cases of such long distance pollen dispersal were described in the literature (Petit & Hampe, 2006; Heslewood *et al*., 2014). However, we do not exclude that more stepping-stone populations of

Zelkova carpinifolia, which connected the regions in the past have already been eliminated due to climate change or anthropogenic influence, and so our results can also illuminate the past gene flow in a long-lived tree.

3.4.3. Incongruent patterns of diversification at nuclear and chloroplast loci

High haplotype diversity was detected in natural populations of *Z. carpinifolia* using chloroplast markers in the recent study of Christe *et al*. (2014). Fifteen haplotypes clustered in two groups, separating western Colchic from eastern Hyrcanian populations. The groups were separated by 19 mutations, and among population differentiation was significant, and constituted about 87% of the total variation. A discrepancy between chloroplast and nuclear markers was shown previously for other tree species (Pakkad *et al*., 2008; Sun *et al*., 2011) and likely corresponds to a difference in seed and pollen dispersal. Chloroplast markers tracking uniparental inheritance revealed significant genetic structuring and differentiation among populations, providing evidence for limited seed dispersal in this species. Fruiting twigs separating from the mother trees can only disperse over very short distances in *Zelkova* species (Hoshino, 1990). However, both pollen and seed production was shown to be irregular in *Zelkova* (Nakagawa *et al*., 1998; Søndergaard & Egli, 2006; Garfi *et al*., 2011). The intensity of fruiting in *Z. carpinifolia* differs from year to year and among individuals, and 30–80% of fruits are empty due to unfavorable environmental conditions during flowering (Gulisashvili, 1961). Nuclear markers are biparentally inherited and track both pollen and seed dispersal. The analysis of ITS variability in populations of *Z. carpinifolia* (Christe *et al*., 2014) did not reveal a clear differentiation between Hyrcan and Colchis regions, which could also be interpreted in the light of pollen mediated gene flow due to wind pollination. Moreover, the greater proportion of pollen flow over the seed flow was indicated in other studies of temperate and tropical tree species (Ennos, 1994; Petit & Hampe, 2006).

Since our results obtained at neutral loci differed from chloroplast data, it should also be taken into account that microsatellites are prone to size homoplasy (Estoup *et al*., 2002) and that evaluation of the genetic diversity could depend on their location in the genome (genic vs. nongenic) (DeFaveri *et al*., 2013), so they would not reflect genome-wide diversity (Vali *et al*., 2008).

3.4.4. Implications for conservation

Despite of *in situ* protection of *Z. carpinifolia* in Nature Reserves, National Parks and Protected areas in Georgia, Iran, and Azerbaijan, the species is still under the threat of human influence and ongoing climate change. It was also shown that very limited *ex situ* collections of this relict species are found in the countries of its origin (Kozlowski *et al*., 2012b). So, e*x situ* and *in situ* conservation planning is still a challenge. Furthermore, abandoned former plantations and clear-cuttings in Talysh lowlands (Azerbaijan) could be used for reforestation purposes (Scharnweber *et al*., 2007). In the planning of conservation strategies, both plastid and nuclear data should be used (Moritz, 1994), and priority should be given to the measure of allelic richness compared with allelic frequencies (Petit *et al*., 1998). Our findings based on nuclear multilocus genotypic data show low values of allelic richness in all studied geographical sites. We identified some populations with non-admixed ancestry based on the distribution of allele frequencies, all of them residing outside the protected areas. None of these populations contained any private alleles or were characterized by higher genetic diversity. Although some other sites exhibit low frequencies of private alleles, no individual site could be indicated as more valuable for conservation. However, they could be the areas close to the source (refugium) populations and could be considered for future investigations. The results suggest that spatially isolated populations are not isolated genetically as a result of gene flow, which prevents inbreeding in and differentiation among the populations; however, high gene flow could be hazardous in terms of outbreeding depression when fitness of the progeny is reduced (Ellstrand, 1992). The reductions in a seed set, production of empty seeds, and irregular flowering in *Zelkova* are usually attributed to unfavorable environmental conditions, but they could be argued to represent the consequences of outbreeding depression as a result of intraspecific gene flow. In conservation genetics, if populations experience outbreeding depression, then management should be directed to reduce the gene flow (Ellstrand, 1992). However, more investigations are required to support the evidence of long-distance gene flow by pollen and the effect of outbreeding depression in *Zelkova* to understand how conservation management should be planned.

CHAPTER 4

Phylogeography and population genetics of the riparian relict tree *Pterocarya fraxinifolia* (Juglandaceae) in the South Caucasus

4.1. Introduction

The present distribution of many plant taxa is, at least to a large extent, a consequence of the contraction and expansion processes that occurred under climatic fluctuations since the Paleogene (66–23.03 Ma) and especially during the Pleistocene (2.59–0.012 Ma), when many plant taxa survived glacial events in so-called "refugia" from which postglacial expansion was initiated (Hewitt, 1999; Hewitt, 2000; Hamrick, 2004; Provan & Bennett, 2008). The genus *Pterocarya* (Juglandaceae) was widely distributed in the Northern Hemisphere in the Paleocene (66–56 Ma; www.fossilworks.org), but, along with other temperate taxa as *Acer*, *Alnus*, *Fagus*, *Fraxinus*, *Platanus*, *Populus*, *Quercus*, *Ulmus*, and *Zelkova*, etc. retreated southwards into refuge areas as the climate became colder and drier (Chaney, 1947; Milne & Abbott, 2002). Currently, *Pterocarya* is represented by five species in East Asia (China and Japan) and one species, *Pterocarya fraxinifolia* (Poir.) Spach. (= *P. pterocarpa* (Michx.) Kunth.; Uotila, 2011), in West Asia.

The fossil record shows that *Pterocarya* was widespread in the Caucasus region in the Oligocene-Miocene (33.9–5.33 Ma; Shatilova *et al.*, 2011; Alizade *et al*., 2014), but now it has a more fragmented distribution. In the South Caucasus, *P. fraxinifolia* mainly occurs in Colchis, extended on the eastern coast of the Black Sea from the eastern Pontic Mountains in Turkey through Rioni lowland of Georgia enclosed by the western Lesser and Greater Caucasus Mountains up to Russia, and Hyrcan, extended on the southern coast of the Caspian Sea from the Lankaran lowland and Talysh Mountains in southeastern Azerbaijan and the Caspian lowland and the northern slopes of Alborz Mountains of Iran up to Turkmenistan, as well as on the southern slopes of the eastern Greater Caucasus mountain range and the Alazan-Agrichay (Alazani) valley (eastern Georgia, northwestern Azerbaijan; Gulisashvili, 1961; Denk *et al.*, 2001; Akhani *et al.*, 2010; Fig. 4.1). Additional populations can be found in northwestern Anatolia (Turkey), southern Turkey and the central Zagros Mts. in Iran (Browicz, 1989; Akhani & Salimian, 2003; Biltekin *et al.*, 2015; Fig. 4.1).

Based on evidence from the fossil record and palaeoclimatic modelling, the Colchis and Hyrcan areas are considered as climate refugia that were formed during the Pliocene (Kolakovsky, 1961) and that allowed the survival of forest taxa during the Last Glacial Maximum (LGM; Grossheim, 1926; Kolakovsky, 1961; Leroy & Arpe, 2007; Akhani *et al.*, 2010; Tarkhnishvili *et al.*, 2012). Although these two regions are often considered to belong to the Euxino-Hyrcanian province of a continuous Euro-Siberian floristic region (Zohary, 1973), Grossheim (1926) suggested that their floras became geographically isolated following the initial uplift and folding of the Lesser Caucasus during the Paleocene-Miocene and the subsequent uplift due to volcanic activity in the Pliocene-Quaternary (Sosson *et al*., 2010), and, as a consequence, evolved independently to a large extent.

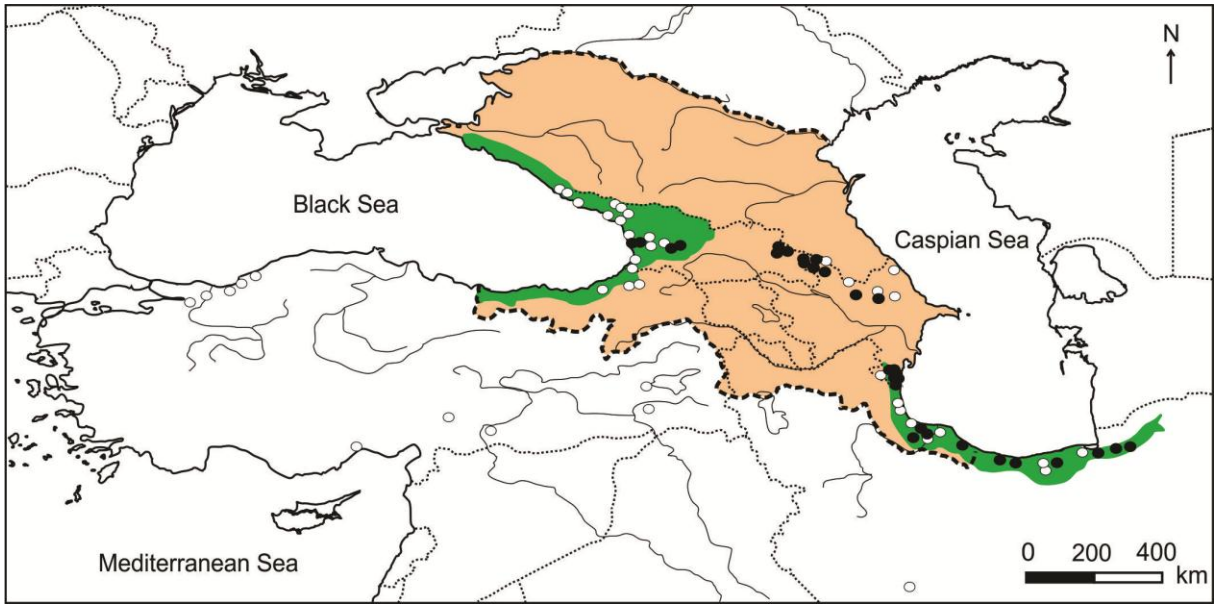

Fig. 4.1. Distribution of *Pterocarya fraxinifolia* in West Asia. White circles represent the distribution according to Browicz & Zielinski (1982) and Akhani & Salimian (2003); black circles represent sampling locations in South Caucasus and northern Iran. The Caucasus ecoregion as designated by the World Wildlife Fund (WWF) is enclosed by the dotted lines. Colchis and Hyrcan areas are shown in green.

The first tree species for which a detailed spatial analysis of genetic diversity across the Caucasus ecoregion, including the regions of Colchis and Hyrcan, was carried out is *Zelkova carpinifolia*. While the maternally inherited chloroplast markers (Christe *et al*., 2014) depicted clearly different haplotype patterns in Colchic and Hyrcanian forests, similar differences were not found using nuclear microsatellite data (Maharramova *et al*., 2015). This discrepancy between the two marker types was attributed to long-distance pollen dispersal in this wind-pollinated tree species and/or the past existence of now vanished stepping-stone

populations. The question arises if the spatial genetic structure of other tree species growing in mesophytic forests along the Black and Caspian Seas is similar to the one observed in *Zelkova carpinifolia*. If the current extension and diversity of the Colchic and Hyrcanian forest communities result from separation as a consequence of the uplift of the Lesser Caucasus (Sosson *et al*., 2010) and a range reduction due to aridification since the Miocene, phylogeographic patterns should be shared among key species of these forests. Also, paths of post-glacial recolonization and refugia of such mesophytic forest species in Western Asia (Tarkhnishvili *et al*., 2012) should be correlated to a considerable extent.

Pterocarya fraxinifolia, the Caucasian wingnut, is a thermophilous deciduous tree species with a lifespan of 200–250 years that grows on well-drained soils of lowland and ravine forests (up to 1,700 m a.s.l.), often along watercourses and in sympatry with other tree species such as *Acer* cp., *Alnus sp.*, *Carpinus* sp., *Quercus* sp., *Ulmus* sp., etc. Trees of *Pterocarya* can grow up to 30 m high and 1.5–2 m in trunk diameter, and hold economic value due to their very light wood that was used in small scale constructions, leaves that contain high amounts of vitamin C, antibiotic juglone and anesthetic agents (Gulisashvili, 1961; Gungor *et al.*, 2007), decorativeness and usage for the fortification of riverbanks. *P. fraxinifolia* used to be more commonly found in lowland areas, but has been cut for agricultural practice, re-channeling of watercourses, tourism development and industrialization purposes (Gulisashvili, 1961; Scharnweber *et al.*, 2007; Akhani *et al.*, 2010). Due to this decline, *P. fraxinifolia*, along with other relict and endemic taxa, is currently protected in National Parks, Protected Areas and State Reserves in Azerbaijan, Georgia and Iran. However, future changes towards drier climates might further endanger the species (Denk *et al.*, 2001) as agricultural alteration of natural landscapes can prevent tree migration (Hamrick, 2004), and low genetic diversity will not allow them to adapt to the changing environment (McLaughlin *et al.*, 2002).

The main aims of the study were: (i) to evaluate the genetic diversity and spatial genetic structure of natural populations of *P. fraxinifolia* across its distribution range (ii) to identify potential past climate refugia of *P. fraxinifolia* in the wider Caucasus and (iii) to discuss implications for conservation of genetic diversity.

4.2. Materials and Methods

4.2.1. Study species and sampling strategy

Pterocarya fraxinifolia is a synchronously monoecious species, with many-flowered pistillate aments at the apex of the flower-bearing twig and many-flowered staminate aments at the side axils of the scales spirally oriented on the same twig (Iljinskaya, 1953). It blooms between mid-April and mid-May and produces wind-dispersed pollen in abundance. The fruit is a winged nutlike drupe that measures with wings up to 4.5 cm wide and is dispersed by both wind and water. Besides sexually, *P. fraxinifolia* also reproduces vegetatively through root sprouting.

Leaf samples of *P. fraxinifolia* were collected from 40 geographical localities in a region that is known as a major biodiversity hotspot in West Asia (Myers *et al.*, 2000), covering the main species distribution area in the South Caucasus and extending to northern Iran: four localities from the Colchis region, ten from the southern slopes of the eastern Greater Caucasus and the adjacent valley (hereafter referred to as "Caucasian"), and 26 from the Hyrcan region (Fig. 4.1, see Table C1 in Appendix C).

Within each sampling location, leaves were collected from trees that were at least 20 m apart and stored in silica gel (see Table 4.1 & 4.2 for sample sizes per location). Except for the Iranian sampling sites, one voucher specimen was collected per site and deposited in the herbarium of the Botanical Garden and Botanical Museum Berlin-Dahlem (BGBM, Germany). Additional samples representing the East Asian species were collected in the arboretum of BGBM: one specimen of *P. tonkinensis* (Franch.) Dode, two of *P. macroptera* var. *insignis* (Rehder & E.H. Wilson) W.E. Manning and two of *P. rhoifolia* Siebold & Zucc. (see Table C2 in Appendix C).

4.2.2. DNA extraction and microsatellite genotyping

Total genomic DNA was extracted from silica-gel dried leaves using the NucleoSpin Plant II kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. Twenty-eight oligonucleotide primer pairs that were developed for the polymerase chain reaction (PCR) amplification of nuclear microsatellites in other members of the Juglandaceae (see Table C3 in Appendix C) were tested for proper amplification of intended target loci in *P. fraxinifolia*. The eight PCR primer pairs that successfully amplified polymorphic

microsatellite loci in *P. fraxinifolia* (see Table C4 in Appendix C) were used for the genotyping of 557 specimens. Only 30 sampling locations with ten or more individuals were used (see Table 4.1).

PCR amplifications of microsatellite loci were carried out in reaction volumes of 15-μL containing 20–40 ng genomic DNA, 0.2 μM each of the forward and reverse oligonucleotide primers, 1 × PCR buffer S (PeqLab, Erlangen, Germany), 1.5 mM $MgCl_2$, 250 μM of each dNTP, 3 mg BSA and 0.45 U Hot *Taq* DNA polymerase (PeqLab, Erlangen, Germany). Either the forward or the reverse PCR primer of each primer pair was labelled with a fluorescent dye (see Table C4 in Appendix C). The PCR temperature profile was as follows: 96°C for 2 min, followed by 35 cycles of 95°C for 30 sec, locus-specific annealing temperature (Table C4 in Appendix C) for 30 sec and 72°C for 45 sec, and final extension at 72°C for 30 min. To avoid non-specific PCR amplification, locus WGA32 was amplified using the following "touchdown" temperature profile: 96°C for 2 min, followed by 10 cycles of 95°C for 30 sec, 60-55°C for 30 sec (every cycle, annealing temperature was reduced by 0.5°C) and 72°C for 45 sec, followed by 25 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 45 sec, and final extension at 72°C for 30 min. PCR products were purified using the Gel/PCR DNA Fragment Extraction kit (Avegene Life Sciences, Taipeh, Taiwan) according to the manufacturer's instructions. Fragment analysis was performed by Macrogen Inc. (Seoul, Korea) using GeneScan 500 LIZ as internal size standard.

4.2.3. Chloroplast DNA sequencing

Universal oligonucleotide primers (see Table C5 in Appendix C) were used for the amplification of the following chloroplast DNA (cpDNA) regions: *trn*S–*trn*G intergenic spacer (IGS) and *trn*G intron, *rpl*32–*trn*L IGS and *trn*L–*trn*F IGS and *trn*L intron. PCR amplifications were carried out for 120 specimens of *P. fraxinifolia* from 40 geographic locations (three per site; see Table 4.3) and five specimens of East Asian species. Reaction volumes of 25-μL contained 20–40 ng genomic DNA, 0.4 μM each of the forward and reverse oligonucleotide primers, 1 × PCR buffer S (PeqLab, Erlangen, Germany), 1.5 mM $MgCl_2$, 250 μM of each dNTP, 5 mg BSA and 0.75 U Hot *Taq* DNA polymerase (PeqLab, Erlangen, Germany). The *trn*S–*trn*G–*trn*G locus was PCR amplified using “protocol 1” from Shaw *et al.* (2005). PCR amplification of *rpl*32–*trn*L and *trn*L–*trn*L–*trn*F used the following temperature profile: 96°C for 2 min, followed by 35 cycles of 95°C for 1 min, 59°C (*rpl*32–*trn*L) or 61°C (*trn*L–*trn*L–*trn*F) for 1 min and 72°C for 2 min, and final extension at 72°C for

15 min. PCR products were purified using the Gel/PCR DNA Fragment Extraction kit (Avegene Life Science, Taipei, Taiwan) and sequenced at Macrogen Inc. (Seoul, Korea) using standard Sanger sequencing.

4.2.4. Statistical analysis

4.2.4.1. Nuclear microsatellites

Microsatellite alleles and genotypes were scored using GENEMARKER 1.95 (SoftGenetics LLC, Pennsylvania, USA). Clonal individuals were detected as identical multilocus genotypes using an identity analysis implemented in CERVUS 3.0.3 (Kalinowski *et al.*, 2007) and multilocus genotype diversity was estimated using a modified Simpson index (Pielou, 1969; Berg & Hamrick, 1994): $D_G = 1 - \{\Sigma[n_i(n_i - 1)]/[N(N - 1)]\}$, where n_i is the number of individuals of multilocus genotype i and N is the total number of individuals. The frequency of clonal individuals per site was calculated as $1 - Ng/Nr$, where Nr is the total number of individuals sampled per site (ramets) and Ng is the number of different genotypes per site (genets) (McClintock & Waterway, 1993; Chung & Epperson, 2000). All subsequent analyses were restricted to genets.

Allelic richnesses (A), private allelic richnesses (P_{AR}), observed heterozygosities (H_O) and expected heterozygosities (H_E) under the assumption of Hardy–Weinberg equilibrium (HWE) were calculated per locus and per sampling location using ARLEQUIN 3.5 (Excoffier & Lischer, 2010). MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.*, 2004) was used to identify null alleles, and CERVUS to calculate null allele frequency (NAF) and the polymorphism information contents (PIC) of the different marker loci (Botstein *et al.*, 1980). Allelic and private allelic richnesses were corrected for sample size differences using HP-RARE 1.1 (Kalinowski, 2005) with rarefied subsamples of six individuals.

ARLEQUIN was used to assess deviations from HWE using a locus-by-locus exact test (1,000,000 Markov chain steps and 100,000 dememorization steps), to calculate Wright's fixation index F_{IS} (Weir & Cockerham, 1984) and to assess pairwise linkage disequilibria (LD) between microsatellite loci using a likelihood ratio test with 10,000 permutations to calculate the significance of the observed likelihood ratios. P–values of the tests were adjusted for multiple comparisons using the method controlling the false discovery rate (FDR; Benjamini & Hochberg, 1995) implemented in R 3.0.2 (R Core Team, 2013). If a significant heterozygote deficiency was detected after correction for multiple comparisons, the

relationship between F_{IS} and F_{ST}, calculated using ARLEQUIN, was evaluated at individual loci using Pearson's product–moment correlation test in R. A significantly positive correlation, with the intercept of the regression line close to zero, would indicate a "Wahlund effect" due to population admixture (Waples, 2015).

An exact test of population differentiation based on genotype frequencies (100,000 Markov chain steps and 10,000 dememorization steps) was performed in ARLEQUIN and pairwise genetic distances between sampling sites were estimated as R_{ST} (Slatkin, 1995) and F_{ST} (Wright, 1951) (10,000 permutations for significance assessment). Correlation between R_{ST} and F_{ST} was evaluated in R using the Mantel test (Legendre & Legendre, 1998) implemented in the package "vegan". Multidimensional scaling of the sampling sites was performed using the pairwise genetic distances and the "cmdscale" routine in R. Pairwise geographic distances between sampling locations were calculated using the GEOGRAPHIC DISTANCE MATRIX GENERATOR 1.2.3 (Ersts, 2013) and the occurrence of isolation-by-distance (IBD) was evaluated by performing a Mantel test in ARLEQUIN with 100,000 permutations in order to assess the significance. The relationships between the genetic diversity estimates (A_R, P_{AR} and H_E) and different geographical parameters (latitude, longitude and elevation) were evaluated by calculating Pearson's product–moment correlations in R.

Population genetic structure was evaluated using non-spatial and spatially explicit Bayesian clustering models, respectively implemented in the programs STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) and TESS 2.3.1 (Chen *et al.*, 2007). Ten independent runs of STRUCTURE were performed with the number of clusters (K) varying from two to 25, a burn-in period of 100,000 iterations and data collection during 1,000,000 iterations. The STRUCTURE model assumed correlated allele frequencies within populations and allowed mixed ancestry of individuals. Although STRUCTURE predicts the "true" number of clusters using the posterior probability of the data for a given K [Ln P (D)], Evanno *et al.* (2005) showed that ΔK, the rate of change in the log probability of the data between successive K values, is a better indicator for the number of clusters. ΔK was calculated using web version 0.6.94 of STRUCTUREHARVESTER (Earl & vonHoldt, 2012). TESS was run using an admixture model with a maximum number of clusters (K_{max}) ranging from two to ten, 100 independent runs for each value of K_{max}, a burn-in period of 30,000 iterations and data collection during 100,000 iterations. Deviance Information Criterion (DIC) values were averaged across all runs for each value of K_{max} and averages were plotted against K_{max}. The "true" number of clusters was selected as the smallest value of K_{max} for which the DIC value reaches a plateau. Outputs of

all STRUCTURE runs and the TESS runs corresponding to the 10% lowest DIC values were permuted using CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007) and visualized using DISTRUCT 1.1 (Rosenberg, 2004).

Analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was performed across all loci in ARLEQUIN using R_{ST} genetic distances, 10,000 permutations to assess the significance of the covariance components and a hierarchical grouping of individual samples according to the results of the Bayesian clustering that considered two regions, one grouping all Colchic–Caucasian sampling locations and the other grouping all Hyrcanian sampling locations. Separate analyses were performed for the individual regions. In addition, the within-population genetic diversities (A_R, P_{AR} and H_E) of these two regions were compared using a two-sample *t*-test in R.

4.2.4.2. Chloroplast DNA

Obtained cpDNA sequences were edited and manually aligned using PHYDE-1 0.9971 (Müller *et al.*, 2010). Aligned sequences of the different cpDNA regions were concatenated in one final alignment. Eight variable mononucleotide repeats and one mononucleotide repeat with an internal substitution were removed from the alignment due to uncertain homology of nucleotide positions. In the final alignment, all insertions–deletions (indels) were considered as single events and coded as single positions. A 1 bp indel within a 7 bp indel in the *trn*S-*trn*G IGS, found in one specimen of *P. fraxinifolia*, was removed from the final alignment but taken into account during haplotype network reconstruction by manually adding a single mutational step. DNA sequences were deposited into GenBank under accession numbers KT455828–KT456202.

General evaluation of cpDNA sequences and haplotype inference were performed in DNASP 5 (Librado & Rozas, 2009), and NETWORK 4.6.1.3 (Bandelt *et al.*, 1999) was used to reconstruct two chloroplast haplotype networks, one with and one without mononucleotide repeats, using the median joining algorithm. Unnecessary links and median vectors were deleted from the network (Polzin & Daneshmand, 2003). Transversions, transitions and indels were given the same weights.

ARLEQUIN was used to calculate haplotype (*h*) and nucleotide (π; Nei, 1987) diversities, the latter both unadjusted and adjusted for multiple substitutions per site using the JC correction (Jukes & Cantor, 1969). Tajima's *D* (Tajima, 1989) and Fu's F_S (Fu, 1997) neutrality tests, with 16,000 simulations to assess the significance, were performed in order to

test whether observed mutations are selectively neutral and/or population size has been constant. An exact test of population differentiation, with 100,000 Markov chains and 10,000 dememorization steps, and hierarchical AMOVA among Colchic–Caucasian and Hyrcanian populations, with 10,000 permutations, were also performed in ARLEQUIN. Finally, occurrence of IBD was evaluated using a Mantel test as implemented in ARLEQUIN with 100,000 permutations to assess the significance.

4.3. Results

4.3.1. Genetic diversity at nuclear microsatellites

Screening a total of 557 specimens for diversity at eight nuclear microsatellite loci from 30 geographical locations across the distribution range of *P. fraxinifolia* in the Caucasus region and Iran revealed a total of 97 different alleles. The total number of alleles per locus (*A*) ranged from 4 (CYC148) to 23 (CYC130) with an average value of 12.125 (SD = 6.289; see Table C4 in Appendix C). Four hundred and thirty-nine different multilocus genotypes were identified. Identical genotypes were detected in 25 locations and the multilocus genotype diversity (D_G) ranged between 0.804 (PT32_IR) and 1 (PT17_AZ, PT31_IR, PT33_IR, PT55_GEO and PT57_GEO) with a mean value of 0.958 (SD = 0.047). Accordingly, the frequency of clonal individuals ranged from 0 (PT17_AZ, PT31_IR, PT33_IR, PT55_GEO and PT57_GEO) to 0.56 in PT32_IR (see Table 4.1) with a mean value of 0.207 (SD = 0.170). The PIC ranged between 0.193 (CYC148) and 0.812 (CYC019) with an average of 0.608 (SD = 0.217), whereas the total gene diversity (H_E) ranged from 0.212 (CYC148) to 0.834 (CYC019) with an average of 0.639 (SD = 0.216; see Table C4 in Appendix C).

The mean allelic richness per sampling location (*A*) ranged from 3.1 for PT32_IR to 7.1 for PT33_IR with an average of 4.7 (SD = 0.98). After correcting for unequal sample sizes, the rarefied allelic richness (A_R) ranged between 2.8 ± 1.1 alleles for PT23_GEO and 4.9 ± 2.0 alleles for PT33_IR with an average of 3.7 (SD = 0.5), whereas the average private allelic richness (P_{AR}) across all sampling locations was equal to 0.07 (SD = 0.08) and relatively high values (P_{AR} > 0.12) were observed for PT18_AZ, PT03_AZ, PT29_IR, PT25_IR, PT33_IR, PT24_IR and PT30_IR. Across loci, the observed heterozygosity (H_O) varied from 0.380 for PT27_IR to 0.719 for PT18_AZ with an average of 0.541 (SD = 0.067) and the expected heterozygosity (H_E) varied from 0.482 for PT23_GEO to 0.710 for PT33_IR with an average of 0.590 (SD = 0.059; see Table 4.1). Significantly higher genetic diversities

($P < 0.05$) were observed in Hyrcanian populations (mean $A_R = 3.9$, SD = 0.5; mean $P_{AR} = 0.1$, SD = 0.08 and mean $H_E = 0.632$, SD = 0.042) compared to Colchic–Caucasian populations (mean $A_R = 3.3$, SD = 0.3; mean $P_{AR} = 0.02$, SD = 0.02 and mean $H_E = 0.579$, SD = 0.063). No correlation was found between the genetic diversity of populations and elevation ($P > 0.05$), but correlations with latitude and longitude were significant ($P < 0.05$): correlations were negative with latitude ($r = -0.6$, -0.7 and -0.5 for respectively A_R, P_{AR} and H_E) and positive with longitude ($r = 0.6$, 0.7 and 0.4 for respectively A_R, P_{AR} and H_E), indicating increase in diversity from north to south and from west to east (see Fig. C1 in Appendix C).

Table 4.1. Genetic diversity of *Pterocarya fraxinifolia* from South Caucasus at eight nuclear microsatellite loci.

No	Site	Region	*N*r	*N*g	D_G	$Freq_C$	*A*	A_R	P_{AR}	H_O	H_E	F_{IS}
1	PT23_GEO	Colchis	20	18	0.989	0.1	3.6	2.8	0.03	0.502	0.482	-0.057
2	PT59_GEO	Colchis	20	11	0.937	0.45	3.9	3.4	0	*0.402*	*0.528*	0.233
3	PT58_GEO	Colchis	10	7	0.911	0.3	3.5	3.4	0	*0.518*	*0.622*	0.179
4	PT57_GEO	Colchis	11	11	1	0	4	3.4	0	0.58	0.568	-0.021
5	PT55_GEO	Caucasus	20	20	1	0	3.8	3	0.01	0.488	0.489	0.003
6	PT56_GEO	Caucasus	20	15	0.968	0.25	4.8	4	0	0.571	0.63	0.088
7	PT54_GEO	Caucasus	20	14	0.958	0.3	4.3	3.5	0.05	0.534	0.587	0.086
8	PT52_GEO	Caucasus	15	13	0.981	0.133	4.1	3.3	0.06	0.529	0.569	0.073
9	PT50_AZ	Caucasus	20	19	0.995	0.05	3.9	3	0.01	0.529	0.507	-0.067
10	PT49_AZ	Caucasus	20	18	0.984	0.1	4.4	3.3	0.04	0.444	0.52	0.109
11	PT48_AZ	Caucasus	20	11	0.905	0.45	4.3	3.5	0.01	0.608	0.561	-0.127
12	PT47_AZ	Caucasus	19	10	0.871	0.474	3.4	3.2	0	0.575	0.539	-0.071
13	PT22_AZ	Caucasus	20	14	0.953	0.3	3.9	3.2	0	0.563	0.534	-0.056
14	PT39_AZ	Hyrcan	19	18	0.994	0.053	5.5	4.1	0.07	0.589	0.625	0.051
15	PT17_AZ	Hyrcan	15	15	1	0	5.5	4	0.04	0.567	0.622	0.082
16	PT04_AZ	Hyrcan	20	14	0.889	0.3	4.5	3.5	0.01	0.549	0.593	0.051
17	PT01_AZ	Hyrcan	20	15	0.958	0.25	4.3	3.5	0.01	0.556	0.61	0.064
18	PT21_AZ	Hyrcan	20	19	0.995	0.05	5	3.8	0.03	0.539	0.618	0.114
19	PT18_AZ	Hyrcan	18	12	0.922	0.333	5.5	4.4	0.12	0.719	0.697	-0.088
20	PT02_AZ	Hyrcan	20	19	0.995	0.05	5.9	4	0.08	0.648	0.622	-0.061
21	PT03_AZ	Hyrcan	20	16	0.974	0.2	5.6	4	0.12	0.617	0.655	0.061
22	PT27_IR	Hyrcan	20	9	0.895	0.55	3.4	3.1	0.05	*0.38*	*0.576*	0.324
23	PT29_IR	Hyrcan	20	16	0.968	0.2	5.6	4.1	0.17	0.533	0.623	0.106
24	PT28_IR	Hyrcan	19	15	0.971	0.211	5.1	3.9	0.08	*0.535*	*0.578*	0.009
25	PT25_IR	Hyrcan	19	15	0.977	0.211	5.8	4.5	0.22	*0.532*	*0.679*	0.216
26	PT32_IR	Hyrcan	18	8	0.804	0.556	3.1	2.9	0.01	0.495	0.524	0.034
27	PT30_IR	Hyrcan	20	15	0.968	0.25	6	4.4	0.22	0.578	0.66	0.077
28	PT31_IR	Hyrcan	18	18	1	0	5.6	3.9	0.07	0.529	0.591	0.065
29	PT33_IR	Hyrcan	17	17	1	0	7.1	4.9	0.28	*0.547*	*0.71*	0.198
30	PT24_IR	Hyrcan	19	17	0.988	0.105	5.4	4.1	0.18	0.476	0.596	0.176
	Mean		557	439	0.958	0.207	4.7	3.68	0.07	0.541	0.59	0.062
	SD				0.047	0.17	0.98	0.52	0.08	0.067	0.059	0.106

Notes: N_r = number of individuals sampled from each site (ramets), N_g = number of individuals with different multilocus genotypes (gamets), D_G = multilocus genotypic diversity, $Freq_c$ = frequency of clonal individuals, *A* = number of alleles (allelic richness), A_R = allelic richness rarefied to a subsample of 6 individuals, P_{AR} = private allelic richness, H_O = observed heterozygosity, H_E = expected heterozygosity, F_{IS} = inbreeding coefficient. For sites significantly deviating from HWP ($P < 0.05$) H_O and H_E values are given in *Italics*. F_{IS} was not significant after correction for false discovery rate ($P > 0.05$) for all populations except PT25_IR and PT33_IR ($P = 0.048$; in *Italics*).

Both CERVUS and MICRO-CHECKER reported the occurrence of null alleles at five loci: CYC130, CYC019, WGA27 and WGA321 exhibited null alleles with moderate frequencies between 0.05 and 0.2, whereas WGA32 exhibited null alleles with a high frequency larger than 0.2. Deviation from HWE occurred at one to four loci in 26 sampling locations after correcting for multiple hypothesis testing. WGA32 and WGA321 deviated significantly from HWE with a heterozygote deficiency in respectively 12 and 8 locations, whereas CYC095 deviated significantly from HWE with a heterozygote excess in 16 locations. Except for these three loci, deviation from HWE of individual loci was usually restricted to only a few sampling locations. Across loci, Wright's fixation index (F_{IS}) ranged from −0.127 for PT48_AZ to 0.324 for PT27_IR with an average of 0.062 (SD = 0.106; see Table 4.1) and, after correcting for multiple hypothesis testing, a significant deviation from zero was found for PT25_IR and PT33_IR. No significant correlations were found between F_{IS} and F_{ST} at individual loci. Among a total of 840 pairwise comparisons for linkage disequilibrium between loci within sampling locations, only 17 indicated significant disequilibrium after adjustment for multiple hypothesis testing.

Due to the high frequency of null alleles or deviation from HWE in a high number of sampling locations, WGA32, WGA321 and CYC095 were excluded from all subsequent analyses.

4.3.2. Genetic structure at nuclear microsatellites

Multidimensional scaling using pairwise estimates of F_{ST} and R_{ST} (see Fig. C2a,b in Appendix C) indicated a differentiation of Colchic–Caucasian and Hyrcanian populations. Based on the posterior probability of the data given K and the ΔK method (see Fig. C3a,b in Appendix C), STRUCTURE predicted the maximum number of clusters to be equal to five. Nevertheless, two large admixed clusters, one comprised of the Colchic–Caucasian samples (predominantly yellow cluster) and the other of the Hyrcanian samples (predominantly blue cluster), can be recognized from the graphical representation of the STRUCTURE results (Fig. C3d in Appendix C, K = 2). The spatially explicit analysis of TESS, in which the DIC value more-or-less started to plateau for K = 4 (Fig. C3c,e in Appendix C), allowed similar observations to be made in addition to a clearer differentiation of the eastern Hyrcanian populations (Fig. 4.2). Hierarchical AMOVA indicated 6.7% of variation between Colchic–Caucasian and Hyrcanian groups, 5.09% variation among sites within these groups and 88.2%

of variation within sampling sites. Separate AMOVAs for the Colchic–Caucasian and Hyrcanian sampling locations resulted in similar estimates of the variance components (see Table 4.2).

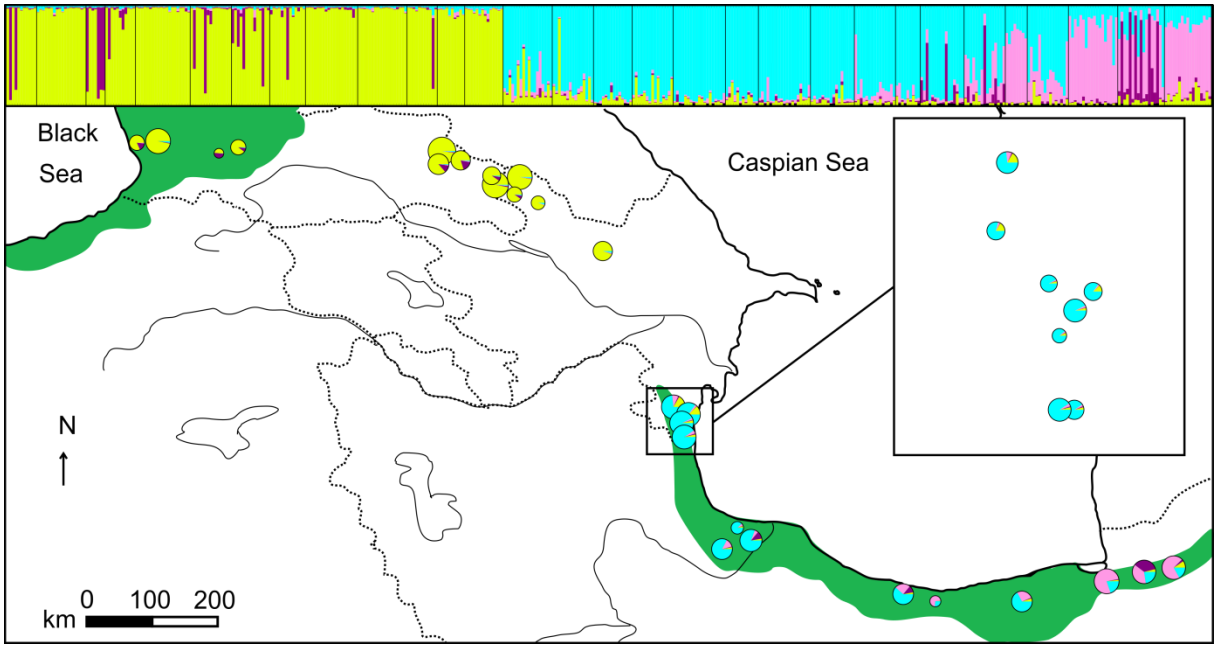

Fig. 4.2. Bayesian clustering results for K = 4 obtained from TESS 2.3.1 and geographical distribution of inferred clusters of the relict tree *Pterocarya fraxinifolia* in South Caucasus and northern Iran. The predominantly yellow cluster comprises locations from Colchis and the eastern Greater Caucasus, the predominantly blue and pink clusters – from Hyrcan. Circles represent sampling locations; circle sizes correspond to the numbers of individuals in each location; circle colours correspond to the colours of the clusters.

Table 4.2. Results of hierarchical AMOVA for *Pterocarya fraxinifolia* populations using five nuclear microsatellite loci.

	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation, %	Fixation indices
Total	Among groups	1	45.313	0.0981	6.71	F_{CT} = 0.06711*
	Among sites within groups	28	96.909	0.0745	5.09	F_{SC} = 0.05460*
	Within sites	848	1093.4	1.2894	88.2	F_{ST} = 0.11804*
	Total	877	1235.6	1.462	100	
CC	Among sites	12	38.358	0.0744	6.14	F_{ST} = 0.06144*
	Within sites	349	396.79	1.1369	93.86	
	Total	361	435.14	1.2113		
H	Among sites	16	58.551	0.0748	5.08	F_{ST} = 0.05082*
	Within sites	499	696.63	1.396	94.92	
	Total	515	755.18	1.4708		

Notes: CC – Colchic-Caucasian and H – Hyrcanian groups of sampling locations (populations). *P < 0.05

Exact tests of population differentiation revealed that PT27_IR is significantly different from nine other sites, namely PT59_GEO, PT23_GEO, PT58_GEO, PT50_AZ, PT48_AZ, PT04_AZ, PT29_IR, PT32_IR and PT24_IR. Pairwise F_{ST} and R_{ST} values were significantly correlated (r = 0.513, P < 0.05), and respectively 365 and 219 comparisons, among a total of 435 F_{ST} and R_{ST} pairwise comparisons, were significantly different from zero (see Table C6 in Appendix C). The lowest significant pairwise R_{ST} value was equal to 0.035 (between PT39_AZ and PT50_AZ) and the highest value was equal to 0.575 (between PT58_GEO and PT01_AZ). A significant correlation between geographic and genetic distances (r = 0.4, P < 0.05) among Hyrcanian sampling locations was revealed using the Mantel test, indicating a pattern of isolation-by-distance.

4.3.3. Genetic diversity at chloroplast loci

The total length of an initial alignment concatenating cpDNA sequence alignments of *rpl*32–*trn*L (845 bp), *trn*L–*trn*L–*trn*F (942 bp) and *trn*S–*trn*G–*trn*G (1,495 bp) was equal to 3,282 bp. After excluding variable mononucleotide repeats and shortening of indels to single nucleotide positions, the total length of the concatenated alignments was reduced to 3,160 bp. The alignment contained six indels and 17 variable sites of which only six were parsimony-informative (three in *rpl*32–*trn*L, two in *trn*S–*trn*G–*trn*G and one nucleotide site in *trn*L–*trn*L–*trn*F region). The total length of the final alignment, including DNA sequences from other *Pterocarya* species, was 3,162 bp and included two additional indels and eight more variable sites. Both neutrality tests suggested that the observed mutations in the cpDNA sequences are selectively neutral (see Table 4.3).

In total, 12 different haplotypes were detected for *P. fraxinifolia* and three for the remaining *Pterocarya* species (Fig. 4.3). The haplotype diversity (h) per sampling location ranged from zero in locations where only a single haplotype was found (32 locations) over 0.667 in locations with two haplotypes (seven locations) to one in PT26_IR with three haplotypes, with an average of 0.142 (SD = 0.291; Table 4.3). The nucleotide diversity (π) per sampling location varied from zero (32 locations) over 0.667 (PT56_GEO and PT17_GEO) and 1.333 (PT20_AZ, PT19_AZ, PT18_AZ, PT27_IR and PT25_IR) to eight (PT26_AZ). However, π changed to zero in PT56_GEO and PT17_GEO, to 0.667 in PT20_AZ, PT19_AZ, PT18_AZ, PT27_IR and PT25_IR, and to 6.677 in PT26_AZ after applying the JC correction for multiple substitutions per site (Table 4.3). Only two haplotypes (H1 and H5) were detected among the Colchic–Caucasian specimens versus 11 among the Hyrcanian specimens.

Table 4.3. Genetic diversity of *Pterocarya fraxinifolia* from South Caucasus at five chloroplast DNA regions.

No	Site	N_i	Haplotypes	h	π	π (JC)	Fu's F_S	Fu's F_S P-value
1	PT23_GEO	3	H1$^{(3)}$	0	0	0	0	—
2	PT59_GEO	3	H1$^{(3)}$	0	0	0	0	—
3	PT58_GEO	3	H1$^{(3)}$	0	0	0	0	—
4	PT57_GEO	3	H1$^{(3)}$	0	0	0	0	—
5	PT55_GEO	3	H1$^{(3)}$	0	0	0	0	—
6	PT56_GEO	3	H1$^{(2)}$, H5$^{(1)}$	0.667	0.667	0	0.201	0.392
7	PT54_GEO	3	H1$^{(3)}$	0	0	0	0	—
8	PT52_GEO	3	H1$^{(3)}$	0	0	0	0	—
9	PT50_AZ	3	H1$^{(3)}$	0	0	0	0	—
10	PT49_AZ	3	H1$^{(3)}$	0	0	0	0	—
11	PT48_AZ	3	H1$^{(3)}$	0	0	0	0	—
12	PT47_AZ	3	H1$^{(3)}$	0	0	0	0	—
13	PT22_AZ	3	H1$^{(3)}$	0	0	0	0	—
14	PT44_AZ	3	H1$^{(3)}$	0	0	0	0	—
15	PT39_AZ	3	H1$^{(3)}$	0	0	0	0	—
16	PT15_AZ	3	H1$^{(3)}$	0	0	0	0	—
17	PT17_AZ	3	H1$^{(1)}$, H6$^{(2)}$	0.667	0.667	0	0.201	0.386
18	PT41_AZ	3	H1$^{(3)}$	0	0	0	0	—
19	PT04_AZ	3	H1$^{(3)}$	0	0	0	0	—
20	PT01_AZ	3	H1$^{(3)}$	0	0	0	0	—
21	PT21_AZ	3	H1$^{(3)}$	0	0	0	0	—
22	PT20_AZ	3	H1$^{(2)}$, H7$^{(1)}$	0.667	1.333	0.667	1.061	0.592
23	PT19_AZ	3	H1$^{(2)}$, H7$^{(1)}$	0.667	1.333	0.667	1.061	0.591
24	PT18_AZ	3	H1$^{(1)}$, H7$^{(2)}$	0.667	1.333	0.667	1.061	0.597
25	PT10_AZ	3	H7$^{(3)}$	0	0	0	0	—
26	PT38_AZ	3	H7$^{(3)}$	0	0	0	0	—
27	PT35_AZ	3	H7$^{(3)}$	0	0	0	0	—
28	PT02_AZ	3	H7$^{(3)}$	0	0	0	0	—
29	PT03_AZ	3	H7$^{(3)}$	0	0	0	0	—
30	PT09_AZ	3	H7$^{(3)}$	0	0	0	0	—
31	PT27_IR	3	H8$^{(1)}$, H9$^{(2)}$	0.667	1.333	1.333	1.061	0.593
32	PT29_IR	3	H9$^{(3)}$	0	0	0	0	—
33	PT28_IR	3	H9$^{(3)}$	0	0	0	0	—
34	PT26_IR	3	H10$^{(1)}$, H11$^{(1)}$, H12$^{(1)}$	1	8	6.677	0.901	0.429
35	PT25_IR	3	H9$^{(2)}$, H13$^{(1)}$	0.667	1.333	0.667	1.061	0.6
36	PT32_IR	3	H14$^{(3)}$	0	0	0	0	—
37	PT30_IR	3	H15$^{(3)}$	0	0	0	0	—
38	PT31_IR	3	H15$^{(3)}$	0	0	0	0	—
39	PT33_IR	3	H15$^{(3)}$	0	0	0	0	—
40	PT24_IR	3	H15$^{(3)}$	0	0	0	0	—
	Mean	120	12 haplotypes	0.142	0.4	0.267	0.165	—
	SD			0.291	1.314	1.078	0.373	—

Notes: N_i = number of individuals used for cpDNA sequencing, Haplotypes– different haplotypes with number of individuals indicated in superscript, h = haplotype diversity, π = nucleotide diversity, π (JC) = nucleotide diversity corrected using the Jukes and Cantor nucleotide substitution model. Fu's F_S test results and P-values are given, Tajima's D statistic was zero for all populations.

The most abundant haplotypes were: H1 (67 specimens), which occurred in all Colchic–Caucasian populations, some Hyrcanian populations, in the East Asian species *P. tonkinensis* and in one specimen of *P. macroptera* var. *insignis*; H7 (22 specimens), which was found only in western Hyrcan; H15 (12 specimens) in eastern Hyrcan; and H9 (10 specimens) in central Hyrcan. All other haplotypes occurred in only one to three specimens. Unique haplotypes were H5 (PT56_GEO) and H6 (PT17_AZ), which are differentiated from H1 through a single indel, H8 (PT27_IR) and H13 (PT25_IR), both differentiated from H9 through two mutational events, H14 (PT32_IR), differentiated from H1 through one mutational event, and H10, H11 and H12 (PT26_IR), which are quite different from one another. The median joining network suggested the existence of two median vectors as unobserved haplotypes (mv1 and mv2, grey circles, Fig. 4.3) through which the Hyrcanian haplotype H7 is connected with other Hyrcanian (H8-H14) and East Asian (H2-H4) haplotypes. Inclusion of mononucleotide repeats resolved four more haplotypes: two haplotypes derived from H1; one haplotype derived from H7; and one haplotype derived from H15.

Table 4.4. Results of hierarchical AMOVA for *Pterocarya fraxinifolia* populations using five chloroplast loci.

	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation, %	Fixation indices
Total	Among groups	1	34.482	0.57393	32.69	F_{CT} = 0.32693*
	Among sites within groups	38	119.5	0.9816	55.91	F_{SC} = 0.83074*
	Within sites	80	16	0.2	11.39	F_{ST} = 0.88607*
	Total	119	169.98	1.75553		
CC	Among sites	13	0.31	0	0	F_{ST} = 0.00000
	Within sites	28	0.667	0.02381	100	
	Total	41	0.976	0.02381		
H	Among sites	25	119.19	1.49094	83.49	F_{ST} = 0.83488*
	Within sites	52	15.333	0.29487	16.51	
	Total	77	134.53	1.78581		

Notes: CC – Colchic-Caucasian and H – Hyrcanian groups of sampling locations (populations). *$P < 0.05$

AMOVA indicated 32.7% of the total genetic differentiation to be due to differences between the groups of Colchic–Caucasian and Hyrcanian sampling locations and only 11.4% of the total variation to be due to differences among specimens within locations. Separate AMOVAs for the Colchic–Caucasian and Hyrcanian sampling locations revealed the absence of differentiation among Colchic–Caucasian sampling locations (Table 4.4). A Mantel test showed the existence of significant IBD among the Hyrcanian sampling locations ($r = 0.5$, $P < 0.05$). An exact test of population differentiation did not reveal any significant difference between pairs of populations.

4.4. Discussion

4.4.1. Genetic diversity and gene flow in the wind-pollinated relict tree

In this study, the genetic diversity and spatial genetic structure of natural populations of the relict tree *P. fraxinifolia* were assessed across its main distribution range in the South Caucasus and northern Iran, including the Colchic and Hyrcanian glacial refuge areas, using nuclear microsatellite and chloroplast DNA markers. Overall, low-to-intermediate levels of genetic diversity were detected in *P. fraxinifolia* using both types of markers. A general trend of lower genetic diversities from the southeast towards the northwest of the sampling area was obvious, with significantly higher levels of genetic diversity in sampling locations from Hyrcan compared to locations from Colchis and the eastern Greater Caucasus. High numbers of chloroplast haplotypes and private alleles at nuclear microsatellites were observed in Hyrcan, where past extinction of many haplotypes is suggested in the chloroplast haplotype network that lacks many mutational steps not represented among the existing Hyrcanian haplotypes. A low level of overall genetic diversity and high number of chloroplast haplotypes were also observed in *Zelkova carpinifolia*, another relict tree species that grows in the area (Maharramova *et al.*, 2015; Christe *et al.*, 2014). However, different chloroplast haplotypes were found in *Z. carpinifolia* sampled from Colchis and Hyrcan, with 19 mutational events separating the haplotypes observed in these two regions according to the chloroplast haplotype network (Christe *et al.*, 2014). *Zelkova* fruits are small drupes that disperse together with the fruit-bearing twigs over only short distances (Hoshino, 1990) and these short dispersion distances may explain the geographical differentiation of chloroplast haplotypes in this species. Fruits of *P. fraxinifolia* are bigger in size, but are adapted for dispersal by wind and water over larger distances. The wide distribution range of haplotype H1 in Colchis, the eastern Greater Caucasus and western Hyrcan may be explained by a past more humid climate, whereas the geographical haplotype differentiation in Hyrcan may be due to the preservation of different haplotypes in several microrefugia.

The higher genetic diversity within sampling locations and lower diversity among sampling locations observed at nuclear microsatellites in *P. fraxinifolia* reflects tendencies towards outcrossing and wind-pollination of many tree species (Hamrick & Godt, 1996; Heuertz *et al.*, 2004) as gene flow by pollen among populations is more restricted than within populations. Contrary to *Z. carpinifolia*, Colchic-Caucasian and Hyrcanian populations of *P. fraxinifolia* are significantly differentiated and this is likely due to restricted gene flow

between these two regions. Although pollen grains of *P. fraxinifolia* tend to be smaller in equatorial diameter (27-34 μm; Iljinskaya, 1953) than those of *Z. carpinifolia* (27-45 μm; Kvavadze & Connor, 2005), the different habitats of the two species may explain the differential wind-dispersal of the pollen grains. *Z. carpinifolia* has a wider ecological amplitude than *P. fraxinifolia* and extends to drier Mediterranean-type temperate local climates, such as those in eastern Colchis (Denk *et al.*, 2001), or xerophytic woodlands, as in the Zuvand highland and the Lesser Caucasus (Gulisashvili, 1961), where pollen grains have a bigger potential to be wind-dispersed over larger distances. The pollen grains of riparian *P. fraxinifolia*, however, are mainly deposited locally due to the humid environmental conditions (Stuchlik & Kvavadze, 1998). The additional differentiation observed in eastern Hyrcan may be due to forest density or other, unknown, barriers that may restrict wind dispersal of pollen grains within this area.

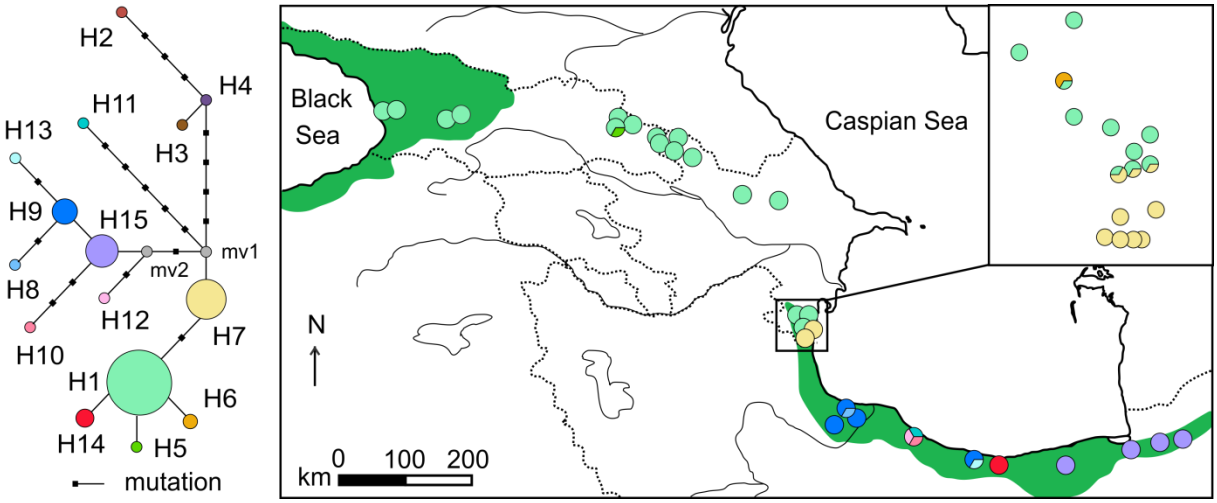

Fig. 4.3. Chloroplast haplotype network reconstructed in NETWORK 4.6.1.3 and geographical distribution of haplotypes of *Pterocarya fraxinifolia* in South Caucasus and northern Iran. Haplotypes H1 and H5–H15 represent *P. fraxinifolia* haplotypes and haplotypes H2, H3 and H4 represent haplotypes of East Asian species of *Pterocarya*. Circle size in network corresponds to haplotype frequency. Haplotypes mv1 and mv2 represent unobserved haplotypes suggested by NETWORK. Lines connecting haplotypes correspond to the number of mutational steps or characters separating haplotypes. Inset shows enlarged area of southeastern Azerbaijan (Talysh, western Hyrcan), where sampling was dense. Circles on the map represent sampling locations; circle sizes are equal (three individuals per location); circle colours correspond to the haplotype colours in the network.

Although neighbouring trees were not sampled, significant fractions of clonal individuals and thus levels of asexual reproduction, known to occur in *P. fraxinifolia* through root sprouting (Gulisashvili, 1961), were revealed in some sampling locations. Considering the low germination rate of seeds produced by *P. fraxinifolia* (Avşar, 2002) and the necessity of an extended cold period to overcome seed dormancy (Cicek & Tilki, 2008), vegetative

reproduction may have been an important means of survival of those *P. fraxinifolia* populations (Venturas *et al.*, 2013).

4.4.2. Past climate refugia for *Pterocarya fraxinifolia*

Whereas *Pterocarya* was widespread in Europe until the Middle and Lower Pleistocene (Guiter *et al.*, 2008; Koutsodendris *et al.*, 2010; Corrado & Magri, 2011, etc.), it persisted in the glacial refugia of West Asia throughout the Quaternary Period (Tarkhnishvili *et al.*, 2012; Biltekin *et al.*, 2015). The existence of Colchic and Hyrcanian climate refugia in the Caucasus was mainly inferred by analyses of the composition of the extant flora (Grossheim, 1926; Kolakovsky, 1961; Zohary, 1973) and by paleoclimatic modelling which confirmed the existence of suitable climates in Colchis and Hyrcan to allow the survival of Caucasian forest tree species during the LGM (Tarkhnishvili *et al.*, 2012). In addition, direct evidence through macrofossils and/or pollen exists that *Pterocarya* occurred in wider riparian communities in the Caucasus during the Pleistocene (Ramezani *et al.*, 2008; Joannin *et al*., 2010; Shatilova *et al.*, 2011; Tagieva *et al*., 2013). However its distribution in the region was affected by climate cooling and aridity during glaciations and climate warming during interglacials and post-glacial, even if the glaciers that existed in the Caucasus never extended to the foothils and lowlands (Milanovsky, 2000).

Assuming that glacial refugia for *P. fraxinifolia* existed in both Colchis and Hyrcan and considering the abovementioned genetic structure of *P. fraxinifolia* populations, postglacial recolonization of the neighbouring regions likely occurred independently from the Colchic and Hyrcanian refuge areas. If cpDNA diversification occurred before the LGM (Gavin *et al.*, 2014), the cpDNA haplotype H1 may have been the only haplotype that persisted in Colchis during the LGM and postglacial expansion of *P. fraxinifolia* from Colchis towards the southern slopes of the eastern Greater Caucasus may have resulted in H1 currently being the dominant haplotype in this region. Generally, populations in refugia or close to refugia are supposed to harbor higher levels of genetic diversity, higher numbers of private alleles but lower levels of spatial structure compared to more distant populations (Lafontaine et al., 2013; Gavin *et al.*, 2014). Therefore, it is also possible that several microrefugia for *P. fraxinifolia* existed in Hyrcan during climate fluctuations as inferred from the observed high number of private alleles and chloroplast haplotypes from which the less diverse area in the north was recolonized. However, more extensive sampling in Colchis is required to confirm the absence of more diverse populations.

4.4.3. Implications for conservation

In the face of global warming, one of the predictions is that the most southern climate refugia will lose large fractions of their diversity as many species will only be able to survive at more northern latitudes (Gavin *et al.*, 2014). Some of the northern *P. fraxinifolia* populations containing chloroplast haplotype H1 (Colchis, Greater Caucasus) are under protection in the Kolkheti National Park (NP) and Lagodekhi Nature Reserve in Georgia and Hirkan (Hyrcan) NP in Azerbaijan. Hyrcanian populations with high numbers of private alleles and chloroplast haplotype H7 are also protected in Hirkan NP. However, populations from Iran that possess more chloroplast haplotypes and private alleles are located outside the protected areas. In order to counter possible losses of genetic diversity due to global warming and/or other human impacts, we propose that in particular Hyrcanian populations, containing higher amounts of genetic diversity, deserve *ex situ* and *in situ* conservation.

CHAPTER 5

General discussion and conclusions

In this study, the genetic diversity and population structure of natural populations of the relict forest trees *Zelkova carpinifolia* and *Pterocarya fraxinifolia* in the South Caucasus were evaluated. The South Caucasus is an important part of the Caucasus ecoregion that has a high biodiversity and is remarkable for its mesophytic relict forests. Studying the population genetics and phylogeography of these relict trees will contribute to their management and to general conservation policies for the Caucasus ecoregion.

Z. carpinifolia and *P. fraxinifolia* are the only extant tree species of their relict genera in the western inland of Eurasia that mainly grow in the mesophytic deciduous broad-leaved forests of the Euxino-Hyrcanian floristic province. Although they may form pure stands, they usually grow in the lowland and foothill mixed forests, on riversides and in ravine forests in low and middle mountain zones. *P. fraxinifolia* grows on a variety of well-drained and moist soils along watercourses and in floodplains, and air humidity is important for its normal development. *Z. carpinifolia* also grows on moist, but not buggy, soils. However, *Z. carpinifolia* also grows under drier conditions, even on the dry stony soils in the Lesser Caucasus and the Zuvand highland in the Talysh Mountains where it develops a shrubby form. The mesophytic forests with these and other relict trees in the lowlands were largely cleared in the past due to the creation of plantations for moist-loving agricultural plants, the construction of roads, industrial development, etc. Current occurrences of *P. fraxinifolia* are patchy, but large forests are still found at the foothills of the Greater Caucasus. *Z. carpinifolia* was also threatened by selective logging and bovine grazing. Despite the national protection of these relict trees, they still suffer from human influences outside the protection areas.

Through an international collaboration, an extensive and representative sampling of populations of *Z. carpinifolia* and *P. fraxinifolia* was carried out between 2010 and 2013 across their main distribution ranges in the South Caucasus and northern Iran, including populations in forests and forest patches as well as tree stands in villages.

The development of next-generation sequencing technologies provided an opportunity to obtain large amounts of DNA sequence data that can be used to understand the phylogenetic relationships within and among different taxa, to study their population genetics and to identify the units important for conservation. Using 454 pyrosequencing a large

number of short sequences of nuclear DNA was obtained for *Z. carpinifolia*, many of which contained short tandem repeats. As a result of primer design and testing, 13 polymorphic nuclear microsatellite markers were developed. New microsatellite markers were not developed for *P. fraxinifolia*. Instead, the markers previously developed for closely related species such as *Juglans nigra* and *Cyclocarya paliurus* were tested for proper PCR amplification in *P. fraxinifolia* and selected based on their exhibited level of polymorphism.

5.1. Genetic diversity in *Zelkova carpinifolia* and *Pterocarya fraxinifolia*

In the present study, low-to-intermediate levels of genetic diversity were revealed at nuclear microsatellite loci in 30 natural populations of *Z. carpinifolia*. The mean number of alleles and the heterozygosity (gene diversity) respectively varied between 2.4-3.6 and 0.425-0.604, and a low number of private alleles was found. Low-to-intermediate levels of genetic diversity at nuclear microsatellites were also detected for *P. fraxinifolia*, in which the number of alleles ranged between 2.8-4.9 and the heterozygosity varied between 0.482-0.711 per population. However, a high number of private alleles was detected in many *P. fraxinifolia* populations, especially those from the Hyrcanian forest. The average number of alleles and heterozygosity per population was significantly higher in *P. fraxinifolia* (t-test, mean A_R = 3.2 and 3.7, mean H_E = 0.50 and 0.59 in respectively *Z. carpinifolia* and *P. fraxinifolia*, $P < 0.05$). In populations of many other temperate trees, the estimated average heterozygosity and the allelic richness were shown to have respective ranges of 0.60-0.80 and 4-7.5 (Bai *et al*., 2010; Mattioni *et al*., 2013; de Lafontaine *et al*., 2013) or even higher (Temunovic *et al*., 2013; Callahan *et al*., 2013). It is, however, impossible to retrieve information about the pre-Quaternary and Pleistocene levels of genetic diversity in these taxa in order to draw conclusions about a possible loss of genetic variation during the Miocene when these trees initially migrated to the area or when they were retreating to refugia during the ice ages. Nevertheless, the absence of inbreeding and deviation from Hardy-Weinberg equilibrium indicate that the current fragmentation of forests may not yet have affected standing levels of genetic diversity, likely due to the long lifespan of the trees and high levels of gene flow. On the other hand, current levels of genetic diversity in the relict tree species could explain the inability of these trees to adapt to the changing environmental pressures and extinction from Europe and North America when the climate became cooler and drier. Their survival was only possible along the Black and Caspian seacoasts where the environment was not affected much by global climate change. The genetic diversity in refugia is usually higher than in the areas

that were colonized from refugia, however when recolonization routes from multiple refugia intersect, the level of genetic diversity rises due to the mixture of different lineages and may even exceed that of the respective refugia (Petit *et al*., 2003). A significantly higher level of genetic diversity at nuclear microsatellite loci is observed in Hyrcan compared to Colchis for *P. fraxinifolia*, supporting the existence of refugia in the region. Such a pattern was, however, not observed for *Z. carpinifolia*. Vegetative reproduction, which is favored in both species, might also be an adaptation of relict trees to prevent fast diversity loss through unsuccessful sexual reproduction and seed establishment, and to conserve existing levels of diversity until the environment is suitable for sexual reproduction. An extreme case of clonal reproduction was revealed in Turkey, where one geographical population in Trabzon, previously described as *Z. carpinifolia* subs. *yomraensis*, was shown to represent a single genetic individual with eight shrubby clones.

5.2. Effects of gene flow on the genetic diversity in *Zelkova carpinifolia* and *Pterocarya fraxinifolia*

High within-population and low among-population differentiation was found in *Z. carpinifolia* and *P. fraxinifolia* at the nuclear microsatellites, which was expected considering the tendency to outcrossing and wind-pollination. Wind-pollination is characteristic for many temperate tree species and allows them to prevent any loss of genetic diversity in populations through continuous gene migration among populations. However, the spatial genetic structure between tree species, even between closely related ones, may differ substantially (Heuertz *et al*., 2004; Hu *et al*., 2008; Temunovic *et al*., 2013). Indications of extensive gene flow by pollen (over 200 km) were found in *Z. carpinifolia*, even if flowering in *Zelkova* species is known to be irregular and pollen production or preservation is low (Stuchlik & Kvavadze, 1993). Despite the Bayesian clustering of populations of *Z. carpinifolia* into two groups, one group including samples from Colchis and eastern Georgia, and the other group with populations from Hyrcan, differentiation of the groups was not significant due to extensive gene flow. Interestingly, a similar structure was found in *P. fraxinifolia*: two differentiated groups of populations with one group containing samples from Colchis and the Greater Caucasus, and the other group with populations from Hyrcan. However, in this case the differentiation between the two groups was significant and constituted about 7% of the total variation. It seems therefore that gene flow by pollen is more restricted across larger geographical distances in *P. fraxinifolia* than in *Z. carpinifolia*, despite the abundant

production of pollen and smaller or equal size of pollen grains in *P. fraxinifolia*. A possible explanation is that air humidity plays a significant role in the ability of pollen to disperse: pollen is dispersed over longer distances in drier environments, such as *Z. carpinifolia* habitats, and over shorter distances in humid air, as in *P. fraxinifolia* habitats. So, the degree of gene flow by pollen is affected by the ecological adaptations of plant species and wind-pollination alone, being an important factor shaping the genetic diversity, is not sufficient to predict the patterns of gene flow. The further substructuring of populations of both species in Hyrcan indicates the possible existence of two gene pools in this region. Also, no patterns of isolation-by-distance were detected among the sampled populations of *Z. carpinifolia*, once again indicating extensive gene flow, whereas significant isolation-by-distance was detected in *P. fraxinifolia* in Hyrcan, where gene flow by pollen is restricted among populations.

Z. carpinifolia was shown to possess a high number of chloroplast DNA haplotypes, even if the average nucleotide diversity was low (15 haplotypes found in 16 populations), and differentiation among the Colchic and Hyrcanian populations based on cpDNA sequences was high (87%; Christe *et al*., 2014). Such contrasting patterns of nuclear and chloroplast differentiation possibly result from a difference in seed and pollen-dispersal in *Z. carpinifolia*. Seed dispersal might be limited to small distances in *Z. carpinifolia*, resulting in strong population structure and phylogeographical patterns at maternally inherited cpDNA markers, but extensive pollen dispersal over larger distances might eliminate this pattern at biparentally inherited nuclear markers. Twelve chloroplast haplotypes were also detected in *P. fraxinifolia*, but across 40 geographical localities, and the average nucleotide diversity was also low. In addition, the differentiation between Colchic and Hyrcan population groups was significant (33%) so that both pollen and seed-dispersal between the regions of Colchis and Hyrcan seem to be restricted in *P. fraxinifolia*. Surprisingly, however, all geographic populations at the northern edge of the distribution range in Colchis and the Greater Caucasus, except for one population with two haplotypes, possessed the single cpDNA haplotype H1, whereas Hyrcanian populations contained 11 haplotypes, including H1. The distribution of a single haplotype among populations from western and eastern Colchis and the Greater Caucasus may indicate that seed dispersal is not restricted and fruits dispersed by wind and watercourses travel large distances. However, such a spread of haplotypes was not observed in Hyrcan. Haplotype H1 may thus have arrived to the area through recolonization combined with a founder event before forests were largely fragmented by human influences.

5.3. The past climate refugia and the history of the Caucasian mesophytic relict forests: future perspectives

As it was described in the introduction, the existence of climate refugia for mesophytic trees in Colchis and Hyrcan is suggested by their modern floral composition, fossil data and species distribution modelling. Populations in refugia are usually genetically more diverse and structured. In addition, it was also shown that areas where recolonizing individuals from refugial populations meet and interbreed may exhibit even higher levels of diversity and stronger population structures. In *Z. carpinifolia*, diverse chloroplast haplotypes suggest ancient intraspecific diversification between the Colchis and Hyrcan regions. However, nuclear markers do not hint at such differentiation and high levels of gene flow may have played a role in the elimination of genetic structure among populations. An increase in pollen-dispersal in forest trees might also be the result of fragmentation, when a barrier for gene flow such as forest density is reduced. In *P. fraxinifolia*, both nuclear and chloroplast data indicate a higher genetic diversity in Hyrcanian forests than in Colchis and the Greater Caucasus. The high number of private alleles and chloroplast haplotypes in Hyrcan populations, serving as evidence for restricted gene flow by both pollen and seeds within the region, show that multiple refugia may have existed in Hyrcan. It is, however, difficult to judge if a refugium for *P. fraxinifolia* may have existed in Colchis from the obtained results. If such a refugium did exist, then haplotype H1 was the only haplotype preserved during the adverse climate conditions and spread to the east through postglacial recolonization events. It is known that when several refugia serve as sources for recolonization, areas where recolonizing individuals or populations meet and mix may have higher levels of genetic diversity than in the actual refugia. So, it is necessary to mention that extant isolated populations from the southern edge of the distribution range (in southeastern Turkey and the Zagros Mountains in Iran) were not included in the study and that their future analysis may shed more light upon the species phylogeography.

Additionally, investigations of the genetic diversity and population structure of other temperate tree and shrub species should be carried out in the future to confirm the existence of past climate refugia in the region. Both shared and endemic species of potential refugia that also occur outside the refugia are of interest, e.g. *Acer* sp., *Albizia julibrissin*, *Alnus glutinosa subsp. barbata*, *Alnus subcordata*, *Betula medwediewii*, *Diospyros lotus*, *Gleditsia caspia*, *Tilia* sp., *Ulmus leavis*, etc. The analysis of the population genetics of species with different reproductive systems, e.g. insect-pollinated *Acer* sp., *Albizia julibrissin*, *Gleditsia caspia*,

Tilia sp., etc., should be carried out to compare how the genetic structure is affected by different reproductive adaptations. Some studies have already reported the unique haplotypes of *Acer campestre* in Colchis (Grimm & Denk, 2014) and *Castanea sativa* in eastern Turkey (Mattioni *et al*., 2013), suggesting the existence of potential refugia, and the genetic diversity of *Gleditsia caspia* was studied in Hyrcan (Azerbaijan) (Schnabel & Krutovskii, 2004) but limited population sampling was carried out. Species distribution modelling based on the distribution of *Abies nordmanniana, Carpinus betulus, Castanea sativa, Fagus orientalis* and *Picea orientalis* and also some animal species (Tarkhnishvili *et al*., 2012) suggested the existence of refugia in the area, but no analyses of genetic diversity of these species in the region was carried out. Species distribution modelling could also be carried out for *Z. carpinifolia* and *P. fraxinifolia*.

5.4. Concluding remarks on conservation

In the present study, no particular geographical locality or population of *Z. carpinifolia* could be indicated as more important from a conservational point of view. However, some populations residing outside the protected areas with relatively large numbers of private alleles, high levels of differentiation and/or high levels of cpDNA variability deserve attention. It should also be noted that high levels of gene flow in *Z. carpinifolia* due to wind-pollination may be a reason for outbreeding depression, possibly reflected in the irregular flowering and the production of empty seeds by *Zelkova*. For *P. fraxinifolia* the areas or populations with high numbers of private alleles at nuclear markers and cpDNA haplotypes in Hyrcan should be considered for both *in situ* and *ex situ* conservation purposes.

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Contribution to Chapter 2:

Maharramova E. H., L. A. Muller, N. Korotkova, and T. Borsch (2014) Development of nuclear microsatellites for the Arcto-Tertiary tree *Zelkova carpinifolia* (Ulmaceae) using 454 pyrosequencing. *Applications in Plant Sciences*, 2(3): 1300072.
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Own contribution: Performed laboratory work and data analysis after primer design and wrote the manuscript.

Contribution to Chapter 3:

Maharramova E. H., H. M. Safarov, G. Kozlowski, T. Borsch, and L. A. Muller (2015) Analysis of nuclear microsatellites reveals limited differentiation between Colchic and Hyrcanian populations of the wind-pollinated relict tree *Zelkova carpinifolia* (Ulmaceae). *American Journal of Botany*, 102(1): 119-128.
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Own contribution: Performed the field work (together with others), laboratory work, data analysis and wrote the manuscript.

Contribution to Chapter 4:

Maharramova E. H., I. M. Huseynova, S. Kolbaia, T. Borsch, and L. A. H. Muller (2015) Phylogeography and population genetics of the riparian relict tree *Pterocarya fraxinifolia* (Juglandaceae) in the South Caucasus (under review in *Journal of Biogeography*).

Own contribution: Performed the field work (together with others), laboratory work, data analysis and wrote the manuscript.

APPENDICES

Appendix A. Supplementary material for Chapter 2

Table A1. Locations and voucher information for populations of *Zelkova carpinifolia* used in this study. Vouchers are deposited at the herbarium of the Botanical Garden and Botanical Museum Berlin-Dahlem (B), Berlin, Germany.

Population	Locality	Geographic coordinates	Collection number
ZE001_AZ	Zuvand Mtn., Lerik, Azerbaijan	N38°43'33" E48°25'53"	EM01
ZE007_AZ	Azfilial, Lankaran, Azerbaijan	N38°40.938' E48°47.222'	EM13
ZE013_AZ	Tezekend, Lankaran, Azerbaijan	N38°39.091' E48°48.284'	EM20
ZE017_AZ	Zungulesh, Astara, Azerbaijan	N38°27.175' E48°47.575'	EM28
ZE021_AZ	Khanbulan, Lankaran, Azerbaijan	N38°39.809' E48°46.649'	EM33
ZE053_IR*	Gorgan, Golestan, Iran	N36°43'35" E54°34'49"	

Note: *used for construction of genomic library

Table A2. *Eco*RI-A and *Mse*I-B primers used for PCR recovery of enriched DNA library.

Primer name	Primer sequence (5'—3')[a]
EcoRI_A001	5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG **ACG AGT GCG T**CT CGT AGA CTG CGT ACC AAT TC-3'
EcoRI_A002	5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG **ACG CTC GAC A**CT CGT AGA CTG CGT ACC AAT TC-3'
EcoRI_A003	5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG **AGA CGC ACT C**CT CGT AGA CTG CGT ACC AAT TC-3'
EcoRI_A004	5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG **AGC ACT GTA G**CT CGT AGA CTG CGT ACC AAT TC-3'
EcoRI_A005	5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG **ATC AGA CAC G**CT CGT AGA CTG CGT ACC AAT TC-3'
EcoRI_A006	5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG **ATA TCG CGA G**CT CGT AGA CTG CGT ACC AAT TC-3'
MseI_B	5'-CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG GAC GAT GAG TCC TGA GTA A-3'

[a]Specimen-specific barcodes (tags) are shown in **Bold**.

Appendix B. Supplementary material for Chapter 3

Table B1. Location information for 30 sampling sites of *Zelkova carpinifolia*.

Sampling site	Country	Locality	Elevation	Latitude	Longitude	Herbaria	Collector(s)	Collection no.	Level of protection
ZE001_AZ	Azerbaijan	Lerik	1205 m	N38°43'33.5"	E48°25'53.1"	B	E.M., G.P., G.K., E.G., H.S.	EM1	Unprotected area
ZE007_AZ	Azerbaijan	Lankaran	72 m	N38°40.938'	E48°47.222'	B	E.M., A.M., H.S.	EM13	Unprotected area
ZE013_AZ	Azerbaijan	Lankaran	–	N38°39.091'	E48°48.284'	B	E.M., A.M., H.S.	EM20	Unprotected area
ZE017_AZ	Azerbaijan	Astara	25 m	N38°27.175'	E48°47.575'	B	E.M., A.M., H.S.	EM28	Hirkan NP
ZE019_AZ	Azerbaijan	Lankaran	9 m	N38°40.814'	E48°48.199'	B	E.M., A.M., H.S.	EM31	Hirkan NP
ZE021_AZ	Azerbaijan	Lankaran	56 m	N38°39.809'	E48°46.649'	B	E.M., A.M., H.S.	EM33	Hirkan NP
ZE027_AZ	Azerbaijan	Astara	11 m	N38°34.188'	E48°49.075'	B	E.M., A.M., H.S.	EM40	Unprotected area
ZE031_AZ	Azerbaijan	Lankaran	143 m	N38°40.591'	E48°44.338'	B	E.M., A.M., H.S.	EM45	Hirkan NP
ZE034_AZ	Azerbaijan	Lankaran	20 m	N38°40.931'	E48°49.731'	B	E.M., A.M., H.S.	EM52	Unprotected area
ZE036_AZ	Azerbaijan	Lankaran	–	N38°38.368'	E48°47.674'	B	E.M., A.M., H.S.	EM54	Hirkan NP
ZE043_AZ	Azerbaijan	Astara	96 m	N38°35.303'	E48°46.094'	B	E.M., A.M., H.S.	EM60	Unprotected area
ZE044_AZ	Azerbaijan	Astara	25 m	N38°35.868'	E48°47.802'	B	E.M., A.M., H.S.	EM62	Unprotected area
ZE051_AZ	Azerbaijan	Lerik	650 m	N38°47.797'	E48°28.086'	NHMF	E. G.	–	Unprotected area
ZE062_AZ	Azerbaijan	Lankaran	43 m	N38°45.919'	E48°45.549'	B	E.M., A.M., H.S.	EM62	Unprotected area
ZE067_AZ	Azerbaijan	Astara	195 m	N38°27.799'	E48°42.635'	B	E.M., A.M., H.S.	EM80	Hirkan NP
ZE069_AZ	Azerbaijan	Astara	252 m	N38°29.862'	E48°44.344'	–	E.M., A.M., H.S.	–	Hirkan NP
ZE072_AZ	Azerbaijan	Lankaran	45 m	N38°51.558'	E48°39.929'	B	E.M., A.M., H.S.	EM94	Unprotected area
ZE074_AZ	Azerbaijan	Lerik	414 m	N38°40.792'	E48°38.335'	B	E.M., A.M., H.S.	EM97	Unprotected area
ZE075_AZ	Azerbaijan	Lankaran	58 m	N38°40.437'	E48°46.919'	B	E.M., A.M., H.S.	EM103	Hirkan NP
ZE053_IR	Iran	Golestan	790 m	N36°43'35"	E54°34'49"	–	M.J.	–	Protected Area
ZE076_IR	Iran	Mazandaran	55-62 m	N36°32'89"	E53°03'32"	B	M.A.R.	–	Unprotected area
ZE077_IR	Iran	Mazandaran	780 m	N36°09'29"	E53°21'34"	B	M.A.R.	–	Unprotected area
ZE079_IR	Iran	Mazandaran	190 m	N36°27'35"	E53°04'83"	B	M.A.R.	–	Unprotected area
ZE054_GEO	Georgia	Akhmeta	470 m	N42°04'52.0"	E45°22'14.9"	NHMF	G.K., D.F.	–	Babaneuri SN R
ZE055_GEO	Georgia	Baghdati	–	N42°02'45.1"	E42°49'35.3"	NHMF	G.K., D.F.	–	Unprotected area
ZE057_GEO	Georgia	Martvili	275 m	N42°24'19.5"	E42°22'42.0"	NHMF	G.K., D.F.	–	Unprotected area
ZE058_GEO	Georgia	Baghdati	–	N42°06'49.3"	E42°47'35.3"	NHMF	G.K., D.F.	–	Unprotected area
ZE059_GEO	Georgia	Baghdati	–	N42°08'35.9"	E42°45'47.5"	NHMF	G.K., D.F.	–	Ajameti NR
ZE060_GEO	Georgia	Vani	100 m	N42°05'18.8"	E42°33'54.8"	NHMF	G.K., D.F.	–	Unprotected area
ZE061_TUR	Turkey	Trabzon	76 m	N40°56'59.1"	E39°51'56.2"	NHMF	I.K.	–	Unprotected area

Notes to Table B1: E.M. – Elmira Maharramova, Botanical Garden and Botanical Museum Berlin-Dahlem (BGBM), Germany & Institute of Botany, Azerbaijan National Academy of Sciences (ANAS); G.P. – Gerald Parolly, BGBM, Germany; G.K. – Gregor Kozlowski, University of Fribourg & Natural History Museum of Fribourg (NHMF), Switzerland; E.G. - Emanuel Gerber, NHMF, Switzerland; H.S. – Hajiaga Safarov, Hirkan National Park, Azerbaijan; A.M. – Ayyub Mutallimov, Institute of Botany, ANAS; M.J. – Mohammed Jafari, University of Tehran, Iran; M.A.R. – Mohammad Amini Rad, Iranian Research Institute of Plant Protection, Iran; D.F. – David Frey, University of Fribourg, Switzerland; I.K. – Ilham Kaya, Yuzuncuyil University, Turkey.
B – Herbarium of the Botanical Garden and Botanical Muzeum Berlin Dahlem, Germany; NHMF – Herbarium of Natural History Museum of Fribourg, Switzerland.

Table B2. Primer combinations used for multiplex PCR amplification of 10 microsatellite loci.

Locus	Primer sequence (5'—3')	Label	Primer mixes
ZMS_12	F: CTTCCAATCATTCCGAGAGC* R: TGACATCTCAAAGGTTGCCA	PET	Mix 1
ZMS_13	F: AACAAAGAATCTTCATCCCC* R: GCATTTGGTTTTACCTAAAGTTACAG	NED	
ZMS_1	F: AAGGTTCATCCCATGAGCAC* R: GTCCAGTCCGGCATCTTTT	6FAM	Mix 2
ZMS_2	F: CAAGCTGCCAATACTCCACA* R: GGTTTGGTTTCTGCTTGGAA	VIC	
ZMS_5	F: TTTTGTTCGTTGTTGCATGTC* R: AGGCTTAGGTTGGGTGGTTT	PET	
ZMS_7	F: TCTCTGTTATGATCACGTCTCCA R: GAGCTAAACCCCATTTCAAATATAA*	NED	
ZMS_3	F: CCCAACAGCACTTGAAGCTA* R: TTTGTGTAGTCTTTGGCCCC	6FAM	Mix 3
ZMS_8	F: ACGAACAAGATCGACAGAGG* R: ATTGGATGGCACACGTAATTC	PET	
ZMS_9	F: CAGGTGATTGGAATTGGAAAA* R: CCTCACAGAGTACGAGGGATG	6FAM	
ZMS_4	F: ACCGTTGGAAACAAAACTCG* R: AGGGCAGTAGAGCTTGATCG	NED	

Notes: F – forward primer, R – reverse primer. * Labeled primer.

Table B3a. Pairwise genetic distances as R_{ST} (below diagonal) and F_{ST} (above diagonal). Distance matrix built using all 495 individuals. Non-significant values are shown in *Italics* ($P > 0.05$).

	ZE001	ZE007	ZE013	ZE017	ZE019	ZE021	ZE027	ZE031	ZE034	ZE036	ZE043	ZE044	ZE051	ZE053	ZE054	ZE055	ZE057	ZE058	ZE059	ZE060	ZE061	ZE062	ZE067	ZE069	ZE072	ZE074	ZE075	ZE076	ZE077	ZE079
ZE001		0.036	0.078	0.203	*0.015*	0.121	0.27	*0.008*	0.131	0.098	0.027	0.105	0.064	0.286	0.134	0.111	0.112	0.225	0.185	0.093	0.253	0.162	0.096	0.036	*0.021*	0.091	0.142	0.054	0.112	0.131
ZE007	0.097		0.14	0.116	0.078	*0.031*	0.276	0.064	0.057	0.083	0.043	*0.03*	*-0.001*	0.189	0.054	0.035	0.091	0.123	0.088	0.047	0.184	0.133	0.042	0.047	*0.013*	0.039	0.08	*0.04*	*0.048*	0.07
ZE013	0.104	0.11		0.245	*0.013*	0.163	0.209	*0.024*	0.17	*0.032*	0.123	0.183	0.151	0.364	0.16	0.165	0.16	0.266	0.285	0.129	0.315	*0.082*	0.152	0.146	*0.041*	0.183	0.235	*0.057*	0.168	0.134
ZE017	0.097	0.101	0.112		0.203	*0.035*	0.368	0.218	0.09	0.1	0.193	*0.02*	*0.048*	*0.03*	0.085	0.162	0.224	0.133	0.051	0.175	0.085	0.097	0.14	0.185	0.112	0.119	0.16	*0.039*	*0.037*	0.115
ZE019	0.068	0.097	0.063	0.079		0.126	0.256	*-0.002*	0.18	*0.032*	*0.034*	0.126	0.107	0.293	0.149	0.13	0.168	0.238	0.222	0.087	0.252	0.096	0.121	0.066	*0.017*	0.11	0.168	*0.025*	0.103	0.106
ZE021	0.115	0.1	0.101	0.043	0.045		0.203	0.124	*0.038*	*0.063*	0.071	*-0.006*	*-0.004*	*0.051*	*0.001*	*0.02*	0.1	*0.045*	*-0.042*	*0.019*	*0.01*	*0.041*	*0.007*	*0.045*	*0.035*	*0.014*	*0.014*	*0.036*	*-0.003*	*0.005*
ZE027	0.175	0.17	0.148	0.156	0.08	0.095		0.247	0.202	0.253	0.239	0.312	0.262	0.41	0.185	0.164	0.184	0.265	0.286	0.119	0.306	0.155	0.138	0.204	0.191	0.247	0.16	0.265	0.259	0.136
ZE031	0.157	0.135	0.138	0.138	0.105	0.088	0.158		0.136	0.056	0.057	0.121	0.071	0.336	0.129	0.113	0.1	0.238	0.246	0.088	0.3	0.153	0.094	0.068	*0.003*	0.103	0.184	*0.042*	0.129	0.123
ZE034	0.054	0.079	0.092	0.067	0.061	0.045	0.12	0.083		0.134	0.158	0.046	*0.006*	0.155	*-0.018*	0.064	*0.019*	0.098	*0.046*	0.081	0.137	*0.071*	0.053	0.096	*0.058*	0.101	0.077	0.088	0.121	0.104
ZE036	0.101	0.055	0.07	0.057	0.03	0.043	0.097	0.132	0.073		0.093	0.068	0.08	0.201	0.084	0.114	0.179	0.167	0.161	0.081	0.161	*-0.015*	0.107	0.121	*0.021*	0.103	0.157	*-0.013*	*0.059*	*0.052*
ZE043	0.093	0.079	0.051	0.067	0.02	*0.018*	0.107	0.069	0.065	0.028		0.095	0.079	0.235	0.124	0.076	0.159	0.204	0.128	*0.024*	0.189	0.137	0.052	*-0.005*	*0.025*	*0.025*	0.059	0.059	*0.043*	*0.053*
ZE044	0.096	0.089	0.092	*0.004*	0.057	0.035	0.142	0.075	0.054	0.05	0.034		*-0.021*	0.103	*0.023*	0.091	0.134	0.133	*0.04*	0.08	0.101	0.108	0.052	0.086	*0.032*	*0.022*	0.093	*0.006*	*0.016*	*0.063*
ZE051	0.085	0.042	0.079	0.039	0.051	*0.011*	0.129	0.063	0.044	0.037	*0.018*	*0.014*		0.12	*-0.011*	*0.034*	*0.043*	0.092	*0.025*	*0.048*	0.124	0.095	*0.017*	*0.034*	*0.007*	*0.023*	*0.062*	*0.02*	*0.036*	*0.066*
ZE053	0.149	0.146	0.182	0.024	0.122	0.034	0.18	0.161	0.116	0.104	0.092	0.047	0.059		0.176	0.192	0.323	0.134	0.043	0.219	0.083	0.195	0.173	0.227	0.197	0.157	0.165	0.124	*-0.008*	0.119
ZE054	0.166	0.119	0.194	0.103	0.138	0.079	0.164	0.155	0.103	0.082	0.116	0.094	0.058	0.131		*0.047*	*0.033*	0.105	*0.039*	*0.046*	0.107	0.081	*0.01*	0.077	*0.034*	0.051	0.074	0.062	0.07	*0.053*
ZE055	0.138	0.097	0.171	0.144	0.11	0.074	0.171	0.18	0.098	0.084	0.102	0.128	0.07	0.132	0.138		0.086	*0.025*	0.075	*-0.002*	0.142	0.114	*0.004*	0.056	*0.048*	0.061	*0.038*	0.096	*0.048*	*0.011*
ZE057	0.178	0.143	0.168	0.141	0.142	0.123	0.175	0.176	0.118	0.119	0.146	0.128	0.116	0.195	0.16	0.119		0.2	0.193	0.088	0.286	0.178	0.055	0.096	0.057	0.123	0.122	0.137	0.188	0.154
ZE058	0.156	0.178	0.248	0.191	0.225	0.186	0.275	0.244	0.166	0.172	0.211	0.201	0.172	0.174	0.212	0.11	0.238		0.076	0.111	0.141	0.146	0.093	0.198	0.142	0.16	0.132	0.136	*0.061*	*0.056*
ZE059	0.095	0.094	0.108	0.054	0.099	0.031	0.148	0.118	0.049	0.052	0.051	0.048	0.03	0.059	0.085	0.051	0.145	0.085		0.072	*0.031*	0.158	*0.03*	0.113	0.105	*0.038*	*0.022*	0.08	*-0.033*	*0.009*
ZE060	0.138	0.085	0.134	0.099	0.106	0.058	0.122	0.124	0.077	0.044	0.079	0.092	0.045	0.107	0.071	0.049	0.115	0.111	*0.017*		0.117	0.091	*-0.019*	*0.014*	*0.022*	*0.017*	*0.012*	0.079	*0.041*	*-0.012*
ZE061	0.292	0.281	0.337	0.235	0.249	0.167	0.282	0.295	0.25	0.218	0.207	0.209	0.205	0.187	0.228	0.185	0.326	0.294	0.146	0.179		0.157	0.099	0.193	0.173	0.106	0.11	0.123	*0.008*	*0.028*
ZE062	0.114	0.123	0.082	0.073	0.044	0.042	0.116	0.126	0.049	0.066	0.063	0.06	0.094	0.118	0.159	0.124	0.135	0.216	0.097	0.114	0.341		0.088	0.138	*0.033*	0.124	0.14	*0.008*	*0.03*	*0.002*
ZE067	0.104	0.055	0.068	0.047	0.046	*0.007*	0.079	0.049	0.041	0.047	0.018	0.029	*-0.004*	0.06	0.079	0.093	0.116	0.188	0.044	0.047	0.218	0.038		*0.015*	*0.028*	*0.01*	*-0.005*	0.078	*0.048*	*0.01*
ZE069	0.091	0.086	0.094	0.065	0.031	*0.014*	0.09	0.116	0.051	*0.008*	*0.016*	0.048	*0.002*	0.096	0.067	0.066	0.122	0.187	0.035	0.044	0.163	0.102	0.037		*0.013*	*0.014*	*0.021*	*0.066*	*0.048*	*0.057*
ZE072	0.069	0.062	0.092	0.046	0.051	0.065	0.136	0.079	0.049	0.057	0.054	*0.018*	0.036	0.097	0.113	0.131	0.115	0.204	0.087	0.112	0.304	0.044	0.037	0.079		*0.026*	0.077	*-0.008*	*0.05*	*0.042*
ZE074	0.181	0.107	0.155	0.064	0.136	0.085	0.197	0.101	0.121	0.087	0.076	0.035	0.049	0.096	0.129	0.19	0.176	0.259	0.092	0.1	0.277	0.132	0.059	0.103	0.056		*0.025*	0.056	*0.028*	*0.035*
ZE075	0.096	0.099	0.069	0.038	0.045	*0.012*	0.062	0.084	0.043	0.061	0.023	0.027	*0.025*	0.053	0.13	0.115	0.138	0.204	0.04	0.068	0.219	0.046	*0.002*	0.041	0.05	0.075		0.118	*0.011*	*0.019*
ZE076	0.109	0.096	0.081	*0.019*	0.057	*0.032*	0.15	0.085	0.074	0.054	*0.027*	*0.006*	*0.025*	0.056	0.117	0.147	0.123	0.229	0.076	0.095	0.291	0.05	*0.026*	0.068	*0.014*	0.035	*0.026*		*0.02*	*0.054*
ZE077	0.174	0.135	0.142	0.119	0.1	0.035	0.11	0.141	0.12	0.085	0.06	0.117	0.054	0.084	0.131	0.105	0.191	0.209	0.064	0.065	0.169	0.133	0.051	0.044	0.149	0.158	0.045	0.096		*0.004*
ZE079	0.135	0.157	0.155	0.094	0.109	0.059	0.127	0.111	0.102	0.095	0.072	0.079	0.075	0.05	0.146	0.113	0.208	0.134	0.029	0.051	0.148	0.119	0.066	0.079	0.114	0.11	0.041	0.091	0.07	

Table B3 b. Pairwise genetic distances as R_{ST} (below diagonal) and F_{ST} (above diagonal). Distance matrix built using gamets only (379 individuals). Non-significant values are shown in *Italics* ($P > 0.05$).

	ZE001	ZE007	ZE013	ZE017	ZE019	ZE021	ZE027	ZE031	ZE034	ZE036	ZE043	ZE044	ZE051	ZE053	ZE062	ZE067	ZE069	ZE072	ZE074	ZE075	ZE076	ZE077	ZE079	ZE054	ZE055	ZE057	ZE058	ZE059	ZE060	ZE061
ZE001		*0.035*	0.062	0.199	*0.01*	0.094	0.253	*-0.028*	0.13	0.057	*0.013*	0.06	0.07	0.34	0.124	0.094	*0.034*	*0.011*	0.105	0.147	*0.027*	0.138	0.114	0.127	0.106	0.077	0.232	0.191	0.106	*0.036*
ZE007	0.068		0.118	0.091	0.08	*0.002*	0.297	*0.002*	*0.049*	0.123	*0.022*	*0*	*-0.017*	0.214	0.144	*0.038*	*0.038*	*0.013*	*0.024*	*0.066*	*0.006*	*0.029*	*0.022*	*0.043*	*0.032*	*0.044*	0.124	*0.064*	*0.042*	*-0.06*
ZE013	0.07	0.086		0.195	*0.023*	0.114	0.149	*-0.01*	0.128	*-0.018*	0.105	0.093	*0.11*	0.359	*-0.015*	0.099	0.102	*0.016*	0.175	0.191	*0.062*	0.152	*0.063*	0.096	0.105	0.11	0.205	0.226	0.112	*0.013*
ZE017	0.087	0.092	0.086		0.184	*0.04*	0.38	0.142	0.08	0.19	0.161	*0.026*	*0.017*	*0.028*	0.144	0.126	0.163	0.11	0.078	0.152	*0.031*	*-0.013*	*-0.014*	0.086	0.146	0.174	0.13	*0.044*	0.15	*-0.198*
ZE019	0.051	0.09	0.032	0.072		0.099	0.255	*-0.022*	0.192	*-0.017*	*0.024*	0.075	0.112	0.338	*0.048*	0.119	*0.06*	*0.015*	0.123	0.174	*0.017*	0.136	*0.093*	0.142	0.112	0.152	0.232	0.211	0.107	*0.015*
ZE021	0.084	0.081	0.047	0.035	0.03		0.219	*0.03*	*0.057*	*0.1*	*0.033*	*-0.004*	*-0.013*	0.106	*0.068*	*-0.004*	*0.014*	*0.026*	*-0.009*	*0.006*	*0.012*	*-0.038*	*-0.04*	*0.015*	*0.003*	*0.059*	*0.058*	*-0.029*	*-0.006*	*-0.263*
ZE027	0.159	0.155	0.083	0.161	0.078	0.09		0.207	0.239	0.23	0.262	0.315	0.29	0.489	0.132	0.137	0.207	0.195	0.308	0.198	0.286	0.284	0.206	0.199	0.172	0.205	0.297	0.326	0.151	*0.182*
ZE031	0.074	0.051	0.06	0.066	*0.051*	*0.026*	0.105		*0.07*	*-0.014*	*0.004*	*0.01*	*0.005*	0.315	*0.054*	*0.022*	*0.013*	*-0.048*	*0.064*	*0.112*	*-0.015*	*0.069*	*0.023*	*0.053*	*0.021*	*0.026*	*0.148*	0.162	*0.043*	*-0.038*
ZE034	0.044	0.071	0.056	0.078	0.058	0.039	0.108	*0.041*		0.183	0.159	*0.031*	*-0.006*	0.179	0.114	0.073	0.112	*0.074*	0.114	0.116	*0.077*	*0.089*	*0.044*	*-0.008*	0.091	*0.007*	0.11	*0.061*	0.098	*-0.106*
ZE036	0.077	0.046	*0.024*	0.071	*0.009*	*0.026*	0.067	*0.03*	0.071		*0.067*	*0.104*	*0.133*	0.37	*-0.01*	0.115	*0.103*	*0.02*	0.16	0.193	*0.037*	*0.133*	*0.061*	0.134	0.111	0.177	0.234	0.247	*0.11*	*0.043*
ZE043	0.066	0.059	*0.022*	0.06	*0.016*	*0.008*	0.096	*0.009*	0.053	*0.003*		*0.054*	*0.069*	0.286	0.115	0.054	*-0.006*	*0.016*	*0.03*	0.068	*0.015*	*0.066*	*0.069*	0.111	0.063	0.112	0.207	0.121	*0.037*	*-0.046*
ZE044	0.06	0.069	0.047	*0.014*	*0.03*	*0.023*	0.126	*0.004*	0.044	*0.03*	*0.017*		*-0.047*	0.169	0.11	*0.047*	*0.061*	*0.002*	*0.015*	0.099	*-0.036*	*0.014*	*-0.014*	*0.012*	0.078	*0.064*	0.149	*0.064*	*0.068*	*-0.108*
ZE051	0.053	0.026	0.039	*0.021*	0.047	*0.002*	0.118	*-0.014*	*0.027*	*0.029*	*0.012*	*-0.014*		0.131	*0.106*	*0.027*	*0.044*	*0.013*	*0.012*	*0.071*	*-0.012*	*0.012*	*-0.003*	*-0.018*	*0.046*	*0.008*	0.094	*0.007*	*0.05*	*-0.179*
ZE053	0.14	0.137	0.157	*0.02*	0.137	0.054	0.211	0.087	0.133	0.123	0.109	0.061	0.05		0.303	0.22	0.284	0.259	0.168	0.206	0.164	*0.013*	*0.072*	0.2	0.242	0.305	0.174	0.067	0.242	*-0.139*
ZE062	0.114	0.12	0.048	0.092	*0.027*	*0.036*	0.096	0.08	0.073	0.054	0.061	0.068	0.088	0.148		*0.063*	*0.1*	*0.018*	0.149	0.146	*0.053*	*0.053*	*-0.044*	*0.09*	0.084	0.153	0.171	0.198	*0.082*	*-0.06*
ZE067	0.087	0.047	0.036	0.029	0.043	*0.001*	0.065	*-0.014*	0.041	0.028	*0.014*	*0.012*	*-0.01*	0.06	0.032		*0.012*	*0.028*	*0.027*	*-0.003*	*0.059*	*0.036*	*0.005*	*0.006*	*-0.005*	*0.03*	0.095	*0.031*	*-0.029*	*-0.167*
ZE069	0.075	0.08	0.051	0.067	0.027	*0.005*	0.075	0.048	0.04	*-0.001*	*0.012*	*0.023*	*0.002*	0.108	0.088	0.029		*0.006*	*0.023*	*0.025*	*0.029*	*0.056*	*0.058*	0.071	*0.044*	*0.063*	0.199	0.105	*0.012*	*-0.067*
ZE072	0.053	0.054	0.071	0.04	0.045	0.056	0.137	*0.018*	0.065	0.051	0.045	*0.013*	*0.025*	0.096	0.053	0.03	0.075		*0.043*	0.087	*-0.013*	*0.066*	*0.024*	*0.034*	*0.042*	*0.035*	0.151	0.109	*0.034*	*-0.079*
ZE074	0.151	0.089	0.118	0.042	0.113	0.061	0.186	*0.043*	0.126	0.072	0.055	0.035	*0.027*	0.073	0.128	0.031	0.081	0.049		*0.029*	*0.02*	*0.012*	*0.035*	*0.057*	0.066	0.094	0.169	*0.021*	*0.03*	*-0.157*
ZE075	0.079	0.085	0.032	0.038	0.04	*0.008*	0.059	*0.03*	0.049	0.046	0.022	0.028	*0.017*	0.062	0.047	*-0.005*	0.037	0.051	0.06		0.104	*0.014*	*0.033*	0.075	*0.029*	0.091	0.131	*0.021*	*-0.005*	*-0.156*
ZE076	0.07	0.073	0.051	*-0.001*	0.043	*0.007*	0.149	*0.022*	0.063	*0.04*	*0.015*	*0.003*	*-0.004*	0.049	0.065	*0.013*	0.045	*0.018*	*0.021*	*0.023*		*0.009*	*-0.003*	*0.058*	0.075	0.083	0.142	*0.065*	*0.073*	*-0.163*
ZE077	0.132	0.105	0.087	0.083	0.085	*0.01*	0.1	0.057	0.08	0.056	0.05	0.073	*0.023*	0.058	0.111	*0.027*	*0.025*	0.116	0.104	*0.016*	*0.052*		*-0.063*	*0.046*	*0.024*	0.133	*0.03*	*-0.075*	*0.018*	*-0.35*
ZE079	0.063	0.095	*0.044*	0.053	*0.05*	*0.008*	0.079	*0.017*	0.052	*0.016*	*0.021*	*0.034*	*0.016*	*0.037*	0.093	*0.017*	*0.012*	0.082	0.089	*-0.01*	*0.046*	*-0.035*		*-0.011*	*-0.01*	*0.096*	*-0.001*	*-0.047*	*-0.008*	*-0.377*
ZE054	0.153	0.119	0.157	0.108	0.141	0.084	0.158	0.086	0.102	0.086	0.108	0.082	0.047	0.127	0.164	0.081	0.063	0.113	0.112	0.13	0.102	0.114	0.115		*0.045*	*0.007*	0.112	*0.044*	*0.04*	*-0.142*
ZE055	0.123	0.097	0.126	0.137	0.094	0.052	0.147	0.088	0.094	0.056	0.088	0.097	0.072	0.142	0.109	0.082	0.055	0.122	0.163	0.098	0.121	0.067	*0.047*	0.138		*0.07*	*0.032*	*0.065*	*-0.02*	*-0.138*
ZE057	0.13	0.11	0.118	0.099	0.112	0.089	0.157	0.096	0.089	0.086	0.118	0.089	0.08	0.171	0.112	0.086	0.094	0.087	0.142	0.116	0.08	0.145	0.134	0.135	0.088		0.174	0.132	*0.068*	*0.02*
ZE058	0.146	0.155	0.212	0.19	0.203	0.17	0.259	0.137	0.173	0.149	0.198	0.179	0.156	0.164	0.221	0.171	0.171	0.196	0.236	0.188	0.207	0.169	0.096	0.204	0.096	0.204		0.084	0.101	*-0.115*
ZE059	0.081	0.082	0.073	0.059	0.096	0.031	0.141	*0.043*	0.054	0.049	0.051	0.043	*0.02*	0.059	0.111	0.036	0.036	0.087	0.078	0.04	0.061	*0.029*	*-0.015*	0.086	0.044	0.113	0.079		*0.046*	*-0.227*
ZE060	0.115	0.07	0.093	0.092	0.097	0.047	0.109	*0.056*	0.073	*0.026*	0.071	0.077	0.04	0.105	0.107	0.039	0.037	0.106	0.087	0.061	0.081	*0.037*	*0.016*	0.069	*0.033*	0.086	0.094	*0.007*		*-0.165*
ZE061	*0.071*	*0.066*	*0.077*	*0.047*	*0.027*	*-0.06*	*0.076*	*-0.021*	*0.032*	*-0.036*	*-0.013*	*-0.031*	*-0.071*	*-0.025*	*0.123*	*-0.018*	*-0.1*	*0.082*	*0.063*	*-0.019*	*0.046*	*-0.144*	*-0.182*	*0.016*	*-0.092*	*0.103*	*0.035*	*-0.086*	*-0.059*	

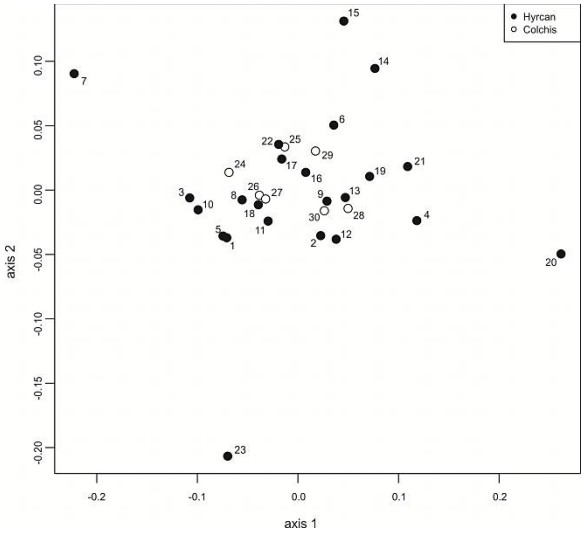

Fig. B1. Multidimensional scaling of 30 sampling sites of *Zelkova carpinifolia* using pairwise R_{ST} as genetic distance (excluding clonal individuals). Circles represent sites. Numbers correspond to the sites as shown in Table 3.1.

Appendix C. Supplementary material for Chapter 4

Table C1. Location information for 40 sampling locations of *Pterocarya fraxinifolia* in South Caucasus and northern Iran. Available vouchers are deposited at Botanical Garden and Botanical Museum Berlin-Dahlem (B), Germany.

Sampling site	Country	District	Elevation (m)	Latitude	Longitude	H	Collection number	Level of protection
PT023_GEO	Georgia	Poti	0	42°12'07.5"N	41°51'20.3"E	B	Gg-11-6	Kolkheti NP
PT059_GEO	Georgia	Poti	3	N42°12.429'	E41°44.258'	B	SK 1302009	Unprotected area
PT058_GEO	Georgia	Baghdati	93	N42°04.106'	E42°49.058'	B	SK 1302010	Unprotected area
PT057_GEO	Georgia	Zestaphoni	140	N42°07'12.6"	E42°57'43.3"	B	SK 1302007	Unprotected area
PT055_GEO	Georgia	Telavi	477	N42°03'46.1"	E45°31'45.2"	B	SK 1302005	Unprotected area
PT056_GEO	Georgia	Telavi	370	N41°59'16.9"	E45°34'38.3"	B	SK 1302006	Unprotected area
PT054_GEO	Georgia	Kvareli	645	N42°01'13.1"	E45°46'08.3"	B	SK 1302004	Unprotected area
PT052_GEO	Georgia	Lagodekhi	550	N41°50'25.8"	E46°18'04.6"	B	SK 1302001	Lagodekhi NR
PT050_AZ	Azerbaijan	Balakan	276	N41°46.035'	E46°20.491'	B	EM 113	Unprotected area
PT049_AZ	Azerbaijan	Zaqatala	565	N41°42.058'	E46°35.479'	B	EM 112	Unprotected area
PT048_AZ	Azerbaijan	Zaqatala	319	N41°36.783'	E46°32.522'	B	EM 111	Unprotected area
PT047_AZ	Azerbaijan	Qakh	277	N41°29.497'	E46°47.666'	B	EM 110	Unprotected area
PT022_AZ	Azerbaijan	Oghuz	1030	N40°59'13"	E47°35'27"	B	GP 13054	Unprotected area
PT044_AZ	Azerbaijan	Ismayilly	600	N40°51.882'	E48°05.504'	B	EM 107	Unprotected area
PT039_AZ	Azerbaijan	Lankaran	97	N38°51.481'	E48°38.277'	B	EM 91	Unprotected area
PT015_AZ	Azerbaijan	Lerik	423	N38°47.975'	E48°30.393'	–	–	Unprotected area
PT017_AZ	Azerbaijan	Lerik	180	N38°44.412'	E48°36.663'	B	EM 49	Unprotected area
PT041_AZ	Azerbaijan	Lerik	414	N38°40.792'	E48°38.335'	B	EM 98	Unprotected area
PT004_AZ	Azerbaijan	Lankaran	265	N38°40.147'	E48°43.602'	B	EM 6	Unprotected area
PT001_AZ	Azerbaijan	Lankaran	9	N38°38.668'	E48°49.150'	B	EM 2	Unprotected area
PT021_AZ	Azerbaijan	Astara	56	N38°36.260'	E48°47.146'	B	EM 67	Unprotected area
PT020_AZ	Azerbaijan	Astara	7	N38°35.922'	E48°48.284'	B	EM 65	Unprotected area
PT019_AZ	Azerbaijan	Astara	85	N38°35.331'	E48°46.122'	B	EM 61	Unprotected area
PT018_AZ	Azerbaijan	Astara	104	N38°35.229'	E48°45.647'	B	EM 58	Unprotected area
PT010_AZ	Azerbaijan	Astara	–	N38°30.854'	E48°49.708'	B	EM 30	Unprotected area
PT038_AZ	Azerbaijan	Astara	88	N38°29.619'	E48°45.051'	B	EM 88	Hirkan NP
PT035_AZ	Azerbaijan	Astara	103	N38°27.307'	E48°43.152'	B	EM 76	Hirkan NP
PT002_AZ	Azerbaijan	Astara	50	N38°27.270'	E48°45.218'	B	EM 4	Hirkan NP
PT003_AZ	Azerbaijan	Astara	38	N38°26.995'	E48°46.550'	B	EM 5	Hirkan NP
PT009_AZ	Azerbaijan	Astara	62	N38°27.159'	E48°47.417'	B	EM 27	Hirkan NP
PT027_IR	Iran	Tulem	−3	N37°20'17"	E49°26'44"	–	–	Unprotected area
PT029_IR	Iran	Rasht	−5	N37°20'14.3"	E49°33'38.7"	–	–	Unprotected area
PT028_IR	Iran	Sardar-e Jangal	100-200	N37°11'54"	E49°11'37"	–	–	Unprotected area
PT026_IR	Iran	Chaboksar	−2	N36°59.901'	E50°31.409'	–	–	Unprotected area
PT025_IR	Iran	Kelarabad	–	N36°42'12"	E51°14'22"	–	–	Unprotected area
PT032_IR	Iran	Nowshahr	200-600	N36°34'20"	E51°35'18"	–	–	Central Alborz PA
PT030_IR	Iran	Savadkuh	320	N36°15'09"	E52°54'06"	–	–	Unprotected area
PT031_IR	Iran	Kordkuy	600	N36°43'09"	E54°06'42"	–	–	Unprotected area
PT033_IR	Iran	Gorgan	790	N36°43'35"	E54°34'49"	–	–	Unprotected area
PT024_IR	Iran	Kamalan	−20	N36°46'24"	E54°56'57"	–	–	Unprotected area

Notes: NP – National Park, NR – Nature Reserve, PA – Protected Area. H – herbarium.

Table C2. List of East Asian *Pterocarya* species collected at the Botanical Museum and Botanical Garden Berlin-Dahlem (BGBM (B); Germany) for cpDNA haplotype network reconstruction.

Species name	Sequence code	Haplotype	Accession number	Herbarium	Origin
Pterocarya tonkinensis	PTT	H1	213148570	B	Unknown
Pterocarya macroptera var. *insignis*	PTI_1	H1	177627480	B	Unknown
Pterocarya macroptera var. *insignis*	PTI_2	H2	177627480	B	Unknown
Pterocarya rhoifolia	PTR_1	H3	186028670	B	Unknown
Pterocarya rhoifolia	PTR_2	H4	135111040	B	Japan: Aonori, lake Towada

Table C3. Nuclear microsatellite loci selected to test for cross-amplification in *Pterocarya fraxinifolia*.

Locus	Forward primer	Reverse primer	Reference
WGA118	TGTGCTCTGATCTGCCTCC	GGGTGGGTGAAAAGTAGCAA	Dangl *et al.*, 2005
WGA321	TCCAATCGAAACTCCAAAGG	GTCCAAAGACGATGATGGA	Dangl *et al.*, 2005
WGA276	CTCACTTTCTCGGCTCTTCC	GGTCTTATGTGGGCAGTCGT	Dangl *et al.*, 2005
WGA202	CCCATCTACCGTTGCACTTT	GCTGGTGGTTCTATCATGGG	Dangl *et al.*, 2005
WGA27	AACCCTACAACGCCTTGATG	TGCTCAGGCTCCACTTCC	Woeste *et al.*, 2002
WGA32	CTCGGTAAGCCACACCAATT	ACGGGCAGTGTATGCATGTA	Woeste *et al.*, 2002
WGA82	TGCCGACACTCCTCACTTC	CGTGATGTACGACGGCTG	Woeste *et al.*, 2002
CYC019	ATTCCCCACCCCCATCTC	CTCCTCCAGCGCACATAA	Fan *et al.*, 2013
CYC095	TGGAACTGGGAACGGTG	ACCCCCTTCGTCGTCTTT	Fan *et al.*, 2013
CYC130	TGCCAAGAGTGACAGATT	GTTGATGATAGTTTGTAGAG	Fan *et al.*, 2013
CYC148	TCCTCCACTTCCAATGAT	AGAGGAGCAAACAAACAT	Fan *et al.*, 2013
CYC015	ACCCCTCAAGTCCCACCA	CCAGATACACATGCACAC	Fan *et al.*, 2013
CYC053	AGATGGCTTTTCAGATTTG	CGGAAACTTGAATCAGAG	Fan *et al.*, 2013
CYC060	CCTCAAGTCTGTGGCTCT	GAACCGAAGCCAGTAAAG	Fan *et al.*, 2013
CYC062	GTGAGAGAATGAAATGAA	TATGTGATTAGATTGAGC	Fan *et al.*, 2013
CYC092	AAGGAGAAAGAGAAAAC	AGAACTGGAGAGGCGAAC	Fan *et al.*, 2013
CYC125	CGTAATGGAATTTAGTCC	GAATACCACCAATCCTTT	Fan *et al.*, 2013
CYC129	GTTATGCTAAAGCCTCGC	GAGGAAAGAAAGAGTTGG	Fan *et al.*, 2013
CYC036	ATCGTCCTGGTG ATGTTG	AGGTCCTCCTTCCTTTGG	Fan *et al.*, 2013
CYC045	GCAAAACATTCTTAGG	ACTTGGTCAAATAGTC	Fan *et al.*, 2013
CYC049	TGCCTCAATCCCAAAGAC	AATTACGCCGAAGGGGTC	Fan *et al.*, 2013
CYC052	CCACTTCGTGATCTGCCG	AGGGAAAGAAAGCGAGAT	Fan *et al.*, 2013
CYC114	AAACTCTCTCTTTCTCAC	CGTTGGTATTTAGGTCTA	Fan *et al.*, 2013
CYC132	AGCCACCGCTAGGAAGCA	GGGCGTTACAGTGGGAGA	Fan *et al.*, 2013
CYC150	AGAGATTAGCTCGGGTCT	GATCCAAAACTGAAGGGA	Fan *et al.*, 2013
PM-CTA24	AAATGGTGAGGAAGTGAAGATATGAA	GCCCCTTATACAGTTCTACCTCTCTC	Grauke *et al.*, 2003
PM-CA12	AGATCGAAAAGCGTGGAGCAAC	ACACCGAATTCTCAATGAGCCAAAC	Grauke *et al.*, 2003
PM-CIN13	CCGCAGATGGTTTGAAGAA	ACAAATTCCTCACTCCGGAG	Grauke *et al.*, 2003

Notes: Loci that were successfully amplified are indicated in *Italics*. References are given below.

References to Table C3

Dangl, G.S., Woeste, K., Aradhya, M.K., Koehmstedt, A., Simon, C., Potter, D., Leslie, C.A. & McGranahan, G. (2005) Characterization of 14 microsatellite markers for genetic analysis and cultivar identification of walnut. *Journal of the American Society for Horticultural Science*, 130, 348-354.

Fan, D.M., Ye, L.J., Luo, Y., Hu, W., Tian, S. & Zhang, Z.Y. (2013) Development of microsatellite loci for *Cyclocarya paliurus* (Juglandaceae), a monotypic species in Subtropical China. *Applications in Plant Sciences*, 1 (1200524).

Grauke, L.J., Iqbal, M.J., Reddy, A.S. & Thompson, T.E. (2003) Developing microsatellite DNA markers in pecan. *Journal of the American Society for Horticultural Science*, 128, 374-380.

Woeste, K.E. (2002) Heartwood production in a 35-year-old black walnut progeny test. *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere*, 32, 177-181.

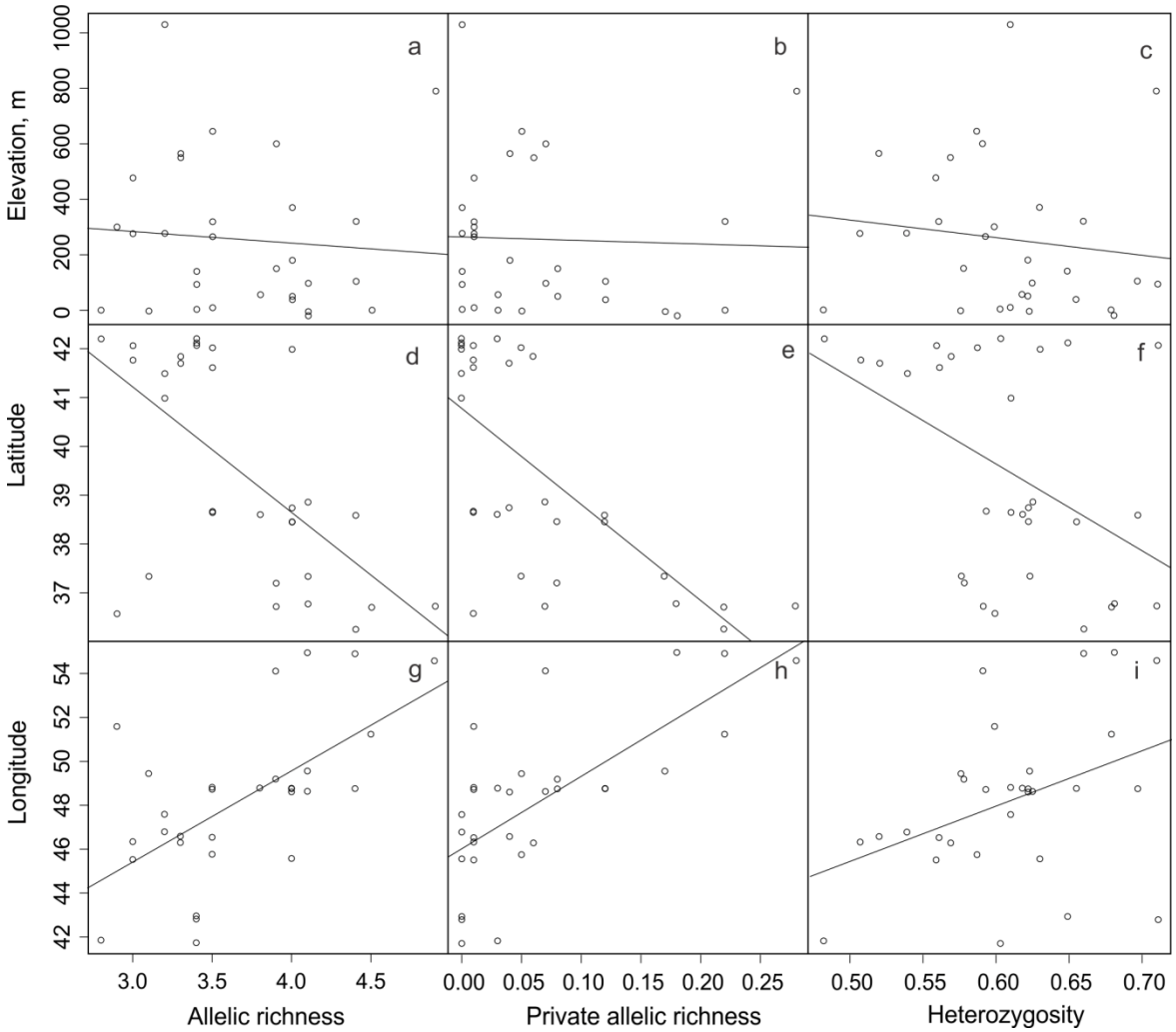

Fig. C1. Relationships between genetic diversity and geographical parameters of the natural populations of *Pterocarya fraxinifolia* in South Caucasus evaluated as Pearson's product–moment correlations. a, b, c – non-significant correlations of the genetic diversity parameters with elevation ($P > 0.05$); d – negative significant correlation of latitude with allelic richness ($r = -0.6$, $P < 0.05$); e – negative significant correlation of latitude with private allelic richness ($r = -0.7$, $P < 0.05$); f – negative significant correlation of latitude with heterozygosity ($r = -0.5$, $P < 0.05$); g – positive significant correlation of longitude with allelic richness ($r = 0.6$, $P < 0.05$); h – positive significant correlation of longitude with private allelic richness ($r = 0.7$, $P < 0.05$); i – positive significant correlation of longitude with heterozygosity ($r = 0.4$, $P < 0.05$).

Table C4. Characteristics of eight nuclear microsatellite loci used for the screening of 557 specimens of *Pterocarya fraxinifolia* from 30 sampling locations in the South Caucasus and northern Iran.

Locus	Primer sequences	Label	*T*a (°C)	Repeat	*A* (total)	*A* (mean)	H_E (total)	PIC	NAF	Missing data (%)
WGA118	F: TGTGCTCTGATCTGCCTCC* R: GGGTGGGTGAAAAGTAGCAA	Yakima Yellow	62	$(TC)_{24}(AC)_8$	9	3.9	0.569	0.537	0.03	1.6
WGA321	F: TCCAATCGAAACTCCAAAGG* R: GTCCAAAGACGATGATGGA	Yakima Yellow	62	$(CT)_{18}$	18	6.067	0.804	0.78	0.196	1.4
WGA27	F: AACCCTACAACGCCTTGATG* R: TGCTCAGGCTCCACTTCC	ATTO 550	62	$(GA)_{30}$	11	3.7	0.493	0.458	0.13	0.7
WGA32	F: CTCGGTAAGCCACACCAATT R: ACGGGCAGTGTATGCATGTA*	ATTO 550	60-55	$(TC)_{19}$	11	5.933	0.831	0.809	0.277	4.1
CYC019	F: ATTCCCCACCCCCATCTC R: CTCCTCCAGCGCACATAA*	6FAM	60	$(CT)_8$	15	6.367	0.834	0.812	0.056	0.7
CYC095	F: TGGAACTGGGAACGGTG* R: ACCCCCTTCGTCGTCTTT	ATTO 565	51	$(GA)_8$	6	3.433	0.611	0.544	0	10
CYC130	F: TGCCAAGAGTGACAGATT* R: GTTGATGATAGTTTGTAGAG	6FAM	55	$(CT)_{13}(AC)_{11}$	23	6.1	0.761	0.728	0.069	0.9
CYC148	F: TCCTCCACTTCCAATGAT* R: AGAGGAGCAAACAAACAT	ATTO 565	57	$(CT)_{17}$	4	1.967	0.212	0.193	0	0.2
Mean					12.125	4.683	0.639	0.608	0.095	2.45
SD					6.29	1.64	0.22	0.22	0.1	3.28

Notes: F – forward primer, R – reverse primer, T_a = annealing temperature, *A* = allelic richness, H_E = expected heterozygosity, PIC = polymorphism information content, NAF = null allele frequencies. *Fluorescently labeled primer.

Table C5. Chloroplast DNA regions and primers used for PCR amplification in *Pterocarya fraxinifolia* and East Asian species.

cpDNA region	Primer name	Primer sequence	Reference
*trn*L intron and *trn*L–*trn*F intergenic spacer	*trn*Lc	CGAAATCGGTAGACGCTACG	Taberlet *et al.*, 1991
	*trn*Lf	ATTTGAACTGGTGACACGAG	Taberlet *et al.*, 1991
*trn*S–*trn*G intergenic spacer and *trn*G intron	*trn*S$^{(GCU)}$	AACTCGTACAACGGATTAGCAATC	Shaw *et al.*, 2007
	*trn*G$^{(UUC)}$	GAATCGAACCCGCATCGTTAG	Shaw *et al.*, 2007
*rpl*32–*trn*L intergenic spacer	*rpl*32-F	CAGTTCCAAAAAAACGTACTTC	Shaw *et al.*, 2007
	*trn*L$^{(UAG)}$	CTGCTTCCTAAGAGCAGCGT	Shaw *et al.*, 2007

References to Table C5

Shaw, J., Lickey, E.B., Schilling, E.E. & Small, R.L. (2007) Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The tortoise and the hare III. *American Journal of Botany*, 94, 275-288.

Taberlet, P., Gielly, L., Pautou, G. & Bouvet, J. (1991) Universal primers for amplification of 3 noncoding regions of chloroplast DNA. *Plant Molecular Biology*, 17, 1105-1109.

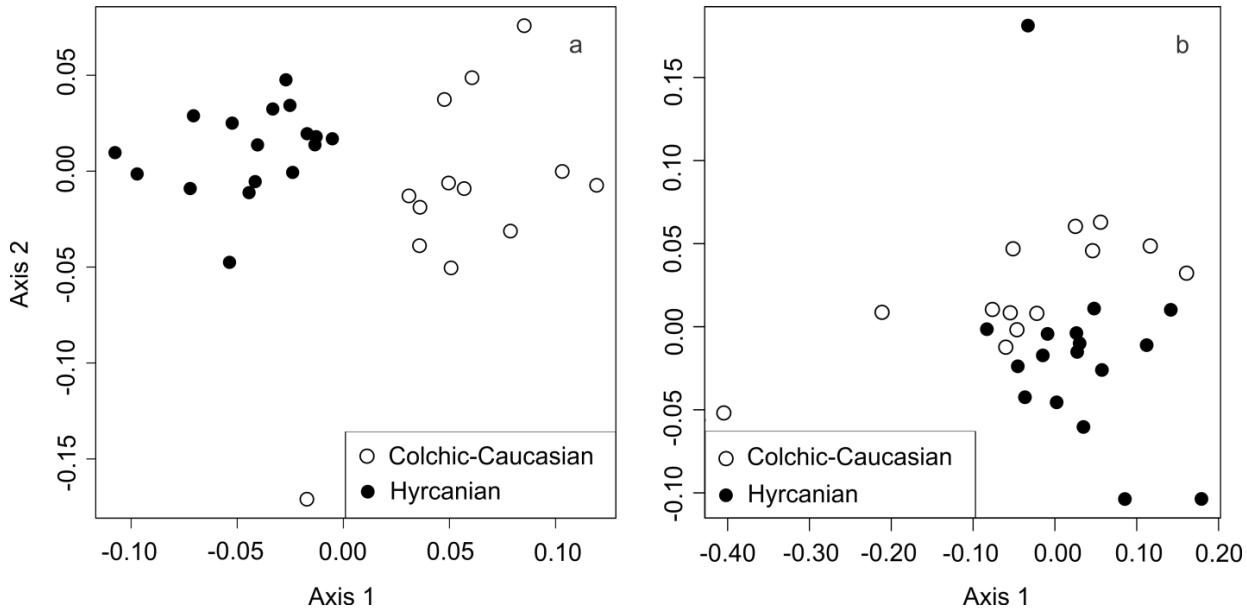

Fig. C2. Multidimensional scaling of population pairwise genetic distances estimated as F_{ST} (panel a) and R_{ST} (panel b). Circles represent populations of *Pterocarya fraxinifolia* from Colchic-Caucasian (empty circles) and Hyrcanian (black circles) groups in South Caucasus.

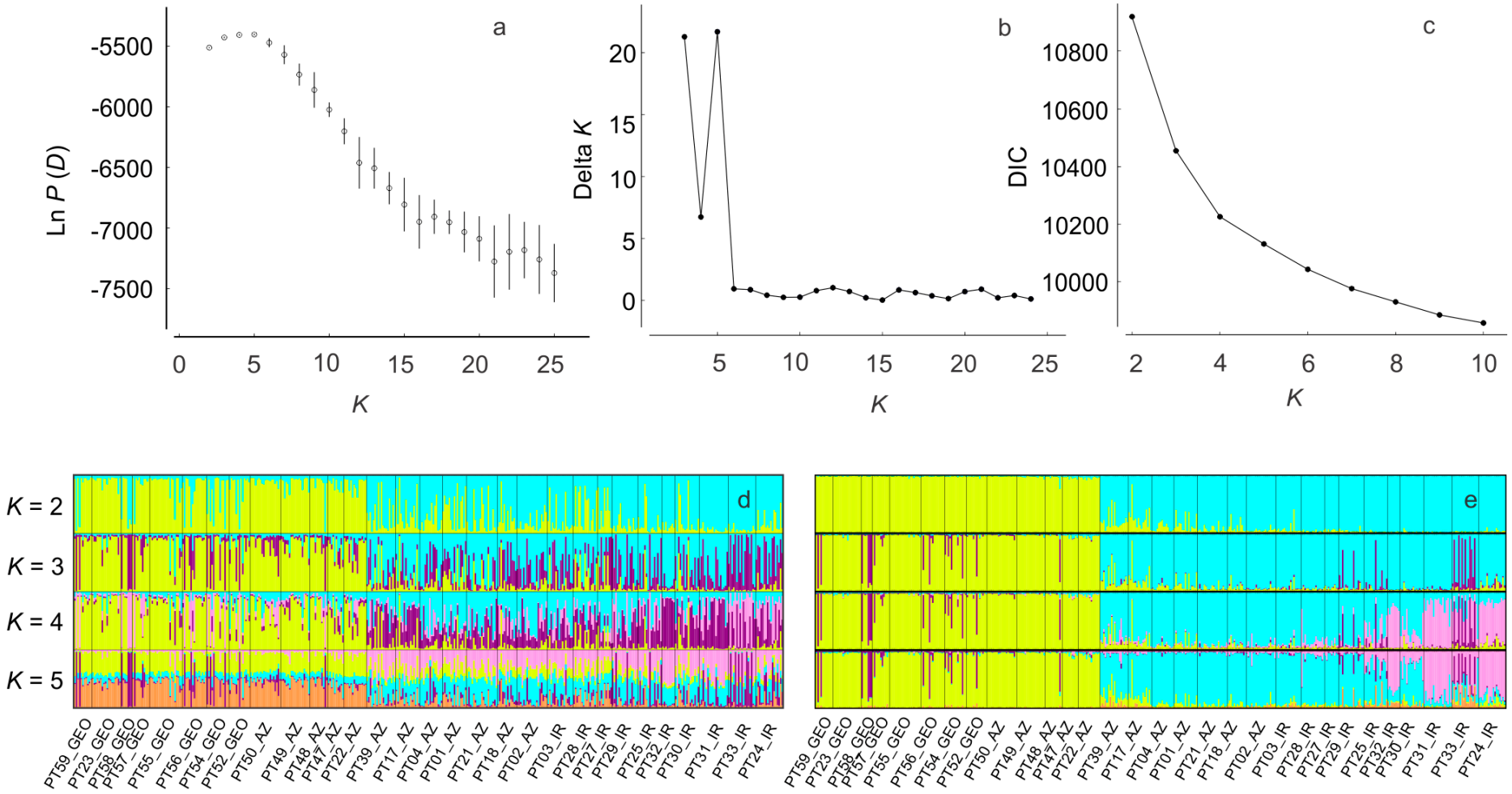

Fig. C3. Genetic structure of populations of *P. fraxinifolia* in South Caucasus and northern Iran inferred using Bayesian clustering implemented in STRUCTURE (panels a, b, d) and TESS (panels c & e). Maximum number of clusters (*K*) is inferred based on the posterior probability of the data for a given *K* (Ln *P* (*D*); panel a), the rate of change in the log probability of the data between successive *K* values (Delta *K*; panel b), and Deviance Information Criterion (DIC; panel c). Panels d & e – graphical representation of individuals' cluster membership coefficients from *K*=2 to *K*=5, where division for *K*=2 corresponds to Colchic–Caucasian (yellow cluster) and Hyrcanian (blue cluster) groups

Table C6. Pairwise genetic distances estimated as R_{ST} (above diagonal) and F_{ST} (below diagonal) for 30 natural populations of *Pterocarya fraxinifolia*. Significant values are shown in *Italics* ($P < 0.05$).

	PT59	PT23	PT58	PT57	PT55	PT56	PT54	PT52	PT50	PT49	PT48	PT47	PT22	PT39	PT17	PT04	PT01	PT21	PT18	PT02	PT03	PT28	PT27	PT29	PT25	PT32	PT30	PT31	PT33	PT24
PT59		0.028	*0.133*	0.04	*0.072*	0.037	0.009	0.02	0.024	*0.083*	0.009	0.013	*0.053*	*0.092*	*0.083*	*0.132*	*0.093*	*0.16*	*0.088*	*0.119*	*0.123*	*0.111*	*0.146*	*0.158*	*0.113*	*0.234*	*0.111*	*0.166*	*0.131*	*0.076*
PT23	-0.012		*0.204*	*0.053*	*0.09*	*0.076*	*0.061*	*0.059*	*0.106*	*0.068*	*0.053*	0.012	*0.045*	*0.094*	*0.071*	*0.191*	*0.144*	*0.191*	*0.144*	*0.156*	*0.169*	*0.14*	*0.187*	*0.2*	*0.138*	*0.258*	*0.135*	*0.221*	*0.179*	*0.139*
PT58	*0.271*	*0.198*		*0.078*	*0.265*	*0.119*	*0.095*	*0.151*	*0.199*	*0.231*	*0.152*	*0.212*	*0.142*	*0.167*	*0.169*	*0.192*	*0.175*	*0.198*	*0.146*	*0.186*	*0.164*	*0.207*	*0.19*	*0.142*	*0.116*	*0.225*	*0.13*	*0.191*	*0.086*	*0.178*
PT57	0.093	0.053	-0.012		*0.094*	0.012	0.033	0.024	*0.057*	*0.058*	*0.055*	*0.071*	0.03	*0.095*	*0.085*	*0.133*	*0.095*	*0.138*	*0.113*	*0.122*	*0.109*	*0.109*	*0.124*	*0.11*	*0.104*	*0.163*	*0.101*	*0.178*	*0.108*	*0.109*
PT55	-0.01	-0.001	*0.301*	*0.132*		*0.036*	*0.088*	*0.06*	*0.068*	-0.001	*0.052*	*0.052*	*0.095*	*0.106*	*0.094*	*0.158*	*0.102*	*0.174*	*0.128*	*0.112*	*0.127*	*0.09*	*0.101*	*0.173*	*0.126*	*0.218*	*0.126*	*0.205*	*0.173*	*0.086*
PT56	-0.028	-0.028	*0.202*	0.066	-0.013		0.026	0.002	*0.023*	*0.04*	0.007	*0.048*	*0.058*	*0.068*	*0.056*	*0.092*	*0.064*	*0.115*	*0.059*	*0.072*	*0.073*	*0.063*	0.035	*0.105*	*0.074*	*0.122*	*0.066*	*0.157*	*0.097*	*0.074*
PT54	0.013	*0.072*	*0.397*	*0.219*	0.053	0.03		0.006	*0.046*	*0.071*	-0.002	0.018	*0.053*	*0.078*	*0.069*	*0.118*	*0.066*	*0.121*	*0.063*	*0.105*	*0.09*	*0.106*	*0.1*	*0.122*	*0.069*	*0.162*	*0.069*	*0.128*	*0.075*	*0.048*
PT52	*0.09*	*0.132*	*0.513*	*0.31*	*0.09*	*0.075*	0.025		-0.001	*0.06*	-0.016	0.015	*0.058*	*0.086*	*0.063*	*0.097*	*0.044*	*0.124*	*0.063*	*0.09*	*0.076*	*0.093*	0.063	*0.116*	*0.099*	*0.174*	*0.09*	*0.176*	*0.111*	*0.089*
PT50	0.024	*0.071*	*0.453*	*0.242*	0.031	0.03	*0.055*	0.027		*0.1*	0.004	*0.065*	*0.107*	*0.144*	*0.134*	*0.15*	*0.093*	*0.189*	*0.106*	*0.14*	*0.132*	*0.148*	*0.118*	*0.18*	*0.165*	*0.247*	*0.155*	*0.218*	*0.167*	*0.116*
PT49	-0.017	-0.015	*0.223*	0.084	-0.025	-0.021	0.046	*0.088*	0.038		*0.069*	*0.043*	*0.067*	*0.081*	*0.067*	*0.151*	*0.098*	*0.14*	*0.129*	*0.098*	*0.113*	*0.076*	*0.103*	*0.142*	*0.094*	*0.181*	*0.104*	*0.194*	*0.145*	*0.077*
PT48	0.014	0.047	*0.415*	*0.222*	0.015	0.006	-0.001	-0.008	0.007	0.02		0.012	*0.093*	*0.08*	*0.072*	*0.117*	*0.061*	*0.147*	*0.049*	*0.102*	*0.086*	*0.114*	0.066	*0.153*	*0.093*	*0.196*	*0.085*	*0.155*	*0.108*	*0.068*
PT47	*0.138*	*0.153*	*0.565*	*0.345*	0.086	*0.093*	*0.074*	-0.012	*0.056*	*0.086*	-0.002		0.037	*0.036*	0.015	*0.089*	*0.047*	*0.096*	*0.048*	*0.068*	*0.077*	*0.061*	*0.102*	*0.123*	*0.076*	*0.202*	*0.067*	*0.141*	*0.126*	*0.055*
PT22	-0.021	-0.011	*0.214*	0.065	0.002	-0.02	0.04	*0.101*	0.054	-0.011	0.045	*0.135*		*0.07*	*0.04*	*0.122*	*0.09*	*0.115*	*0.11*	*0.098*	*0.106*	*0.075*	*0.149*	*0.088*	*0.082*	*0.159*	*0.081*	*0.169*	*0.12*	*0.103*
PT39	0.043	*0.074*	*0.264*	*0.182*	*0.054*	*0.042*	0.01	-0.016	*0.035*	*0.056*	-0.027	-0.028	*0.061*		0	*0.051*	*0.057*	*0.029*	*0.031*	*0.018*	*0.037*	0.018	*0.091*	*0.059*	0.018	*0.135*	*0.036*	*0.11*	*0.071*	*0.062*
PT17	*0.098*	*0.141*	*0.376*	*0.272*	*0.104*	*0.096*	0.042	-0.002	*0.072*	*0.105*	-0.001	-0.023	*0.12*	-0.026		*0.049*	*0.049*	*0.042*	*0.032*	*0.022*	*0.039*	0.019	*0.066*	*0.056*	0.024	*0.117*	*0.027*	*0.12*	*0.09*	*0.07*
PT04	*0.18*	*0.174*	*0.446*	*0.3*	*0.13*	*0.132*	*0.159*	*0.106*	*0.166*	*0.12*	*0.106*	*0.075*	*0.125*	0.032	*0.055*		-0.013	-0.002	0	-0.003	-0.016	0.004	0.051	0.02	*0.047*	*0.167*	0.023	*0.089*	*0.089*	*0.075*
PT01	*0.223*	*0.218*	*0.574*	*0.381*	*0.156*	*0.165*	*0.181*	*0.092*	*0.153*	*0.147*	*0.126*	*0.071*	*0.166*	0.027	*0.048*	-0.014		*0.027*	0.01	0.019	-0.006	0.017	0.038	*0.029*	*0.046*	*0.141*	0.017	*0.082*	*0.074*	*0.045*
PT21	*0.117*	*0.129*	*0.359*	*0.245*	*0.099*	*0.097*	*0.085*	*0.064*	*0.129*	*0.091*	*0.058*	0.043	*0.084*	0.023	*0.044*	-0.021	0.005		*0.03*	0.001	0.002	0.017	*0.086*	0.013	*0.031*	*0.147*	*0.032*	*0.1*	*0.076*	*0.078*
PT18	*0.165*	*0.173*	*0.48*	*0.329*	*0.142*	*0.12*	*0.098*	0.024	*0.13*	*0.133*	0.02	0.004	*0.155*	-0.03	-0.023	0.047	*0.057*	0.024		0.011	0.005	*0.031*	0.022	*0.049*	*0.034*	*0.155*	0.017	*0.072*	*0.063*	*0.053*
PT02	*0.06*	*0.093*	*0.346*	*0.226*	*0.055*	*0.057*	0.037	-0.017	0.023	*0.06*	-0.026	-0.034	*0.067*	-0.013	-0.008	0.013	-0.006	0.017	-0.015		0	-0.007	*0.042*	*0.023*	*0.026*	*0.134*	*0.026*	*0.116*	*0.072*	*0.061*
PT03	*0.118*	*0.142*	*0.394*	*0.272*	*0.1*	*0.102*	*0.09*	0.019	*0.078*	*0.1*	0.027	-0.01	*0.107*	0	0.005	-0.014	-0.024	0.002	-0.01	-0.02		0.016	0.035	0.013	*0.027*	*0.128*	0.014	*0.083*	*0.061*	*0.054*
PT28	*0.062*	*0.053*	*0.327*	*0.161*	0.016	0.024	*0.099*	*0.095*	*0.079*	-0.003	0.043	*0.081*	0.035	-0.014	0.046	0.032	*0.068*	0.03	*0.085*	0	0.001		*0.063*	0.026	0.02	*0.138*	0.016	*0.093*	*0.08*	*0.049*
PT27	*0.198*	*0.204*	*0.528*	*0.365*	*0.135*	*0.148*	*0.145*	*0.108*	*0.159*	*0.128*	*0.058*	0.033	*0.207*	-0.017	-0.019	*0.08*	*0.154*	*0.074*	0.032	0.011	0.03	*0.116*		*0.082*	*0.064*	*0.14*	0.041	*0.14*	*0.103*	*0.092*
PT29	0.057	*0.097*	*0.242*	*0.171*	0.084	0.063	0.02	0	0.063	0.079	0.014	0.006	0.049	0.002	0.006	0.007	0.004	-0.002	-0.003	0.004	0.001	0.001	0.036		*0.035*	*0.13*	*0.037*	*0.108*	*0.06*	*0.091*
PT25	*0.099*	*0.141*	*0.342*	*0.251*	*0.108*	*0.099*	0.038	0.024	*0.111*	*0.104*	0.028	0.007	*0.101*	-0.014	-0.013	0.025	0.032	0	-0.013	0.001	0.005	0.031	0.011	-0.016		*0.08*	-0.008	*0.056*	0.029	*0.036*
PT32	*0.087*	*0.156*	*0.369*	*0.235*	*0.152*	*0.106*	*0.074*	*0.142*	*0.127*	*0.131*	0.056	*0.197*	*0.158*	*0.053*	*0.088*	*0.306*	*0.351*	*0.234*	*0.204*	*0.119*	*0.191*	*0.211*	*0.2*	0.093	*0.115*		*0.089*	*0.142*	*0.136*	*0.135*
PT30	0.024	0.01	*0.22*	*0.093*	0.004	-0.005	*0.06*	*0.083*	*0.07*	-0.013	0.023	*0.079*	-0.005	0.015	*0.065*	0.037	*0.089*	0.018	*0.087*	0.028	0.041	-0.026	*0.107*	0.011	0.038	*0.18*		0.024	0.014	-0.029
PT31	*0.061*	*0.096*	*0.349*	*0.224*	0.048	*0.062*	0.035	0.01	*0.05*	*0.051*	-0.022	-0.011	*0.053*	0.006	0.015	-0.004	-0.011	-0.007	0.018	-0.011	-0.009	-0.011	0.032	-0.001	-0.017	*0.156*	*0.038*		*0.082*	*0.043*
PT33	0.016	0.016	0.018	-0.004	0.052	0.012	0.054	*0.106*	*0.102*	0.028	0.047	*0.116*	0.013	*0.077*	*0.123*	*0.14*	*0.163*	*0.114*	*0.131*	*0.112*	*0.134*	0.024	*0.142*	0.074	*0.097*	0.079	*0.042*	*0.103*		*0.066*
PT24	-0.01	-0.002	*0.17*	0.07	-0.019	-0.01	0.03	0.052	0.024	-0.022	-0.007	0.042	-0.019	0.039	*0.068*	0.038	0.067	0.022	*0.083*	0.026	0.05	-0.049	0.081	0.033	0.043	*0.116*	*0.04*	0.016	0.027	