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DISSERTATION

Titel der Dissertation

Phylogeography, Flowering Phenology and Cytonuclear Interactions of *Populus alba* and *P. tremula*: Two European Hybridising Forest Trees

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Content

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"I saw a fox far away near the forest side, but when it came closer I noticed it wasn't a fox at all
but a dog.
That goes to show how clever the fox was."

Antti Sadinmaa

Abstract

During the long glacial periods of the Quaternary, European forests were considerably more restricted than today because of the Mediterranean Sea in the south and unsuitable environment in the north. Two ecologically important tree species of the genus *Populus*: the white poplar (*Populus alba,* L.) and the European Aspen (*Populus tremula,* L.), are distributed along European floodplain areas and adjacent upland forests, respectively. Both have extended their natural ranges to the present area after the last glaciation. Lacking fossil pollen data for poplar hampers the search for glacial refugia – these refugia are assumed in South-eastern Europe, but there is no convincing proof of this hypothesis yet.

The central aim of the first chapter was to clarify the picture of postglacial recolonisation and the reconstruction of refugia of *P. alba* and *P. tremula* in the light of hybridisation of the two species. I investigated 26 populations located in Central and South-eastern Europe using maternally inherited chloroplast (cp) markers. Phylogeographic structure was found for *P. alba* with low diversity in Eastern Europe versus high diversity in Italy and Central Europe. In contrast, lack of phylogeographic structure seems to prevail for *P. tremula,* as can be expected for a boreal forest tree. Two main refugia were identified for *P. alba* in Italy and Romania, but in contrast *P. tremula* probably recolonised its present habitats in Central Europe from several refugia near the former ice cap. I assume separate disconnected refugia for *P. alba* and *P. tremula* and suggest an immigration scenario involving the mixing of colonization routes and interspecific introgression to be responsible for the observed patterns.

In order to test whether these patterns can be useful in assigning individuals to populations, the genetic diversity of *P. alba* of Malta was assessed. Altogether 38 samples from the two different, although neighbouring islands of Malta and adjacent regions within the Mediterranean were analysed. Nuclear microsatellite analysis (nuSSR) revealed that the 28 sampled trees of Malta belonged to one clone. Chloroplast data suggested relatedness of the Maltese clone to Italian *P. alba* samples. However, nuclear data revealed additional admixture through pollen from North Africa. Human activities for ornamental purposes can be considered the main cause for introducing this clone in the 16th century.

In order to find a more formal way of assigning individuals to species and hybrids when sampling individuals within hybrid zones, morphological traits were

tested. The main purpose of assessing the utility of leaf morphological parameters for species assignment was to establish an easy measurable trait, which can be applied in the field. I present a method of separating *P. alba*, *P. ×canescens* (Aiton, Sm) and *P. tremula* in an Austrian hybrid zone using the degree of "lobedness" of the leaves, supported by two additional parameters, which identify *P. ×canescens*, the hybrid taxon. The statement in the literature that “lobedness” is a distinctive character in white poplar is now measurable by the HF index and the L1/L2 ratio. Furthermore, the two-step sampling approach was successful in dealing with heterophylly and identifying the most valuable type of shoot for morphometry in the studied taxa.

Another aspect of hybridisation in nature is that plant species have to overlap in their flowering time in order to hybridise. *P. alba* and *P. tremula* are dioecious trees occurring in several hybrid zones across Europe. Here I used a small scale approach to study prezygotic barriers to gene flow based on flowering phenological observations. Flowering assessment was carried out in two consecutive years in three study plots and a total of 27 trees. Overall flowering period varied between individuals and years. However, in both years male trees started flowering earlier than females of both species on each site (protandry). Here, overlapping flowering times due to local climatic and site conditions between species on different sites facilitated hybridisation. In fact, I observed not very strong barriers to gene flow, although year to year climatic variation can influence this pattern.

In order to test for postzygotic barriers to gene flow I studied the interaction between cytoplasmic and nuclear gene markers. Chloroplast and nuclear DNA of altogether 541 individuals in 23 populations of *P. alba* and *P. tremula* in Central Europe were investigated. Within *P. alba,* stronger geographic structuring was found compared to *P. tremula* based on cpDNA and twenty nuSSRs, presumably due to recolonisation from disconnected glacial refugia. Conversely, in *P. tremula* genetic variation was more evenly distributed across the studied populations. Hybridisation was observed in both directions in zones of contact. Significant cytonuclear interactions were detected within hybrids (*P. ×canescens*) and *P. alba* at six and four nuclear loci, respectively. Although factors like assortative mating and migration may play a role in their origin, those are likely to affect all loci. Thus, selective mechanisms are a more likely explanation for these patterns. Linkage of microsatellite markers to certain genes involved in cytonuclear processes might cause the observed disequilibria, especially in

early generation hybrids carrying relatively large chromosome blocks inherited from each parental species.

Zusammenfassung

Während der langen Eiszeiten des Quartärs waren die Europäischen Wälder stärker eingeengt als heutzutage, wegen dem Mittelmeer im Süden und der unwirtlichen Umweltbedingungen im Norden. Zwei ökologisch wichtige Baumarten der Gattung *Populus*: Weisspappel (*Populus alba,* L.) und Zitterpappel (*Populus tremula,* L.), sind entlang von Europäischen Auwaldlandschaften beziehungsweise in angrenzenden höher gelegenen Wäldern verbreitet. Beide haben ihr natürliches Verbreitungsgebiet nach der letzten Eiszeit auf ihr heutiges Areal ausgedehnt. Fehlende Pollendaten für Pappeln erschwert die Suche nach Glazialrefugien – diese Refugien werden in Südeuropa vermutet, jedoch fehlen bislang überzeugende Beweise für diese Hypothese.

Das Hauptziel des ersten Kapitels war die Klärung der postglazialen Rückwanderung nach der letzten Eiszeit und die Rekonstruktion von Refugien von *P. alba* und *P. tremula* im Licht von Hybridisierung der beiden Arten. Ich untersuchte 26 Populationen aus Mittel- und Südosteuopa mithilfe von mütterlich vererbten Chloroplastenmarkern (cpDNA). Phylogeografische Strukturen wurden für *P. alba* gefunden, mit niedriger Diversität in Osteuropa und hoher Diversität in Italien und Mitteleuropa. Für *P. tremula* wurde keine Struktur gefunden, was bei einer borealen Baumart durchaus erwartet werden kann. Zwei Hauptrefugien können demnach für *P. alba* in Italien und Rumänien vermutet werden, hingegen hat *P. tremula* ihre heutigen Standorte in Mitteleuropa von mehreren Refugien nahe am früheren Eisschild zurückerobert. Ich vermute deshalb gesonderte Refugien für *P. alba* und *P. tremula* und schlage ein Immigrationsszenario vor, das die Vermischung der Wanderungswege und interspezifische Introgression für das beobachtete Muster verantwortlich macht.

Um zu testen, ob dieses Muster geeignet ist, um Individuen einer bestimmten Population zuzuordnen, wurde die genetische Diversität von *P. alba* auf Malta untersucht. Insgesamt wurden 38 Proben von den beiden benachbarten Inseln und Nachbarregionen innerhalb des Mittelmeergebietes getestet. Die Untersuchung mittels Kernmikrosatelliten (nuSSR) ergab, daß die 28 untersuchten Bäume von Malta einen Klon darstellten. Chloroplastenmarker deuten auf die Verwandtschaft des Klons mit italienischen *P. alba* Proben hin. Allerdings stammt väterlicher Anteil vermutlich auch

von Pollen aus Nordafrikanischen Populationen. *P. alba* gibt es auf Malta vermutlich seit dem16. Jh. Wahrscheinlich sind menschliche Aktivitäten und die Funktion des Baumes als Zierpflanze für dessen Ursprung und Verbreitung auf Malta verantwortlich.

Um einen formalen Weg zu finden, Individuen den Arten und dem Hybriden bei Beprobungen in Hybridzonen zuzuordnen, wurden morphologische Merkmale getestet. Hierbei wurde versucht abzuschätzen, inwiefern blattmorphologische Merkmale für die Artzuordnung brauchbar waren, zugleich aber sollten es einfache Merkmale sein, die auch im Freiland gemessen werden können. Ich stelle eine Methode vor, die *P. alba*, *P. ×canescens* (Aiton, Sm) und *P. tremula* in einer Hybridzone in Österreich unterscheiden kann. Dabei wird die „Gelapptheit“ der Blätter gemessen, und unterstützt von zwei zusätzlichen Merkmalen identifizieren diese den Hybriden *P. ×canescens*. Die Anmerkung in der Literatur, daß “Gelapptheit” ein ausgeprägtes Merkmal für die Weißpappel ist, kann nun über den HF-index und das L1/L2 Verhältnis gemessen werden. Darüber hinaus konnte gezeigt werden, daß die zweistufige Vorgehensweise zur Probensammlung erfolgreich war, um die Heterophylie zu behandeln. Hier wurden die nützlichsten Zweigtypen für Morphometrie in den untersuchten Arten identifiziert.

Für die Hybridsierung von zwei Pflanzenarten müssen diese in ihren Blütezeiten überlappen. *P. alba* und *P. tremula* sind diözische Bäume, die mehrere Hybridzonen in Europa bilden. Hier verwendete ich einen kleinräumigen Ansatz, um präzygotische Barrieren für den Genfluß zu studieren, der auf blühphänologischen Beobachtungen basiert. Die Beobachtungen dazu wurden in zwei aufeinanderfolgenden Jahren auf drei Standorten und insgesamt an 27 Bäumen durchgeführt. Die Gesamtblühdauer variierte zwischen Individuen und Jahren. Jedoch blühten in beiden Jahren die männlichen Bäume vor den weiblichen in beiden Arten und auf allen Standorten (sog. „Protandrie“). Lokales Klima und unterschiedliche Standortseigenschaften ermöglichten ein Überlappen der Blühzeiten der beiden Arten. Insgesamt wurden keine sehr starken Barrieren gegenüber Genfluß beobachtet, jedoch beeinflussen jährliche Kimavariationen dieses Muster.

Um postzygotische Barrieren gegenüber Genfluß zu testen, untersuchte ich die Interaktion zwischen zytoplasmatischen und nuklearen Genmarkern. In dieser Studie wurde cpDNA und Kern-DNA von insgesamt 541 Individuen aus 23 Populationen von *P. alba* und *P. tremula* aus Mitteleuropa untersucht. Innerhalb von *P. alba* wurden stärkere geografische Strukturen gefunden, als für *P. tremula*, vermutlich aufgrund von Wiedereinwanderung aus unterschiedlichen Glazialrefugien. Im Gegensatz dazu war

die Variation der cp-DNA einheitlicher über die untersuchten Populationen verteilt. Hybridisierung wurde in beide Richtungen in den Kontaktzonen der beiden Arten beobachtet und die meisten Hybriden waren aus der F1 oder F2 Generation. Signifikante zytonukleare Interaktionen wurden innerhalb der Hybride und in *P. alba* an sechs beziehungsweise vier Kernmarkern festgestellt. Obwohl Faktoren wie nicht-zufällige Paarung und Migration eine Rolle spielen können, wirken diese eher auf alle Genorte gleichzeitig. Daher sind wahrscheinlich selektive Mechanismen eine bessere Erklärung für die beobachteten Muster. Kopplung von Mikrosatelliten mit bestimmten Genen, die in zytonukleare Interaktionen involviert sind, könnten das beobachtete Ungleichgewicht erzeugt haben; speziell in Hybriden der ersten Generationen, die relativ große Chromosomenblöcke enthalten, die sie von ihren Elternarten geerbt haben.

General introduction

Species description

Populus species are diploid (2n=38) with relatively small genome sizes (550Mb). This and several other advantages make the genus a "model forest tree" (Bradshaw et al. 2000) and the sequencing of its whole genome was completed in 2006 (Tuskan et al 2006). Central Europe is host to two ecologically important tree species of the genus *Populus*: European Aspen (*Populus tremula*) and white poplar (*Populus alba*). Both have extended their natural ranges to this area after the last glacial maximum (LGM). The genus *Populus* together with *Salix* and *Chosenia* belong to the family of Salicaceae. *P. alba* and *P. tremula* are dioecious, outcrossing European forest trees. *P. tremula* is a pioneer species distributed from Europe to Siberia, missing only in the South of the Iberian peninsula (Fig. 1). Aspen reaches into the submontane and montane zones (Adler et al.1994) up to 1360 m (altitude) in the northern parts of the Alps, and up to 2000 m in the Central Alps. *P. alba* (white poplar) is considered a pioneer species in lowland floodplain areas of Europe (except in the northern parts), Western Asia and Northern Africa and reaches up to 500 m. *Populus ×canescens* is a natural hybrid between the two species *(= P. alba × P. tremula)* and it forms where the habitats of *P. tremula* meet with those of *P. alba* (Adler et al. 1994). *P. ×canescens* trees are located predominantly in the vicinity of *P. alba* trees in the floodplain forest (Lazowski 1997); this is supported by Lexer et al (2005) stating that introgression is mostly unidirectional from *P. tremula* via pollen towards *P. alba.*

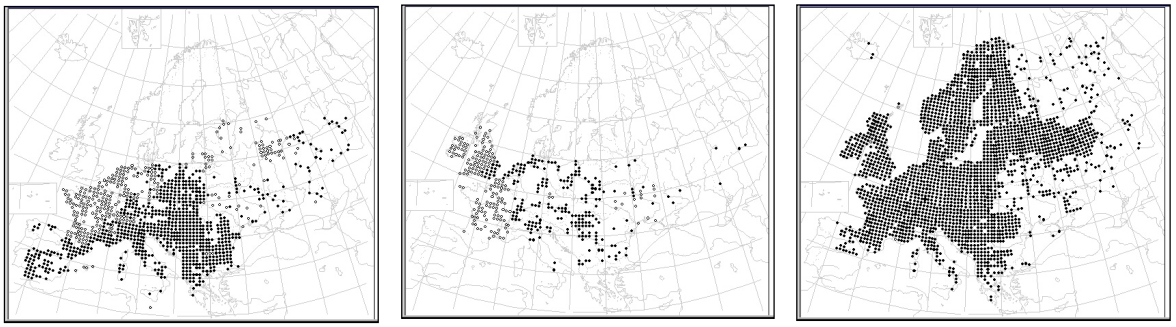

Fig. 1 European distribution range of *Populus alba*, *Populus* ×*canescens* and *Populus tremula*; from left to right (taken from the Florae Europaea, Royal Botanic Garden Edinburgh, Inverleith Row, Edinburgh, EH3 5LR, United Kingdom; http://rbg-web2.rbge.org.uk/FE/fe.html)

Pollen record

Fossil pollen records and charcoal remains indicate the survival of trees at isolated favourable spots at the southern edge of the permafrost line at the time of the LGM between 17,000 and 25,000 years ago (Taberlet 1998). For instance Willis et al. (2000) dated charcoal remains of *Populus* on a rockshelter in Slovenia back to approx. 25,000 years BP, and 7 tree species (*Pinus, Picea, Betula, Juniperus, Salix, Larix* and *Carpinus*) in Hungary back to more than 17,000 years ago. Data indicate a more open environment and a low tree density (Willis et al. 2000). In other words, as steppe and tundra dominated Central Europe, trees must have survived the glaciations in small populations located in sheltered microclimates without leaving much evidence in the pollen record (Stewart & Lister 2001). Comps et al (2001) have studied European beech, a wind pollinated species, for which a detailed pollen record is available, and found no hybridisation between closely related species that could blur the picture. This is in contrast to *Populus*, where rare pollen data is available in Europe, because European poplar pollen is less well conserved compared to other species; that is because of low content of sporopollin in the exine layer of the pollen grain (Havinga 1984, in Bittkau 2002). Lacking pollen data for poplar hampers the search for glacial refugia (Huntley & Birks 1983). Although in low concentration (in general 1 %, seldomly 5 %), there is evidence for poplar pollen 9.000 BP along the north-western rims of the Alps in the vicinity of the maximum extension of the ice sheet during the LGM (Huntley & Birks 1983). This points to survival of poplars in the Northern Alpenvorland together with southern refugia (Burga & Perret1998, in Bittkau 2002). To complicate matters the European poplar species can not be easily differentiated on the

basis of pollen data. This would be important to know for reconstruction of ice age refugia, because the two species prefer contrasting climatic environments. *P. alba* is considered a floodplain pioneer species up to 600 m altitude, prefers rather warm temperature compared to *P. tremula* which grows as an upland pioneer species up to 2000 m and can withstand severe frost. All evidence for poplar pollen in early Holocene most probably stems from *P. tremula,* the only poplar species with a boreal distribution in Europe.

Biogeography and climatic influences

During the last glaciation, ice covered large parts of Northern Europe, including Scandinavia and Northern Britain, as well as mountain ranges such as the Alps and the Pyrenees (Taberlet 1998). European forests in that period were considerably more restricted than today because the Mediterranean Sea in the south prevented southward escape of forest tree species from northern rough climate (Petit et al 2003). Southern and parts of Central Europe were dominated by steppe vegetation (Elenga et al. 2000, in Palmé 2003) and the more northern parts by tundra (Huntley & Birks 1983).

Phylogeographical studies of taxa with a history of range changes during the Quaternary ice ages represent insights into the evolution and dynamic nature of species (Widmer & Lexer 2001). After the retreat of the ice sheet some of the populations expanded, but others became extinct or survived by shifting their altitudes (Petit et al 2003). The present genetic structure of populations is defined by the number and location of refugia during quaternary cold periods, together with human influence. Taberlet et al. (1998) suggest three main refugia around the Mediterranean, evident from 10 model plant and animal taxa; however postglacial colonization routes differed widely between species. Warm-loving trees conform well to this pattern e.g. *Quercus sp*. (Dumolin-Lapègue et al. 1997), whereas the survival of more boreal distributed taxa is attributed to more diffuse populations further north (*Acer sp., P. tremula*, Bittkau 2002). The phylogeographic pattern of *Acer sp*. does not fit into the scheme of three main refugia in Southern Europe, because the potential of peripheral regions of the Alps has been underestimated (Bittkau 2002). Brewer et al. (in Bittkau 2002) indicate that the Alps were of great importance as refugia for non-thermophilous species like *Acer*, *Alnus*, *Betula*, *Corylus*, *Fraxinus* und *Hedera* and had taken a pronounced part in recolonialization of Central Europe.

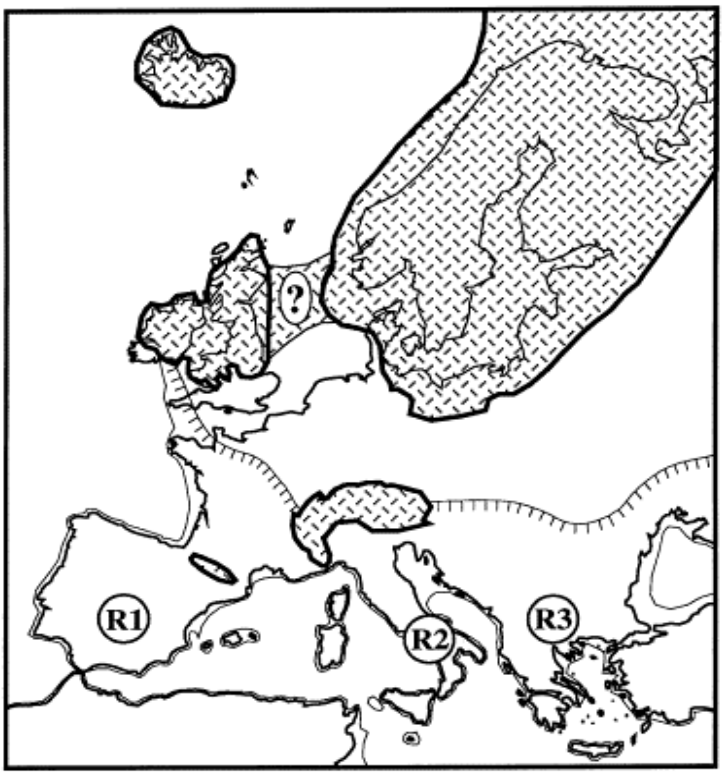

Fig. 2 Maximum extension of the ice sheet in Europe during the last cold period, 20.000 –18.000 y ago (redrawn from Frenzel et al. 1992; Lundqvist &Saarnisto 1995). R1, R2 and R3 indicate three main potential refugia in Portugal-Spain, in Italy and in the Balkans, respectively. The southern limit of the permafrost is indicated by the scaled line. Lowered sea shore is shown by the thinner line at the 100 m submarine contour (taken from Taberlet et al. 1998).

However the existence of northern refugia is controversially discussed (Willis et al. 2000, Carcaillet and Vernet 2001, Stewart and Lister 2001). During the LGM, trees grew as far north as Hungary, probably in micro-environmentally favourable sites (Willis et al. 2000). This is supported by pollen climate reconstructions for the Holocene, revealing major spatial and seasonal differences in temperature trends within a remarkably balanced regional and annual energy budget (Davis et al. 2003). Eastern areas, close to the former ice sheet of the Alps, provided an important cold-stage refugium for the European flora and fauna (Willis et al. 2000).

The genera *Salix* (Palmé *et al.* 2003) and *Populus* have been assumed to have survived not only in refugia in Southern Europe, but also in climatic suitable habitats in the north. Therefore, populations of both taxa were not as isolated compared to those of thermophilous species with restricted refugia in Southern Europe. Divergence during the LGM has been blurred due to high gene flow. The influence of genetic drift might have been of minor importance (Bittkau, 2002). However, phylogeographic patterns are species specific, because of dispersal rate, competition with other species and occasional local extinctions, therefore research on particular species is required to inquire historical influences on the present distribution of the species of interest (Comes & Kadereit 1998).

Speciation and hybridisation

The formation of new species is an interesting issue for biologists and speciation remains a big question, even after 200 years of debate (Darwin 1859, in Hewitt 2001). One of the forces influencing speciation is hybridisation. In general, 25% of the plant species are affected by hybridisation and introgression (Mallet 2005) and hybridisation is not thought to be a dead-end in evolution anymore (e.g. Gömöry & Schmidtova 2007). In the evolution of plants it has long been recognized that hybridisation can lead to new phenotypes and even to new species (hybrid speciation, Turelli et al. 2001, Mallet 2007). Experimental hybridisation is used as a tool in plant breeding and has long been used in *Populus*, because it is relatively easy to perform interspecific crossings within the genus. By artificial hybridisation, numerous economically important clones were derived and planted worldwide e.g. for biomass production, the pulp and paper industry, and agroforestry. Hybridisation in *Populus* is known to have played a major role during evolution of the genus *Populus* (Hamzeh & Dayanandan 2004) and is considered the main reason for controversial species numbers within the genus. From 22 (Eckenwalder 1996) up to 85 species were proposed, some representing either hybrids or polymorphic variants of single diverse taxa (Dickmann & Kuzovkina 2008).

Two concepts about the origin of hybrid zones have been debated – primary divergence and secondary contact (Edler 1982 in Hewitt 2001). The latter accounts for most of the hybrid zones in the Alps and along the Pyrenees, because they could not have been there during the LGM and must have only formed around 9,000 BP (Hewitt 1985, in Hewitt 2001). Thus the role of geography in speciation through the Pleistocene

has been considered (Hewitt 2001). Postglacial colonization routes were shaping contact zones of divergent lineages, which establish hybrids or even new species. Although the aforementioned might be true for most of the present European tree species, some studies suggest refugia closer to the ice caps for non-thermophilous species like *P. tremula* (Bittkau, 2002), and *Salix sp*. (Palmé, 2003). Present-day zones for *P. ×canescens* hybrids are located in floodplain areas close to mountain ranges, because of the ecological requirements of the parental species *P. alba* and *P. tremula*, respectively.

Phenological observations

The European Aspen (*P. tremula*) and the white poplar (*P. alba*) are deciduous, dioecious and wind-pollinated tree species. The flowers appear in predominantly unisexual pendulous catkins before the leaves in early spring – only a few hermaphrodite catkins are observed. Fruits of both species consist of dehiscing capsules, which open at maturity and release large quantities of seeds which are dispersed by wind and water.

The two species have wide overlapping geographic ranges in floodplain areas and adjacent mountain regions. Hybridisation in nature requires that species have an overlap in flowering time. Hungarian studies of flowering times of *P. alba* and *P. tremula* have revealed that phenograms of populations on sandy sites have a larger overlap compared to populations on water influenced sites, thus hybridisation is expected more frequently on sandy ground (Bartha 1989). Protrandy has already been observed in *Populus* (Farmer 1966, in Bartha 1989), as well as for other species e.g. *Quercus sp*. (Bacillieri et al. 1995). Furthermore, Vanden Broeck (2003) did not find significant protrandy in the first year of observations, but in the second year male flowers of *P. ×canadensis* and *P. nigra* ripened and shed pollen a few days before females; this is to ensure that enough pollen is in the air when female flowers open. Pollen is viable for a few days, whereas females are receptive only for 20-30 hours (Baumeister 1964, in Bartha 1989). The onset of flowers in the poplar plantation in the Belgian study started after a temperature peak (Vanden Broeck 2003), therefore fluctuations in flowering time from year to year is expected. *P. alba* is considered to flower from March to April, *P. tremula* flowers in March and *P. ×canescens* in early February (Humphries et al 2006). Interestingly, the hybrid is the one that is considered to flower first.

Comparison of cytoplasmic and nuclear DNA-Markers

Organelles (e.g. chloroplasts and mitochondria) are inherited via the cytoplasm from the parents to the offspring. In most of the species they are maternally inherited, only in few systems paternally inherited organelles are known. As plastids in angiosperms are usually maternally inherited, they are moved by seeds only. Maternal inheritance of the chloroplast was proved for the genus *Populus* by Mejnartowitz (1991), Rajora & Dancik (1992). Seed movement in *Populus* is more restricted compared to pollen and for that reason chloroplast haplotypes usually give a more pronounced geographic pattern than nuclear genotypes. Cytonuclear disequilibrium arises when alleles or genotypes of nuclear loci and cytoplasmatically inherited organellar DNA are non-randomly associated (Asmussen et al. 1987). The level of disequilibrium is affected by various evolutionary forces, such as migration, hybridisation, selection and drift (Asmussen et al. 1989). Empirical studies have been done commonly in hybrid zones between two or more taxa using diagnostic nuclear and cytoplasmic markers (Paige et al. 1991, Lexer et al. 2005). Cytonuclear disequilibrium was also used to study a hybrid zone of e.g. deep-sea mussels to identify immigrants vs. hybrids (Won et al. 2003). Hybrid zones can serve as natural laboratories for studying the role of hybridisation in the evolution of *Populus* species (Lexer et al. 2005); therefore they can assist in assessing the contribution of cytonuclear interactions to the process. Moreover, hybrid zones can be dynamic centres to investigate ecological and evolutionary questions on plants and their associated communities (Whitham et al. 1999).

Aims

The main aim of this study is to use the genus *Populus* as a "model system" to investigate interactions between hybridisation and backcrossing and to assist in understanding the function of genes. Criteria are postulated to compare refugia and migration paths of both *P. alba* and *P. tremula;* this may inform the ongoing search for adaptation abilities to climate change.

To formulate and test particular hypotheses regarding the role of hybridisation, migration, selection and drift in the evolutionary history of each of these two species, the combined analysis of cytoplasmic and nuclear markers as well as flowering

phenology is essential. In order to support the chloroplast patterns of diversity, the genetic diversity in the nucleus of the respective taxa was studied. This is important because these two species are known to hybridise in nature, and thus cytoplasmic *and* nuclear markers are required to obtain a full picture of their evolutionary histories. The two types of marker complement each other well: cytoplasmic (plastid DNA) markers allowed me to study phylogeographic structure in each species, and nuclear markers enabled me to assess the degree to which phylogeographic patterns seen in one species were affected by historical interactions with the other.

In order to assign hybridising species appropriately, leaf morphological markers were measured and subjected to statistical tests, in order to evaluate their usefulness, either as single characters, or as a combination of traits. The basic approach may help to overcome current difficulties in species differentiation and description of species boundaries, which may in part be due to introgression. On the other hand, this investigations may show how introgression effects traditional botanical classification, as only one example of morphological and/or adaptive traits.

Phenological observations were performed in order to analyse flowering times of the parental species vs. hybrids, and in order to test for prezygotic barriers to gene flow. Hybridisation in nature requires that species have an overlap in flowering time, predominantly occurring in the vicinity of floodplain areas and mountain regions for the studied system. To gain knowledge of the potential to hybridise and assess the strength of temporal barriers to gene flow I investigated different sites consisting of pure species and hybrids.

Following these ideas, the study can be divided into two major parts:

First, the distribution of genetic diversity in space (biogeographical aspects) was investigated to learn more about present day phylogeographic patterns, migration and history of the species complex of the two European poplar species *P. alba* and *P. tremula*.

Second, the two species are known to hybridise in nature, and thus several key-aspects related to hybridisation (morphology, flowering phenology, cytonuclear interactions) were looked at.

The study is presented here as five chapters either published, submitted or in preparation for journal publication:

The first chapter uses plastidic DNA markers (cpPCR-RFLPs) to establish a full picture of Central European phylogeographic patterns and possible refugia of *P. alba* and *P. tremula* and their hybrid, focussing on the maternal lineage because among most of the angiosperms chloroplasts are inherited exclusively maternally. The second chapter explores the genetic diversity of *P. alba* on a Mediterranean Island (Malta) and tries to relate the trees to the established phylogeographic pattern using chloroplast markers and nuclear microsatellites. Chapter 3 investigates the possibility to distinguish *P. alba*, *P. tremula* and their hybrid using basic morphometric measurements. In order to assess hybridisation potential in nature, flowering phenological observations were explored and related to climatic data (Chapter 4). Finally, the comparison of cytoplasmic *and* nuclear DNA markers were used in Chapter 5 in order to recognize interactions of the different genomes during hybridisation and to obtain a full picture of their evolutionary histories.

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Chapter 1 – Phylogeography of *Populus alba* (L.) and *Populus tremula* (L.) in Central Europe: secondary contact and hybridisation during recolonisation from disconnected refugia.

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Abstract

The central aim of this paper is to clarify the picture of postglacial recolonisation and the reconstruction of refugia of *P. alba* (L.) and *P. tremula* (L.) in the light of hybridisation of the two species. We focussed our study on Central and South-eastern Europe including reference samples from Spain, Sweden and Northern Africa. We investigated 414 individuals of 26 populations using restriction fragment length polymorphisms (PCR-RFLPs) in six maternally inherited chloroplast (cp) markers. Altogether 57 haplotypes were analysed of which 4 indicated hybridisation events in the past. Phylogeographic structure was found for *P. alba* with low diversity in Eastern Europe versus high diversity in Italy and Central Europe. A lack of phylogeographic structure was assessed for *P. tremula* as expected for a boreal forest tree and diversity was evenly distributed in the studied populations. Two main refugia were identified for *P. alba* in Italy and Romania. A previously described hybrid zone between species in Central Europe turned out also to be a zone of contact between southern and eastern chloroplast lineages in *P. alba*. In contrast, *P. tremula* recolonised its present habitats in Central Europe from several refugia near the former ice cap. We assume separate disconnected refugia for *P. alba* and *P. tremula* and suggest an immigration scenario involving the mixing of colonization routes and interspecific introgression to be responsible for the observed patterns.

Keywords: Postglacial recolonisation, Hybridisation, PCR-RFLPs, cpDNA, *Populus alba*, *Populus tremula*

Introduction

Phylogeographic patterns in Europe are highly influenced by the ice ages, especially affecting Northern Europe and the mountain chains (Taberlet et al. 1998). Forests were much more restricted than today, because of the climatic conditions and after the retreat of the ice shields, migration shaped today's species distribution ranges (Petit et al. 2003). Although detailed postglacial migration routes differ between species, they can be illustrated by ten model plant and animal species (Taberlet et al. 1998). Thermophilous taxa fit into these models (e.g. *Quercus* spp., Dumolin-Lapègue et al. 1997; *Populus nigra*, Cottrell et al. 2005), while boreal taxa apparently survived further north (*Acer* sp. and *P. tremula*, Bittkau 2002; *Betula* sp., Maliouchenko et al. 2007). Boreal populations of the genus *Salix* and *Populus tremula* (Petit et al. 2003) do not show high differentiation, in line with the assumption of additional northern refugia close to the ice shields. Fossil pollen records and charcoal remains provide evidence for the survival of trees on favourable sites along the southern edge of the permafrost during the LGM (Taberlet et al. 1998).

Although poplars have dispersal distances of up to 16 km (Slavov et al. 2004), seedling establishment relies on connections between patches of suitable habitat types. This is particularly true for tree species bound to river ecosystems. The Danube in Europe provides an uninterrupted drainage system and connects sites close to the former ice sheet of the last glacial maximum (LGM) with warm and dry areas in the Southeast of Europe, providing a corridor in periods that are warmer or cooler than today. Depending also on their specific dispersal abilities, high mountains or large water bodies most often represent barriers for movements of plants and animals; i.e. affecting seed viability after travelling by wind or submersion in water or even salt water.

In contrast to other species with detailed pollen records (e.g. oak, *Quercus* spp., Dumolin-Lapègue et al. 1997), *Populus* pollen is less viable, and fossil pollen data are scarce. Lack of pollen data complicates the search for glacial refugia for members of the genus (Huntley and Birks 1983): there is, for example, some evidence for pollen in low concentrations (1-5%) from 9,000 BC onwards, along the north-western rims of the Alps, in close proximity to the then glaciated Alps themselves (Huntley and Birks 1983). Although terrestrial plants are sessile, they can overcome long distances via pollen and seed dispersal, sometimes exhibiting surprisingly high migration speeds (e.g. *Quercus* sp. up to 500 m yr^{-1} and *Alnus* sp. up to 2000 m yr^{-1} of range expansion after

the LGM, Huntley and Birks 1983). Like in most angiosperms, chloroplasts are maternally inherited in *Populus* (Rajora and Dancik 1992), thus they facilitate the understanding of migration events in the past and can serve as a proxy for lacking pollen data.

During the entire Pleistocene, range expansions and contractions of different species took place and influenced present-day patterns of diversity in many taxa (Widmer and Lexer 2001). These past demographic processes often led to hybridisation and are now more and more acknowledged to play a major role in the evolution of the genus *Populus* (Hamzeh and Dayanandan 2004). During the course of speciation, geographic and/or reproductive isolation at the intra-specific level are the main driving forces and species formation is understood as a by-product of intra-specific evolution (Stearns and Hoekstra 2005). Hybridisation occurs in two scenarios in which reproductive isolation is not complete: In the first one, species are separated geographically but not reproductively, thus hybridisation occurs after secondary contact of the species. This concept explains most of the hybrid zones in the Alps and in the Pyrenees, where divergent lineages have come into contact during postglacial recolonisation, thereby forming hybrids or even new species (Hewitt 2001). A second scenario involves hybrid zones between divergent populations in the process of parapatric speciation, but distinguishing this scenario from secondary contact is not trivial (Coyne and Orr 2004). Further research is required to estimate species interaction models and accurate timing of contact and divergence more reliably (Comes and Kadereit 1998; Coyne and Orr 2004) and natural hybrid zones provide the opportunity to study such evolutionary processes and concepts (Lexer et al. 2005, 2007).

Hybridisation is not easy to detect, neither morphologically nor genetically. The status of *Populus* hybrids has been discussed for decades and still new concepts emerge (Eckenwalder 1996; Dickmann and Kuzovkina 2008). The grey poplar (*P.* ×*canescens*, Aiton, Sm) is a natural hybrid between white poplar (*P. alba*, L.) and European aspen (*P. tremula*, L.). Morphological distinction between them is based mainly on leaves and flower catkin bracteoles, but the variability within each species is enormous, and intermediate forms exist (Fischer et al. 2005; Eckenwalder 1996). *P.* ×*canescens* occurs where the habitats of both parental species come into contact (Fischer et al. 2005) to form hybrid zones. More frequently, grey poplar is found close to the floodplain forests of *P. alba* (Lazowski 1997). To our knowledge, hybrid fitness has never been estimated directly, but population genetic work indicates the presence of many recombinant

individuals in hybrid populations so it appears that F1 fitness is not seriously impaired (Lexer et al. 2005). Also, *P.* ×*canescens* exhibits slightly increased vegetative fitness compared to *P. alba* in the Danube floodplain forest as indicated by greater levels of asexual reproduction (van Loo et al. 2008).

Both parental species are pioneer trees. *P. alba* is a characteristic of floodplain forests, *P. tremula*, on the other hand, is a pioneer species of upland forests, with greater ecological amplitude/plasticity. Both species and their hybrids are usually diploid (van Dillewijn 1940).

Although the genus *Populus* has been studied intensively in the past, and is now considered as a model forest tree (Tuskan et al. 2006), there is a lack of knowledge about historical range expansions of white poplar and European aspen (Lexer et al. 2005, 2007), which is why the objectives of this study are i) to trace the postglacial migration routes of *P. alba* and *P. tremula*, ii) to discuss the possible survival of the species in a Danube refugium during the LGM, and iii) to explore the extent to which species interactions such as hybridisation and introgression may influence phylogeographic inference based on cpDNA markers in these species. The limited data currently available on patterns of cpDNA diversity in parapatric *P. alba* and *P. tremula* in Central Europe suggest that introgression of the chloroplast DNA molecule is rare (Lexer et al. 2005). Thus, our expectation at the outset was that a comparative phylogeographic analysis of the two species should be feasible, as long as the possibility of occasional introgression is taken into account in the analysis.

Materials and Methods

Plant material

Species identification was based on botanical criteria (Fischer et al. 2005). Hybrids were identified based on morphology as well, based on the expectation that leaf morphology can predict the genotype reasonably well as long as many independent traits are considered simultaneously (Lexer et al. 2009)

As leaf dimorphism (heterophylly) is a further issue in the genus *Populus* (Bartels 1987), species identification was performed considering the appearance of the entire tree, including long and short shoots. Fully developed leaves on both short and long shoots were assessed for the following three morphological traits: leaf shape, leaf margin and pubescence, based on the current excursion flora of Austria (Fischer et al. 2005). Typical leaves for *P. alba* were 3-5 lobed and coarsely toothed. Leaves from the

long shoots had downy-white undersides and those from short shoots were downy-grey. *P. tremula* leaves were round with a sinuate margin and short blunt teeth, and neither short nor long shoots had a downy surface. The hybrid *P. ×canescens* had triangular to ovoid leaf shapes with lobes or irregularly spaced, coarse dentation. The undersides of leaves from long shoots were downy-grey and those of leaves from short shoots were slightly downy-grey to glabrous. Those criteria were utilized to identify samples from our own collections and from those obtained by our collaborators (for details of sampling see Appendix S1).

Leaves and buds were obtained from altogether n=414 trees, grouped into 26 populations. 19 populations dominated by *P. alba* (281 individuals) and 7 populations dominated by *P. tremula* (133 individuals) were studied. Leaf morphological traits were assessed to estimate the proportion of hybrids (*P. ×canescens*) in each population. Distances between samples in a population were as large as possible in order to avoid sampling of closely related individuals or clones (especially *P. ×canescens* can form large clones, van Loo et al. 2008). Leaf material was dried in silica gel. Small branches with buds were stored at -20°C.

Study sites

The location of the sampled populations is given in Fig. 3 and the numbers correspond to sampling information given in Appendix S1. Natural populations in Central and South-eastern Europe were intensively sampled. Samples from Italy and Czech Republic were obtained from clone collections and provided by collaborators there. Those samples were grouped into populations according to original collection sites; those collections were established in order to cover high genetic diversity and thus original collection sites had a wide geographic distribution. Reference populations with low numbers of individuals from Spain, Morocco, Tunisia and Sweden completed the picture. The Spanish and the Italian samples had been chosen on the basis of their variation in chloroplast microsatellite patterns (S. González-Martínez and S. Castiglione, personal communications).

DNA extraction

DNA was extracted from approximately 10 mg of dry, or 50 mg of fresh material, using the DNeasy 96 plant kit (Qiagen) or the CTAB method (Doyle and Doyle 1987). Polymerase chain reaction (PCR) and PCR-RFLP conditions were as

given in Lexer et al. (2005), using the six primer pairs and five enzymes listed in Table 1. Primer pairs were chosen among the 'universal' ones listed at: http://bfw.ac.at/bfwcms.web?dok=4961 (Heinze 2007). Amplification and restriction products were visualised in 2.5 % agarose gels stained with ethidium bromide. Fragment scoring was done manually, except for the fragment II (rpl16R1516 + rpl16F71R), which showed a high variability (Table 1) with apparent 16 base-pair repeats. Therefore, for this fragment, one primer was marked with fluorescent dye (D2, D3 or D4, Sigma-Proligo, USA) and the restriction products were analysed on a Beckman CEQ 8000 automatic sequencer for exact length determination.

Table 1 Patterns of variation obtained with different primer-restriction enzyme combinations in *P. alba* and *P. tremula*

Fragment number	Forward Primer	Reverse Primer	Observed length (bp)	Restriction enzymes	Variable fragments	Characters
I	trnDP	trnTP	1066	HinfI	2	a,b,c,d / a,b,c
II	rpl16R1516	rpl16F71R	1180	EcoRI+HhaI	1	a,b,c,d,e,f,g,h
III	rpl16ex1f	rps3r2	800	EcoRI+HhaI	1	a,b,c
IV	rps3f2	ccmp10R	1020	HhaI+SspI	1	a,b,c,d
V	atpBSAM	rbclSAM	850	EcoRI	1	a,b
VI	ccmp10R	trnHM	600	MspI	2	a,b,c,d,e,f / a,b

Data analysis – gene diversity and differentiation; phylogeography

Diversity values (gene diversity and haplotypic richness) and distance measures were calculated. Gene diversity (diversity of unordered alleles, h_T) is frequency dependent and accounts for relative diversity in a population. Haplotypic richness is equal to allelic richness in the case of chloroplasts. Haplotypic richness (diversity of ordered alleles, v_T) is dependent on the sample size (Table 2). Both measures h_T and v_T were calculated using the programs HAPLODIV (Pons and Petit 1995) and CONTRIB (Petit et al. 1998). Haplotypic diversity requires standardisation for the smallest sample size (rarefaction - five in our case), which is implemented in HAPLODIV.

Table 2 Diversity statistics of 414 individuals in 26 populations, sorted by decreasing haplotypic richness, given for *P. alba* and *P. tremula* separately. Average sample size per population for *P. alba* was 14.8 and for *P. tremula*, 19

Species	Population	No. of haplotypes[a]	No. of polymorphic sites	Gene diversity[b]	Haplotypic richness[c]	P[d]
P. alba	Viennese Forest_c	8 (10)	7	0.955 (0.059)	4.556	0.6
P. alba	Central Italy	5 (6)	3	0.933 (0.121)	4.333	0
P. alba	Southern Italy	5 (6)	3	0.933 (0.121)	4.333	0
P. alba	Czech Republic_alba_c	12 (23)	7	0.917 (0.034)	4.251	0.26
P. alba	Spain	6 (8)	5	0.892 (0.111)	4.107	0
P. alba.	Tunisia	4 (6)	6	0.866 (0.129)	3.667	0
P. alba	Vienna Danube_c	11 (41)	5	0.741 (0.053)	3.181	0.44
P. alba	Western Hungary_c	4 (8)	4	0.750 (0.139)	3.143	1
P. alba	Eastern Croatia_alba	3 (5)	7	0.800 (0.164)	3.000	0
P. alba	Northern Austria_alba_c	7 (32)	4	0.681 (0.139)	2.865	0.38
P. alba	Eastern Hungary_c	4 (28)	6	0.616 (0.077)	2.575	0.89
P. alba	Central Romania	6 (27)	4	0.495 (0.109)	2.323	0
P. alba	Central Croatia_c	3 (9)	4	0.416 (0.190)	2.111	0.78
P. alba	Western Romania	2 (17)	2	0.441 (0.097)	1.872	0
P. alba	Eastern Romania	3 (12)	2	0.318 (0.163)	1.833	0
P. alba	Southern Hungary_c	3 (29)	2	0.305 (0.100)	1.725	1
P. alba	Crete	1 (7)	0	0.000	1.000	0
P. alba	Northern Italy	3 (4)	1	0.833 (0.222)	n.incl	0
P. alba	Morocco	1 (3)	0	0.000	n.incl	0
P. tremula	Northern Alps	6 (12)	3	0.879 (0.046)	3.874	0
P. tremula	Czech Republic_trem	8 (22)	6	0.865 (0.040)	3.835	0
P. tremula	Eastern Croatia_trem_c	5 (10)	2	0.822 (0.096)	3.532	0.4
P. tremula	Eastern Alps	5 (16)	3	0.825 (0.044)	3.474	0
P. tremula	Central Alps	3 (8)	2	0.464 (0.200)	3.000	0
P. tremula	Northern Austria_trem_c	10 (62)	7	0.675 (0.044)	2.827	0.27
P. tremula	Sweden	2 (3)	3	0.666 (0.314)	n.incl	0

[a]sample size in brackets; [b]standard deviation in brackets; [c]rarefaction to 5 individuals; [d]portion of morphological hybrids, 0 means no hybrids, 1 means 100% hybrids; c indicates presence of *P. ×canescens* based on botanical identification in the field; n.incl., populations not included in the calculation of haplotypic richness because of too small sample size

Genetic distance was calculated between pairs of haplotypes (the number of different alleles between two RFLP haplotypes) using analysis of molecular variance (AMOVA), as implemented in the software ARLEQUIN (Schneider et al. 2000). Different hierarchical levels were tested and significance was calculated at the $p<0.001$ level after 10000 permutations.

The relationship between populations was calculated in PHYLIP (Felsenstein 2004) based on Reynolds, Weir and Cockerham‘s genetic distance (1983). An unrooted UPGMA tree was constructed, and branch support was assessed using 1000 bootstraps in the SEQBOOT software of the PHYLIP package. Shorter distances are expected for geographically closer populations under a model of isolation-by-distance. Interspecific hybridisation can disrupt such within-species patterns because hybridisation tends to be a localized phenomenon. Thus, hybridisation can affect branching patterns detected in distance-based analysis.

The presence of phylogeographic structure was estimated by measures of differentiation - the proportion of diversity between populations to total variation, termed G_{ST} for unordered and N_{ST} for ordered alleles, respectively. The relationship between both measures can reveal whether phylogeographic structure is present in a set of populations (Pons and Petit 1996); it was calculated for each species separately in HAPLODIV (Pons and Petit 1995) and PERMUT (Pons and Petit 1996). Phylogeographic structure is evident if $N_{ST} > G_{ST}$ i.e. if the proportion of more closely related haplotypes in one population is higher than the proportion of unrelated haplotypes. Significance was tested at the $p<0.05$ and $p<0.01$ levels after 10000 permutaions.

Relationships among haplotypes were assessed on the basis of a median-joining network, using the software NETWORK (http://www.fluxus-engineering.com/, Bandelt et al. 1999). The program searches for a minimum spanning tree with the shortest possible connections with the help of median vectors, which act as starting points for connections between haplotypes. In this analysis, the data set was reduced from 57 to 30 haplotypes by excluding singletons for clarity. Haplotype frequency maps were established in order to allow geographic and historic interpretation essentially following Posada and Crandall (2001).

The haplotype networks were also employed in assessing the impact of hybridisation on phylogeographic inference, as haplotypes are characteristic and clearly

separated according to species (Lexer et al. 2005). Haplotypes of apparent morphological hybrids were scrutinized for their position in the network, and congruence between our species designations and the haplotype positions in the network was assessed.

Results

Altogether ten populations were affected by hybridisation, whereas 16 populations did not show any sign of hybrids based on the morphological identification criteria. Proportions of morphological hybrids in each population varied considerably from 27-100%. Hybridisation appeared to be more frequent in *P. alba* with eight out of 16 populations harbouring morphological hybrids (Table 2). For *P. tremula*, two out of seven populations contained morphological hybrids. No morphological signs of hybridisation were detected where both species occurred isolated from each other; i.e. Romania and Crete for *P. alba*, Central and Northern Alps and Sweden for *P. tremula.*

Molecular variation

Six PCR-RFLP fragments yielded 35 length polymorphisms, combining into 57 haplotypes in the 26 populations (414 individuals). Haplotype descriptions are given in Appendix S2. Total diversity for *P. alba* was h_T =0.898 and v_T=0.907 for unordered and ordered alleles, respectively. The values for *P. tremula* were slightly higher, h_T =0.905 and v_T=0.915. Diversity values for populations are listed in Table 2, sorted by decreasing haplotypic richness. The highest haplotypic richness was found in population Viennese Forest_c (4.5 haplotypes after rarefaction) followed by the Mediterranean populations. Central European populations yielded higher haplotypic richness compared to South-eastern European ones. Calculation of molecular variance (AMOVA) revealed great and significant differentiation among species of 54.5 %, consistent with earlier reports of very low levels of chloroplast DNA introgression (Lexer et al. 2005). Differentiation between populations was higher in *P. alba* (36.3 %) than in *P. tremula* (18.6 %). Within populations, *P. tremula* had higher diversity (81.4 %) than *P. alba* (63.7 %).

Geography and genetic relationships

The UPGMA tree revealed separation of most populations of *P. tremula* from those of *P. alba* with moderate bootstrap support. Populations of *P. alba* were further split into several groups (Fig. 1), the groupings within the Danube drainage system receiving 65-100% bootstrap support. Two of the *P. alba* groups had evidence for the presence of hybrids based on our morphological identification. The Danube drainage system clade had the highest proportion of intermediate morphotypes (51.4 %) followed by the Central European group of *P. alba* with 24.5 % of intermediate morphotypes. The Italian group of *P. alba* was well defined by private haplotypes and populations from Northern and Central/Southern Italy were clearly separated (Fig. 1). No morphological evidence for hybridisation was found in Italian populations. The remaining samples of *P. alba* from the Mediterranean basin (Crete, Tunisia and Morocco) grouped together with the clade containing mainly populations of *P. tremula* with low bootstrap support. A conspicuous result was the positioning in close proximity of the Swedish and Spanish samples within the *P. tremula* group, as the Spanish samples comprised *P. alba* morphotypes.

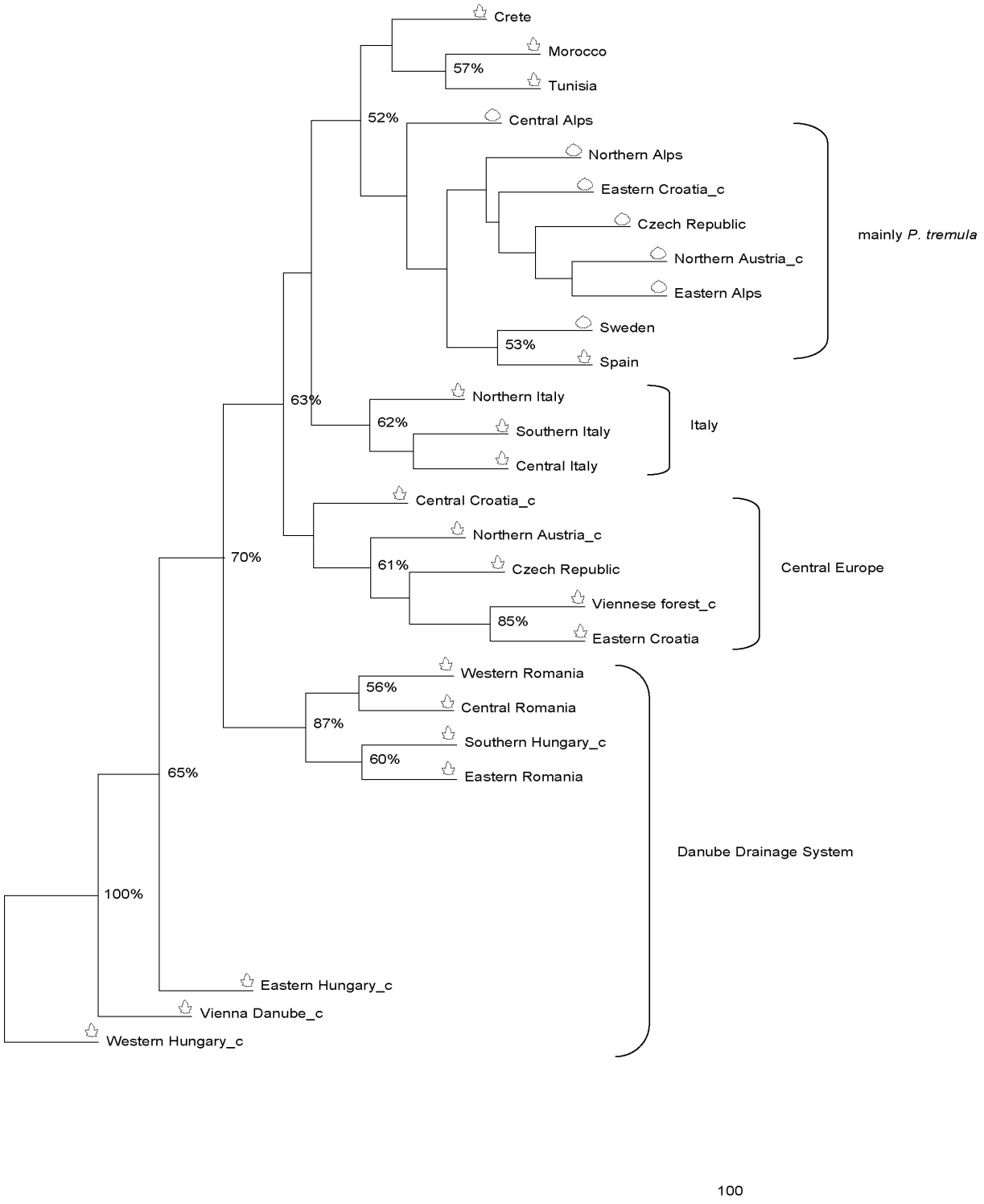

Fig. 1 Relationships among 26 populations visualised in an unrooted UPGMA tree based on pair wise population distance values, revealing four main groups dominated by species and geographical relationship of *P. alba*, *P.* ×*canescens* and *P. tremula* supported by bootstrap analyses based on 1000 replications (only values higher than 50% are shown). Species origin of the sampled populations is indicated by leaf symbols: lobed for *P. alba* and round for *P. tremula*. c indicates the presence of *P.* ×*canescens* in the respective populations based on morphological identification

Phylogeographic patterns

Comparing N_{ST} and G_{ST} revealed a highly significant phylogeographic structure for *P. alba* (Table 3), where related haplotypes were more frequently found within the same population. The Italian populations were dominated by three haplotypes (Figs 2 and 3, yellow) exclusively distributed in Italy, whereas two additional Italian haplotypes were also present in Croatia, Hungary, Austria and the Czech Republic. *P. alba* populations along the Danube were dominated by two exclusive haplotypes (H21, H15). Populations from smaller rivers in the Danube basin were characterised by additional haplotypes (H4 in Central Croatia and Northern Austria, Fig. 3). In populations from Northern Austria and the Czech Republic, one Italian haplotype H5 (yellow) occurred frequently, indicating admixture from this refugial area. In contrast, no significant difference between N_{ST} and G_{ST} was detected in *P. tremula* (Table 3), indicating a weak phylogeographic structure in this species. Two haplotypes (H41, H45) dominated *P. tremula* in the majority of the populations. North African populations of *P. alba* were grouped with the *P. tremula* clade, and were characterized by two haplotypes (H39, H57) separated from other haplotypes by six mutational steps. The sample from Crete was fixed for a single haplotype (H30).

Table 3 Chloroplast diversity statistics and genetic differentiation for ordered and unordered alleles following Pons and Petit (1996) for *P. alba* and *P. tremula*, including population size and the number of haplotypes considered

Species	Number of populations	Number of haplotypes	h_S[a]	h_T[a]	G_{ST}[a]	v_S[b]	v_T[b]	N_{ST}[b]
P. alba	19	57	0.626	0.898	0.303	0.461	0.907	0.492
P. tremula	7	57	0.772	0.905	0.147	0.724	0.915	0.208

[a] Unordered haplotypes: h_S, within-population diversity; h_T, total diversity; G_{ST}, genetic divergence. [b] Ordered haplotypes: v_S, within-population diversity; v_T, total diversity; N_{ST}, genetic divergence. The difference in genetic divergence when estimated using unordered *vs.* ordered haplotype information (G_{ST} *vs.* N_{ST}) was highly significant ($p<0.01$) in *P. alba*, but not significant in *P. tremula*

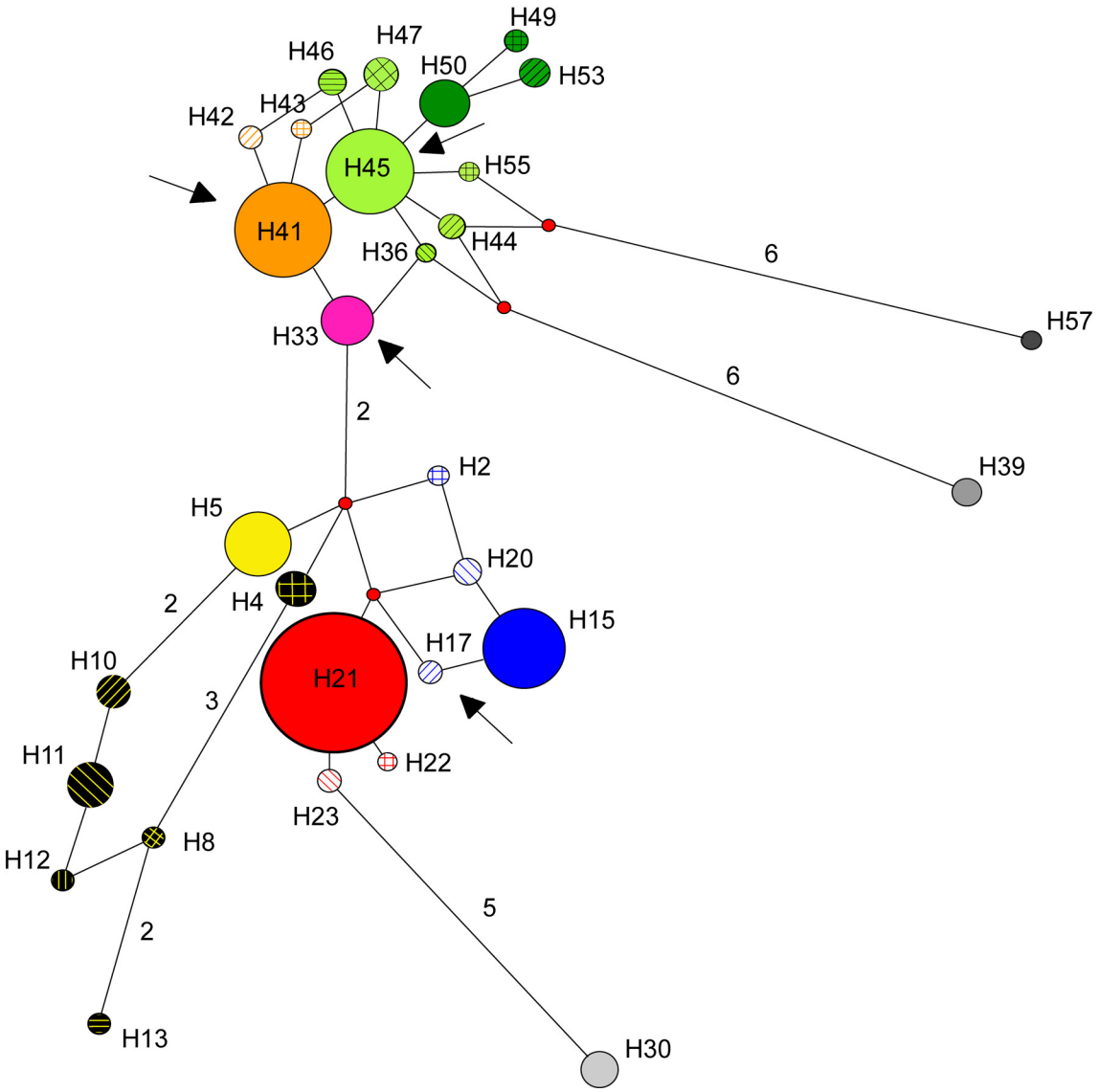

Fig. 2 Median-joining network of 30 haplotypes, without singletons. Colours and patterns correspond to Fig.3 with *P. alba* in red, blue and yellow and *P. tremula* in green and orange. Size of circles correspond to number of individuals in a given haplotype and number of mutation steps between haplotypes is one except for those indicated along the connections. Haplotypes found in hybrid morphotypes are indicated by arrows

Network supports relationship of haplotypes

Grouping of the haplotypes (omitting singletons) within a mutational framework resolved them into network-type clusters (Fig. 2). Haplotypes from H1 to H30 were typical for *P. alba*, whereas haplotypes H31 to H57 were typical for *P. tremula*. The haplotypes in the network are clearly split into two clades. Haplotypes in the upper

clade predominantly appeared in populations of *P. tremula*. The haplotypes in the lower clade were mainly found in populations of *P. alba*. Only few haplotypes occurred in both species (indicated by arrows in Fig. 2). Haplotype H33, connecting both clades, was distributed in three populations of *P. tremula* in Central Europe and in Sweden and additionally appeared in the Spanish *P. alba* population.

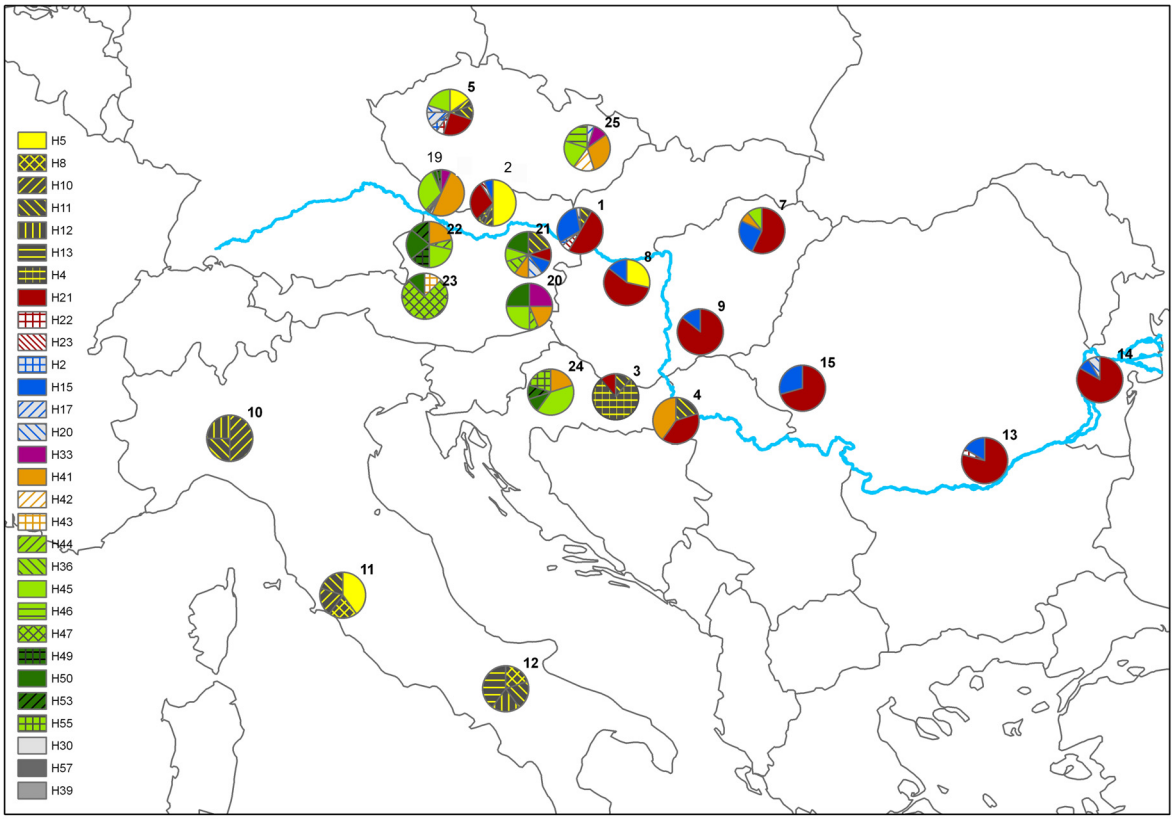

Fig. 3 Geographic distribution of chloroplast PCR-RFLP haplotypes for *P. alba* and *P. tremula* in Central, Southern and South-eastern Europe. Colours and patterns of the segments correspond to the haplotype network of *P. alba* (red, blue, yellow) and *P. tremula* (green, orange). Frequencies for populations from Sweden, Spain, Greece, Tunisia and Morocco are illustrated in the Appendix S3

Discussion

The two poplar species in this study (*P. alba* and *P. tremula*) hybridise in nature, thus hampering field identification, if only single traits are considered. Morphological species identification based on many traits revealed a substantial proportion (22.2%) of potential hybrids in the studied populations (Table 2). Of course this can only be a very coarse assessment of hybridisation, because only a limited number of genes are

involved in characterizing leaf morphology. A more profound assessment of hybridisation was undertaken in previous studies of our groups in a subset of populations of this study based on microsatellites. Those revealed hybridisation in three populations (Lexer et al. 2005, 2007; van Loo et al. 2008), suggesting that both directions of hybridisation are possible in this species complex.

Hybridisation is complicating our attempts to reconstruct refugia and migration routes for the two species. Furthermore it is difficult to locate refugia based on poplar and aspen pollen, because pollen data are scarce in the fossil record (Huntley and Birks 1983). Moreover, species distinction of pollen is nearly impossible in European *Populus* species (R. Litschauer, BFW Vienna, personal communication). Therefore criteria for possible glacial refugia can currently only be derived from genetic data. The following criteria based on haplotype networks and diversity values can be applied (Posada and Crandall 2001): high-frequency and central haplotypes can be considered ancient, and populations carrying these haplotypes should be taken into consideration as representing possible refugia (or direct descendants of refugial populations). These "rules" have been used extensively in the literature and rely on the assumptions underlying the coalescent process, hence they are associated with uncertainty. Haplotype diversity is another clue; it should be higher in possible refugia, unless it can be attributed to meeting points of zones of different postglacial routes (Petit et al. 2003).

In our study we found strong phylogeographic structure within *P. alba*, supported by the *G*st/*N*st test, but only weak structure within *P. tremula*. We conclude that *P. alba* populations have different phylogeographic histories, because they survived the Quaternary glaciations in disconnected refugial areas. This is visible from the separate lineages characterizing the Italian Peninsula and the Danube catchment (Figs 2 and 3). Furthermore, the phylogenetic tree separated Central European and South-eastern populations of *P. alba* from Mediterranean and Italian populations with 63% bootstrap support (Fig. 1).

Because of the thermophilous nature of *P. alba*, its survival during the LGM must have been further south and/or southeast compared to its present distribution, as shown for other thermophilous species such as oaks (*Quercus* sp., Dumolin-Lapègue et al. 1997). Northern habitats of *P. alba* were recolonised primarily via the Danube corridor as shown in Fig. 3 where Austrian haplotypes are related to those from further downstream along the Danube. For the Central European distribution range of *P. alba* this is plausible because ecologically the species is dependent on large rivers like the

Danube. A similar phylogeographic pattern has recently been shown for barbel fish (*Barbus barbus,* Kotlík and Berrebi 2001), a species in the Danube drainage system dominated by one major mitochondrial haplotype that originated in the Black Sea. A conspicuous result is that haplotypes from Italy were detected in Northern Austria and Czech Republic, but no Central European haplotypes were found in Italy. This pattern indicates that Italian populations contributed to the colonization of Central Europe as well.

As a result of various recolonisation routes towards the north, mixing of haplotype lineages was detected in Austrian, Czech, Hungarian and Croatian populations of *P. alba*. In those populations the proportion of admixed Italian haplotypes was 25-50%. The remaining portion was made up by Eastern European or local private haplotypes. Mixing of lineages was likely to happen in those populations, because habitats were newly established there during the melting process of the Alpine glaciers. For establishing new populations in that critical phase two processes have to be considered. First, as the whole genus *Populus* has wind-dispersed seeds and pollen, long distance movements during recolonisation must be expected (Nathan 2006). In some cases, rare long distance dispersal events play a key role in structuring maternally inherited genes (Le Corre et al. 1997). Moreover, a suture zone was previously proposed for another 'fluviphilous' tree species, *Fraxinus excelsior*, in the north of the Pannonian basin (Hungary and Slovakia; Heuertz et al. 2004), on the basis of nuclear microsatellite data. Especially the Eastern Alps were considered as a glacial refugium for several plant taxa (Niklfeld 1972, 1974). Secondly, newly arisen mutations might have taken advantage of a founder effect and changing river dynamics (see also *Populus nigra*; Cottrell et al. 2005), which may explain high levels of cpDNA diversity in Austria. For instance, five haplotypes exclusive to the Vienna Danube_c population were present at low frequency. However, we do not expect a refugium in that area for *P. alba* due to its thermophilous nature. High diversity supported Italy as a refugial area for *P. alba*, with the presence of three exclusive haplotypes. On the contrary, diversity was surprisingly low in Romanian populations but the occurring haplotypes were central in the network and highly frequent in the populations, thus suggesting a possible refugium in South-eastern Europe as well. In this case, historic events and/or human impact may have caused the loss of genetic diversity (bottleneck) in Romanian populations.

We detected no phylogeographic structure for *P. tremula* in Central Europe, which supports the hypothesis that there were several connected refugia during the Quaternary glaciation cycles. This appears to have led to the relatively even haplotype distribution observed among the populations in our study. *P. tremula* as a boreal species followed the pattern of other boreal species with a more northern distribution and refugia and without clear phylogeographic patterns, i.e. *Betula pendula* (Maliouchenko et al. 2007) and *Salix caprea* (Palmé et al. 2003).

A refugium for *P. tremula* can be assumed close to the ice shields of the LGM. We conclude that recolonisation from those refugia happened fast and thus mixing of lineages from several refugia led to the current picture of high haplotypic diversity in the studied forests. Survival close to the ice shield is supported by the current distribution of *P. tremula* trees from 200-2000 m above sea level. The ability of the species to cope with a great variety of ecological conditions and especially its tolerance towards cold temperature supports our conclusions. This is also supported by the rare but available pollen data from the early Holocene from Central and Northern Europe (Huntley and Birks 1983) together with macrofossil data of *Populus* wood dating back to 25,000 BC on protected sites in Slovenia (Willis et al. 2000). Further arguments for the potential survival of trees near the ice shield can be derived from Davis et al. (2003), who found evidence for substantial local and seasonal climatic differences within a balanced regional and annual climate. Altogether this feeds the ongoing debate about possible northern tree refugia (e.g., Willis et al. 2000, 2004; Carcaillet and Vernet 2001; Stewart and Lister 2001).

One of the most striking factors besides seed dispersal and mutations in shaping the phylogeographic pattern of the *P. alba – P. tremula* species complex was hybridisation. The alternations of cooling and warming during the Pleistocene provoked seesaw movements of the tree species according to their ecological requirements either as one of the first colonizers (*P. tremula*) or trailing the recolonisation front line in some spatial and temporal distance (*P. alba*). Therefore we believe that hybrids already have formed during earlier recolonisation processes. The formation and 'good' separation of the species over a long evolutionary time period is supported by the clear separation of the two clades in the haplotype network. However single haplotypes were able to jump across this divide, appearing in morphological hybrids or the other species (indicated with arrows in Fig. 2).

Perhaps the most striking case of haplotype sharing observed in the present study is that between Spanish *P. alba* and Swedish *P. tremula*. We can only offer chloroplast capture or ancient haplotype sharing as possible explanations (see discussion in Rieseberg and Soltis 1991). *P. alba* in a possible western refugium may have captured chloroplasts from more widespread *P. tremula* there, but further sampling is required to support this result. Also, our UPGMA tree is unrooted, thus we cannot infer the direction of chloroplast capture from it. Nevertheless, the haplotype network (Fig. 2) provides indications for the identity of ancestral and derived haplotypes in each species. We also have to consider human transfer of material, but for the studied species this seems very unlikely, as they have not been of major commercial interest.

A second unusual case of haplotype sharing is the placement of the North African *P. alba* haplotypes into the *P. tremula* clade. Morphologically these two populations represented two subspecies of *P. alba*, according to herbarium specimens from the Natural History Museum in London (UK): *P. alba* ssp. *hickeliana* (BM000794106) and *P. alba* ssp. *subintegerrima* (BM000794108). Genetically the two taxa were characterized by two distinct haplotypes, separated by 6 mutational steps (Fig. 2 and Appendix S3), and both were placed within the *P. tremula* clade. Again, recurrent interspecific gene flow or shared, ancestral polymorphism are possible explanations. We suspect that these species share haplotypes more frequently than generally assumed - at least in the extreme western and eastern refugia; a situation already encountered in hybridising oak (Dumolin-Lapègue et al. 1997), birch (Palmé et al. 2004) and ash species (Heuertz et al. 2006). Analysis of more samples from the Eastern Mediterranean (e.g., Turkey) would be necessary to complete the picture.

Looking further into the past of Central Europe reveals that towards the end of the Pliocene, climate was warmer and drier than today with poplars distributed along the coast of the Pannonian sea (Starmühlner and Aschenbrenner 1972). Tests of past population demography of *P. tremula* based on 77 genes revealed a severe bottleneck at the beginning of the Pleistocene (Ingvarsson 2008), thus suggesting that *P. tremula* was more widespread in the Pliocene. During the first cooling in the Pleistocene, thermophilous species were pushed further south and only cold tolerant lineages survived in Central Europe. Such historical processes may have played a role in shaping today's distribution ranges and hybrid zones in this area. As an example we propose an immigration scenario for Central Europe on the basis of our findings (Fig. 4). We

assume that *P. tremula* survived the last glaciation near the ice shield, close to the permafrost line. Thus, with progressive climate warming, this species was able to establish itself quickly in the area. Later, *P. alba* arrived mainly from South-eastern Europe and replaced *P. tremula* on the most favourable sites (e.g., population Vienna Danube_c), but in the more temperate hills (Viennese Forest_c), hybridisation and introgression occurred.

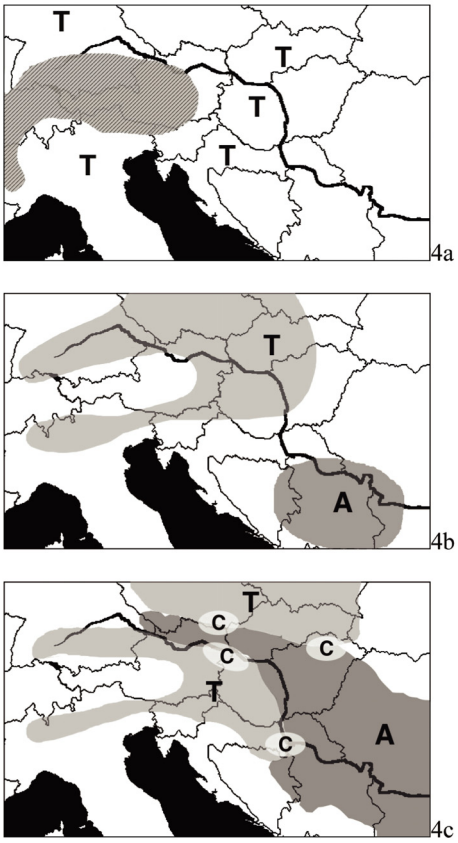

Fig. 4 Immigration scenario for *P. alba* and *P. tremula* in Central Europe after the last glacial maximum. **(a)** Possible refugia for *P. tremula* (T) in close proximity to the ice shield (hatched area). **(b)** Range expansion of *P. tremula* (T) while *P. alba* (A) was still situated in its refugia. **(c)** Immigration of *P. alba* (A) upstream the Danube: replacement of and hybridisation with *P. tremula* (T) and forming *P.* ×*canescens* (C)

For explaining these patterns, we need to know more about the time of species divergence between *P. tremula* and *P. alba*. The exact timing of divergence is unknown and the two species may have repeatedly hybridised in previous interglacial periods. Our study points out that many more interesting insights may be gained from expanding our outlook further into the past (Hewitt 2004), thereby revealing species evolutionary histories with repeated interconnections that may have left their marks in today's genomes.

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Appendix

Appendix S1 Sampling locations and number of haplotypes for the sampled populations

Species	Country	No.	Population	Origin*	Collaborators, providing samples	Vouchers**	n	1	2	3	4	5	6
P. alba	Austria	1	Vienna Danube_c	NP	Own sampling (*Lexer et al.* 2005, 2007)	S	41	1	1	0	0	0	0
P. alba	Austria	2	Northern Austria_alba_c	NP	Own sampling	S	32	0	0	0	1	16	0
P. alba	Croatia	3	Central Croatia	NP	Kajba D., Bogdan S. (University of Zagreb, HR)	S	9	0	0	0	7	0	0
P. alba	Croatia	4	Eastern Croatia_alba	NP	Kajba D., Bogdan S. (University of Zagreb, HR)	S	5	0	0	0	0	0	0
P. alba	Czech Republic	5	Czech Republic_alba_c	CC	Cvrčková H., Máchová P. (FGMRI, CZ)	N	23	0	1	1	0	3	1
P. alba	Greece	6	Crete	NP	Van Loo M. (RGB Kew, GB)	S	7	0	0	0	0	0	0
P. alba	Hungary	7	Eastern Hungary_c	NP	Bartha D. (NYME, HU)	S	28	0	0	0	0	0	0
P. alba	Hungary	8	Western Hungary_c	NP	Bartha D. (NYME, HU)	S	8	0	0	0	0	2	0
P. alba	Hungary	9	Southern Hungary_c	NP	Benke A. (ERTI Sarvar, HU)	S	29	0	0	0	0	0	0
P. alba	Italy	10	Northern Italy	CC	Castiglione S. (UniSa, I)	N	4	0	0	0	0	0	0
P. alba	Italy	11	Central Italy	CC	Castiglione S. (UniSa, I)	N	6	0	0	0	0	2	0
P. alba	Italy	12	Southern Italy	CC	Castiglione S. (UniSa, I)	N	6	0	0	0	0	0	0
P. alba	Romania	13	Central Romania	NP	Nica M.S., Ipati A. (ICAS, RO)	S	27	0	0	0	0	0	0
P. alba	Romania	14	Eastern Romania	NP	Nica M.S., Ipati A. (ICAS, RO)	S	12	0	0	0	0	0	0
P. alba	Romania	15	Western Romania	NP	Nica M.S., Ipati A. (ICAS, RO)	S	17	0	0	0	0	0	0
P. alba	Spain	16	Spain	NP	González-Martínez S. (INIA, E)	N	8	0	0	0	0	0	0
P. alba	Tunisia	17	Tunisia	NP	Own sampling	S	6	0	0	0	0	0	0
P. alba	Morocco	18	Morocco	NP	Own sampling	S	3	0	0	0	0	0	0
P. tremula	Austria	19	Northern Austria_trem_c	NP	Own sampling	S	62	0	0	0	0	0	0
P. tremula	Austria	20	Eastern Alps	NP	Own sampling	S	16	0	0	0	0	0	0
P. tremula	Austria	21	Viennese Forest_c	NP	Own sampling	S	10	0	0	0	0	0	0
P. tremula	Austria	22	Northern Alps	NP	Own sampling	S	14	0	0	0	0	0	0
P. tremula	Austria	23	Central Alps	NP	Own sampling	S	10	0	0	0	0	0	0
P. tremula	Croatia	24	Eastern Croatia_trem_c	NP	Kajba D., Bogdan S. (University of Zagreb, HR)	S	10	0	0	0	0	0	0
P. tremula	Czech Republic	25	Czech Republic_trem	CC	Cvrčková H., Máchová P. (FGMRI, CZ)	N	22	0	0	0	0	0	0
P. tremula	Sweden	26	Sweden	NP	Own sampling	C	3	0	0	0	0	0	0

No.	Population	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
1	Vienna Danube_c	0	0	0	0	3	0	0	0	11	0	0	0	1	1	18	0	2	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
2	Northern Austria_alba_c	0	0	0	2	1	0	0	0	2	0	0	0	0	0	9	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	Central Croatia	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	Eastern Croatia_alba	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
5	Czech Republic_alba_c	0	0	1	1	2	0	0	0	0	0	2	0	0	1	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	Crete	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0
7	Eastern Hungary_c	0	0	0	0	0	0	0	0	7	0	0	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
8	Western Hungary_c	0	0	0	0	0	0	0	0	1	0	0	0	0	0	4	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	Southern Hungary_c	0	0	0	0	0	0	0	0	4	0	0	1	0	0	24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	Northern Italy	0	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	Central Italy	0	1	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	Southern Italy	1	1	0	0	1	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	Central Romania	0	0	0	0	0	0	0	0	4	1	0	0	0	0	19	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
14	Eastern Romania	0	0	0	0	0	0	0	0	1	0	0	0	0	1	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	Western Romania	0	0	0	0	0	0	0	0	5	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	Spain	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	1	0	1	1	0	0	0	0
17	Tunisia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0
18	Morocco	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0
19	Northern Austria_trem_c	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	4	0	0	1	0	0	0	0	30	0
20	Eastern Alps	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	3	0
21	Viennese Forest_c	0	0	0	0	2	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0
22	Northern Alps	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0
23	Central Alps	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
24	Eastern Croatia_trem_c	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
25	Czech Republic_trem	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	6	3
26	Sweden	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0

No.	Population	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57
1	Vienna Danube_c	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	Northern Austria_alba_c	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	Central Croatia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	Eastern Croatia_alba	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	Czech Republic_alba_c	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0
6	Crete	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	Eastern Hungary_c	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
8	Western Hungary_c	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	Southern Hungary_c	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	Northern Italy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	Central Italy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	Southern Italy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	Central Romania	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	Eastern Romania	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	Western Romania	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	Spain	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
17	Tunisia	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2
18	Morocco	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	Northern Austria_trem_c	1	1	19	0	0	1	0	2	0	0	2	0	0	0	0
20	Eastern Alps	0	1	4	0	0	0	0	4	0	0	0	0	0	0	0
21	Viennese Forest_c	0	0	1	0	0	0	0	2	0	0	0	0	0	0	0
22	Northern Alps	0	1	3	0	0	0	2	3	0	0	2	0	0	0	0
23	Central Alps	1	0	0	0	6	0	0	1	0	1	0	0	0	0	0
24	Eastern Croatia_trem_c	0	0	4	0	0	0	0	1	0	0	1	0	2	0	0
25	Czech Republic_trem	0	0	4	4	0	0	0	0	1	0	0	0	0	0	0
26	Sweden	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0

Sample size (n). * Origin of samples (NP natural population, CC clone collection); ** Available voucher specimens (S silica-gel dried leaves, C clone collection, N no material available

Appendix S2

Haplotype definition of 8 variable fragments.

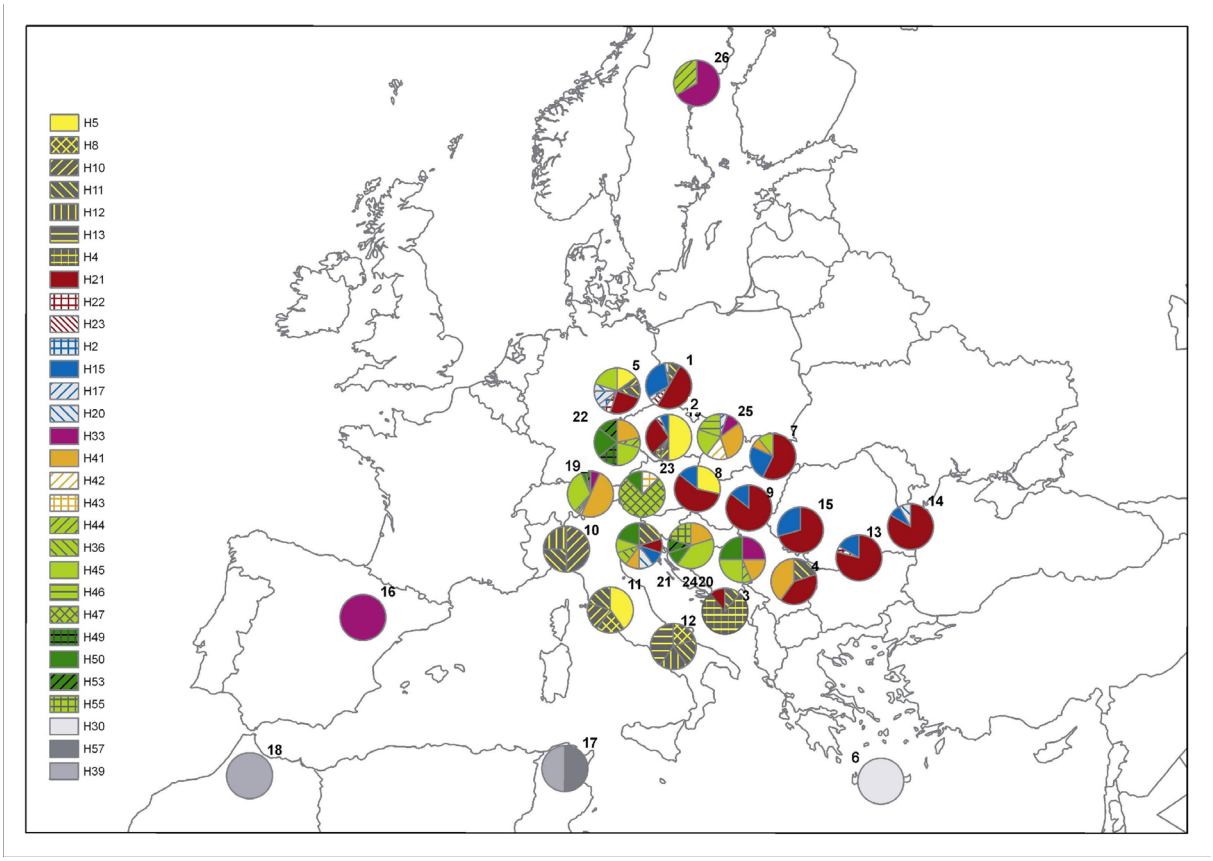
	I-1	I-2	II	III	IV	V	VI-1	VI-2
H1	a	a	a	a	a	a	a	b
H2	a	a	a	a	b	a	a	b
H3	a	a	a	a	b	a	b	b
H4	a	a	a	a	b	a	c	b
H5	a	a	a	a	b	b	b	b
H6	a	a	a	a	b	b	d	b
H7	a	a	a	a	c	a	d	b
H8	a	a	a	a	c	a	e	b
H9	a	a	a	a	c	b	b	b
H10	a	a	a	a	c	b	c	b
H11	a	a	a	a	c	b	d	b
H12	a	a	a	a	c	b	e	b
H13	a	a	a	a	d	a	f	b
H14	a	a	a	a	d	b	d	b
H15	a	a	a	b	a	a	a	b
H16	a	a	a	b	a	a	a	c
H17	a	a	a	b	a	a	b	b
H18	a	a	a	b	a	a	c	b
H19	a	a	a	b	a	b	a	b
H20	a	a	a	b	b	a	a	b
H21	a	a	a	b	b	a	c	b
H22	a	a	a	b	b	a	d	b
H23	a	a	a	b	b	b	c	b
H24	a	a	a	b	c	a	a	b
H25	a	a	a	b	c	a	d	b
H26	a	a	a	b	c	b	c	b
H27	a	a	a	b	c	b	d	b
H28	a	a	b	a	b	a	b	b
H29	a	a	b	b	a	a	a	b
H30	a	a	f	b	b	b	c	b
H31	a	a	f	b	b	a	c	b
H32	a	b	a	a	b	a	b	b
H33	a	b	b	a	b	a	b	b
H34	a	b	b	a	b	a	c	b
H35	a	b	b	a	b	b	b	b
H36	a	b	c	a	b	a	b	b
H37	a	b	c	a	a	a	a	b
H38	a	b	e	a	b	a	c	b
H39	a	c	h	a	b	a	b	a
H40	a	c	h	a	c	a	c	a
H41	b	b	b	a	b	a	b	b
H42	b	b	b	a	b	a	c	b
H43	b	b	b	a	b	b	b	b
H44	b	b	c	a	b	a	b	a
H45	b	b	c	a	b	a	b	b
H46	b	b	c	a	b	a	c	b
H47	b	b	c	a	b	b	b	b
H48	b	b	c	a	c	a	c	b
H49	b	b	d	a	a	a	b	b
H50	b	b	d	a	b	a	b	b

H51	b	b	d	a	b	a	c	b
H52	b	b	d	a	b	b	b	b
H53	b	b	e	a	b	a	b	b
H54	c	b	e	a	b	a	c	b
H55	c	b	c	a	b	a	b	b
H56	d	b	g	c	b	a	c	a
H57	d	b	g	a	b	a	c	a

Fragment number corresponds to the primer combinations in Table 1

Appendix S3

Geographic distribution of chloroplast PCR-RFLP haplotypes in Europe and Northern Africa. Colours and patterns of the segments correspond to the haplotype network of *P. alba* (red, blue, yellow), *P. tremula* (green, orange), *P. tremula* from Sweden (pink), *P. alba* from Greece and Northern Africa (grey) and *P. alba* from Spain (pink).



Chapter 2 – Investigations into the origin of the only *Populus alba* (L.) clone present in Malta

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Abstract

Populus alba (L.) was present in the Mediterranean basin already 6000 ago and the earliest record of *P. alba* on Malta is known from the end of the 16th century. Some studies from other Mediterranean islands suggest that clonal growth in *P. alba* can be very extensive. We investigated 38 samples from Malta and neighbouring regions within the Mediterranean in order to assess genetic diversity and the origin of Maltese *P. alba* trees. Nuclear microsatellite analysis revealed that all 28 trees from the two islands of Malta belonged to one clone. Chloroplast data suggested relatedness of the Maltese clone to Italian *P. alba* samples. However, nuclear data suggested additional admixture through pollen from North Africa. For the arrival of this clone in Malta, human introduction is the most likely explanation, because alternative scenarios like autovegetative propagation or arrival by seed seem unlikely.

Key words: clonal growth, Mediterranean basin, *Populus alba*, White poplar, nuclear microsatellites, PCR-RFLPs

Introduction

The first fossil records of the genus *Populus* are from around 40-50 million years ago (Stettler 1996). It first appeared in North America, and the first records in Europe appeared around 10 million years later (Collinson 1992). Notwithstanding its presence in Europe, until recently there were doubts as to whether *P.s alba* was autochthonous to certain parts of the Mediterranean, since it is often considered as a cultivated or sub-spontaneous species in the Western Mediterranean basin (Roiron et al. 2004). It was also known to be a popular tree in Roman times and was often planted in public places, thus giving the notion that it might have been spread by the Romans themselves in certain regions.

The discovery of fossil evidence in Southern France in the last decade, in the form of leaf imprints preserved in a travertine sequence from the Early Holocene, confirmed the presence of this species in the Mediterranean at least 6000 years before the arrival of the Romans (Roiron et al. 2004). Moreover, the discovery of unique chloroplast DNA haplotypes in samples taken from *P. alba* populations found in Sardinia, not only confirm the nativity of these trees in certain Mediterranean islands (Brundu et al. 2008), but also indicate that these trees could be relicts of a native flora that arrived here much earlier than previously thought. The same hypothesis may be deduced from the specific chloroplast types (Chapter 1) in samples from the Mediterranean, exhibiting large mutational differences to Central European trees of both *P. alba* and *P. tremula*.

The flora of the Maltese islands is quite similar to that of its closest neighbour Sicily, nevertheless there are numerous species that are found in Malta but absent from Sicily. Some of these are species of North African origin, others being species endemic to the Maltese islands, while others still are found in the Maltese islands and in one or two other places in the Mediterranean (Cassar et al. 2008).

Changes in sea level in the Central Mediterranean at various stages in time were mainly responsible for the arrival of the various biota that are nowadays found in Malta. Whilst there is plenty of evidence for the formation of temporary land bridges between Malta and Sicily, there is no very strong evidence for a land bridge with North Africa (Schembri 1997). The best possible time for this to happen was during the Messinian marine regression, whereby most of the Mediterranean dried up following the severing of the link with the Atlantic at Gibraltar (Cassar et al. 2008). This of course does not count

out the possibility of species having reached the islands by natural means or through humans, deliberately or accidentally.

The Maltese Islands are composed predominantly of marine sedimentary rock formed during the Oligo-Miocene era (30–5 million years BP) (Schembri 1997). The first evidence of dry land was in the late Miocene, around 10 million years ago. The remaining rock was composed after this, during the quaternary. According to a review on these quaternary deposits, traces of many different species of plants and trees, including evidence of riverine ecosystems with species belonging to the genus *Fraxinus* were found in Malta during this phase, however no records or traces of *Populus* were found (Hunt 1997). Nevertheless many of these riverine and wetland species became extinct by the end of the Pleistocene due to a change in climate (Schembri 1994).

The first reliable record of *P. alba* in Malta is in 1567 in a garden in Ghajn Filep (today part of Marsa) belonging to the then Grand Master of the Hospitaleer knights of the then Order of St John of Jerusalem (Grech 2001). Notwithstanding its presence in a private garden, the species was subsequently recorded growing in certain valleys including Wied Hazrun, in 1621 (Grech 2001). Up till 1927, it was extant in 6 locations in Malta and another 3 in Gozo (Borg 1927). By 2007, of the original populations recorded in 1927, only 4 in Malta survived. There were also a number of new populations never before recorded (Tabone 2007) however some of these most probably were planted by man.

Thus the goals of this studies were i) to estimate genetic diversity of *P. alba* stands in Malta and ii) to derive structures and relatedness to other Mediterranean regions. Chloroplast and microsatellite DNA (Chapter 1 and Brundu et al. 2008) were chosen as methods for this purpose because of their usefulness for phylogeography and assignment studies.

Materials and Methods

Study site

For this study we analysed 28 samples from the two (of three) islands of Malta (Fig. 1) and additional 10 reference samples from the Mediterranean basin.

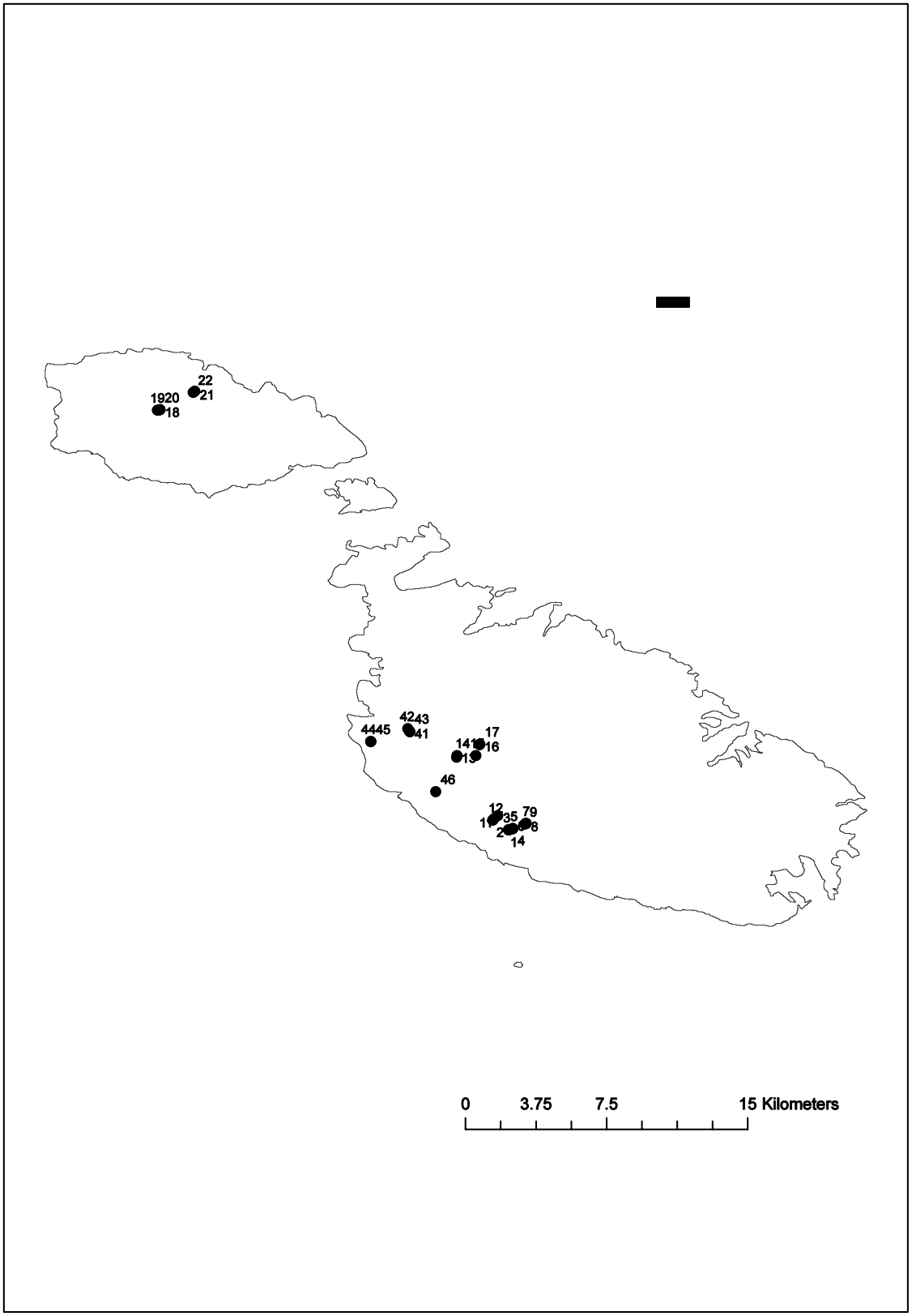


Fig. 1 Sampling sites of 28 trees of *P. alba* on the two islands of Malta

Molecular analysis

DNA was extracted from approximately 10 mg of dry material, using the DNeasy 96 plant kit (Qiagen) or the CTAB method (Doyle and Doyle 1987). Chloroplast DNA PCR-RFLP (polymerase chain reaction - restriction fragment length polymorphism) conditions were as given in Lexer et al. (2005), using six primer pairs (trnDP-TP, rpl161516-rpl16F71R, rpl16ex1f-rps3r2, rps3f2-ccmp10R, atpBSAM-rbclSAM, ccmp10R-trnHM) and five enzymes listed in Chapter 1. Primer pairs were chosen among the 'universal' ones listed at: http://bfw.ac.at/bfwcms.web?dok=4961 (Heinze 2007). Scoring of the fragments were done as described in Chapter 1.

For nuclear microsatellite analysis we used the M13-labelled primer method (Boutin-Ganache et al. 2001). PCR conditions were as follows: for a 15 µl reaction volume (1 µl DNA and 14 µl reaction mix) we had a final concentration of 2 mM Mg, 0.1 pM M13 labelled primer, 0.1 pM reverse primer, 0.02 pM forward primer including M13 extension, 0.1 mM dNTPs, 0.01µl Polymerase (Phire hot start, Finnzymes). The reaction was performed on a MJ research PTC-100 thermal cycler. We used a touch down procedure with the following settings: 20 pre-cycles from 70°C to 50°C and 30 additional cycles with 55°C annealing temperature. Fifteen primer pairs were chosen based on previous results (Lexer et al. 2005, 2007) from different original sources listed in Table 1.

Table 1 Details for 15 nuclear microsatellite primers located on 8 different chromosomes, including their repeat motive and original source

Nuclear microsatellites	Motive	Linkage group	Resource
ASP_112322	AG	6	d
GCPM_1192	AC	5	a
ORPM_202	TAA	8	c
ORPM_214	AG	18	c
ORPM_25	TA	5	c
ORPM_29	AC	11	c
ORPM_344	GA	10	c
ORPM_60	AAT	x	c
ORPM_82	CT	5	c
PMGC_0639	GA	x	a
PMGC_2558	GA	x	a
WPMS_09	GTC	6	b
WPMS_14	CGT	5	b
WPMS_16	GTC	7	b
WPMS_17	CAC	5	b

a, http://www.ornl.gov/sci/ipgc/ssr_resource.htm; b, van der Schoot et al. 2000; Smulders et al. 2001; c, Tuskan et al. 2004; d, personal communication Cottrell J.; x, linkage group unknown

Statistical Analysis

Descriptive statistical values were calculated in MSA (Microsatellite analyser version 4.05, Dieringer and Schlötterer 2003). Standard allelic richness was calculated based on the minimum number of sampled individuals for each locus. For populations with a smaller sample size than specified, allelic richness was not determined.

We used equation (1) of Parks and Werth (1993) to calculate the probability P_{gen} of an identical multilocus genotype (MLG) to result from an independent sexual reproduction event. We considered two scenarios, for the first we used allele frequencies

of the total sampling area to calculate P_{gen} for the Malta genotype. In the second scenario we used only the samples from Italy and Malta to calculate P_{gen}.

In order to assign the Maltese samples to surrounding regions of the Mediterranean, we analysed the data with the software STRUCTURE (Falush et al. 2003). The analysis parameters were set to 10000 runs in the burn-in period, then 50000 iterations, under the admixture model. The prior for population clusters, K, was set to vary between one and seven, and ten repetitions were run at each K. The Structure Harvest web service (http://taylor0.biology.ucla.edu/struct_harvest/ accessed 2010 06 16) was used to deduce the most likely number of K, following the rationales in the STRUCTURE manual, and the procedure suggested by Evanno et al. (2005).

Results

Our analysis of 15 nuclear microsatellites yielded an average of 4.5 alleles per locus in a total number of 38 samples in the Mediterranean basin (Table 2). All samples from the Maltese archipelago turned out to have identical microsatellite patterns. For one locus we found a private allele on Malta, which did not occur elsewhere. Allelic richness was rather low with an average of 1.42 for all populations and over all loci, due in part to the low number of samples in the reference populations, and the single clone in Malta (Table 3). One striking result was that we found only one genotype within the Maltese samples, whereas all of the 10 reference samples showed distinct genotypes. We calculated the probability that we sampled identical genotypes by chance. We encountered an extremely low genotype probability (P_{gen}) when considering all Mediterranean samples ($2.20\times e^{-122}$) and a slightly higher value for P_{gen} using only Italian and Maltese samples ($4.35\times e^{-94}$). These low values suggest that the origin of the identical genotypes from sexual recombination is very unlikely. The probability to obtain identical multi-locus genotypes (MLGs) by chance is very low when a high number of loci is analysed.

Table 2 Number of alleles for 15 microsatellite loci and 6 *P. alba* populations

	Malta (28)	Crete (2)	Italy (3)	Tunisia (2)	Morocco (2)	Portugal (1)	Total
PMGC_2558	1	1	n.d.	n.d.	1	n.d.	2
GCPM_1192	1	2	1	1	1	1	2
ORNL_202	2	3	2	2	2	2	7
ORNL_029	2	3	3	1	1	1	4
PMGC_0639	2	3	3	4	2	1	8
ORNL_60	2	4	1	1	1	n.d.	4
WPMS_14	2	3	2	3	3	2	9
WPMS_09	1	2	2	1	n.d.	1	3
ORNL_344	1	1	1	1	1	1	1
ORNL_214	1	2	2	1	1	1	2
ORPM_25	2	2	1	1	1	2	2
WPMS_16	2	3	3	3	2	1	7
ASP_112322	2	4	3	2	2	2	11
WPMS_17	2	3	2	1	1	n.d.	5
ORPM_82	1	1	1	1	1	1	1
Total	24	37	27	23	20	16	68

Table 3 Allelic richness 15 microsatellite loci and 6 *P. alba* populations

	Malta (28)	Crete (2)	Italy (3)	Tunisia (2)	Morocco (2)	Portugal (1)	Total
PMGC_2558	1,00	1,00	n.d.	n.d.	1,00	n.d.	1,27
GCPM_1192	1,00	1,67	1,00	1,00	1,00	1,00	1,20
ORNL_202	1,51	2,00	2,00	2,00	2,00	2,00	1,66
ORNL_029	1,51	1,83	1,73	1,00	1,00	1,00	1,60
PMGC_0639	1,51	1,83	1,60	2,00	2,00	1,00	1,74
ORNL_60	1,52	2,00	1,00	1,00	1,00	n.d.	1,56
WPMS_14	1,51	1,83	1,50	1,83	1,83	2,00	1,64
WPMS_09	1,00	1,50	1,50	1,00	n.d.	1,00	1,07
ORNL_344	1,00	1,00	1,00	1,00	1,00	1,00	1,00
ORNL_214	1,00	1,50	1,33	1,00	1,00	1,00	1,08
ORPM_25	1,52	1,50	1,00	1,00	1,00	2,00	1,51
WPMS_16	1,52	1,83	1,60	1,83	2,00	1,00	1,61
ASP_112322	1,51	2,00	1,73	1,50	1,50	2,00	1,74
WPMS_17	1,51	1,83	1,53	1,00	1,00	n.d.	1,59
ORPM_82	1,00	1,00	1,00	1,00	1,00	1,00	1,00
Average	1,31	1,62	1,30	1,21	1,22	1,07	1,42

For chloroplast data, we found a total of 6 haplotypes (Table 4). Those are very distantly related according to a median joining network (Fig. 2 in Chapter 1). The Maltese samples exhibit haplotype H12, which was found also in Southern Italy. Haplotypes of the Southern Mediterranean (Portugal, Morroco, Algeria, Tunisia and Crete) belonged to haplotypes with a deep (ancient) split from the rest of the European samples (see Chapter 1).

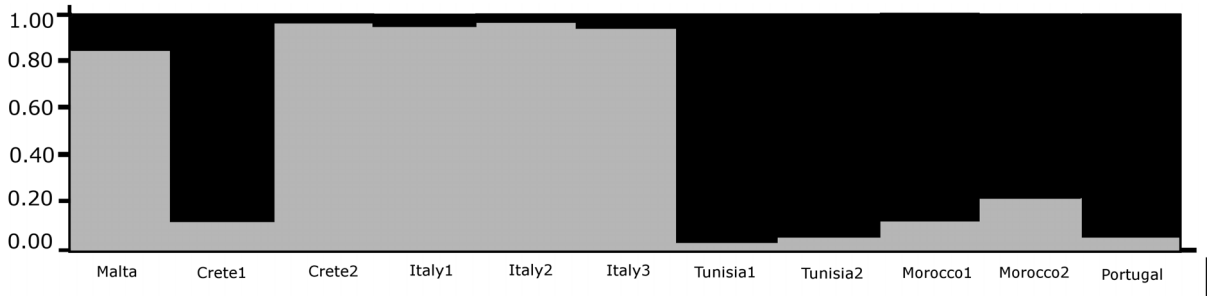
Table 4 Allele size ranges per locus and population. Allele size ranges (bp) of 15 microsatellite markers and one chloroplast marker of 6 Mediterranean populations of *Populus alba* (sample size is given in brackets per population)

Name of marker	Type of marker	Total size range[a]	Malta (28)	Crete (2)	Italy (3)	Tunisia (2)	Morocco (2)	Portugal (1)
ASP_112322	SSR	165-205	165/179	167-205	173-198	165-183	171-197	169/179
GCPM_1192	SSR	211-213	213	211-213	213	211	213	211
ORNL_029	SSR	196-226	196/226	196-226	196-198	196	196	196
ORNL_060	SSR	220-238	220/226	220-238	226	220	220	
ORNL_202	SSR	216-286	226/264	226-261	226-261	252-261	282-286	216/252
ORNL_214	SSR	180-182	180	180-182	180-182	180	180	180
ORNL344	SSR	273	273	273	273	273	273	273
ORPM_25	SSR	227-237	227/237	227-237	237	227	227	227/237
ORPM_82	SSR	183	183	183	183	183	183	183
PMGC_0639	SSR	107-125	107/111	111-121	117-125	119-125	117-119	121
PMGC_2558	SSR	166-168	166	168			168	
WPMS_09	SSR	247-265	247	247-265	247-249	247		247
WPMS_14	SSR	225-259	238/241	238-253	238-244	235-245	225-259	238/253
WPMS_16	SSR	167-254	194/206	194-254	167-200	188-200	185-194	194
WPMS_17	SSR	133-145	137/139	137-145	137-139	133	143	
rpl16	cp	438-470	450	438	450	463-470	470	470
haplotypes	cp	7,12,13,30,39,57	12	30	7,12,13	57,39	39	39

[a] For haplotypes, designations are according to Chapter 1

The STRUCTURE runs clearly identified only two apparent source populations that were evident from all the runs, independent of K. The samples from Malta clustered with the Italian ones, while there was a separate cluster consisting of the North African (Tunisia, Morocco) and the single Portuguese sample. Interestingly, each of the clusters contained one of the two samples from Crete. An example of such a run with K=2 is shown in Fig. 2.

Fig. 2 STRUCTURE bar plot of a run at K=2. Each bar represents one sample. Shadings show assignment proportion to each of two assumed source populations. Order of samples (black and grey bars): Malta, Crete 1, Crete 2, Italy 1, Italy 2, Italy 3, Tunisia 1, Tunisia 2, Morocco 1, Morocco 2, Portugal



Discussion

Based on our molecular analysis we found that all the *Populus alba* samples on the islands of Malta belong to only one clone. All the genotypes of the 28 samples were identical and the probability of observing identical genotypes by chance was very low. All of these samples were taken from different valley systems, of which only few are connected downstream. This is a striking result because we analysed several stands on different islands. It is even more extreme than what Brundu et al. (2008) found for the same species in Sardinia - with rather old clones ranging up to several kilometres along rivers, and resembles the situation in Southern Spain (L. Santos del Blanco and E. Hidalgo, personal communication). Furthermore Brundu et al. (2008) reported low portions of viable seed production and no sign of seedlings, suggesting that clonality in *P. alba* in the Mediterranean might be the only chance for the species to survive.

We assume the Maltese clone to originate (with higher probability) from Italy, because it carries an Italian chloroplast haplotype. However, nuclear data suggest an additional, though low, admixture with North African alleles: most of the nuclear microsatellite alleles are similar to Italy, and the STRUCTURE runs also suggest this as a general conclusion, but a small portion is identical to the typical North African alleles

(Table 4). These seem to suggest that the clone is more typical for Southern Italy in general, or Sicily in particular. It is more likely for African pollen to cross the Mediterranean sea than for seeds to travel over sea and still be viable afterwards. Also it is likely that there is ongoing pollen exchange between Italy and North Africa and because of low sample numbers we might not have captured rare traces of North African alleles in Italy.

We see several possible scenarios for the arrival of this clone in Malta. If it is similar to genotypes in Southern Italy (or Sicily), it could have arrived over a land bridge during the Quaternary. The last time the sea level fell between Malta and Sicily was during the Pleistocene with the last Glacial Maximum occurring between 22,000 to 17,000 years ago before humans settled on the islands (Cassar et al. 2008). These arrived in the Holocene, around 7500 years ago (Hunt and Schembri 1999). The probability of a single clone of *P. alba* crossing over between Sicily and Malta by autovegetative propagation across a temporary land bridge seems rather unlikely. Moreover, there is no evidence of this species in Quaternary deposits.

A second scenario would involve airborne seed(s) that travelled across the sea. Again, that would imply that only one individual seed arrived here or at least only one established itself. Again, the probability of this occurring seems low.

The third scenario would be that someone brought it as a cutting from a tree (or as a potted tree or as a bare root plant) originating just across the sea. *P. alba* is not as easily propagated by cuttings as e.g. *P. nigra*. But it seems that the Maltese clone is especially suited for vegetative propagation, which has been performed successfully for the last 100 years. As shown for the case of an *Ulmus* clone going back to Roman times and associated with viticultural practices (Gil et al. 2004), human beings were capable of organising such long-distance plant transport two thousand years ago.

Given the low probability of a single seed or clone arriving in Malta by natural dispersal, we propose to consider the possibility of the reduction of a formerly genetically more diverse population down to a single clone during history. This clone, probably restricted to a single location initially, would then have been dispersed by humans (or as a result of their e.g. agricultural activity), as it is now present in different small valleys, and on two different islands. Physical agents (wind and water), or animals, are less likely to have resulted in such a dispersal pattern. It is unlikely that any animal would have spread (root) cuttings – there is no such example in the *Populus* literature.

Evidence for *P. alba* clones spreading locally (by root suckers) is numerous, however, and includes molecular studies (van Loo et al. 2008).

We cannot, however, with the limited set of comparison clones available for this study, determine whether the Maltese clone is typical for Southern Italy (or Sicily). If the Maltese clone will indeed turn out to be more related to trees from the Italian mainland or further up the peninsula (in any further studies), then it could not have arrived there naturally at all. The distances for seed dispersal would have been too large. The only other feasible option would be for it to have arrived here through direct human intervention, which in the light of the evidence we have so far could very likely be the case. For all scenarios involving the active human introduction of this clone, including Roman civilization, the knights of St. John are more likely to have been involved, because the first record of the species presence was as a tree found in the private garden of the then grandmaster. Again, it could have spread from there by human intervention, and once established in the river systems, by autovegetative propagation along the valleys.

The present study shows how molecular genetic evidence can inform botanical, paleontological, and archival studies, resulting in a nice example of interdisciplinary research on the origin of a species on an island.

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Chapter 3 – Computer-assisted morphometry in a Central European poplar (*Populus*) hybrid zone

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Abstract

We present a sound method of separating *P. alba* (L.), *P. ×canescens* (Aiton, Sm) and *P. tremula* (L.) in an Austrian hybrid zone. Statistical analyses demonstrate that the parental species can be discriminated by a method that is based on measuring the degree of "lobedness" of the leaves, supported by two additional parameters, which identify *P. ×canescens*, the hybrid taxon. The statement in the literature that “lobedness” is a distinctive character in white poplar is now measurable by the HF index. Furthermore a two-step sampling approach was successful in dealing with heterophylly and identifying the most valuable type of shoot for morphometry in the studied taxa.

Keywords: Austria, *Populus* hybrid zone, morphometry, leaf trait measurements

Introduction

In order to describe hybridisation, it is first of all necessary to understand species formation and how species boundaries develop. Currently, from 22 (Mayden 1997, 2002) to 26 different species concepts (Wilkins 2006) are proposed, some of them reach back to Aristotle and Linnaeus, based on morphology (reviewed in Wilkins 2006), others emerged with the use of molecular genetic methods. From a biological species concepts point of view, a species consists of populations able to interbreed with each other. On the other hand, evolutionary species concepts try to look into the past to evaluate relationships between species and/or biological units and their historical development. For the evaluation of today's species, studies implement crossing experiments, assessment of reproductive barriers and studies of hybrid zones. Species concepts, and a clear definition of species, are needed in order to label biological units in e.g. assessing biodiversity, and for management plans for conservation purposes, as well as in evolutionary studies. For practical reasons, it is desired to have at hand parameters that can be assessed in the field to differentiate between species and their hybrids.

On the one hand, hybridisation is an important process in nature. In general, 25% of the plant species are affected by hybridisation and introgression (Mallet 2005). Hybridisation is considered to play an important role in the evolution of plants (Gömöry & Schmidtova 2007) and can lead to new phenotypes and even to new species (hybrid speciation, Turelli et al. 2001, Mallet 2007). From the ecological point of view it has recently been shown that hybridisation is one of the factors responsible for the success of invasive species (Tiébré et al. 2007, Ellstrand & Schierenbeck 2000). On the other hand, experimental hybridisation is used as a tool in plant breeding and has long been used in *Populus*, because it is relatively easy to perform interspecific crossings within the genus. Hybridisation in *Populus* is also the main reason for controversial species numbers within the genus, as 22 species (Eckenwalder 1996) up to 85 species were proposed (Dickmann & Kuzovkina 2008). The recently completed genome sequence of a *Populus trichocarpa* tree (Tuskan et al. 2006) has revealed that recent genome duplication has happened in the family of *Salicaceae*. Consequently, evolving gene reorganization following this process may have contributed to the current difficulties in species differentiation and description of species boundaries.

One way to tackle this problem is to find appropriate parameters in e.g. floral (Aldridge 2005), or leaf (*Quercus*, Gömöry & Schmidtova 2007) morphological markers, measure them and subject them to rigorous statistical tests, in order to evaluate their

usefulness, either as single characters, or as a combination of traits. This is what we tackle in this paper for a *Populus* hybrid zone in Central Europe.

The genus *Populus* belongs to the family of *Salicaceae* together with the genus *Salix* (Dickmann & Kuzovkina 2008). Members of the genus *Populus* are fast growing deciduous trees with wind-dispersed pollen and seeds. Three organs are mainly used for species identification, namely flower bracteoles, buds and leaves.

Firstly, the flowers are arranged in catkins on dioecious trees. Our species of interest are *P. alba*, *P. tremula* and their hybrid *P. ×canescens*, and they differ in their catkin appearance. The female and male catkins in *P. tremula* look almost alike in colours of brown and grey and they are very soft due to their deeply incised and hairy bracteoles. On the other hand, female catkins of *P. alba* and *P. ×canescens* are green with fragile bracteoles bearing only few hairs. Male catkins of *P. alba* and *P. ×canescens* may appear similar to *P. tremula* on first sight but with a distinctive character of the bracteole margin. In *P. alba* the bracteoles are entire or slightly dentate, in contrast to the deeply incised and hairy bracteoles in *P. tremula*. In *P. ×canescens* bracteole margins are moderately incised (Fischer et al. 2005).

Secondly, winter buds are a further character for identification of the three species described in the literature. They are different in shape and pubescence (Fischer et al. 2005). *P. alba* has ovoid and thick buds, which are grey in colour. Buds of *P. tremula* are more slender, pointed at the tip and brown (Fischer et al. 2005, Humphries et al. 2006). *P. ×canescens* appears closer to *P. alba* with ovoid and slightly pubescent buds (Fischer et al. 2005, Humphries et al. 2006).

Thirdly, leaf characters are widely used in trees to identify species. In poplars, mainly leaf shape and pubescence are used and they differ between short and long shoots. This is because there is pronounced heterophylly between preformed and neoformed leaves. Preformed leaves are present within winter buds and appear on short shoots and early long shoots. Neoformed leaves appear on late long shoots within the crown as well as on ground shoots. This phenomenon affects the whole genus, which is why taxonomists agreed to use short shoots for identification of the species. There is only one exception where neoformed leaves are more diagnostic taxonomically – namely the lobed neoformed leaves of *P. alba* and its hybrids (Bartels 1987, Eckenwalder 1996). Therefore it is the "typical" leaves that are used in literature for species identification in the *Populus alba-tremula-×canescens* complex. Those leaves appear on the long shoots in *P. alba* with 3-5 lobes (Fig. 1) and downy white hairs on the lower surface (Humphries et al. 2006). Typical leaves of *P. tremula* occur on the short shoots and are broadly ovoid (Fig. 1), with a point at the leaf

apex and regular dentate margins, paler below than above but without hairs. Typical leaves of *P.* ×*canescens* on long shoots appear triangular or ovate with coarse irregular dentate margins and grey hairs below (Fischer et al. 2005, Humphries et al. 2006). The leaves of short shoots are grey pubescent in *P. alba* and without hairs in *P.* ×*canescens* and *P. tremula*.

In the literature, the shape of the flower bracteoles is proposed as the strongest character to resolve the *P. alba-tremula-*×*canescens* complex (Fischer et al. 2005). Thus, lacking flowers it remains challenging to determine species accurately. As a fact, for tree species leaves are the most interesting characters due to practical reasons. Flowers are usually only available for a limited period of time, they are difficult to sample, because they appear in the top of the crown and trees have delayed flowering. For example, *P. alba* usually flowers in March and April in Central Europe and starts flowering only at the age of 10-15 years (Dimitri & Halupa 2001), which is even early in their lifetime, compared to other tree species. In contrast, leaf characters that unambiguously identify the species and their hybrid would provide a practical and fast method for species identification in early stages in tree development, available throughout the whole growing season.

An interesting approach to morphology was recently proposed by Lexer et al. (2009), based on elliptic Fourier transformation of leaf outlines.

In this study, we measured six leaf morphological parameters in the two species and their intermediate hybrid, the *Populus alba-tremula-*×*canescens* complex and suggest a more traditional way of analysing the key differences between these taxa.

The goals were i) to develop a method for unambiguous identification of the species based on leaf characters and ii) to evaluate the developed sampling scheme in a statistical way by sampling trees along a hybrid zone in Central Europe.

Material and Methods

P. alba (white poplar) and *P. tremula* (European aspen) occur parapatric in Austria, but come into contact in various locations. A large hybrid zone in the Danube floodplain was described by Lexer et al. (2005, 2007). In this paper, we focus on a population at the northern edge of the natural range of *P. alba* in Austria, within a *P. tremula*-dominated hybrid zone (Fig. 1). The two hybrid zones are connected via the river Kamp emptying into the Danube. The sampled area is characterized by an elevational gradient from high to low of *P. tremula/P.* ×*canescens/P. alba*. *P. tremula* occurs upland, whereas *P. alba* is mainly situated in the bottom of the valley.

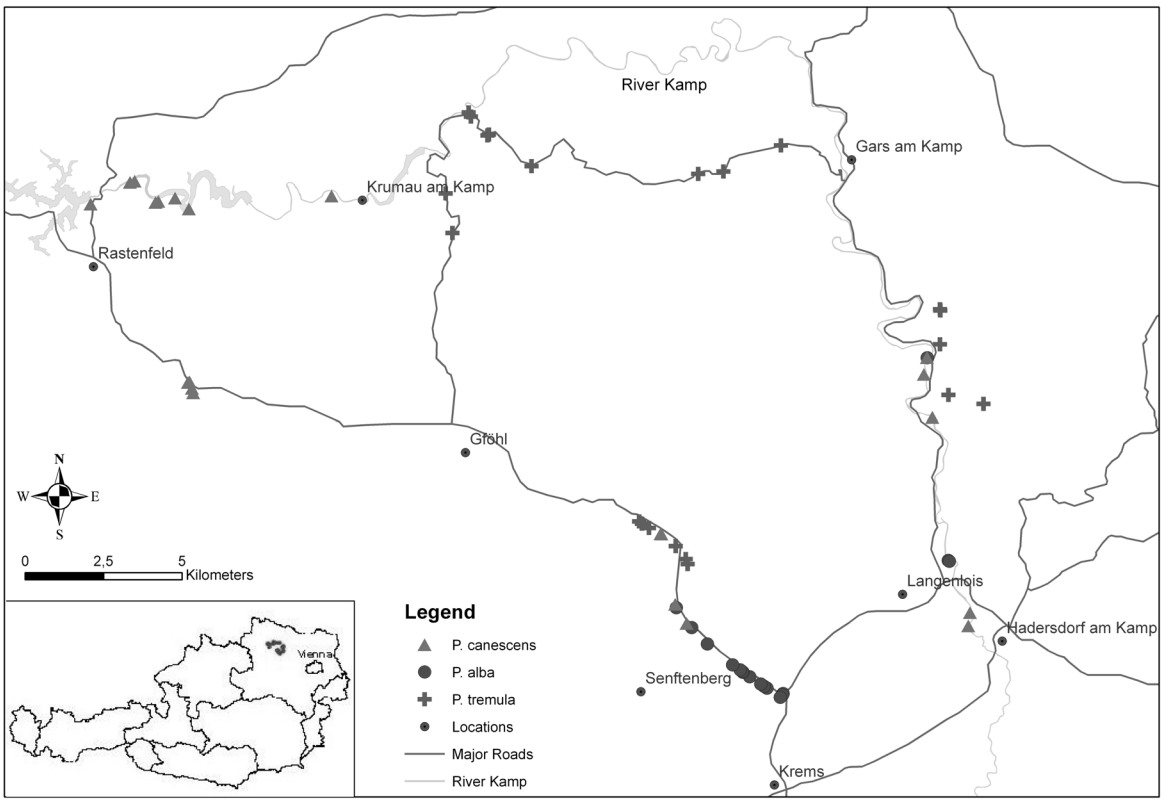


Fig 1 Distribution of *P. alba*, *P. tremula* and *P.* ×*canescens* in the study area in Northern Austria, mainly sampled along the river Kamp

The sampling area was located in Northern Austria and comprised about 50x50 km. The elevation ranged from 200-670 m above sea level. Trees were mainly collected along linear transects usually following a road and/or the river Kamp (Fig. 1). Locations of the trees were taken with a GPS device. In a first step a total of six trees, two per taxon were sampled. Trees and their leaves were initially identified as one of the three taxa, based on the current Excursion Flora of Austria (Fischer et al. 2005), considering the appearance of the entire tree, including long and short shoots. For each tree four to seven long and short shoots were collected and one leaf per shoot was selected for measurements. For short shoots one leaf of the bunch was taken and for long shoots every 9th leaf from the new annual shoot was taken (n=63). To ensure well developed and entire leaves sampling was performed in September, when leaf growth and development was completed. Sampled leaves are stored in the herbarium at the Federal Research and Training Centre for Forestry, Natural Hazards and Landscape in Vienna, Department of Genetics. Both surfaces were scanned with a scanner (HP Scanjet 3670) set to 300x300 dots per inch (dpi) resolution. The following 6 leaf parameters were measured (Fig. 2):

1. Base angle: the angle between the first vein and the main vein

2. Peak angle: the angle formed by the leaf tip

3. L1: the distance of the starting point and endpoint of the first vein on the left site [mm]

4. L2: the distance of the starting point and endpoint of the main vein [mm]

5. Leaf area [mm²]

6. Circle diameter: the diameter of the smallest circle which covers the leaf area exactly [mm]

a) b)

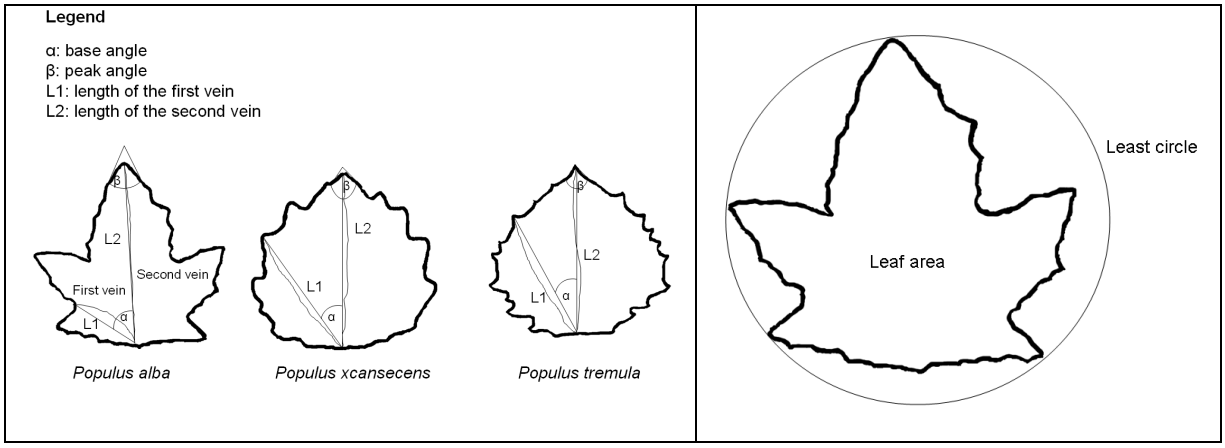


Fig 2 Leaf parameters (a), leaf area and the circle diameter (b)

L1, L2, the base and the peak angle as well as the circle diameter were measured by GIMP 2.4.6 graphical software (http://www.gimp.org).

The leaf area was calculated by ImageJ (Abramoff et al. 2004). Both the leaf area and the circle area defined the HF index, being calculated as their ratio, which relates to the "roundness" *vs.* "lobedness" of the leaf.

After the analysis of those first-step samples we decided to perform a second collection which consisted of altogether 94 trees (*P. alba* 20, *P. ×canescens* 32, *P. tremula* 42) that was processed in the same way. That time we collected only from the “typical” shoot of the species as supported by a discriminant analysis of the first-step samples (see results Table 3). In that way, a species assignment of the trees was performed based on the appearance of the whole tree (habitus; Fischer et al. 2005), and then it was decided which kind of leaves to take. Thus leaves of *P. alba* were sampled from long shoots and leaves of *P. ×canescens* and *P. tremula* were taken from short shoots.

The statistical analysis was performed with the R statistical software packages (R Development Core Team 2008). Here, differences between the mean values were calculated between all species combinations and are given together with confidence intervals and

significance levels. In order to compare all pairs of means among the groups (species), we performed analysis of variance with Tukey's post-hoc honestly significant difference test. This test corrects for mulitple testing arising by comparing all pairs of means, i. e. it holds the experimentwise type I error rate at the nominal significance level of 0.05.

A discriminant analysis was performed using the software STATGRAPHICS Plus (ver. 4, Centurion XV, StatPoint Technologies, Inc.) in order to check for the correct assignment to one of the three groups.

Results

For the first step (assessing short shoots vs. long shoots in all taxa), morphometric analysis was performed based on five parameters of altogether 63 leaf specimens of 6 trees within the hybrid zone (Table 1). The HF index was significantly different between all three taxa. Morphometric measurements for the samples of the second step are listed in Table 2 based on 94 specimens (one leaf per tree). On average, values differed clearly between *P. alba* and *P. tremula* and mean values for the hybrid *P. ×canescens* resided between the pure species, except for the peak angle, where *P. ×canescens* had the lowest value (Table 2). Significant differences between species combinations were detected in three parameters (base angle, L2, HF index), the other two were not significant (Table 2 and S1). The calculated HF index again showed significant differences among all three species (Table 2). The parameter L2 (the length of the main vein) was significant for distinguishing *P. tremula* at the 5% level, and the base angle significantly distinguished *P. alba* from both other taxa (Table 2).

Table 1 Mean values and standard deviation of leaves of short (SS) and long shoots (LS) per each of six trees, based on one leaf per each shoot of 4-7 shoots per type of shoot and tree

	A44 SS[6]	A44 LS[5]	A5 SS[5]	A5 LS[5]	C15 SS[5]	C15 LS[4]	C73 SS[5]	C73 LS[5]	T20 SS[7]	T20 LS[5]	T64 SS[6]	T64 LS[5]
Base angle												
mean	66,8	85,7	71,1	62,2	57,2	76,5	65,8	54,6	65,9	69,4	40,9	55,9
SD	15,9	8,7	14,1	8,9	6,8	8,4	13,6	16,5	8,8	17,5	6,5	20,4
Peak angle												
mean	84,8	82,8	79	58,4	113,3	93,9	75,1	72,7	101,4	90,7	89,6	93,3
SD	8,8	9,8	11,9	11	8,9	5,1	10,1	4,5	12,8	7,3	12,3	12,8
L1												
mean	21,6	20,9	18,7	45,8	14,4	14,9	17	27,4	23,3	25,9	38,2	31
SD	10,7	5,2	11,7	6,6	5,1	5,1	6,1	8,7	12,1	11,6	6,9	11,5
L2												
mean	53,3	64,9	61,8	84,3	60,6	71,4	44,6	48	58,4	62,6	51,6	48,8
SD	1,9	2,4	7,2	18,3	16,6	3,9	3,8	5,9	5,2	2	6,7	3,6
HF index												
mean	0,721	0,728	0,586	0,617	0,645	0,602	0,856	0,884	0,891	0,836	0,817	0,787
SD	0,058	0,045	0,035	0,101	0,033	0,035	0,05	0,069	0,043	0,026	0,05	0,059

A *P. alba*, C *P.* ×*canescens*, T *P. tremula,* Statistics based on different numbers of shoots per tree indicated by superscript numbers in each column

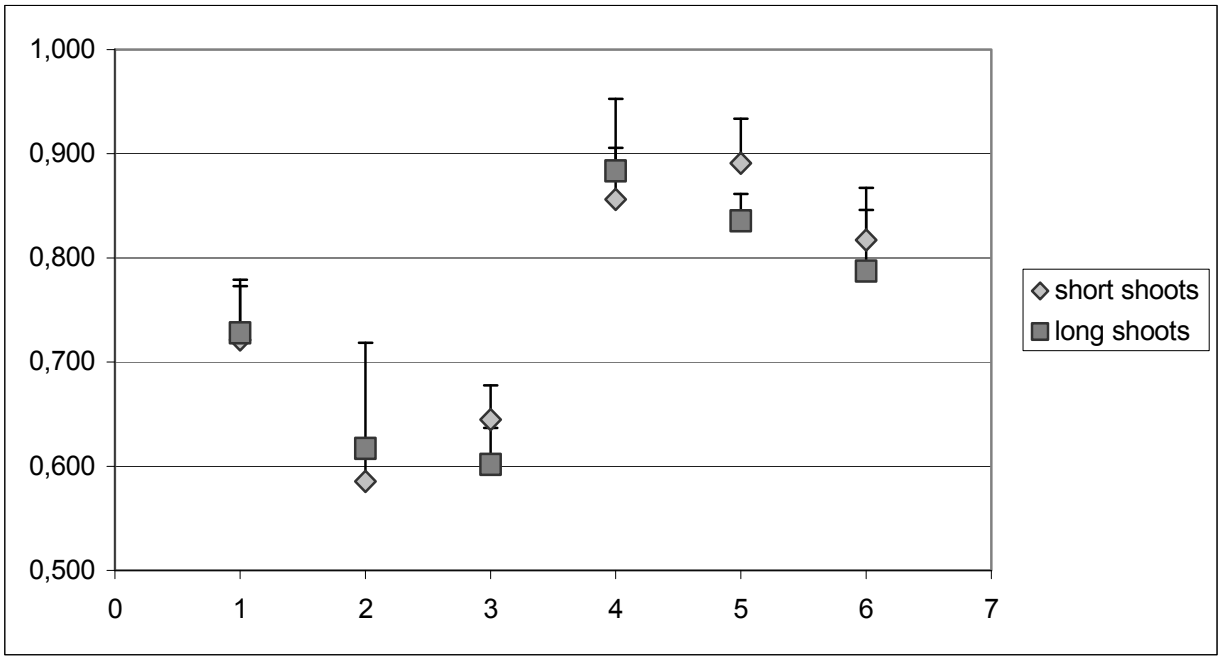
Table 2 The mean value and standard deviation of six measured parameters in all three taxa and 94 trees including base angle, peak angle, L1 (first vein), L2 (second vein), and HF index as a measure of lobedness

	P. alba	*P. ×canescens*	*P. tremula*
Base angle			
mean	71,8*	58,5	52,1
SD	12,6	18,9	17,1
Peak angle			
mean	81,6	77,7	85,8
SD	15,1	21,4	19,1
L1			
mean	14,8	27,9	31
SD	11,05	12,1	13,8
L2			
mean	68,7	65,3	56,4*
SD	12,7	9,8	9,3
HF index			
mean	0,586*	0,688*	0,753*
SD	0,060	0,066	0,038

*Significant values at $p < 0,05$

On the one hand, each of the measured parameters taken for itself did not show specific values for each taxon, except the HF index (Fig. 3). For the HF index, trees one and two (of the first set) were clearly different from trees five and six indicating clear separation of the pure species. But hybrid specimens were assigned to one of the pure species as well – tree number three to *P. alba* and tree number four to *P. tremula*, respectively.

Fig. 3 Mean values and standard deviation of the HF index differentiating the parental species, given for leaves of short (diamonds) and long shoots (squares) of two trees per taxon



P. alba (1,2), *P.* ×*canescens* (3, 4), *P. tremula* (5, 6). Standard deviation is indicated as vertical line

On the other hand, combining all five parameters using a discriminant analysis allowed to distinguish three groups. The analysis of all leaves per tree without separation of long and short shoots revealed a total correct classification of 76.2 % (Table 3a). Nevertheless misclassification into the other pure species was observed in three individuals. When only leaves from long shoots were considered the total correct classification was more successful (79.3%), but misclassified individuals into the other pure species were still recognised. When only short shoots were analysed a clear increase of correctly classified individuals was observed (91.4%) but misclassifications into the other pure species could not be eliminated. Combining leaves from long shoots for *P. alba* and short shoots for *P.* ×*canescens* and *P. tremula* led to slightly lower total correct classification (90.9%), but misclassifications into the other pure species were eliminated, thus indicating that the correct classification can be substantially increased by sampling the „typical" leaves of the three taxa. Consequently we decided to design the second sampling scheme according to the results of the first one and sampled the typical leaves of 94 trees as described above. The lower total correct classification of 73.4% of the

measured leaves was compensated by the fact that no misclassifications into the other pure species was observed.

Table 3 Discriminant analysis of leaf morphology in *P. alba*, *P. ×canescens* und *P. tremula* for the first (a, long shoots LS and short shoots SS, respectively) and the second (c) sampling scheme

		Predicted species			Percent of cases correctly
Species	**Group size**	***P. alba***	***P. ×canescens***	***P. tremula***	**classified**
a)		**SS+LS**	**SS+LS**	**SS+LS**	**76,2%**
P. alba	21	16 (76,2%)	3 (14,3%)	2 (9,52%)	
P. ×canescens	19	3 (15,8%)	14 (73,7%)	2 (10,5%)	
P. tremula	23	1 (4,4%)	4 (17,4%)	18 (78,3%)	
b)		**LS**	**SS**	**SS**	**90,9%**
P. alba	10	10 (100%)	0	0	
P ×canescens	10	0	8 (80%)	2 (20%)	
P. tremula	13	0	1 (7,7%)	12 (92,3%)	
c)		**LS**	**SS**	**SS**	**73,4%**
P. alba	20	18 (90,0%)	2 (10%)	0	
P. ×canescens	32	5 (15,6%)	17 (53,1%)	10 (31,3%)	
P. tremula	42	0	8 (19,0%)	34 (81,0%)	

a, leaves of long and short shoots per tree based on 63 specimens of 6 trees; b, leaves of long shoots for *P. alba* and short shoots for *P. ×canescens* and *P. tremula* based on 33 specimens of 6 trees; c, leaves of long shoots for *P. alba* and short shoots for *P. ×canescens* and *P. tremula* based on 94 trees

Discussion

The morphological leaf traits that we measured and calculated can group individuals into the three taxa with statistical confidence and correspond well with the initial classification in the field. The two-step sampling approach supported by the discriminant analysis turned out to be an adequate way to deal with the matter of heterophylly in the genus. First, the separate consideration of leaves of the two shoot types was clearly necessary (evident when comparing to results obtained by mixing shoot types for each species). Second, through selection of the “typical” shoots per taxon the

best results (regarding avoidance of misclassification into the other species) were achieved. Our sampling of pre- and neoformed leaves according to the overall appearance of the tree in the field is reasonable as long as several traits are considered. Out of the 5 calculated parameters that we used for taxon comparisons, one was most striking in identifying all three taxa. The HF index turned out to be most valuable because it described the incision of the leaves most accurately and was significant in all three groups of comparison (*P. alba - P. ×canescens, P. tremula - P. alba, P. tremula - P ×canescens,* Tablr S1). In addition L1 and L2 also measure leaf lobedness which is one of the best leaf characters to distinguish between the three taxa (Fischer et al. 2005, Humphries et al. 2006) and can easily be measured in the field. Especially L2 was recognized differentiating *P ×canescens* from *P. tremula*, whereas the base angle was valuable in differentiating *P ×canescens* from *P. alba*. The ratio L1/L2 may serve a similar purpose as the HF index, as it also depends on the depth of the lobes.

In contrast to our basic approach, Lexer et al (2009) used linear discriminant analysis of complex morphometric traits, based on elliptic Fourier analysis. They revealed that leaf shape (length/width), lobation and leaf tip are the major characters differentiating the pure species. Furthermore, Lexer et al. (2009) found hybrids characterised by various phenotypes and those were distinguished by complex and composite aspects of lobation and leaf architecture.

Assessment of some additional characters (pubescence and leaf petioles) was considered in our study, but rejected for several reasons. According to some authors (Fischer et al. 2005, Humphries at al. 2006) pubescence of several organs i.e. leaves and leaf petioles, buds and shoots are useful for identification purposes. However, a closer look at these references reveals that they concede that pubescence changes during the year, i.e., any comparison would have to take the seasonal variation at the collection site into account. Moreover, we noticed that pubescence was a difficult character to measure in the *P. alba - tremula - ×canescens* complex. Not like in *Quercus* species, where hairs are relatively large and more spaced (Gömöry and Schmidtova 2007), in *P. alba* they are very small and dense. It is not possible to count them under the binocular microscope, especially in dry herbarium specimen. We also experimented with digital image processing to detect colour differences caused by the degree of pubescence. There are several limitations to that option though: it is depending on the selection of the leaf area to measure and the age of the leaf. Furthermore, colour values should preferentially be measured on fresh leaves and not with herbarium material, like in our study. Consistent

with our doubts, a recently published taxonomy states the pubescence in *Populus* as a controversial character (Dickmann & Kuzovkina 2008), because of the manifold expression of this trait within a species. In general, pubescence or hairiness in plants has long been acknowledged for its protective function against drought in warm climates (Picotte et al. 2009), against herbivory (Lill et al. 2006, Hoof et al. 2008), against UV-radiation at higher altitudes (Uribe-Sales et al. 2008) and against salt incrustation of the stomata (Morrison 2002). Thus, pubescence has a strong environmental component, rather than being a purely genetic trait. In fact, we observed such biogeographical differences in *P. tremula* between Central and South-eastern Europe. Leaves from Croatia had hairs on the upper and lower surfaces, whereas *P. tremula* from all other regions were without hairs (unpublished results). Based on this character alone, it would not be possible to distinguish *P. ×canescens* from *P. tremula* in that region. However a very recent study of Lexer et al. (2009) managed to visualize differences in leaf surfaces of *P. tremula* and *P. alba* by using scanning electron microscopy, thereby providing an objective tool to measure leaf pubescence.

Furthermore, we did not use leaf petioles, because they are controversially treated in the literature as well. Humphries et al. (2006) stated that leaf petioles are laterally flattened in both parental species. Dickmann & Kuzovkina (2008) divided the section of *Populus* into two groups according to the petiole cross section, i.e. round for white poplars and flattened for aspens. Fischer et al. (2005) do not mention this character at all.

But most importantly, how distinct were the two species and their hybrid, based on our results? *P. alba* and *P. tremula* were significantly different in three of the measured characters with large differences in their means (Table 2). But single morphotypes within the species occurred with sometimes strong deviation from the mean. Between *P. alba* and *P. ×canescens*, base angle and HF index were significantly different. *P. ×canescens* as a taxon was intermediate in all characters, except the peak angle, between the two species. However, single trait values may approach or reach those of one of the parent species, which is reasonable if such traits are inherited rather independently from each other in further hybrid generations. This is in line with earlier findings for molecular markers (Lexer et al. 2005, 2007), which have also been shown to be independently inherited, especially if they are situated on different chromosomes. It is also in line with the independent factors obtained after elliptic Fourier transformation of leaf outlines by Lexer et al. (2009).

Overall, the weakest distinction was between *P. tremula* and *P. ×canescens* in the measured population (Table 3c). First, this could have biological reasons, if backcrosses to *P. tremula* occurred among the material. Lexer et al. (2005) genetically described a high number of backcrosses towards *P. alba* within a *P. alba*-dominated Austrian hybrid zone. In contrast, the measured population of this study was situated at the northern edge of the natural range of *P. alba* within a *P. tremula*-dominated hybrid zone. Thus, backcrosses towards *P. tremula* are more likely to happen there, but molecular studies yet have to prove this assumption. Second, the similarity of *P. tremula* and *P. ×canescens* in this study might be due to the selection of characters that were measured and calculated. There may be additional informative characters, or alternative calculated parameters or ratios that bring out differences in a more pronounced way. However, to validate this approach, it is most important to measure additional populations and check whether the characters that were introduced here are useful in different biogeographical contexts.

We believe that with this method, single trees can be assigned correctly to one of the three taxa of this complex. First, the characters used in the literature were fine-tuned, because the term "3-5 lobed" (Fischer et al. 2005, Humphries 2006) is rather vague and does not consider the depth of the lobes. We measured the depth of lobes by the HF ratio, describing the fact that the more a leaf is incised, the smaller is the ratio between leaf area and the area of the circumscribing circle. Second, the traits were subjected to statistical treatment and the levels of usefulness of the characters were evaluated for all the three comparisons. Third, two new measures (base angle and peak angle) were identified which have not yet been described in the literature until recently ('leaf tip', Lexer et al. 2009) as differentiating the taxa. The statistical treatment shows that the base angle supported the other values, but would not on its own allow a good distinction of all taxa.

Now this method has to be validated in a broader sample of individuals of different geographical regions. Furthermore, we would like to expand our investigation and compare the results with genetic analysis.

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Table S1 Differences in mean values and significance at the 5% level for all 3 species combinations and altogether 94 trees

	Differences in mean values	Confidence intervals	Significance
Base angle			
can-alba	-13,2807	-24,9486 – -1,6128	*
trem-alba	-19,7330	-30,8539 – -8,6122	*
trem-can	-6,4523	-16,0573 – 3,1527	ns
Peak angle			
can-alba	-3,8796	-17,0981 – 9,3389	ns
trem-alba	4,2615	-8,3373 – 16,8602	ns
trem-can	8,1411	-2,7404 – 19,0226	ns
L1			
can-alba	3,0756	-5,6835 – 11,8347	ns
trem-alba	6,1600	-2,1884 – 14,5084	ns
trem-can	3,0844	-4,1261 – 10,2949	ns
L2			
can-alba	-3,4250	-10,5265 – 3,6765	ns
trem-alba	-12,2548	-19,0233 – -5,4863	*
trem-can	-8,8298	-14,6757 – -2,9839	*
HF index			
can-alba	0,10169	0,06422 – 0,13917	*
trem-alba	0,16671	0,13100 – 0,20243	*
trem-can	0,06502	0,03417 – 0,09587	*

Significance levels: * $p<0,05$; ns, non significant

Chapter 4 – Assessing the hybridisation potential of *P. alba* (L.) and *P. tremula* (L.) by means of flower phenological observations

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Abstract

In order to hybridise, plant species have to overlap in their flowering time. *Populus alba* (L.) and *P. tremula* (L.)are dioecious trees occurring in several hybrid zones across Europe. Observation of a total of 25 trees in-situ on three study sites in two consecutive years was performed. Additionally, detached twigs of 13 trees were observed ex-situ. The total flowering period varied between individuals and years. However, in both years male trees started flowering earlier than females of both species on each site (protandry). The calculation of growing degree days revealed higher temperature thresholds for *P. alba* and the hybrid and lower thresholds for *P. tremula*. Divergent flowering periods between two species can be seen as an evolutionary barrier against hybridisation. Here, overlapping flowering times due to local climatic and site conditions between species on different sites facilitated hybridisation influenced by year to year climatic variation.

Keywords: flowering phenology, prezygotic barrier, *Populus,* hybrid zone, protandry

Introduction

Populus alba and *Populus tremula* are deciduous, dioecious and wind pollinated tree species belonging to the family of *Salicaceae*. This family is characterized by its catkins (Figs. 1a-d), emerging before the leaves in spring. Both species produce an enormous amount of seeds within their fruits which are capsules. Seeds are embedded within white cotton helping them to disperse by wind and water (Fischer et al. 2005). From an ecological point of view, both trees are pioneer species at the beginning of the forest succession. *P. alba* is a thermophilous species and grows in lowland floodplain forests up to 600 m a.s.l. On the other hand, *P. tremula* is a boreal forest tree which has a high climatic adaptability and a wide ecological amplitude; it grows from 200-2000 m a.s.l. in Central Europe. Both species have overlapping distribution ranges in Europe, where lowland floodplains come in contact with hills and mountains, representing potential habitats for *P. ×canescens* (Aiton, Sm), the hybrid of the two species.

Figure 1 Sequence of flowering stages for (a) male and (b) female catkins of *P. alba*, and for (c) male and (d) female catkins of *P. tremula*

(a)

(b)






(c)

(d)

The biological species concept defines a species different populations able to interbreed with each other (Mayden 2002). During the process of speciation, barriers evolve preventing gene flow between previously interbreeding populations. Several such species isolating barriers have been described acting before or after mating and can be extrinsic or intrinsic (Rieseberg & Willis 2007). Most of the time more than one type of barrier is involved in preventing species from interbreeding, but still there is a lack of knowledge about the relative contribution of each of the barriers and about the order and speed they evolved (Coyne & Orr 2004).

The role of hybridisation during speciation can be very complex, because there are various processes involved. First, the integrity of two hybridising species in sympatry can be enhanced by reinforcement, where unfit hybrids are selected against, thereby strengthening prezygotic barriers (Rieseberg & Willis 2007). This mode of speciation occurs in cases where gene flow is still present between species, but is thought to

enhance isolation between them. For example, applying different fertilizer treatments on grass populations caused divergence of flowering times within plots towards their boundaries; reinforcement is thought to be responsible for the observed pattern of within plot variation (Silvertown et al. 2005). Second, the break down of barriers between hybridising species can lead to increased variation in the recipient species through introgression by backcrossing to one of the parental species (Hegarty & Hiscock 2005). Furthermore the exchange of adaptive alleles between species can be facilitated through break down of barriers as well (reviewed in Rieseberg &Willis 2007, Lexer et al. 2009). Such adaptive alleles are thought to be sheltered from further recombination by clonal growth, which is very prominent in the hybrid *P.* ×*canescens* (Van Loo et al. 2008). In contrast, deleterious mutations cannot be purged during clonal reproduction (Orr 2000). However clonality may contribute to the persistence of hybrids but with both positive and negative effects on fitness. Finally hybrids can evolve into new species themselves by "hybrid speciation" which has been increasingly acknowledged to play a decisive role in the evolution of the angiosperms (Stebbins 1959, Rieseberg 1997, Hegarty & Hiscock 2005). In the course of hybrid speciation, populations of intermediate or transgressive phenotypes evolve into a new species by adopting intermediate or extreme environments. At the same time they are reproductively separated from the parental species, as it is known from hybrid sunflowers which colonized novel habitats (Rieseberg et al. 2007). In order for two species to hybridise, several prerequisites have to be met.

First, a phylogenetically close relationship might favour natural hybridisation of two species. The two studied species are very closely related (Hamzeh & Dayanandan 2004, own unpublished data) and their hybridisation was already described by Smith (1804 in the Flora Britania (Fl. brit. 3:1080), USDA 2010) as *P.* ×*canescens* and studied in detail in 1940 by van Dillewijn (van Dillewijn 1940). But also intersectional hybrids occur within the genus *Populus*, in some cases even in natural stands – although those species are not closely related (Eckenwalder 1996).

Second, different aspects regarding the pollen and its fertility have to be considered. Because pollen of poplar is very light and wind dispersed, travel distance can be very large. Nevertheless, there is probably a certain maximum distance for the travelling pollen still to be fertile when reaching the female flower.

Third, reproductive barriers (pre- and postzygotic) play a major role in speciation and thus also in hybridisation (Rieseberg & Willis 2007). Prezygotic barriers occur before pollination, caused by behavioural, temporal or spatial barriers or after pollination,

caused by intrinsic genetic or biochemical barriers in order to prevent the formation of a viable zygote. Postzygotic barriers involve hybrid inviability or hybrid sterility caused by intrinsic or extrinsic factors including the loss of fitness in any of the subsequent generations (Coyne & Orr 2004). Spatial barriers between hybrids and parental species can be formed by extrinsic geographical barriers, but also by habitat preferences (Rice 1987, Fry 2003). Speciation of hybrids by temporal (flowering time) isolation from their parents has been described for several species (reviewed in Gross & Rieseberg 2005). But if temporal barriers are absent between two species flowering times are synchronized, facilitating successful hybridisation. If potentially hybridising species occur in neighbouring habitats, flowering time has to be different in order to maintain species delimitation. In case of overlapping flowering times hybridisation of two species can take place. Because in *Populus* female and male trees exist, flowering times have to overlap regarding certain combinations for successful hybridisation. Either male *P. alba* may interbreed with female *P. tremula* or vice versa. In this respect also the phenomenon of protandry has to be mentioned which is the flowering of male flowers prior to the female flowers. It is thought to ensure that there is enough pollen in the air at the time female flowers reach receptivity. This protandry can vary from year to year (Vanden Broeck et al. 2003). Furthermore, local climatic conditions had a high impact on the variation of flowering time assessed in Hungary (Bartha 1989). In that study, flowering overlap was caused by differences in soil because of delayed flowering of *P. tremula* on loamy soils and earlier flowering of *P. alba* on sandy soils, which correspond to cold vs. warm conditions, respectively. Such processes likely enhance the hybridisation potential.

Extrinsic barriers, if existing long enough, can evolve into intrinsic (genetic) barriers in the absence of gene flow, but do not have to necessarily (Coyne & Orr 2004; Gross & Rieseberg 2005). However, both types of barriers (extrinsic and intrinsic) prevent hybridisation through accumulation of differences between species (Rieseberg & Willis 2007). If genetic barriers are porous, as suggested by Lexer et al. (2005, 2007) for *P. alba* and *P. tremula* and differences are not distinctive enough, hybridisation can occur. But if genetic barriers like cytonuclear incompatibilities (e.g. cytoplasmic male sterility) are too strong, hybrids fail to survive (Bomblies 2010). On the other hand, when temporal or spatial barriers vanish for example during migration after the last glaciation or due to future climate change species are likely to interbreed.

Finally, mechanisms like dispersal and germination of hybrid seeds are important for successful establishment of hybrids in a population. Despite the huge number of seeds

produced in a poplar tree (50 million were estimated by Vanden Broeck et al. 2005 for *P. nigra*) only a small fraction usually survives the first summer. They also reported that continuous water supply is essential for the survival of the seedlings in the first and second year and only about every 10-20 years new seedlings were established depending on climatic condition and river morphology (reviewed in Vanden Broeck et al. 2005). No data is currently available for *P. alba* and *P. tremula* in this respect but for *P. alba* similar obstacles like in *P. nigra* can be assumed, whereas seedlings of *P. tremula* are dependent on open soil and rather have to fight against competing grass. On the whole it seems more difficult for hybrids to be formed in natural populations than to persist once being established (e.g. clonal growth).

Thus the aims of this study were to assess i) if hybridisation of *P. tremula* and *P. alba* is possible on the basis of overlapping flowering time, ii) if hybridisation is possible in both directions and iii) if climatic conditions influence the flowering time of both species.

Material and Methods

Observation of flowering behaviour was already prepared in 2007, when branches with flower buds were collected at four different locations. A total of 13 trees (8 branches per tree) were observed ex-situ under controlled conditions in a climate chamber (16 hours of daylight; day temperature was 20°C and night temperature was 15°C, 70% relative humidity). Phenological data of the ex-situ observations were collected three times per week and for each of the branches separately. Those observations were the basis for the description of the 6 flowering stages (Table 1) and were defined for male and female catkins separately. The studied species differed in their catkin morphology (Fig. 1), but not in their functioning and flowering mechanism, therefore the scheme was used for both species.

Table 1 Description of flowering stages of male and female catkins of *P. alba* and *P. tremula*

Male catkins			Female catkins		
Score	Description of state	Pollen shedding*	Score	Description of state	Receptivity*
1	Bud closed	0	1	Bud closed	0
2	Buds opening, catkins visible at the apex	0	2	Buds opening, catkins visible at the apex	0
3	Catkins clearly visible and elongate, some red stamens shed their pollen	25	3	Catkins clearly visible, pollen grains can hide inside the bracteoles	25
4	Catkins full length reached and flexible, pollen sacks turn yellow, full shedding	100	4	Catkins full length reached, styles visible	100
5	Catkins long and thin, pollen sacks turn black, weakly shedding	25	5	Basal flowers pollinated and ovaries swell, apical flowers still receptive	25
6	Catkins wither and fall down, not shedding	0	6	Catkins loose bracteoles, all ovaries swell	0

* the percentage of „pollen shedding“ and „receptivity“ is given as an estimate [%]

For the observation of the flowering behaviour on standing trees (in-situ) altogether 25 trees at three locations were selected, situated around Vienna (Fig. 2). For the population Vienna forest (A) eleven trees, for Vienna-Mariabrunn (B) four trees and for Vienna Danube (C) ten trees were studied. Each population was visited once a week and the stage of flowering was assessed using a binocular. Stages were assessed using the whole appearance of the tree, not on certain branches separately. For the male trees the beginning of pollen shedding was defined by the moment when the first catkins showed

their red stamens. The end of male pollen shedding was defined when all the catkins had shed their pollen. In the same way female trees were treated. Thus the beginning of the female receptivity was defined as soon as the first catkins on the tree did reach stage 3. The end of the female receptivity was defined only when all female catkins were reaching stage 5 (Table 1).

The ex-situ observations of flowering phenological stages were easier to perform compared to the observations in-situ on standing trees because the catkins within the climate chambers could be examined in more detail and therefore studied every second day. The single flowering stages could be investigated more precisely, e.g. the intensity of pollen shedding could be observed by touching the catkins and although towards the end of flowering when they looked already withered, there was still pollen shedding from them. For the female flowers e.g. the swelling of the fruit defining the end of female receptivity could be seen more directly by inspecting the catkins in the climate chamber. In contrast the exact timing of flowering on the standing trees was more difficult to see in such a detail, therefore it was reasonable to observe them once a week. Altogether the time spent on the observations of ex-situ flowers was much shorter than for the in-situ observations.

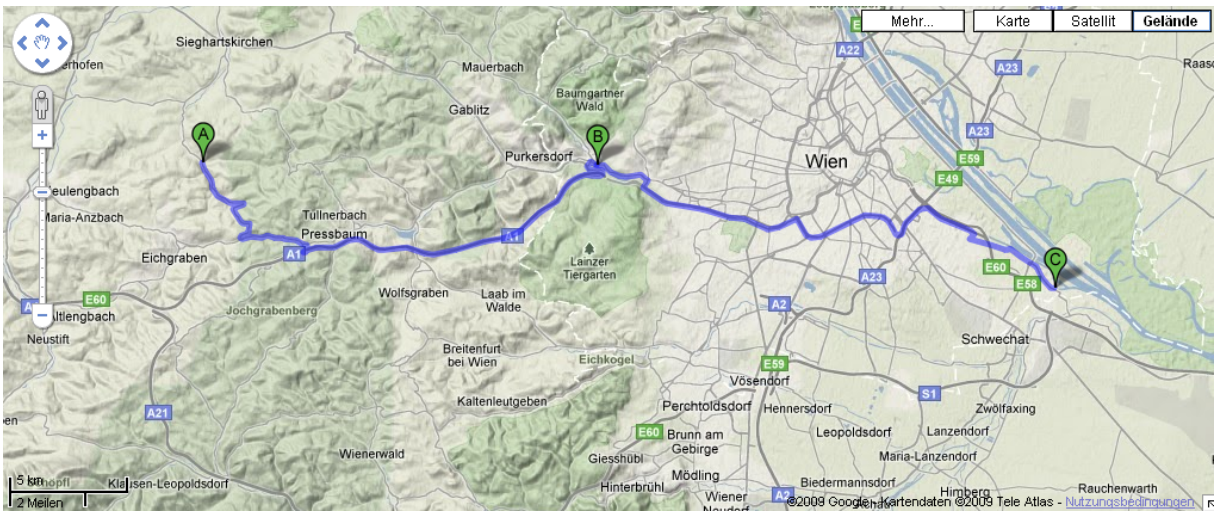


Fig. 2 Selected locations for flowering phenological observations: A, Vienna Forest; B, Vienna-Mariabrunn; C, Vienna Danube (map created with "google maps, 2009")

Statistical analysis

Phenological data of the ex-situ observations were analysed on a three days per week basis and for in-situ observations data were analysed on a one day per week basis using the program SYNCHRO.SAS (Zas et al. 2003). Therefore each stage had to be

defined by a percentage value (Table 1). Following parameters were calculated: beginning, end and period of pollen shedding and female receptivity of each of the trees given by means and standard deviation for each species and for the two sexes separately; diagrams for flowering synchronity of male and female trees (Matziris 1994); phenograms for male and female trees; Pearson's correlation coefficient (Gömöry et al. 2003); probability values for the Pearson correlation are computed by treating
$t = (n-2)1/2 \ ([(r2)/(1-r2)])1/2$
as coming from a t distribution with (n-2) degrees of freedom, where r is the sample correlation (Raf Zas, personal communication).

Climatic data were kindly provided by the ZAMG (Zentralanstalt für Meteorologie und Geodynamik) within a cooperation agreement with the Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW) for the closest available weather station near the study plot. Temperatures were summed up as mean daily temperature starting from 1st of January every year, using effective temperature i.e. means were only summed up when they were higher than 5°C (growing degree days >5°C, following Codesido et al. 2005).

The comparison of temperature sum with flowering phenological observations are illustrated by two graphs plotting the growing degree days against the start and peak day (stages 2 and 4) of the flowering of trees on each study site. The first graph of Fig. 7 shows values for male trees focusing on between species differences between study sites. The second graph focusing on within species differences, in this case on female *P. alba* between study sites.

Results

The observation of flowering phenology in-situ was conducted in two consecutive years in 2008 and 2009 at three sites for altogether 25 trees. The total flowering period varied between individuals and years (Table 2). In 2008 male trees flowered for 12-32 days and female trees flowered for 13-28 days. In 2009 the total flowering period was shorter and comprised 20-22 days for male and female trees. As a comparison in the climate chambers with standardized conditions male catkins flowered for 13-21 days and female catkins flowered for 9-21 days. The mean flowering period was shortest for *P. alba* and longest for *P. tremula* in both years of in-situ observation and in both sexes. Within species male trees of *P. alba* flowered for a shorter period than female trees and in 2008 male trees of *P. tremula* flowered for a longer period than female trees.

Table 2: Mean period of pollen shedding and female receptivity (D) and the variation of the start of flowering (V) from the first to the last tree in the two species and the hybrid for both years across all locations.

		D		V	
		2008	**2009**	**2008**	**2009**
P. alba	M n=6	18.2 (SD 3.1)	12.8 (SD 2.8)	5.5	12
	F n=7	19.3 (SD 5.0)	15.9 (SD 3.7)	5.5	12
P. ×canescens	M n=4	19.1 (SD 4.7)	16.3 (SD 4.9)	6	5.5
	F n=1	13.5	16.5	0	0
P. tremula	M n=4	27.2 (SD 3.6)	16.4 (SD 4.7)	11.5	9.5
	F n=3	19.7 (SD 1.2)	16.5 (SD 0)	5.5	0

M male; F female; n number of individuals studied; SD standard deviation

The flowering period started at day 51 in 2008 and was delayed in 2009 starting at day 72 due to lower temperatures (according to ZAMG data). However in both years male trees started flowering earlier in all taxa and all three locations (protandry, Fig. 3). In 2008 protandry was more pronounced as in 2009. Because of the delayed start of flowering in 2009 the total flowering period was shortened compared to the year 2008 (Fig. 3). Variation of the length of the flowering period on an individual basis can be seen in Fig. 4 for both sexes in both years.

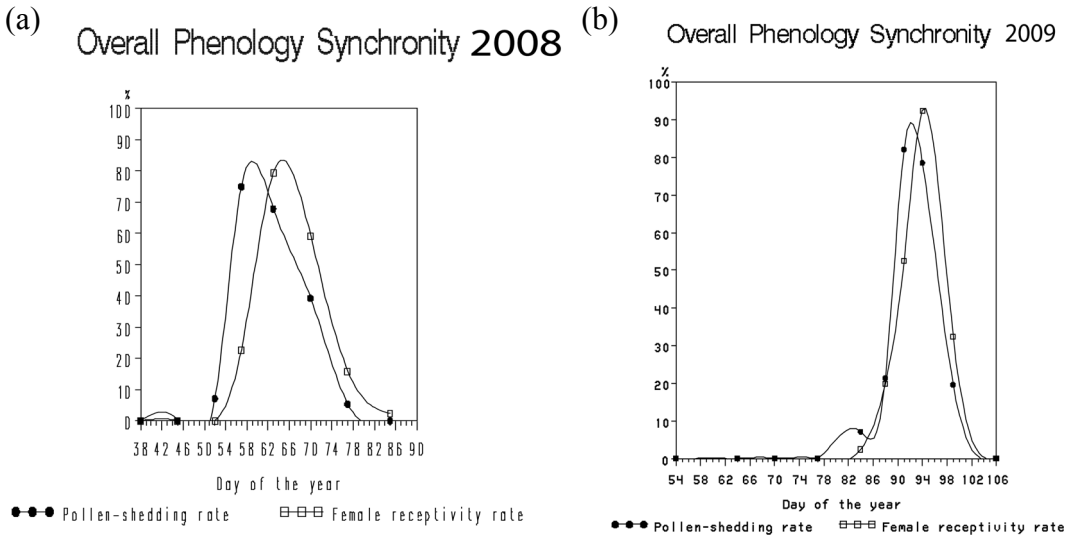
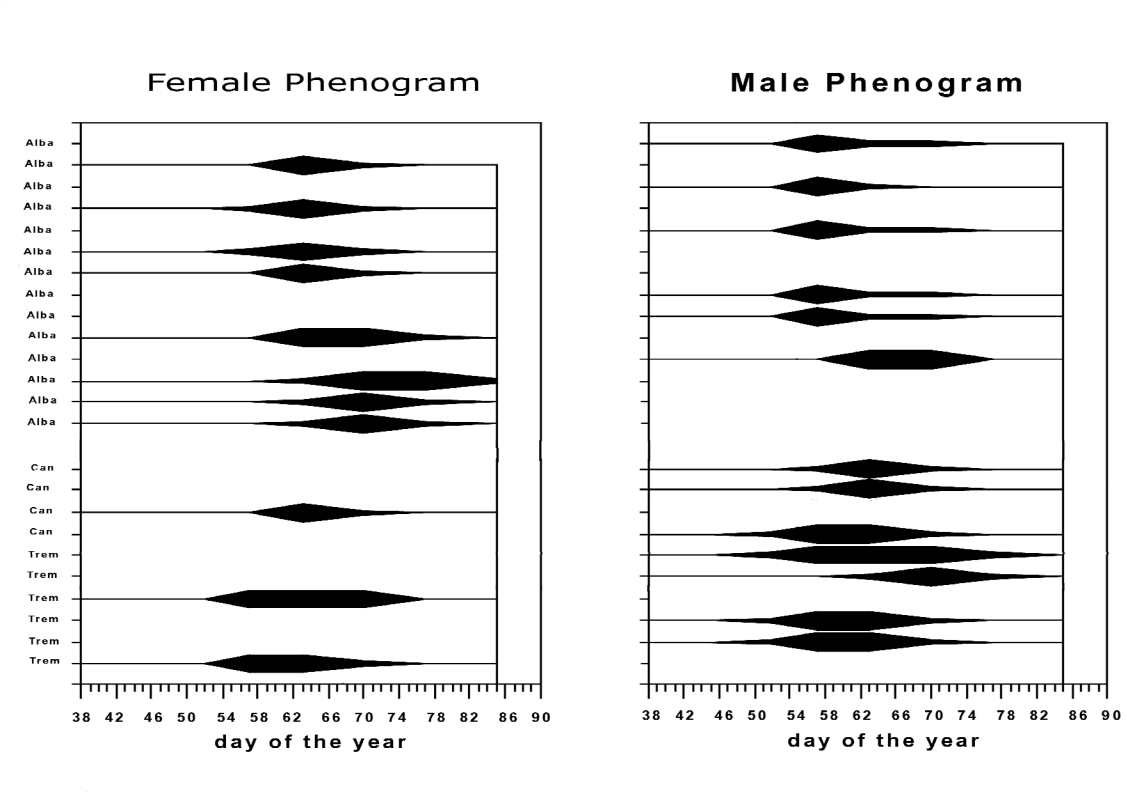


Fig. 3 Flowering synchronity of *P. tremula* and *P. alba* in both years of observation and on all locations

(a)



(b)

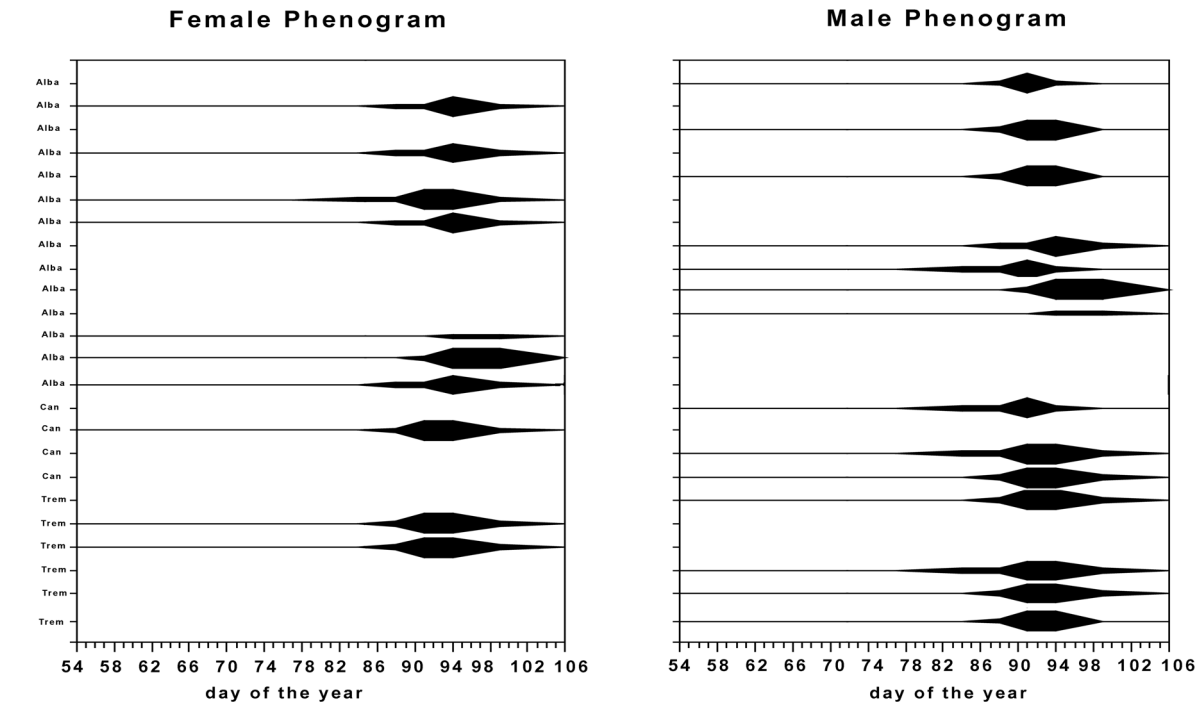


Fig. 4 Phenograms for female and male trees of *P. alba* (Alba), *P.* ×*canescens* (Can) and for *P. tremula* (Trem) (a) for the year 2008 and (b) for the year 2009. trem, *P. tremula*, can, *P.* ×*canescens*, alba, *P. alba*

At the location Vienna Forest (A) protandry was observed most clearly. Here both species and their hybrid were present and the corresponding phenograms illustrate a certain flowering chain (Fig. 5): first were the male *P. tremula* to flower, closely followed by female *P. tremula*. Male *P alba* were located at the end of the chain followed by females and hybrid trees were in between. There was more pronounced overlap between *P. tremula* females with P. *alba* males than vice versa, therefore both combinations were again analysed with the SYNCHRO software for all individuals in order to visualize their potential for hybridisation.

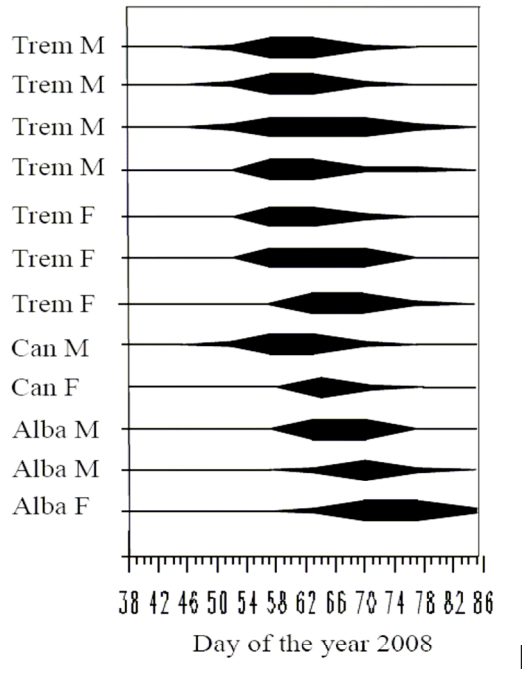


Fig. 5 Phenogram for location A (Vienna Forest) for all taxa and sexes.
trem, *P. tremula*; can, *P. ×canescens*; alba, *P. alba*; M, male trees; F, female trees

Either flowering periods of female trees of *P. alba* and male trees of *P. tremula* need to overlap, or those of male *P. alba* with female *P. tremula* have to overlap in other to facilitate hybridisation. Fig. 6 illustrates both flowering synchronity graphs in both directions. Although the graph for male *P. alba* and female *P. tremula* shows protandry more clearly (Fig. 6a) also the other combination (6b) shows overlap, but the flowering of males peaks later than that of females.

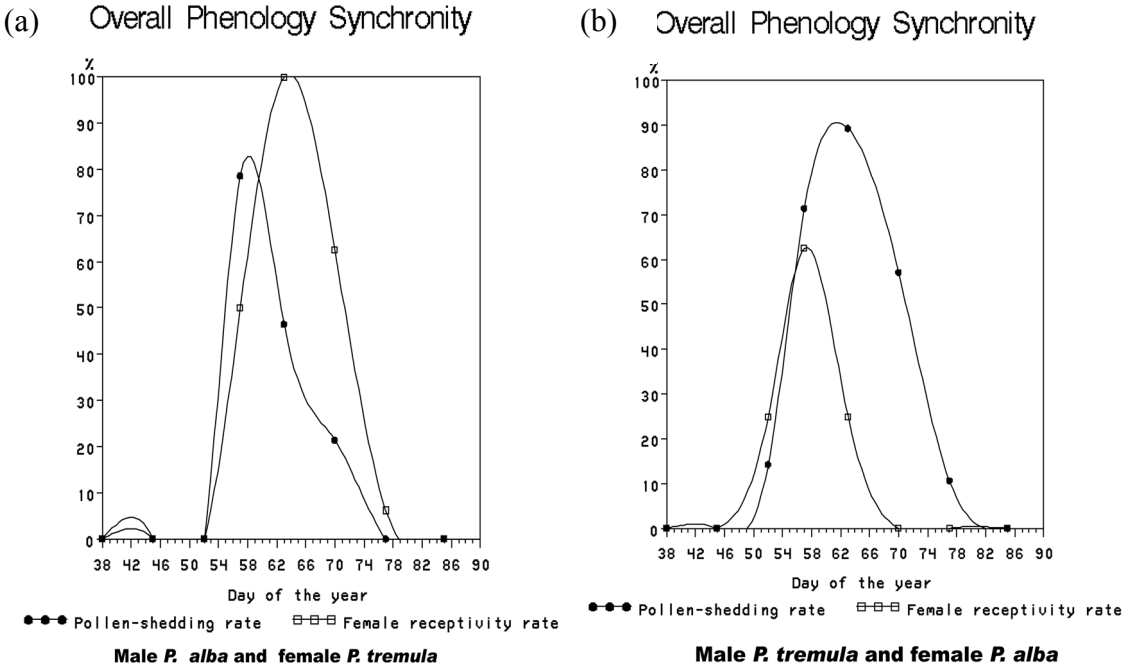


Fig. 6 Flowering synchronity for (a) male *P. alba* and female *P. tremula* and (b) male *P. tremula* and female *P. alba*

The calculation of Pearson's correlation coefficient allowed the establishment of two scenarios. First, three groups overlapped in the year 2008 and second, all four groups overlapped in the year 2009. For the first scenario the three overlapping groups were: the female *P. tremula* with male *P. tremula*, the female *P. tremula* with the male *P. alba* and the female *P. alba* with male *P. tremula*. These results highlight that within *P. tremula* overlap and hybridisation in both directions was possible. Surprisingly, within *P. alba* no significant overlap was detected, most likely reflecting the action of protandry.

In the year 2009 all four groups of combinations overlapped significantly (within *P. tremula*, between male *P. tremula* and female *P. alba* and between female *P. alba* and male *P. tremula* and within *P. alba*). Here especially tree no. 18, a male *P. alba*, was highly significant in all combinations (Table 3b).

Table 3: Pearson's correlation coefficient between male and female trees of all taxa for (a) the year 2008 and (b) the year 2009 including significance tables for each year (trem, *P. tremula*, can, *P. ×canescens*, alba, *P. alba*)

(a)

	M	*trem*	*trem*	*trem*	*trem*	*can*	*can*	*can*	*alba*	*alba*	*alba*	*alba*	*alba*	*alba*	*alba*
F	No.	5	6	12	15	2	13	16	9	11	17	18	22	25	27
trem	4	0,98	0,98	0,85	0,98	0,82	0,98	0,82	0,49	0,11	0,82	0,82	0,82	0,81	0,82
trem	7	0,84	0,84	0,98	0,84	0,76	0,84	0,76	0,76	0,57	0,76	0,76	0,76	0,63	0,76
trem	10	0,43	0,43	0,73	0,47	0,76	0,43	0,76	0,98	0,82	0,11	0,11	0,11	-0,1	0,11
can	14	0,63	0,63	0,61	0,63	0,97	0,63	0,97	0,83	0,33	0,12	0,12	0,12	0,09	0,12
alba	1	0,07	0,07	0,59	0,13	0,29	0,07	0,29	0,79	1	0,07	0,07	0,07	-0,2	0,07
alba	3	0,07	0,07	0,59	0,13	0,29	0,07	0,29	0,79	1	0,07	0,07	0,07	-0,2	0,07
alba	8	-0,1	-0,1	0,33	0,04	0,07	-0,1	0,07	0,47	0,8	-0,1	-0,1	-0,1	-0,3	-0,1
alba	19	0,63	0,63	0,61	0,63	0,97	0,63	0,97	0,83	0,33	0,12	0,12	0,12	0,09	0,12
alba	21	0,8	0,8	0,74	0,8	1	0,8	1	0,79	0,29	0,36	0,36	0,36	0,33	0,36
alba	24	0,8	0,8	0,74	0,8	1	0,8	1	0,79	0,29	0,36	0,36	0,36	0,33	0,36
alba	26	0,63	0,63	0,61	0,63	0,97	0,63	0,97	0,83	0,33	0,12	0,12	0,12	0,09	0,12

No., Sample number; F, female trees, M, male trees

	M	*trem*	*trem*	*trem*	*trem*	*can*	*can*	*can*	*alba*	*alba*	*alba*	*alba*	*alba*	*alba*	*alba*
F	**No.**	**5**	**6**	**12**	**15**	**2**	**13**	**16**	**9**	**11**	**17**	**18**	**22**	**25**	**27**
trem	**4**	***	***	***	***	***	***	***	*	n.s.	***	***	***	***	***
trem	**7**	***	***	***	***	***	***	***	***	**	***	***	***	***	***
trem	**10**	*	*	***	*	***	*	***	***	***	n.s.	n.s.	n.s.	n.s.	n.s.
can	**14**	***	***	**	***	***	***	***	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
alba	**1**	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.	***	***	n.s.	n.s.	n.s.	n.s.	n.s.
alba	**3**	n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.	***	***	n.s.	n.s.	n.s.	n.s.	n.s.
alba	**8**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	***	n.s.	n.s.	n.s.	n.s.	n.s.
alba	**19**	***	***	**	***	***	***	***	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
alba	**21**	***	***	***	***	***	***	***	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
alba	**24**	***	***	***	***	***	***	***	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
alba	**26**	***	***	**	***	***	***	***	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Significance values for 2008: *p<0,05; ** p<0.01; *** p<0,001; n.s., non significant

(b)

	M	*trem*	*trem*	*trem*	*trem*	*can*	*can*	*can*	*alba*	*alba*	*alba*	*alba*	*alba*	*alba*	*alba*
F	**No.**	**5**	**6**	**12**	**15**	**2**	**13**	**16**	**9**	**11**	**17**	**18**	**22**	**25**	**27**
trem	**4**	1	0,98	1	0,98	1	0,98	0,76	0,48	0,61	0,76	0,82	0,98	0,98	0,81
trem	**7**	1	0,98	1	0,98	1	0,98	0,76	0,48	0,61	0,76	0,82	0,98	0,98	0,81
can	**14**	1	0,98	1	0,98	1	0,98	0,76	0,48	0,61	0,76	0,82	0,98	0,98	0,81
alba	**1**	0,82	0,8	0,82	0,78	0,82	0,8	0,29	0,76	0,78	0,29	1	0,78	0,78	0,34
alba	**3**	0,61	0,59	0,61	0,48	0,61	0,59	0,09	0,98	1	0,09	0,78	0,48	0,48	0,14
alba	**8**	0,48	0,46	0,48	0,35	0,48	0,46	-0,08	1	0,98	-0,08	0,76	0,35	0,35	-0,04
alba	**19**	0,82	0,8	0,82	0,78	0,82	0,8	0,29	0,76	0,78	0,29	1	0,78	0,78	0,34
alba	**21**	0,98	1	0,98	0,97	0,98	1	0,8	0,46	0,59	0,8	0,8	0,97	0,97	0,79
alba	**24**	0,82	0,8	0,82	0,78	0,82	0,8	0,29	0,76	0,78	0,29	1	0,78	0,78	0,34
alba	**26**	0,82	0,8	0,82	0,78	0,82	0,8	0,29	0,76	0,78	0,29	1	0,78	0,78	0,34

No., Sample number; F, female trees, M, male trees

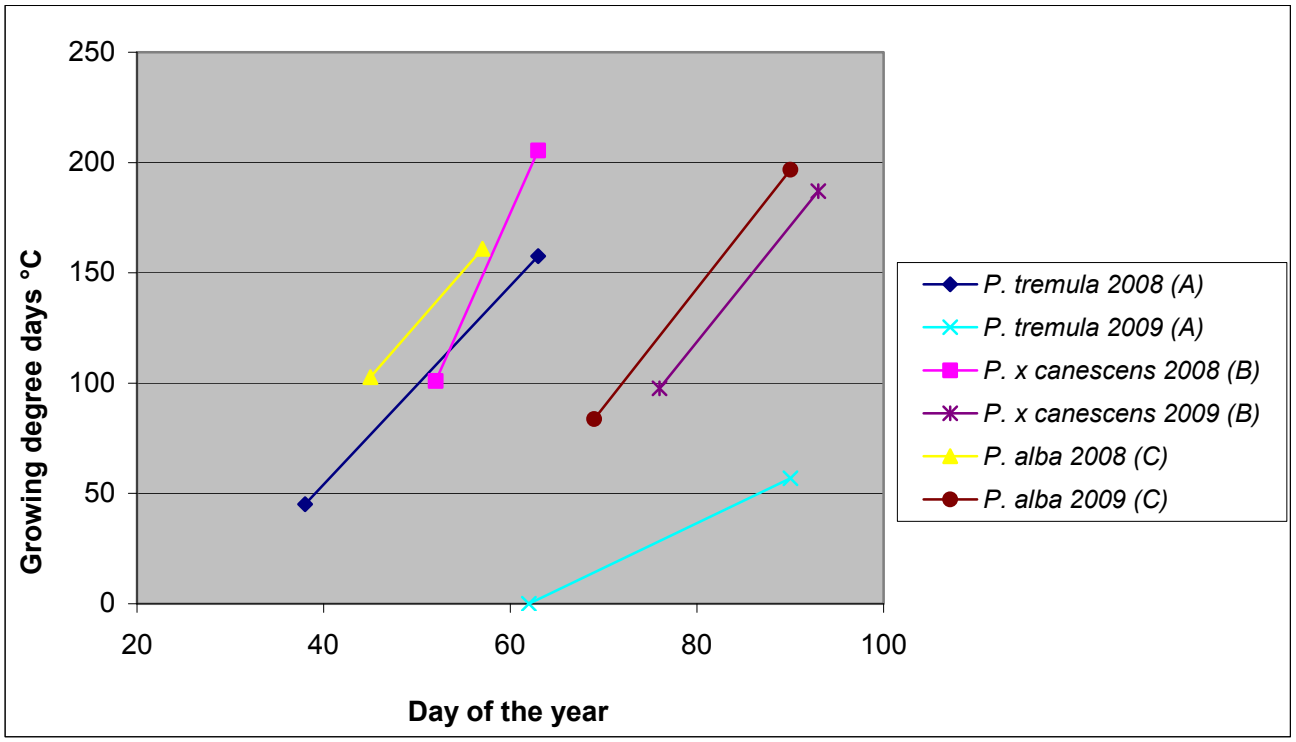
F	M No.	*trem* 5	*trem* 6	*trem* 12	*trem* 15	*can* 2	*can* 13	*can* 16	*alba* 9	*alba* 11	*alba* 17	*alba* 18	*alba* 22	*alba* 25	*alba* 27
trem	**4**	***	***	***	***	***	***	***	*	**	***	***	***	***	***
trem	**7**	***	***	***	***	***	***	***	*	**	***	***	***	***	***
can	**14**	***	***	***	***	***	***	***	*	**	***	***	***	***	***
alba	**1**	***	***	***	***	***	***	n.s.	***	***	n.s.	***	***	***	n.s.
alba	**3**	**	**	**	*	**	**	n.s.	***	***	n.s.	***	*	*	n.s.
alba	**8**	*	*	*	*	*	*	n.s.	***	***	n.s.	***	n.s.	n.s.	n.s.
alba	**19**	***	***	***	***	***	***	n.s.	***	***	n.s.	***	***	***	n.s.
alba	**21**	***	***	***	***	***	***	***	*	**	***	***	***	***	***
alba	**24**	***	***	***	***	***	***	*	***	***	n.s.	***	***	***	n.s.
alba	**26**	***	***	***	***	***	***	*	***	***	n.s.	***	***	***	n.s.

Significance values for 2008: *p<0,05; ** p<0.01; *** p<0,001; n.s., non significant

The calculation of the growing degree days for all three locations for both years is illustrated in Fig. 7. Here a comparison of temperature sums with start and peak day of flowering on each study plot is shown. The graph highlights that on the study plots Vienna-Mariabrunn (B) and Vienna Danube (C) similar temperature sums independent from a certain day of the year (Fig. 7a) were required in order to reach a certain stage. Both study plots were dominated by *P. ×canescens* and *P. alba* and a temperature threshold of around 100°C was identified. A lower flowering threshold for the study plot Vienna Forest with predominantly *P. tremula* was found. Furthermore the beginning of the flowering at the location Vienna Forest also indicated a higher variation between the years than the other study plots regarding the dependence of flowering stages from growing degree days. Male *P. tremula* even flowered at mean day temperatures constantly below 5°C in the year 2009.

The graph for female *P. alba* shows that trees from Vienna Danube flowered earlier than *P. alba* from Vienna Forest. Again trees flowered after they were exposed to a certain temperature sum (Fig. 7b) – mostly between 100 –150°C. A clear hint for protandry was here the difference between male *P. alba*, which already flowered at 80-100°C growing degree days and the female *P. alba*, which only flowered at 100-150°C growing degree days. The study site Vienna Danube in 2009 exhibited this trend most clearly with male trees starting at 80°C after 65 days and female trees at 150°C after 80 days.

(a)



(b)

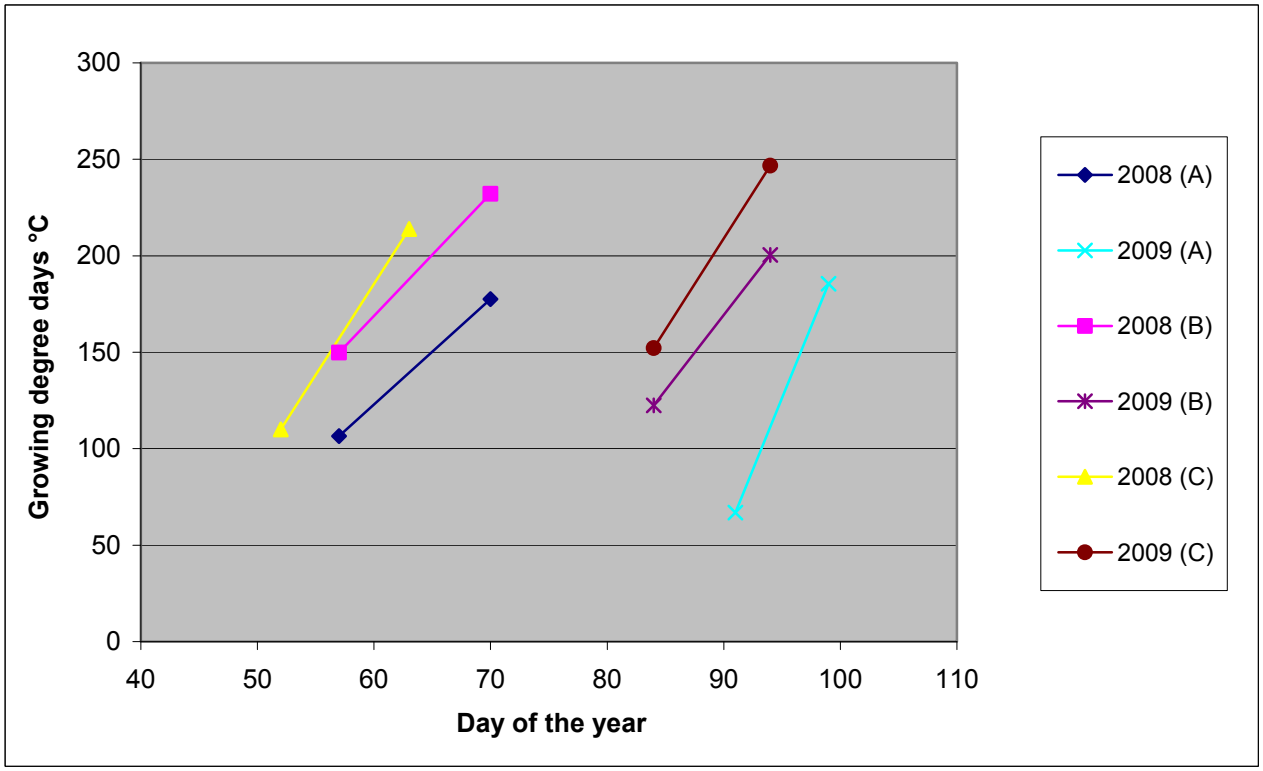


Fig. 7 Comparison of growing degree days with start (first point) and peak day (second point) of flowering on each study plot in both years. The graphs show (a) male trees of all species: *P. tremula* – Vienna Forest (A), *P.* ×*canescens* – Vienna-Mariabrunn (B), *P. alba* – Vienna Danube (C) with a temperature threshold at 100°C for start of flowering for male *P. alba* and *P.* ×*canescens* and (b) only female *P. alba* on all study plots (A, B, C) with a temperature threshold at 80-150°C. Different colours correspond to different study plots in different years.

Discussion

In order to study the hybridisation potential of two poplar species, flowering phenological measures were assessed at three sites. Members of the genus *Populus* are dioecious thus male and female trees had to be considered separately. On the one hand flowering period of male and female trees varied in length but male trees were always flowering first within species and within study plots. This protandry was already reported to happen in different tree species (e.g. in *Populus nigra*, Vanden Broeck et al. 2003; in *Quercus sp.* Bacilieri et al. 1995).

Different measures have been used in this study to illustrate overlap of flowering. Graphs generated with the software SYNCHRO showed protandry more precisely, whereas Pearson's correlation coefficient expressed direct overlap of flowering periods of single trees. But according to the protandry theory, male trees would have to flower before the female trees in order to be successful in pollination. Probably the optimum is a slight protandry followed by a certain period of overlap. Most probably the time of overlap plays a role, in which single individuals with longer flowering phase might be more successful in hybridisation than trees with shorter periods (Fig. 4a).

Pearson's correlation coefficient might not show protandry accurately but it is certainly useful to illustrate that varying climatic conditions in certain years can cause a clear shift of flowering behaviour. This was shown for the spring 2009 where Pearson's correlation coefficient showed a significant total overlap of all trees. However this total overlap can be assumed uncommon due to exceptional delayed spring temperatures in that year, whereas the year 2008 might rather provide the rule. Most probably also the wind direction during flowering plays a role when considering hybridisation direction.

Moreover specific properties of the studied poplar species enhance or reduce their hybridisation potential. Basically because of the prolonged flowering period of male *P. tremula* compared with male *P. alba* (Table 2), the former ones might be potentially more often involved in pollination of the other species than the other way around (Table 3a and 3b). In addition the variation of the start of flowering seems to play a role in male trees, i.e. in 2008 an increase of the flowering period was observed from *P. alba* (5.5 days), *P.* ×*canescens* (6 days) to *P. tremula* (11.5).

Divergent flowering periods between two species can be seen as an evolutionary adaptation against hybridisation (Coyne & Orr 2004). The observed flowering chain at the Vienna Forest study site illustrated that only certain individuals of different species overlap with others. More often trees were grouping within species, where male trees

flowered earlier than females. When study sites were combined, the potential of hybridisation was higher because flowering times of different species overlapped more often. Male *P. tremula* from study site A and male *P. alba* trees from study site C flowered simultaneously and thus a common pollen pool was provided for female trees of both species.

Regarding the direction of hybridisation Lexer et al. (2005) found *P. tremula* pollen introgressing into *P. alba* more frequently based on nuclear microsatellites and chloroplast markers, probably because the hybrid population was located within a *P. alba* dominated hybrid zone. Within this thesis, populations were studied in both *P. alba*- and *P. tremula*- dominated hybrid zones and no clear preference of any direction of gene flow was observed using molecular-genetic techniques (Chapter 5).

Climatic and site conditions influenced the flowering time of the species in this study. When removing climatic and site differences between the species (climate chamber experiment) the typical flowering chain of the species was shown, similar to the observations of all species at one site. Here the overlap of male and female trees within species was clear, although with differences on the individual basis. Moreover the beginning of the flowering period varied in different years, because it was temperature dependent (Fig. 7). The temperature sum gave the start point and was reached in different years at different dates. Because of faster warming of the location Vienna Danube (Fig. 7b) *P. alba* trees flowered earlier than those at the Vienna Forest site and temperature sums were relatively constant along the years. A site dependent shift of flowering times was shown in Hungary (Bartha 1989), where due to a faster warming of sandy soils the flowering periods overlapped with the other species on loamy soils.

As shown in several studies of the model tree *Populus*, flowering is influenced by genetic components to a certain degree (Mohamed et al. 2010). Whereas the onset of flowers is under strong genetic control, the timing of spring bud flush and flowering is influenced to a great part by temperature (Mohamed et al. 2010). Two studies on phenological traits in the Swedish aspen (SwAsp) collection along a latitudinal gradient showed significant correlations for bud set but not for bud flush (Hall et al. 2007, Luquez et al. 2007). It would be interesting to study flowering phenology in the SwAsp collection as well, in order to better understand individual based differences. The observed individual based differences within species in this study might lead to assortative mating, influenced by long versus short flowering period (compare Fig. 4a and 4b)

One more noticeable morphological characteristic of poplars might also increase the potential of hybridisation. In fact a prolonged flowering period is facilitated by the organization of single flowers along the catkins and a temporal variation in their flower opening, with the flowers opening earlier at the base than at the top of the catkins. Seed development started earlier at the base, whereas flowers at the top were still receptible and pollen was still shedding from the top whereas the base was already withered.

In general the following flowering scenario can be established (Fig. 8): for the year 2008 broad overlap for both species in both directions for 2.5 weeks was identified and for 1.5 weeks for the year 2009.

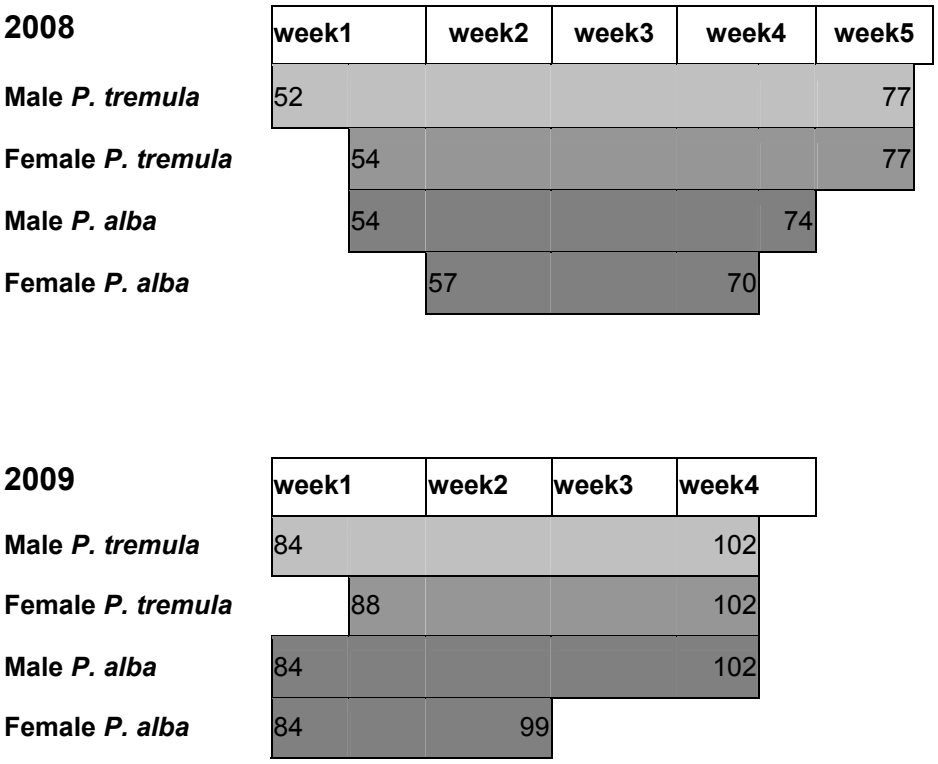


Fig. 8 Flowering scenario of *P. alba* and *P. tremula* in both years of observation for each week of flowering with numbers giving the start end end day of flowering.

Conclusion

In fact the most important influences on flowering were the site and the frequency with which the species occur on one site and surrounding (see also Lepais et al. 2009), in that the concentration of con-specific and non conspecific pollen is an important factor in the fertilization process of hybrids (Van den Broeck et al. 2005). In this study, an increased potential for hybridisation was identified in the direction of male *P. alba* with

female *P. tremula* in both years. Although in the second year of observation total flowering time for both species was shortened and shifted for *P. alba*, the mentioned direction of hybridisation was more likely. Whether there is a necessity of overlap or protandry for successful hybridisation, cannot be concluded from this study, though. Observation of flowering phenology in common garden experiments (e.g. SwAsp collection) would have the power to more precisely dissect individual based genetic factors from environmental influences on the timing of flowering.

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Chapter 5 – Cytonuclear interactions as a driving force preventing the establishment of hybrids in two European poplar species

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Abstract

Hybridisation of species can lead to new interactions between the nuclear and the cytoplasmic genomes. Due to maternal inheritance of both mitochondria (mt) and chloroplasts (cp) in angiosperms, nuclear DNA gets combined with mt and cpDNA of the other species in hybrid genotypes. This may affect the formation and function of e.g. photosynthetic or respiratory enzymes encoded by both the nuclear and the cytoplasmic genomes, thus they must complement each other well.

Chloroplast and nuclear DNA of altogether 541 individuals in 23 populations of *Populus alba* (L.) and *P. tremula* (L.)were investigated in Central Europe. Based on 57 chloroplast haplotypes, two clades, one for each species, were detected. Within *P. alba,* clear geographic structure was found, presumably due to recolonisation from disconnected glacial refugia. Conversely, in *P. tremula* cpDNA variation was more evenly distributed across the studied populations. In addition 20 nuclear microsatelles (SSRs) revealed stronger geographic structuring within *P. alba* as well, compared to *P. tremula*. Hybridisation was observed in both directions in zones of contact and most hybrids appeared to be early generation hybrids.

Significant cytonuclear interactions were detected within hybrids (*P.* ×*canescens* Aiton, Sm) and *P. alba* at six and four nuclear loci, respectively. Although factors like assortative mating and migration may play a role in their origin, those are likely to affect all chromosomes. Thus, selective mechanisms are a more likely explanation for these patterns. Linkage of microsatellite markers to certain genes involved in cytonuclear processes might cause the observed disequilibria, especially in early generation hybrids carrying relatively large chromosome blocks inherited from each parental species.

Keywords: cytonuclear interactions, hybridisation, *P. tremula*, *P. alba*, nuclear microsatellites, chloroplast PCR-RFLPs, postzygotic barriers

Introduction

Already in 1987 (Asmussen et al. 1987) the first model for assessing cytonuclear interactions (the non-random mating of cytonuclear genotypes) was established. Some theoretical studies followed and described models for interpreting cytonuclear interactions in hybrid zones (Goodisman et al. 1997, Dakin 2006). Others used field data in order to investigate cytonuclear interactions and also focused on hybrid zones using genetic markers (Paige et al. 1991, Won et. al. 2003, Leaché et al. 2007, Mir et al. 2007, Lajbner et al. 2009). Some studies point out that genetic data alone is not sufficient in some cases, but additional ”field data“ is required in order to explain mechanisms leading to cytonuclear disequilibrium more accurately (Latta et al. 2001, Monsen et al. 2007).

Cytonuclear disequilibrium occurs if the cytoplasmic genomes of one species combine non-randomly with the nuclear genome of the other species. Among the evolutionary forces involved in this process are hybridisation, migration, assortative mating, selection and genetic drift. Such uneven distribution of cytonuclear combinations is likely to occur on different levels, such as the population level, the species level, and on the level of hybrids. Overrepresentation of one combination in one population is likely to contrast with a high deficit of the same combination in a different population, which can appear as the consequence of co-adaptation of the cytonuclear combination to the local environment (Asmussen 1987). Thus, epistatic effects are likely to influence the fitness of the individuals, in cases where a disruption of the co-adapted genotypes leads to functional incompatibilities (Dowling et al. 2007). For instance, several photosynthetic proteins are encoded in the nucleus and transported into the chloroplast. This can be the basis of a wide range of interactions between nuclear products with those from the chloroplasts and thus several possibilities are provided for epistatic interactions to influence the fitness and the cytonuclear disequilibrium (Sambatti et al. 2008). Hybrids are likely to react in a very strong way to epistatic effects, because divergent genetic backgrounds are combined. There are numerous possibilities for interaction provided between those different genomes, often described as disharmonic. Hybrids do not automatically have low fitness, but hybrids can be both fit and unfit. Burke et al. (1998) discovered advantageous and non- advantageous cytonuclear interactions in several *Iris* crosses. In naturally hybridising *Ipomopsis* species, hybrids were able to perform better in hybrid habitats than each of the parents alone, but it depended strongly on the direction of the cross (Campbell & Waser 2001).

As a model for hybridising tree species *P. alba* and *P. tremula* were chosen. The cytoplasm, including the haploid chloroplast genome, is maternally inherited in the genus *Populus* (Rajora & Dancik 1992a, Rajora et al. 1992b) and therefore non-recombining. Markers have been chosen within the chloroplast genome, but for the interpretation of the results also the mitochonrial genome has to be considered, because it is also maternally inherited in *Populus* and thus linked to the chloroplast genome. In contrast, the nuclear genome is inherited biparentally and recombination occurs. Due to different inheritance of cell organelles and nucleus, diversity studies using markers of both genomes can elucidate the evolutionary history of the species. On the one hand, cytoplasmic (e.g. chloroplast) markers allow studying the geographic distribution of genetic diversity and the historic relationship between populations (phylogeographic patterns). On the other hand, possible influences on the phylogeographic pattern of one species by historic interaction with the other species (such as hybridisation) can be identified using nuclear markers.

Chloroplasts are inherited through the maternal species when hybridisation occurs between the two pure species. During backcrossing towards the paternal species through recurrent pollen immigration "chloroplast capture" occurs and the chloroplast of the maternal species appears in the morphologically opposite species. Chloroplasts in *Populus* are mainly species specific (Lexer et al. 2005 and Chapter 1) and only occurs rarely in the other species.

Several cases of cytonuclear associations can occur in populations consisting of both pure species and hybrids. Non-random associations are detected when either *P. tremula* nuclear alleles (11) occur more often than randomly expected together with species specific *P. tremula* haplotypes and *P. alba* nuclear alleles (22) with species specific *P. alba* haplotypes. Such homospecific organelle-nuclear combinations are considered the rule for pure parental individuals. Other combinations of heterospecific nuclear alleles with different chloroplast haplotypes are typical for hybrid individuals.

The multi-locus genotype resulting from nuclear markers determines the pure species and the composition of the hybrids together with their hybrid generation. The haplotype (cytotype) in hybrids is determined by the "mating behaviour" of the parents.

Resulting from this, following questions arrise: Which species is the mother? Which genotypes within the fathers are preferred by those mothers? Such considerations are important from an epistatic point of view, because if cytotypes and nuclear genotypes do not fit certain functions cannot be met (Asmussen et al. 1989).

The aims of this study were i) to estimate the extent of hybridisation across a broad geographic scale, because previous studies have focussed on spatially well defined hybrid zones, here I am interested in patterns at a larger scale and ii) to determine the role of cytonuclear interactions within the evolution of the species and the hybrids.

Methods

Study sites and plant material

Altogether 541 individuals out of 23 populations were available for this study, partly corresponding to the ones studied in Chapter 1. They originated in hybrid zones (17 populations) and reference regions (six populations). The morphological identification of both pure species and the hybrids followed the criteria as described in Chapter 1.

Molecular analysis

DNA-extraction and the analysis of chloroplast fragments followed the method described in Chapter 1. The nuclear DNA was genotyped using 20 nuclear microsatellites originating from different resources (Table 1). One marker per chromosome was chosen (two for chromosome 5) in order to ensure even distribution and independence of the markers. From the database of poplar microsatellites of the International *Populus* Genome Consortium (IPGC) the microsatellite markers listed in Table 1 were chosen. PCR amplification was performed using the M13-method (Boutin-Ganache et al. 2001) and fragments were analysed on a Beckmann-Coulter 8000 automated sequencer as described in Chapter 2.

Table 1 Description of 20 microsatellites studied in *P. tremula* and *P. alba* in Central Europe including locus name, average length of the fragments, type of repeat motive, linkage group (or chromosome number) and the resource.

Locus	Length (bp)	Motive	Linkage group	Resource
GCPM_1719	203	AAG	1	a
GCPM_1158	225	CTG	2	a
PTR_4	160	TC	3	b
ORPM_220	190	TTTA	4	f
WPMS_14	245	CGT	5	e
WPMS_17	140	CAC	5	e
ASP_112322	180	AG	6	h
WPMS_16	145	GTC	7	e
PTR_8	140	(A)11(CT)8	8	d
PTR_2	250	TGG	9	c
ORPM_344	288	GA	10	f
ORPM_29	245	AC	11	f
PMGC_2885	317	GA	12	a
WPMS_20	252	TTCTGG	13	e
GCPM_1812	220	GGT	14	a
GCPM_1894	186	TAT	15	a
ORPM_86	204	CTT	16	f
Yin_2	257	AAC	17	g
ORPM_214	165	AG	18	f
ORPM_206	196	GCT	19	f

a	http://www.ornl.gov/sci/ipgc/ssr_resource.htm
b	Suvanto et al. 2005
c	Dayanandan et al. 1998
d	Rahman et al. 2000
e	van der Schoot et al. 2000, Smulders et al. 2001
f	Tuskan et al. 2004
g	Yin et al. 2009, starting at position 4919779 on chromosome 19
h	personal communication J. Cottrell

Statistical analysis

The relationship of chloroplast haplotypes was estimated constructing a median-joining network using the Software NETWORK (http://www.fluxus-engineering.com/, Bandelt et al. 1999). This program searches for the „minimum spanning tree“ with the shortest possible connections using median vectors, providing starting points for connections between haplotypes and is described in more detail in Chapter 1. The

haplotypes were defined as species-specific using the haplotype network in Chapter 1 (Fig. 2), because this was important for the calculation of cytonuclear disequilibrium.

For the nuclear microsatellites the Software MSA (Dieringer & Schlötterer 2003) was used to calculate diversity parameters for each locus and separately for species and hybrids. Hardy-Weinberg-equilibrium and F-statistics of Weir and Cockerham (1984) together with significance levels were calculated in GENEPOP 4.0 (Rousset 2008).

In order to estimate the extend of hybridisation of both species the software STRUCTURE (Falush et al. 2003) was used, which is a Bayesian clustering method using *a posteriori* probabilities for the assignment of one individual into a certain predefined number of clusters (K). The settings for the calculations were 10000 burn-in and 10000 MCMC repetitions for K=2 and 10 repetitions per K. The probabilities of those two assumed clusters defined the two pure species *P. alba* and *P. tremula*. Each individual was assigned to one of the pure species with a probability of $p < 0.1$ for *P. alba* and with $p > 0.9$ for *P. tremula*. All individuals with a probability in between those two values were considered hybrids ($0.1 < H < 0.9$), because they were assigned with more than 10% to both clusters.

Additionally STRUCTURE was used to estimate population structure within each species. Settings were similar as above except K was set from 2-20 for *P. alba* individuals only and also for *P. tremula* individuals only. The Structure Harvest web service (http://taylor0.biology.ucla.edu/struct_harvest/ accessed 2009 10 19) was used to deduce the most likely number of K, following the procedure suggested by Evanno et al. (2005).

In order to estimate different hybrid generations within the data set the software NEWHYBRIDS (Version 1.1 beta (2003), Anderson 2002) was used. Detailed information was gained about the admixture proportions of both gene pools within each individual. This program allowed the assignment of each individual based on the expected proportions of genotypes (posterior-probability) of the predefined hybrid class. With a posterior probability of $p > 0.5$ the individual was assigned to a certain hybrid class (Berthier et al. 2006). Six species-level genotypic classes were defined here: P1 (pure *P. tremula*), P2 (pure *P. alba*), F1 (first generation hybrids), F2 (second generation hybrids, crossing of F1x F1), Bx1 (backcross of F1 hybrids towards pure *P. tremula*) and Bx2 (backcross of F1 hybrids towards pure *P. alba*). Table 2 defines the composition the 6 classes. The four columns explain the proportion of loci within one individual

possessing 0, 1 or both alleles of one species (allele 1 specific for *P. tremula*, allele 2 for *P. alba* and the genotypes 12, 21 defined hybrids).

Table 2 The proportion of loci possessing 0, 1 or both alleles of one species defined for six hybrid classes used in the software NEWHYBRIDS; allele 1 specific for *P. tremula*, allele 2 for *P. alba* and the genotypes 12, 21 defined hybrids; . P1 (pure *P. tremula*), P2 (pure *P. alba*), F1 (first generation hybrids), F2 (second generation hybrids, crossing of F1x F1), Bx1 (backcross of F1 hybrids towards pure *P. tremula*) and Bx2 (backcross of F1 hybrids towards pure *P. alba*).

	11	12	21	22
P1	1.0	0.0	0.0	0.0
P2	0.0	0.0	0.0	1.0
F1	0.0	0.5	0.5	0.0
F2	0.25	0.25	0.25	0.25
Bx1	0.5	0.25	0.25	0.0
Bx2	0.0	0.25	0.25	0.5

As suggested in the manual of the software (Anderson 2002), several runs were performed until the results were stable. The single runs were terminated after 70.000 Markov chains.

The calculation of the normalized cytonuclear disequilibrium (D') was performed with the software CNDm (Basten & Asmussen 1997) using chloroplast haplotypes and nuclear genotypes.

The settings within the software CNDm was: number of batches =100 and number of observations per batch= 1000. In order to find the right method for calculating D', three prerequisites had to be considered:

1. Pooling of alleles: Before starting the analysis the observed alleles for each locus had to be summarized into species specific "synthetic" alleles (Mir et al. 2007; Latta et al. 2001). Thus all loci were treated as diallelic ("1" *P. tremula* typical allele and "2" *P. alba* typical allele). For chloroplast data the haplotypes were classified according to their appearance within the haplotype network, either within the *P. tremula*- or within the *P. alba*-clade. Thus for each locus only six different combinations were possible (11t, 12t, 22t for individuals carrying *P. tremula* chloroplasts, und 11a, 12a, 22a for individuals with *P. alba* chloroplast

haplotypes). The pooling procedure had the advantage to filter the D' for each species and the hybrid for cases where a high number of alleles and haplotypes occurred. This was important because single and low frequent genotypes and haplotypes would not yield enough counts in order to detect a pattern. Furthermore the pooling reduced the number of D's per locus and led to more precise estimations about the reaction of loci with respect to cytonuclear disequilibrium than without pooling.

2. For the pooling procedure species specific nuclear alleles were defined based on both clear frequency differences and diagnostic alleles. Two thirds of the alleles were diagnostic and one third was frequency dependent (e.g. 1:10 for the allele 189 at the locus WPMS_16 – this allele occurred 30 times in *P. tremula* individuals and 384 times in *P. alba* individuals).
3. Selection of data sets for the calculation of D':
   a) First, the whole dataset of 541 individuals was chosen, in order to detect the extent and direction of cytonuclear disequilibrium at different loci
   b) Second, based on the probabilities calculated in the software STRUCTURE with K=2, one hybrid and two pure species groups were defined. For these three groups D' was calculated again for each locus, especially focusing on mulitlocus-hybrids in order to explore which loci exhibit significant D'. Here D' was calculated using both species specific clades and haplotypes separately.
   c) For hybrid populations D' was calculated at all loci in order to assess the main evolutionary factors shaping those populations.

As an alternative method, clusters defined by STRUCTURE were used to calculate D'. For *P. tremula* no clear population structure was suggested, therefore D' was only calculated for *P. alba*. The software STRUCTURE clearly defined four clusters as the best fitting number of subpopulations for all *P. alba* individuals. Subsequently synthetic alleles over all loci were assigned to each of the clusters. The four clusters defined four geographical groups: cluster 1 defined a subgroup of individuals within one Northern Austrian population, cluster 2 defined Central European individuals, cluster 3 defined Romanian individuals and cluster 4 defined Tunisian individuals. Each of the individuals was defined by the proportion of each cluster. Thus if one individual had more than 90% of one cluster, it was defined as homozygous for that specific cluster (11, 22, 33, 44). If proportions of one individual

were divided between two or more clusters, the two highest proportions were taken resulting in heterozygous individuals (12, 13, 14, 23, 24, 34). For the chloroplast data haplotypes were assigned to haplotype lineages according to results in Chapter 1. For all *P. alba* individuals four lineages were defined: one for Italian haplotypes (yellow, see Fig.2 in Chapter 1), one for Romania and the Danube drainage system (red), one for Central Europe and the Danube drainage system (blue), and one for Tunisia, which was more closely related to *P. tremula* haplotypes. Frequencies were assessed for each of the synthetic nuclear genotype × chloroplast lineage combinations (altogether 28 cytonuclear combinations were present).

Results

The chloroplast haplotypes were assigned to species specific clades following the method described in Chapter 1. All of the 20 microsatellites were highly variable from three to 27 alleles and varied between the three taxa (Table 3). The expected heterozygosity was higher than the observed in several cases and resulted in significant deviations from Hardy-Weinberg equilibrium (18 loci in *P. alba*, 15 in *P. ×canescens* and 17 in *P. tremula*). This can be caused by the Wahlund effect, when single populations consist of subpopulations harbouring different allele frequencies resulting in low total heterozygosity. The F_{ST}-value was high for 12 loci suggesting that the respective marker was useful for differentiating the species (loci with $F_{ST} > 0.2$: WPMS_16, WPMS_14, WPMS_17, WPMS_20, PTR_8, ORNL_344, ORNL_214, GCPM_1158, GCPM1894, ORPM86, ORPM_206, Yin2)

Table 3 Genetic variability of 20 nuclear microsatellites in *P. alba* (258 individuals), *P. ×canescens* (48 individuals) and *P. tremula* (235 individuals)

	P. alba					*P. ×canescens*					*P. tremula*					Overall	
Microsatellites	A	H_E	H_O	F_{is}	HWE	A	H_E	H_O	F_{is}	HWE	A	H_E	H_O	F_{is}	HWE	F_{is}	
GCPM_1719	14	0,873	0,807	0,076	**	9	0,85	0,913	-0,075	n.s.	7	0,646	0,576	0,106	**	0,073	**
GCPM_1158	7	0,486	0,602	-0,239	**	5	0,536	0,83	-0,557	**	9	0,465	0,601	-0,294	**	-0,295	**
PTR_4	17	0,891	0,86	0,035	**	15	0,885	0,917	-0,037	**	11	0,592	0,445	0,249	**	0,107	**
ORPM_220	4	0,152	0,069	0,546	**	4	0,386	0,262	0,325	**	6	0,609	0,569	0,065	**	0,166	**
WPMS_14	16	0,706	0,696	0,015	**	13	0,847	0,739	0,128	**	21	0,662	0,593	0,103	**	0,066	**
WPMS_17	9	0,581	0,401	0,31	**	9	0,711	0,444	0,377	**	12	0,615	0,556	0,096	**	0,212	**
ASP_112322	27	0,821	0,729	0,112	**	16	0,898	0,814	0,095	**	14	0,82	0,838	-0,022	*	0,048	**
WPMS_16	15	0,287	0,184	0,359	**	13	0,673	0,66	0,02	n.s.	21	0,818	0,793	0,031	**	0,099	**
PTR_8	12	0,187	0,069	0,63	**	9	0,736	0,532	0,279	**	12	0,784	0,594	0,243	**	0,307	**
PTR_2	14	0,78	0,749	0,04	**	8	0,816	0,395	0,519	**	10	0,68	0,546	0,197	**	0,153	**
ORPM_344	7	0,449	0,174	0,613	**	6	0,732	0,556	0,243	*	8	0,673	0,53	0,213	**	0,352	**
ORPM_29	24	0,782	0,523	0,331	**	17	0,809	0,553	0,318	**	25	0,727	0,677	0,068	**	0,206	**
PMGC_2885	8	0,591	0,481	0,187	**	10	0,73	0,568	0,224	**	12	0,571	0,556	0,025	n.s.	0,117	**
WPMS_20	5	0,056	0,022	0,609	**	7	0,521	0,5	0,04	n.s.	12	0,627	0,556	0,113	**	0,136	**
GCPM_1812	12	0,814	0,409	0,499	**	8	0,804	0,478	0,408	**	8	0,648	0,619	0,045	n.s.	0,298	**
GCPM_1894	15	0,752	0,677	0,101	**	7	0,707	0,63	0,109	n.s.	9	0,213	0,139	0,348	**	0,153	**
ORPM_86	4	0,307	0,249	0,189	*	2	0,497	0,574	-0,158	n.s.	6	0,452	0,452	-0,007	n.s.	0,043	n.s.
Yin_2	3	0,063	0,047	0,246	*	6	0,648	0,512	0,212	*	5	0,468	0,47	-0,005	n.s.	0,056	**
ORPM_214	8	0,272	0,28	-0,029	n.s.	6	0,601	0,442	0,268	**	5	0,258	0,148	0,427	**	0,215	**
ORPM_206	6	0,1	0,094	0,056	n.s.	5	0,531	0,439	0,176	n.s.	6	0,543	0,439	0,192	**	0,173	**
Total		0,499	0,407	0,183	**		0,699	0,592	0,153	**		0,593	0,535	0,099	**		

A, number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity, F_{is}, inbreeding coefficient; HWE, deviation and significance of Hardy-Weinberg equilibrium for each locus. ** $p<0.01$, * $p<0.05$, n.s. non significant

For the total set of 23 populations, the proportion of hybrids and the number of loci were estimated and revealed 8 populations with more than 10% of hybrids, 8 populations with 2-10% of hybrids and six populations with no hybrid individuals (Table 4). Two of the reference populations revealed low (3%) and high (33%) proportions of hybrids, Umea_t and Tunisia_subintegerrima_a, respectively. Whereas Northern Croatia_t, which was considered a hybrid population at first, did not show any signs of hybridisation according to the 20 studied nuclear microsatellites.

Table 4 Descriptive statistics of populations of *P. tremula, P. alba* and *P. ×canescens* including the proportion of hybrid individuals in each population according to nuclear admixture proportion (calculated in STRUCTURE) and number of loci exhibiting significant cytonuclear disequilibrium.

Population	N	Portion of hybrid individuals	No of loci with significant D'
Danube Delta_t	14	0,43	**8**
Tunisia subintegerrima_a	3	0,33	**0**
Central Croatia_a	23	0,22	**0**
Northern Croatia_a	19	0,21	**5**
Viennese Forest_c	27	0,19	**16**
Czech Republic_a	23	0,17	**0**
Central Alps_t	24	0,17	**0**
Western Hungary_c	62	0,11	**17**
Brasov_t	13	0,08	**0**
Northern Austria_a	32	0,06	**0**
Western Romania_a	17	0,06	**0**
Czech Republic_t	22	0,05	**0**
Danube Vienna_a	48	0,04	**0**
Central Romania_a	27	0,04	**0**
Eastern Alps_t	28	0,04	**0**
Umea_t	30	0,03	**0**
Northern Austria_t	59	0,02	**0**
Eastern Romania_a	12	0	**0**
Maramures_t	4	0	**0**
Northern Croatia_t	23	0	**0**
Northern Alps_t	14	0	**0**
Oeland_t	14	0	**0**
Tunisia hickeliana_a	3	0	**0**
Total	541		

Populations dominated by morphologically identified individuals of t, *P. tremula*, a, *P. alba*, c, *P. ×canescens*

The analysis in STUCTURE (Falush et al. 2003) assigned the majority of the individuals to the class of pure species (258 *P. tremula*, 235 *P. alba*, Fig. 1). Altogether 48 individuals were considered hybrids consisting of more than 10% of the other species. Those 48 hybrids belonged to 17 populations, whereas 6 populations did not show any sign of hybridisation; these were consequently treated as the reference populations.

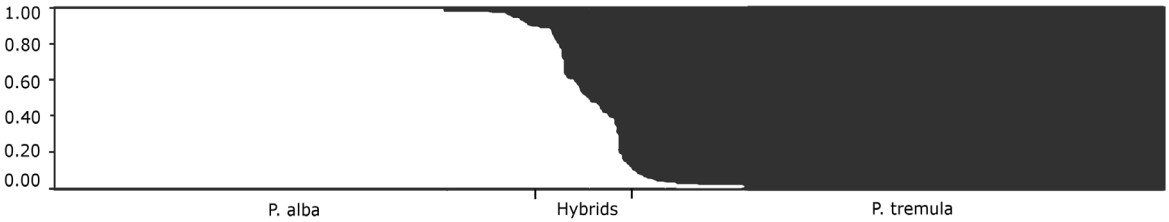

Fig. 1 Distribution and admixture proportions for both parental gene pools of *P. alba* (white) and *P. tremula* (black) in all 541 individuals for K=2. Altogether 48 hybrid individuals were identified with admixture proportions between 10-90%.

Five different runs were performed in NEWHYBRIDS and did not yield any differences, therefore only the results of one run are presented here. The individuals were assigned to six genotypic classes and most of them belonged to the pure species groups (258 individuals within the P1 group of pure *P. tremula*, 235 individuals within the P2 of pure *P. alba*) with 97-99% of assignment probability. The remaining 48 individuals were divided between the four hybrid classes with the majority of individuals identified as F2 hybrids (assignment probability of Q=0.52-0.99, Fig. 2).

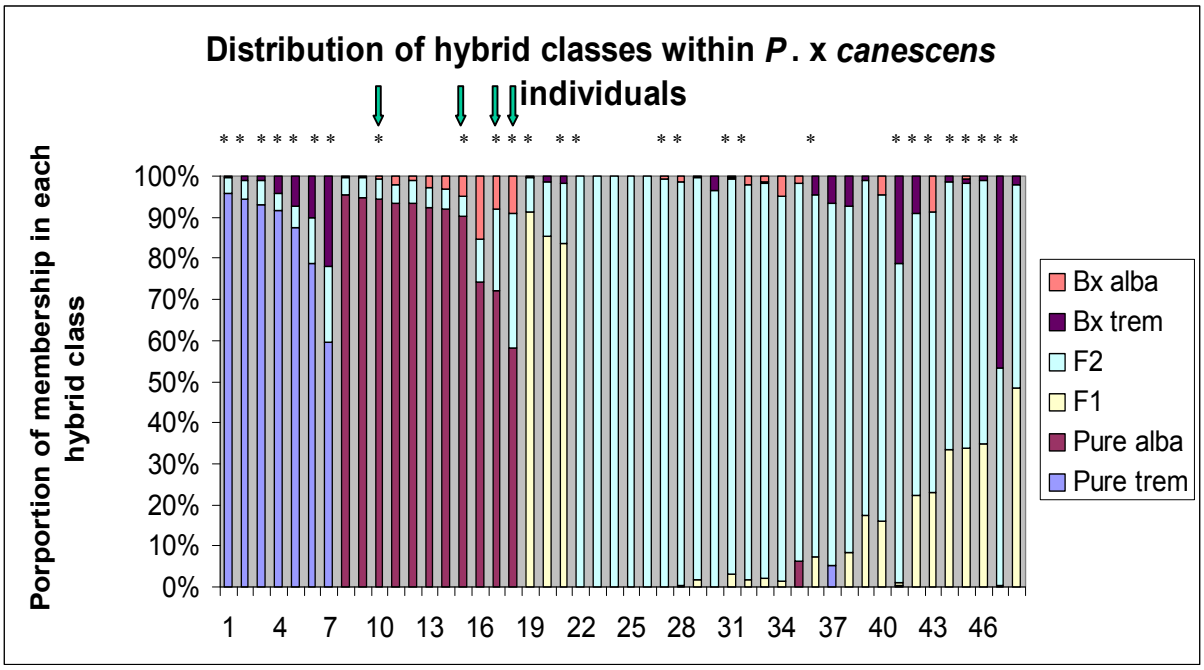

Fig. 2 Distribution of hybrid individuals within 6 hybrid classes. * individuals carrying chloroplast haplotypes of the *P. tremula*-clade. Arrows indicate four individuals with *P. tremula* haplotype and *P.* ×*canescens* nuclear genome occurring within the population Northern Croatia_a

For the whole dataset a highly significant disequilibrium ($p<0.001$) for the species-level homozygotes coupled with con-specific chloroplasts was observed (Table 5): i.e. pure nuclear *P. tremula* genotypes (11) were significantly associated with haplotypes of the *P. tremula*-clade (CPt) – the same was true for nuclear *P. alba* genotypes (22) and haplotypes of the *P. alba*-clade (CPa). For the admixed nuclear genotypes (12) significant association of 13 loci with chlorotypes of the *P. tremula*-clade and only 5 loci with chlorotypes of the *P. alba*-clade was observed. For the remaining two loci no significant association was observed.

The calculation of cytonuclear disequilibrium (D') within the group of hybrids using haplotype clades revealed clear deviation of random distribution at 6 loci (Table 5). The results of calculations using "singular" haplotypes instead of haplotype clades revealed cytonuclear disequilibrium at 10 loci. Significant association with pure *P. alba* genotypes were found at six loci, with pure *P. tremula* at 3 loci and with intermediate genotypes at 5 loci. Several haplotypes were affected from significant associations - H15, H17, H33, H41 und H45. H15 represents the second most frequent *P. alba* haplotype, and H41 and H45 are the most frequent *P. tremula* haplotypes. H33 connected the *P. alba* and *P. tremula* clades within the haplotype-network, and was associated most often with intermediate genotypes. Altogether cytonuclear disequilibrium was more often detected among particular haplotypes and those associations did not occur at random. Most probably migration movements between Central Europe and Spain played a role for the patterns concerning haplotype H33 (see also Chapter 1), however, migration should result in cytonuclear disequilibrium at all loci (Asmussen et al. 1987, Latta et al. 2001). The most common haplotype within *P. alba* (H21) did not show significant D', because it was more evenly associated with different genotypes and also occurred frequently with *P. tremula*-alleles.

As a comparison D' was calculated within the groups of pure species – only at four loci within *P. alba* significant deviations were observed. In contrast the group of *P. tremula* individuals did not show any deviations of random cytonuclear genotype combinations.

Table 5 Positive and negative associations at 20 nuclear microsatellite loci and two chloroplast clades for three datasets: whole dataset (541 individuals), hybrids (48 individuals), *P. alba* (235 individuals). Within each locus pure individuals and hybrids were grouped according to synthetic allele assignment

	Whole dataset			Hybrids		*P. alba* data set		
	CPt	CPa		CPt	CPa	CPt	CPa	
GCPM_1719								
11	0.81	-0.81	****			1	-1	*
12	-0.28	0.28	***			-1	1	*
22	-0.76	0.76	***					

PTR_4									
11	0.92	-0.93	****						
12	-0.16	0.16	*						
22	-0.87	0.87	***						
WPMS_14									
11	0.86	-0.86	****						
12	0.19	-0.19	*						
22	-0.90	0.91	****						
WPMS_17									
11	0.81	-0.81	***						
12	0.49	-0.49	***						
22	-0.64	0.64	****						
ASP_112322									
11	0.93	-0.93	***						
12	-0.32	0.32	***						
22	-0.91	0.91	***						
WPMS_16									
11	0.88	-0.88	****	0.78	-0.78	**			
12	-0.17	0.17	ns						
22	-0.90	0.90	****				-0.4	0.40	*
PTR_8									
11	0.81	-0.81	****						
12	0.61	-0.61	***						
22	-0.81	0.82	****	-0.86	0.86	***			
PTR_2									
11	0.74	-0.74	****						
12	-0.27	0.27	***						
22	-0.76	0.76	***						
ORPM_344									
11	0.96	-0.96	***						
12	0.66	-0.66	***						
22	-0.87	0.87	****						
ORPM_206									
11	0.97	-0.97	***						
12	0.61	-0.61	***						
22	-0.80	0.80	****	-0.41	0.41	*			
PMGC_2885									
11	0.39	-0.39	***						
12	-0.29	0.29	***						
22	-0.65	0.65	***						
ORPM_214									

11	0.95	-0.95	****						
12	0.63	-0.63	***						
22	-0.88	0.88	****						
GCPM_1158									
11	0.94	-0.94	***				0.79	-0.79	*
12	0.01	-0.01	****						
22	-0.94	0.94	***						
WPMS_20									
11	0.97	-0.97	***						
12	0.79	-0.79	***	0.39	-0.39	*			
22	-0.91	0.91	****	-0.48	0.48	**			
GCPM_1894									
11	0.92	-0.92	***						
12	0.09	-0.09	***						
22	-0.81	0.81	****	-0.51	0.51	*			
ORPM_86									
11	0.72	-0.72	***						
12	0.33	-0.33	***	0.38	-0.38	*			
22	-0.71	0.71	****	-0.61	0.61	**			
ORPM_220									
11	0.59	-0.59	***						
12	0.74	-0.74	***						
22	-0.69	0.69	****						
ORPM_29									
11	0.91	-0.91	****				0.48	-0.48	*
12	-0.08	0.08	n.s.						
22	0.85	-0.85	****						
GCPM_1812									
11	0.77	-0.77	****						
12	0.28	-0.28	***						
22	-0.83	0.83	****						
Yin_2									
11	0.97	-0.97	****						
12	0.51	-0.51	**						
22	-0.92	0.92	****						

*	<0.05
**	<0.01
***	<0.001
****	<0.0001
n.s.	non significant

The significant D' was calculated within four populations: Viennese Forest_c, Western Hungary_c, Northern Croatia_a, Danube Delta_t (Table 6). Within the populations Western Hungary_c and Viennese Forest_c, the species-specific homozygotes were responsible for the significant D'. Within the population Danube Delta_t, also species-specific homozygotes were dominant but at some orders of magnitude less so. Within the population Northern Croatia_a intermediate genotypes were associated with *P. tremula* haplotypes and pure *P. alba* with *P. alba* haplotypes (22a). All four individuals indicated with arrows in Fig. 2 (*P. tremula* haplotype and *P. ×canescens* nuclear genome) resided within the population Northern Croatia_a.

Table 6 Significant associations within four hybrid populations of *P. tremula* and *P. alba* in different genotype classes (A-E) according to their composition and occurrence of D' (see footnote).

	No. of loci with significant D'				
	A	B	C	D	E
Western Hungary_c	5	10	0	2	0
Viennese Forest_c	0	13	1	1	1
Danube Delta_t	0	3	2	1	2
Northern Croatia_a	0	0	0	2	3

A, all genotypes affected (11, 12, 22); B, only homospecific organelle-nuclear combinations affected (11, 22); C, only *P. tremula* genotypes affected (11); D, only hybrid genotypes affected (12); E, only *P. alba* genotypes affected (22)

Calculations for cytonuclear disequilibrium for synthetic nuclear x chloroplast lineage combinations for *P. alba* lineages revealed four significantly positive and four significantly negative associations. Italian homozygous genotypes (within the population Northern Austria_a) were positively associated with the Italian chloroplast lineage and negatively associated with Romanian and Central European haplotype lineages. Furthermore Romanian homozygotes were positively associated with Romanian haplotypes, but negatively associated with the Italian haplotype lineage. Additionally Tunisian nuclear homozygotes were positively associated with Tunisian haplotype

lineages and negatively associated with the Italian haplotype lineage. Only one result was surprising: the highly significant ($p > 0.01$) positive association for one group of individuals heterozygous for the Romanian and Tunisian nuclear gene pool with the Romanian haplotype lineage ($D' = 0.738$).

Discussion

Cytonuclear disequilibrium is defined by the non-random association of the nuclear genome with the cytoplasmic genomes(chloroplast and mitochondria). Analysing the nuclear genome using mulitlocus genotypes allowed the assessment of pure species and hybrids. The assessment of the chloroplast genome offered conclusions about the mating patterns, i.e. if both sexes of both species were involved in mating patterns. Strong cytonuclear disequilibrium observed consistently across loci are driven by migration and assortative mating. Both mechanisms can be detected in hybrid zones, e.g. *Hyla* tree frogs were studied, where males of one species only interbreed with the females of the other species (Lamb & Avise 1986, Arnold 1993). Whereas the evolutionary dynamics of disequilibria caused by selection and/or genetic drift are more locus specific.

Genetic drift results in low level of normalized D', caused mainly by founder effects and diminishes after few generations. One study of *Pinus ponderosa* showed that the observed disequilibrium was caused by genetic drift in a non-hybridising population (Latta et al. 2001). Genetic drift affected some loci at random and did not affect others. Selective factors do not affect all loci similarly, but only those which were associated with important genes. But fitness effects would show along different genomes.

In this study first the whole dataset was considered as a "large hybrid zone" in order to estimate effects of cytonuclear interactions regarding single loci. Significant occurrence of species-specific homozygotes of pure species with their species specific chloroplast haplotypes (11t und 22a) suggested selection against hybrids. Here almost all loci were affected, but patterns did not direct in one direction, compared to the *Hyla* example. The intermediate genotypes (12), when looking at the whole dataset, were associated in 13 loci with *P. tremula* haplotypes and only in 6 loci with *P. alba* haplotypes. These results refer to the fact that hybridisation in the majority of loci seems more common into the direction of *P. tremula* cytoplasm, compared with *P. alba* cytoplasm. The lower association of *P. alba* haplotypes with intermediate genotypes compared with *P. tremula* haplotypes suggested stronger barriers of female *P. alba*

towards *P. tremula* genotypes, whereas *P. tremula* seems to mate rather randomly. The flower phenological observations in two consecutive years point in the same direction (Chapter 4). The overlap of flowering of male *P. alba* with female *P. tremula* seemed more likely than the other way around in the studied populations.

Furthermore, the extent of D' tells something about the age of existing interactions. The higher the D'-value, the older and more stable the existing disequilibrium and the lower the D'-value the younger is considered the disequilibrium (Asmussen et al. 1987). In this study for *P. alba* an average value of 0.26 for 5 loci and for *P. tremula* a value of 0.46 at 13 loci was detected. Following the proposed principles of Asmussen et al. (1987) rather young interactions between intermediate genotypes and *P. alba* chloroplast haplotypes can be assumed, because of a low average D'-value for *P. alba*. In contrast higher average D' values for *P. tremula* indicated older interactions. However this argument has to be taken with caution, since I had to collapse nuclear markers into synthetic alleles.

Reducing the dataset and only using the *P. ×canescens* mulitlocus genotypes yielded cytonuclear interactions at a lower number of loci – species-level homozygotes coupled with con-specific haplotypes occurred predominantly within *P. alba*, and intermediate nuclear genotypes occurred predominantly with *P. tremula* haplotypes. When only looking at hybrids, significant D' occurred in 6-10 loci and 5 haplotypes. The most frequent *P. alba* haplotype was not affected but both affected ones belonged to the Danube lineage (red, Chapter 1).

Differences in the compatibility of certain genotype-cytoplasmic-combinations during hybridisation could be attributed to the fact that within the dataset of hybrids with singular haplotypes, intermediate genotypes were predominantly associated with *P. tremula* haplotypes. As the STRUCTURE results support the fact that the extent of hybridisation was rather low in the studied populations, selection against hybrids can be assumed. On the one hand genetic markers can explore "real" incompatibilities of genes from different organelles/compartments, like one of the most important example of a nuclear and chloroplast encoded enzyme – RUBISCO (Sambatti et al. 2008), if markers and genes are linked. On the other hand, those genes are most probably highly conserved among different species, in order to maintain their function. The question here is, if also combinations of such genes encoding enzymes of different compartments are highly conserved as well. However, the effects of very small differences e.g. in non-coding regions have not been sufficiently studied until now and even epigenetic variations can

be responsible for resulting fitness differences (Sambatti et al. 2008). Incompatibility could influence certain traits of plants, in order to keep the species integrity despite gene flow. Positive D' would in this case suggest a certain compatibility of certain haplotypes with certain genotypes.

As a comparison, D' was calculated at the population level. As reported by Latta et al. (2001) a good basis for studying hybrid populations can be the investigation of non-hybrid populations, because D' can be caused by genetic drift without hybridisation (Latta et al. 2001). In that case some loci were affected at random, and others did not show any disequilibria; small populations were more affected than larger ones. In *Pinus sylvestris*, Muona and Smidt (1984) showed that the youngest population exhibited the highest variance of D' between different pairs of loci. In our study only four populations showed significant D', all of them were influenced by hybridisation. Non-hybridising populations were not affected by cytonuclear disequilibrium. In one population (Central Romania_a) different lineages of haplotypes within the *P. alba* clade occurred, but no significant association with nuclear genotypes existed. Due to the high frequency of species-specific homozygotes within the populations Western Hungary_c and Viennese Forest_c, migration and assortative mating of the parents can be assumed as the main factors. Within the population Danube Delta_t, cytonuclear disequilibrium was affecting a low number of loci, but interactions were evenly distributed among all genotype classes. Within the population Northern Croatia_a, cytonuclear disequilibrium occurred, because of species-specific homozygotes associated with *P. alba* haplotypes (22a) and intermediate genotypes associated with *P. tremula* haplotypes (12t), but again only affecting a low number of loci.

Considering the whole dataset cytonuclear disequilibrium was not evenly distributed over all loci, thus selective mechanisms can be assumed (Asmussen et al. 1989), if the affected loci were linked to genes under selection. This cannot be directly derived from this study, but it is possible that loci affected by cytonuclear disequilibrium are linked to chromosome blocks under selection. Those blocks are supposed to be formed in the hybrid offspring and are larger in first generation hybrids and their size will decrease in later generations and backcrosses (Pfaff et al. 2001, Lexer et al. 2007). An interesting hint could be locus WPMS_16, with significant D' in the whole dataset, within the hybrids, the *P. alba* dataset, and within three of four hybrid populations showing significant overall D'. Within the hybrids at this locus *P. tremula* genotypes more often occur with *P. tremula* cytotypes than randomly expected (Table 5). A small

portion of genes within the hybrids can show positive interactions and represent the raw material for adaptive evolution of hybrids (Rieseberg et al. 1996), to look for loci linked to such genes using genome-wide studies of cytonuclear interactions seems rewarding in this respect.

The fact that only 9% of the analysed individuals were defined as hybrids points to selection against hybrids. The formation of hybrids seem possible with respect to prezygotic barriers: flower phenological observations showed overlap of flowering times of both species in both directions, but highly influenced by locality and weather conditions during spring (Chapter 4). It seems that the limiting factor is the survival of hybrids, because first generation hybrids were more common than backcrosses to any of the parents. As shown in Fig. 2 first and second generation hybrids are the majority, indicating that hybrid formation and fertility might not be the limiting factor. But as almost no backcrosses were found, there must be some intrinsic cause for that. Postzygotic barriers e.g. negative cytonuclear interaction or epistasis among nuclear markers might be a possible explanation.

In a studied population of deep-sea mussels, the main forces for shaping the population were recent immigration and hybridisation (Won et al. 2003). Furthermore, a large proportion of individuals had species-specific organelle-nuclear combinations suggesting selection against hybrids and fitter parents than offspring. In general, hybridisation requires the successful mating of genomes from well differentiated parental species (Rieseberg et al. 1996). External factors mainly influence the genetic isolation of species, i.e. if species with divergent ecological adaptation meet along an environmental gradient (e.g. divergent adaptation combined with mating patterns). Breaking apart or loosing those habitat differences might lead to softening of reproductive barriers. In this study, gene flow within the species was larger than between species; this probably contributes to the maintenance of species barriers. The good differentiation of species (into three taxa) was evident from a high value of F_{ST}= 0.28; whereas differentiation within species was only F_{IS}= 0.14. Despite gene flow, both studied species still occur as independent species. This can be due to well defined historical separation, which is also evident from the well separated chloroplast data exhibiting species specific haplotype clades. Thus hybridisation seems to be of rather recent origin in the studied populations or in the studied region.

A further phenomenon leading to cytonuclear disequilibrium is assortative mating as for example the admixture of genetic lineages during postglacial recolonisation or

hybridisation. Assortative mating as a result of migration caused by the ice ages could have led to backcrossing towards the immigrating male species through constant pollen flow and replacement of the nuclear genome of the local species; i.e. “chloroplast capture” (e.g. in Spain, where H33, an old haplotype with a large distribution area prevails). This may also be true for four individuals within the population Northern Croatia_a, where a large proportion of *P. alba* specific alleles were associated with *P. tremula* specific haplotypes (Fig. 2). By way of immigration of *P. alba* pollen, hybridisation and constant backcrossing towards *P. alba* could have occured. However, assortative mating was most likely not the main cause shaping the overall patterns of this study, because not all loci were affected from cytonuclear disequilibrium.

Finally, environmental factors might interact with cytoplasm and thus shaping cytonuclear interactions. Recently ecological speciation was studied in hybrid sunflower by transplant experiments, revealing that the parental species’ cytoplasm was strongly locally adapted (Sambatti et al. 2008). They speculate that the chloroplast genome is responsible for the local adaptation patterns and the mitochondrial genome for cytonuclear interactions. If the cytoplasm is reacting to environmental factors, the species providing the chloroplast is interacting more closely with environment than the male contributing species. E. g. hybrids with *P. tremula* chloroplasts might be adapted rather to *P. tremula* typical habitats. This seems to be true for certain populations within this study (e.g. Central Croatia_a), consisting of pure *P. alba* and hybrids carrying *P. alba* chloroplasts – thus being better adapted to *P. alba* habitats. But for other populations it is difficult to assess, because the four populations exhibiting most of the hybrid genotypes (Viennese Forest_c, Western Hungary_c, Northern Croatia_a, Danube Delta_t) carry haplotypes from both species (Fig. 3). Thus environmental conditions of those populations seem to consist of patches suitable for both species and their hybrids.

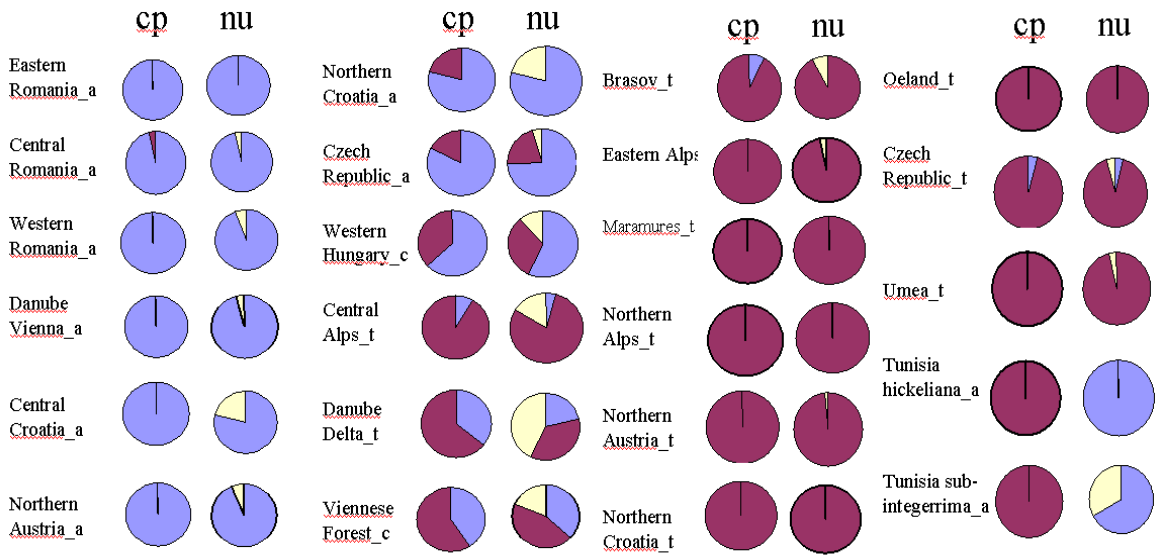

Fig. 3 Chloroplast and nuclear admixture proportion of each population. Haplotype assignment of chloroplast data was performed according to Chapter 1, where two main clades were identified (blue, *P. alba*-clade; purple, *P. tremula*-clade). Nuclear admixture derived from the STRUCTURE-run (K=2) and proportion of hybrids classified according to Fig.1 (this Chapter) – blue , pure *P. alba*; purple, pure *P. tremula*, yellow, *P.* ×*canescens*

Considerations about the utilized method of calculating cytonuclear disequilibrium

At the time when models of cytonuclear disequilibrium were developed (Asmussen et al. 1987) only few possibilities of simultaneous investigations of nuclear and cytoplasmic markers in a species or species complex existed (Latta et al. 2001). Nowadays molecular genetic methods are not limited any more. Even the observation of genome-wide cytonuclear interactions would be feasible, but the software for analysing such data is not available yet. The frequently used software for detailed tests is CNDm (Basten & Asmussen 1997), but unfortunately the capacity is restricted to 9 nuclear alleles per locus. Moreover, the input of data as a matrix of cytonuclear frequencies of individual combinations is very time consuming and inconvenient.

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General discussion

Here I present the first phylogeographic study looking at both *P. tremula* and *P. alba* as well as their hybrid *P. ×canescens* based on maternally inherited plastid DNA markers. The results revealed a clear separation of the two parental species into two main clades – only occasionally haplotypes were shared between the two species, and hybrids carried both *P. alba* typical and *P. tremula* typical haplotypes. These findings suggest rather stable species established long time ago and hybridisation upon secondary contact. Zones of contact, where hybridisation occurs, are formed locally and have been recognized in this study wherever habitats of both parental species meet. In general those habitats of each parental species differ clearly representing low elevation floodplains for *P. alba* and higher elevation and more boreal conditions for *P. tremula. P. ×canescens* is supposed to occur on intermediate sites, but this has not been tested by controlled experiments. Within this study, zones of contact were identified in three regions in Central Europe including Eastern Austria, Western Hungary and Northern Croatia. Altogether this may represent one vast hybrid zone, but sampling was not organized along one transect, but rather as disjunct populations. This probably was the reason why different regional patterns of hybrid generations and directions of introgression were discovered.

For *P. alba* separate refugia can be postulated, one in Italy and one for South-eastern Europe, and different recolonisation trajectories shaped the composition of today's populations. For instance part of one population in Northern Austria consists of Italian colonizers, but the time of their arrival cannot be derived from the data – interestingly, however, also nuclear microsatellites identified them as a separate gene pool (unpublished results). I obtained a different picture of no clear patterns for *P. tremula,* and haplotypic diversity was evenly distributed across the studied populations. This pattern may partly have arisen from surviving populations near the ice sheet. *P. tremula* probably was one of the first colonizers of newly established habitats after the retreat of the glaciers. Furthermore *P. tremula* has a great ecological amplitude and thus is able to cope with different climatic conditions, making it an ideal pioneer species for recolonising open landscapes. This might well have happened shortly after the peak of the cold periods during the Pleistocene.

A practical test for the usefulness of the described phylogeographic pattern emerged with the case of tree samples from the Maltese islands. Here, an attempt was

made to relate *P. alba* on Malta to the established phylogeographic pattern for Europe. The relatedness of *P. alba* on Malta to the close penninsula of Italy was unambiguous based on chloroplast markers, but paternal contribution from Northern Africa seems possible. The assessment of genetic diversity of *P. alba* of the two islands of Malta within the Mediterranean sea was somewhat surprising, because all 28 sampled trees consisted of the same mulitlocus genotype. Clonal propagation of *P. alba* was already reported to be high on other Mediterranean islands (Brundu et al. 2008), but at least several clones were found in those studies. The Maltese clone is likely a human introduction to the Archipelago and might represent the best clonal reproducer as a remainder of few introduced individuals, because *P. alba* in general is not easily propagated vegetatively (personal observation). However, it would be interesting to estimate the age of the clone using the methods proposed by Mock et al. (2008) and Ally et al. (2008).

When sampling individuals within hybrid zones, species assignment can be ambiguous. In order to find a more formal way of assigining individuals to species and hybrids, morphological traits were tested. The utility of leaf morphological parameters for species assignment was shown in one population. Here, more traditional leaf measurements were used compared to recently suggested approaches by Lexer at al. (2009). The main attempt was to establish an easily measurable trait, which can be applied in the field as well as in the lab. Me and my co-authors succeeded in confirming previous results, in that lobedness is one of the best measures to distinguish between *P. alba*, *P. tremula* and their hybrid. But additionally we introduced a field measure of lobedness (i.e. L1/L2, which is the ratio of the first to the main vein), describing the three taxa very well.

Finally, the model system *Populus* is very useful in studying different aspects of evolution, because a wide variety of markers have been developed to choose from. Furthermore a high number of studies are available for comparison with non-model species within the genus. Here I used two different approaches to explore the role of hybridisation and backcrossing for speciation:

First I used a small scale approach to study prezygotic barriers to gene flow based on flowering phenological observations. Temporal barriers like divergent flowering time between the two species has to be considered in two scenarios. Either both species occur within the same habitat, in this case overlap of flowering time was less pronounced. The alternative case is where species occur in different habitats but close enough to interact

and exchange pollen and seeds. Influenced by soil and climate, overlap is more pronounced in the second case. Hybrids appeared to be intermediate in flowering, thus once established they could represent a stepping stone for the introgression of advantageous alleles from one species into the other (Barton 2001). Altogether flowering phenology does not seem to represent a very strong barrier to gene flow in the studied species pair.

Second I studied cytonuclear interactions as an indicator of postzygotic barriers to gene flow on a broad geographic scale. Here I used several resources for nuclear microsatellite markers (Table 1, Chapter 5). The results revealed low proportions of hybrids, thus strong barriers to gene flow can be assumed. Because early generation hybrids (F1s and F2s) were more frequently observed than backcrossed individuals, some kind of epistatic nuclear-nuclear or cyto-nuclear interaction can be assumed to act on certain combinations of genotypes. Different mechanisms would have to be involved in the formation of a new stable hybrid species. One of those mechanisms would be reproductive isolation towards the parent species, which could become complete through the mating of F1s and F2s among themselves, without backcrossing towards the parental species (Barton 2001). Such reproductive isolation most likely arises from chromosomal changes or ecological divergence (Rieseberg 2003). Thus different habitat requirements of the hybrid compared to the parental species could facilitate hybrid speciation (e.g. hybrid sunflower species, Rieseberg et al. 1996, 2003), assigning ecological adaptation a key role during hybrid speciation. Moreover, hybrids have to escape from the vast majority of presumably unfit recombinants in order to reach population level. This could be facilitated by asexual reproduction (Barton 2001). Here the clonal growth of *P.* ×*canescens*, which is more pronounced in the hybrid than in *P. alba* (van Loo et al 2008) could present a mechanism to overcome unfavourable environmental conditions and/or unsuitable mating associates (e.g. other less fit hybrids) in order to secure persistence of beneficial allele combinations for a longer period of time.

General conclusion

The chosen system of the two poplar species allowed me to contribute towards the understanding of the role of hybridisation in evolution. With the use of chloroplast markers I could show that both species had different evolutionary histories influenced by the ice ages, consequently exhibiting different migration activities. A range wide

collection of both species and hybrids would be important to assess if patterns detected for Central and South-eastern Europe also hold for Western Europe; especially for the Iberian Peninsula, representing a refugium for several other species as well.

The influence of hybridisation on the migration and distribution of current population patterns seems to be high in zones of contact but hybrids might not be able to establish themselves on a broader geographic scale. A further possibility for future work on hybrid zones would be to look further into the past when assessing and locating possible hybrid zones, because hybrid zones may have moved as the parental species migrated during glaciation cycles.

Slight temporal barriers to gene flow could only be detected for individuals of both species occurring on the same site. In contrast, for neighbouring habitats influenced by different environmental factors overlap of flowering of both species was revealed. In order to study more deeply the genetic influence on flowering within the studied species and the role of hybrids, common garden experiments would be necessary using controlled crosses between the two species to produce hybrids with known pedigree.

In general controlled crosses for the studied species pair, which could answer rather basic questions about the direction of hybridisation, but also various more advanced evolutionary questions, in particular speciation and adaptation to e.g. “hybrid” environments, are missing. It is an excellent challenge for the future to try and establish the exact pedigrees of hybrids in the wild with the help of “genomic” methods, and in this way to refine and deepen the insights into the functions of individual genes, as well as their interplay, in local adaptation and overall species evolution.

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Projects

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08/2003-03/2005	EU-project “Realising Ash’s Potential”: using microsatellites in reproductive material (Austrian seed harvest populations) and gene flow studies.

Courses and lab visits

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08/2008	International Workshop ‘Population, Quantitative and Comparative Genomics of Adaptation in Forest Trees’ Centro di Ecologia Alpina, Monte Bondone, Trento, Italy. In collaboration with the University of California, Davis (Department of Plant Sciences). Course Instructors: David Neale (UCDavis), Jill Wegrzyn (UCDavis), Andrew Eckert (UCDavis) and Santiago Gonzalez-Martinez (INIA, Spain).
05/2007	Lab visit at the Jodrell Laboratory, Royal Botanic Gardens, Kew, England. Instructor: Christian Lexer; Statistical analysis of chloroplast fragments in *Populus*
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Publications and presentations

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Fussi B., Koziel A., Heinze B., 2007: Selection of seed orchard parents in common ash (*Fraxinus excelsior*): A genetic comparison of seed orchard composition with commercial seed lots.
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Poster presented at the 57. Tagung 2006 der Vereinigung der Pflanzenzüchter und Saatgutkaufleute Österreichs, HBLFA Raumberg-Gumpenstein, 21.-23. November 2006. Tagungsband S. 96

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