Uniwersytet im. Adama Mickiewicza w
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Bartosz Łabiszak

Genetic variation and population history of peat-bog pine (*Pinus uliginosa* N.) as compared to closely related species from the genus *Pinus*

Zmienność genetyczna i historia populacji sosny błotnej (Pinus uliginosa N.) względem spokrewnionych gatunków rodzaju Pinus

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Promotor

prof. dr hab. Witold Wachowiak
Zakład Ekologii Roślin i Ochrony Środowiska
Instytut Biologii Środowiska, Wydział Biologii
Uniwersytet im. Adama Mickiewicza w Poznaniu

Recenzenci

prof. dr hab. Emilia Alicja Brzosko
Katedra Biologii i Ekologii Roślin
Wydział Biologii,
Uniwersytet w Białymstoku

prof. dr hab. Jakub Sawicki
Katedra Botaniki i Ochrony Środowiska
Wydział Biologii i Biotechnologii
Uniwersytet Warmińsko-Mazurski w Olsztynie

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Spis treści

I. Abstract

The understanding of processes that shape genetic diversity of species is crucial in research focused on their evolutionary history and adaptive capabilities of populations in the face of ongoing climate change. Recent developments in analytical methods and fast growing genomic resources facilitate population genetics and genomics studies in non-model plant species. This is especially true for ecologically and economically important forest tree species, which still lack proper assessment of their evolutionary history. Peat bog pine (*Pinus uliginosa* N.) is one of the most intriguing among all native Polish pines. Interestingly, the taxonomic position of this species is not fully resolved yet, and due to the similarities of some morphological and genetic traits, it is often grouped with dwarf mountain pine (*Pinus mugo*) and mountain pine (*Pinus uncinata*) into so-called *Pinus mugo* species complex. The current geographic range of peat bog pine is restricted to a few well-known, isolated stands in Poland, Czech Republic, Germany, and Ukraine. Destruction of its primary habitat by drying out of the bog areas is currently accelerated by climatic changes, which poses a severe threat of its extinction.

Major goal of my doctoral dissertation was to assess the structure and level of genetic variation of *P. uliginosa* populations and gain better knowledge about the processes involved in shaping its genetic structure. This knowledge can greatly aid the development of proper conservation management strategies for this pine. Furthermore, it was important to resolve the complex taxonomy of *P. uliginosa* within a closely related group of pines, especially in light of an earlier hypothesis regarding the hybrid origin of peat bog pine. In my research, I utilized polymorphism data from nuclear, plastid, and mitochondrial genomes obtained from several populations of peat bog pine, as well as reference populations of its close relatives. By analyzing genetic data with the use of population genetic and biological modeling methods, I was able to: assess the influence of geographic isolation on the contemporary genetic structure of peat bog pine; understand the relationship between demographic changes and the level of genetic variation in *P. uliginosa*; understand the speciation model of pines within the *P. mugo* complex. Moreover, I developed novel genetic markers in mitochondrial genome, that is inherited in pines in maternal line and distributed by seeds at relatively short geographical areas, that are especially valuable and useful in phylogeographical studies of related pines. Using the markers I formulated guidelines for better management of genetic resources of this endangered species. The results of my PhD thesis broaden current understanding of the demographic and evolutionary process underlying levels of genetic variation in *Pinus* species, establishing the groundwork for development of proper conservation management strategies for these species in face of ongoing climate change.

II. Streszczenie

Poznanie procesów kształtujących zmienność genetyczną gatunku jest istotne dla zrozumienia jego przeszłości ewolucyjnej a także możliwości adaptacji populacji w obliczu postępujących zmian środowiskowych. Rozwój metod analitycznych i wzrastające zasoby genomowe pozwalają na badania z zakresu genetyki i genomiki populacyjnej u nie-modelowych gatunków roślin, w tym ważnych ekologicznie gatunków drzew leśnych, o wciąż słabo poznanej historii ewolucyjnej. Sosna błotna (*Pinus uliginosa* N.) jest najbardziej intrygującym spośród czterech rodzimych gatunków sosen w Polsce. Pozycja taksonomiczna tego gatunku nadal pozostaje w pełni nierozstrzygnięta. Z uwagi na liczne podobieństwa morfologiczne i genetyczne, wraz kosodrzewiną (*Pinus mugo*) i sosną hakowatą (*Pinus uncinata*) włączana jest najczęściej w obręb tzw. kompleksu gatunków *Pinus mugo*. Aktualnie jej zasięg ograniczony jest do kilku izolowanych stanowisk m.in. w Polsce, Czechach, Niemczech i na Ukrainie. Postępujące wraz ze zmianami klimatycznymi osuszanie torfowisk wysokich, będących naturalnym siedliskiem tego gatunku, stwarza poważne ryzyko jego wyginięcia.

Głównym celem mojej pracy doktorskiej było poznanie zmienności genetycznej sosny błotnej oraz procesów historycznych kształtujących strukturę populacji, co ma szczególnie istotne znaczenie w kontekście opracowania skutecznych strategii ochrony zasobów genowych tego gatunku. Kolejnym ważnym aspektem pracy było ustalenie pozycji taksonomicznej *P. uliginosa* w odniesieniu do innych blisko spokrewnionych gatunków sosen, zwłaszcza w kontekście testowania hipotezy o potencjalnie hybrydowym pochodzeniu sosny błotnej. W pracy wykorzystałem zmienność rejonów jądrowego, plastydowego i mitochondrialnego DNA szeregu populacji sosny błotnej i zbadałem polimorfizm tych samych rejonów u innych blisko spokrewnionych taksonów referencyjnych. Analiza danych genetycznych z wykorzystaniem szeregu metod modelowania biologicznego pozwoliła na określenie: wpływu izolacji geograficznej na kształtowanie struktury populacji sosny błotnej, wpływu czynników demograficznych na poziom zmienności genetycznej tego gatunku oraz modelu specjacji w obrębie kompleksu *P. mugo*. Dodatkowo, w ramach pracy opracowano nowe markery genetyczne mitochondrialnego DNA, szczególnie użyteczne w badaniach filogeograficznych sosen. Uzyskane wyniki badań przyczyniają się do poszerzenia dotychchczasowej wiedzy na temat procesów demograficznych i ewolucyjnych leżących u podstaw obserwowanej zmienności genetycznej taksonów rodzaju *Pinus,* stanowiąc jednocześnie podstawę opracowania strategii gospodarowania ich istniejącymi zasobami genetycznymi w obliczu zmian środowiskowych.

III. Introduction

Forest trees are foundation of several terrestrial ecosystems, and they play an important role in processes involved in carbon and water cycles, carbon dioxide sequestration, soil erosion mitigation, biodiversity harboring and they serve as the source of economically important natural resources (FAO 2018). Tree species maintain high intra- and low interpopulation genetic variation, even between populations separated by vast geographic distances (Petit and Hampe 2006). Genetic diversity of trees is shaped mainly by their unique life history traits, such as: longevity and large seed production, effective pollen and/or seed dispersion mechanisms, high selection on seedlings, and consequently low frequency of offspring reaching maturity, high phenotypic plasticity and adaptability, facilitating survival despite environmental changes perceived during their long lifetime (Petit and Hampe 2006).

The anthropogenic pressure is increasing in recent years and affects levels of genetic diversity in forest tree species. Mainly, its influence is negative and leads to a decrease in genetic variation of populations and their lower adaptability. Major human-caused threats include: habitats destruction, fragmentation, pollution, and environmental changes due to unsuitable use and exploitation of land and ongoing climate changes (van Mantgem et al. 2009, Shearman et al. 2012, Fettig et al. 2013, Riitters et al. 2016). Some human activities may lead to an increase in levels of genetic diversity, when populations of different origins meet in artificial secondary contact zones or when hybrid zones between related but long isolated species are created (Schaberg et al. 2008, Grabenstein and Taylor 2018). Nevertheless, by disruption of coadapted gene complexes and further shifts in the adaptive optima of population, this may lead to outbreeding depression and can cause genetic erosion, which remains an important issue in the context of conservation of natural hybrids (Allendorf et al. 2010, Allendorf et al. 2012, Stronen and Paquet 2013).

The genetic diversity of trees is shaped by the interplay between demographic processes, such as past changes of population size and distribution or levels of inter- and intrapopulation gene flow, and the evolutionary forces involved in the generation and maintenance of genetic variation of species, in both neutral background and genomic regions affected by selection (Hewitt 2004, Neale and Ingvarsson 2008, Sork 2015, Ellegren and Galtier 2016). Changes of species' ranges during the glacial cycles of Pleistocene have left significant imprints on the contemporary distribution and levels of genetic diversity of populations in the Northern Hemisphere and are well studied in several ecologically important tree species (Hewitt 1999, Hewitt 2004, Stewart et al. 2010). Long-lasting geographic isolation with lack of gene flow between populations might increase their levels of genetic diversity and facilitate further species divergence. At the same time, secondary contact zones, where individuals from closely related but previously isolated species met and interbreed, could enable interspecific gene flow. Recently, many studies indicate the important role of hybridization in shaping the genetic diversity of plant species, including trees and significance of this process in speciation (Arnold 2007, Gao et al. 2012, Abbott et al. 2013, Schumer et al. 2014).

Genetic markers offers insights into the evolutionary history of species and enable **the analysis of past gene flow, population migrations routes and range shifts, and assessment of the effects of admixture and introgression on the genetic structure of populations.** The technological progress and the availability of high-throughput sequencing methods for whole genomes in recent years have greatly improved the research of inter- and intraspecies genetic diversity. The access to genomic resources facilitates now comparative genetic and genomic studies of closely related species, helps searching for signatures of selection, identifying genomic regions involved in local adaptation to specific environmental niches or finding regions associated with the maintenance of species integrity. However, studies of many non-model plant species, especially conifers with large and structurally complex genomes (from several to dozens of billions base pairs), are still challenging and require the development of suitable molecular tools for DNA polymorphism analyses (Petit and Vendramin 2007, Roy et al. 2010, Whitlock et al. 2010). Such research will significantly improve the current understanding of the evolutionary processes involved in shaping the genetic diversity of populations and aid the development of effective forest tree management strategies and breeding programs, which are crucial in face of ongoing climatic changes (Possingham et al. 2001, Pautasso 2009).

Peat bog pine (*Pinus uliginosa* N.) grows in Central and Eastern Europe and its current distribution is restricted to a few isolated stands, best described in Poland (Boratyński 1994, Boratyński, Boratyńska et al. 2003). Due to the ecological specialization, small population sizes and habitat loss as a consequence of peat bog drainage, this species is at risk of extinction (Holt 1990; Weltzin et al. 2003; Peterson et al. 2005; Audrey et al. 2009). The taxonomic position of this species is not fully resolved yet, and due to the similarities of some morphological and genetic traits, it is often grouped with dwarf mountain pine (*Pinus mugo*) and mountain pine (*Pinus uncinata*) into so-called *Pinus mugo* species complex. Proper taxonomic delineation of species within this complex is an issue of great importance in the conservation context, especially for endangered and protected peat bog pine. The current understanding of evolutionary history of species within this group was limited to several studies, mainly based on the analysis of the morphology of cones and needles, isoenzymes, and small sets of molecular markers (Siedlewska 1995, Prus-Glowacki, Bujas et al. 1998, Boratyńska and Boratyński 2007, Boratynska and Lewandowska 2009, Wachowiak and Prus-Glowacki 2009, Wachowiak et al. 2011). The results of those studies indicated the intermediate character of peat bog pine compared to Scots pine and the other pines from *P. mugo* complex. However, the lack of high resolution diagnostic markers made the proper phylogenetical relationship analyses of this group especially difficult. Furthermore, previous research did not provide a clear answer to the question, whether similar level of genetic diversity within this pine complex reflects recent divergence of taxa and incomplete ancestral lineage sorting or is rather a result of interspecific gene flow (Wachowiak et al. 2011, Wachowiak et al. 2018). Additionally, despite being protected and enlisted in the Polish Plant Red Book (Kaźmierczakowa et al. 2014) comprehensive assessment of the phylogeographical relationships of remnant populations of this species and level of their genetic diversity have not been conducted thus far. Such assessments are needed to implement proper conservation practices, to evaluate the adaptability potential of this endangered species and its extinction risk due to environmental changes. Therefore, the major goal of my research during PhD studies was to better understand the evolutionary process and population history of peat bog pine, to: **1) evaluate the genetic structure and levels of genetic diversity of isolated populations of this species; 2) examine the phylogenetic relationships of taxa within the *P. mugo* complex; 3) test the alternative speciation model for peat bog pine and compere its level of genetic diversity with closely related pines from *P. mugo complex* and Scots pine (*Pinus sylvestris*).** To address my research objectives, the following hypotheses were tested:

H1: Level of genetic diversity in peat bog pine is low, as a result of geographic isolation and small population size, compared to reference taxa characterized by larger geographic ranges.

H2: Peat bog pine populations are strongly genetically differentiated due to the long-lasting isolation and separate population histories.

H3: Genetic diversity patterns clearly delineates peat bog pine from reference taxa.

H4: Peat bog pine originated as a result of homoploid hybrid speciation.

To accomplish those objectives, I used polymorphism data of inter- and intra-species variation from several molecular markers at chloroplast, mitochondrial and nuclear genomes, characterized by different mode of inheritance and dispersal mechanisms in pines. Individuals from 6 well-known populations of peat bog pine, as well as several reference populations of its close relatives including Scots pine (*P. sylvestris*), dwarf mountain pine (*Pinus mugo*) and mountain pine (*Pinus uncinata*) and the outgroup maritime pine (*Pinus pinaster*) were used. Relatively recent divergence time between those closely related species (~ 5 Mya), weak reproductive barriers, and similar genetic variation at neutral loci but at the same time phenotypical and ecological differentiation makes these pines suitable model for testing the aforementioned research hypothesis

The first part of my PhD thesis answered the question regarding the influence of geographic isolation on shaping the genetic structure of peat bog pine populations (Łabiszak et al. 2019, *AoB Plants*). The currently observed island like distribution of peat bog pine populations might reflect either their past long-lasting isolation or a relatively recent split of much wider distribution range and subsequent decrease in population size. Those alternative scenarios will differ in the effects that they imposed on the levels of genetic diversity and population structure of peat bog pine – populations will be greatly differentiated as a results of stronger genetic drift in small populations or they will share a common history and overall similar genetic background (Frankham 1996, Frankham et al. 2010).

In genetic structure studies in many wind pollinated species, mitochondrial DNA (*mt*DNA) markers that are maternally inherited and dispersed by seeds on relatively short distances, are preferred over markers dispersed by the pollen (Tóth et al. 2017). However, due to low mutation rate of mitochondrial genomes, their repeated content varying in size (up to 30 kbp) and large size comparable to bacterial genomes (~4-5 Mbp), studies of population structure of pines were previously hindered by the lack of such suitable molecular markers (Soranzo et al. 2000, Smith 2016, Toth et al. 2017). Therefore, development of novel *mt*DNA markers based on polymorphism detected in sequenced fragments of mitochondrial DNA of pines was a crucial step in the first part of my PhD thesis (Łabiszak et al. 2019, *AoB Plants*). Based on the new marker set, I was able to infer the presence of a strong and statistically important genetic structure in peat bog pine populations. Especially surprising was the detection of striking genetic differentiation among populations, expressed by the high genetic distance of populations from relatively close geographic proximity (< 80 km). Despite the small population size, the overall level of genetic diversity measured by the numbers of haplotypes and frequency of singletons was the highest in peat bog pine compared to the other studied species of *P. mugo* complex. The results also suggest long periods of isolation as likely cause of substantial differentiation of peat bog pine populations and possible interspecific gene flow from populations of other pines. Signatures of hybridization events were also indicated by the widespread sharing of some haplotypes by peat bog pine and dwarf mountain pine, followed by reported hybridization in secondary contact zones between those species (Łabiszak et al. 2019, *AoB Plants*).

In the second part of my PhD thesis, I examined the influence of demographic processes on the patterns of genetic diversity in peat bog pine (Łabiszak et al. 2020, *Journal of Systematics and Evolution*). Population genetics and conservation genetic studies focus heavily on the understanding of the mechanisms that shape the genetic diversity of small and isolated populations, where effects imposed by the genetic drift are especially severe. Drift leads to random loss of alleles regardless of their impact on fitness of individuals and further depletion of genetic variation due to the fixation or loss of some polymorphisms. Another consequence of small numbers of individuals is inbreeding – mating between close relatives, which has a dramatic influence on the fitness reduction of individuals that lowers the adaptability of whole populations (Allendorf et al. 2012, Frankham 2015). Those genetic factors, when combined with environmental changes and habitat loss are especially dangerous for endangered species, because they are more likely to fall into a feedback cycle called "extinction vortex", which greatly elevate their extinction risk (Fagan and Holmes 2006, Frankham et al. 2010). However, some specific life traits of conifer trees, including long lifespan, long generation time, and high outcrossing rate mitigate to some extent the negative consequences of genetic diversity loss imposed by fragmentation (Pautasso 2009, Lowe et al. 2015).

To verify the hypothesis of lower genetic diversity in peat bog pine as a result of geographic isolation and small population size, compared to reference taxa characterized by much wider distribution range and bigger census population sizes, I used nuclear (*n*SSR) and chloroplast microsatellite markers (*cp*SSR) (Łabiszak et al. 2020, *Journal of Systematics and Evolution*). The results of the *n*SSR data showed level of genetic variation in *P. uliginosa* comparable to other studied pines and F_{IS} coefficient indicates lack of signature of inbreeding (Wright 1922, Wright 1933). However, *cp*SSR data showed a significant depletion of genetic diversity in peat bog pine, with both the lowest number of *cp*DNA haplotypes (including species- specific haplotypes) and lowest haplotype diversity among the studied pines as well as those from other studies. Additionally, the distribution of identical alleles and haplotypes shared among individuals within the same population indicates small number of effective pollen donors within populations. This suggests mating between closely related individuals, which is in line with the observed small census population sizes and similar estimates of effective population sizes of peat bog pine (Łabiszak et al. 2020, *Journal of Systematics and*

Evolution).

Furthermore, by combining demographic modeling within Approximate Bayesian Computation (ABC) framework with species distribution modeling based on paleoclimatic data I was able to answer the question regarding possible scenarios that shaped the contemporary island-like distribution and patterns of genetic variation in peat bog pine. Best supported by the data was the model with a drastic genetic bottleneck dated at the last glaciation (~ 26,400 – 33,000 years ago). The results of species distribution modeling are also in agreement with ABC results, confirming the substantial population contraction after Last Glacial Maximum (Łabiszak et al. 2020, *Journal of Systematics and Evolution*).

The final part of my PhD thesis presents the results of phylogenetic analysis of taxa within *P. mugo* complex and testing of alternative models of speciation of peat bog pine (Łabiszak and Wachowiak, 2021, *Forests*). In recent years there is growing interest among evolutionary biologist in hybridization, especially in light of its ability to create a genetic novelty (on which selection may operate) or its role in the origin of hybrid species (Grant and Grant 2002, Baack and Rieseberg 2007, Schumer et al. 2014, Ru et al. 2018). Several theoretical models now take into account the role of gene flow and introgression in speciation (Nosil 2008, Nosil et al. 2009, Feder et al. 2012, Martin, Dasmahapatra et al. 2013). Lack of complete reproductive barriers between emerging species makes investigation of the relationship among closely related species difficult, and such species due to morphological similarities are often grouped together in taxonomically challenging species complexes. There are several examples of such complexes in *Pinus*, e.g. in aleppo pine (*P. halpensis*), Khasi pine (*P. kesiya*), lodgepole pine (*P. contorta*) and jack pine *(P. banksiana*) (Bucci et al. 1998, Yeh et al. 2007, Businský et al. 2014). Additionally, the well-documented hybrid speciation in this genus was described in the case of *P. densata* from Qinghai–Tibet Plateau (Gao et al. 2012). Earlier reports that addressed the evolutionary history within *P. mugo* complex were focused mainly on the alternative speciation hypothesis of the origin of *P. uliginosa*, considered either as a marginal population of *P. uncinata*, a hybrid between *P. mugo* and *P. uncinata* and/or *P. mugo* and *P. sylvestris* (Krzakowa 1984, Lewandowski et al. 2000, Boratyńska and Boratyński 2007, Businský and Kirschner 2010, Wachowiak et al. 2011). However, at this moment, the importance of hybridization in the context of the phylogenetic relationship between peat bog pine and its congeners is not fully resolved.

Sequence data from 48 nuclear genes (with no signature of selection) obtained from four studied pine species and maritime pine (*P. pinaster*) as an outgroup were used to reconstruct the phylogeny of the studied pines. Firstly, I conducted maximum-likelihood (ML) analysis on a concatenated sequence set composed of 48 neutrally evolving nuclear loci to reconstruct the phylogeny of the studied species on an individual level. Then, to test whether reticulation events were present in the evolutionary history of the studied species, an analysis based on individual genes was performed to reconstruct species tree. Finally, I looked at the overall pattern of genetic variation at within and between species levels (i.e. presence of polymorphisms shared between species or fixed differences between species) and the divergence to the outgroup. To address the questions regarding the speciation model that lead to the emergence of peat bog pine, in light of earlier hybrid origin hypotheses, I used the coalescent framework to test the fit of empirical data to those simulated under 16 different speciation scenarios. The results did not confirm the pure hybrid origin of *P. uliginosa* as interspecific hybrid of other taxa form *P. mugo* complex or between them and *P. sylvestris.* However, the evolutionary history within this pine group is heavily influenced by interspecific gene flow after divergence and past reticulation events. After the divergence of *P. sylvestris* and common ancestor of the taxa from the *P. mugo* complex dated about 5.9 Mya, the two subsequent divergence events within the pine complex were relatively recent. Initially, *P. uliginosa* split from the common ancestor about 4 Mya and then those two pines further diverged around 2 Mya, which led to the emergence of contemporary *P. mugo* and *P. uncinata* (Łabiszak and Wachowiak, 2021, *Forests*).

Conclusions

The outcome of my PhD thesis provided insights into the evolutionary and demographic processes that shape the genetic diversity in natural populations of studied forest tree species. Then it revealed how the interplay between geographic isolation and hybridization affects the relatively unknown evolutionary history of *Pinus* species and helped in the qualitative and quantitative assessment of genetic diversity levels of peat bog pine. Finally it provided the information about genetic structure and demography of the populations crucial for the proper conservation management strategies for this species. The most likely explanation of the patterns of genetic variation in peat bog pine is long-lasting isolation, possibly predating the Last Glacial Maximum with multiple fluctuations in population size associated with the transient character of peat bog habitats, and the dependence of such habitats on glacial cycles during Pleistocene **(Łabiszak et al. 2019, *Aob Plants,* Łabiszak et al. 2020, *Journal of Systematics and Evolution*)**. Genetic diversity of peatbog pine was influenced by genetic drift and inbreeding, along with gene flow from related pines, which is reflected in difference in estimates based on markers of different origin. The observed and projected habitat loss, decreased seed production and poor natural regeneration demands serious conservation efforts to ensure the species further existence **(Łabiszak et al. 2020, *Journal of Systematics and Evolution*)**. Peat bog pine originated by divergence within the *P. mugo* complex, but the evolutionary history of this complex is heavily influenced by the interspecific gene flow (also with *P. sylvestris*) and phylogenetic relationships are mainly reticulate **(Łabiszak and Wachowiak, 2021, *Forests*)**. Due to their relatively recent divergence and similar genetic variation at neutral loci, these pines are particularly hard to delineate using neutral markers alone. Nevertheless, considering their phenotypical and ecological differentiation, those pines could be a useful model to search for genetic basis of adaptive variation and genomic regions involved in maintenance of the species integrity.

IV. Wprowadzenie

Gatunki drzew leśnych stanowią podstawę wielu ekosystemów lądowych, pełniąc istotną rolę w procesach związanych z obiegiem wody i węgla, wychwytywaniem dwutlenku węgla z atmosfery, ochroną gleby przed erozją, utrzymaniem bioróżnorodności, a także dostarczaniem surowców naturalnych o ogromnym znaczeniu ekonomicznym (FAO 2018). Drzewa charakteryzują się wysoką wewnątrzpopulacyjną zmiennością genetyczną przy jednoczesnej niewielkiej zmienności międzypopulacyjnej, nawet w przypadku odległych geograficznie populacji (Petit i Hampe 2006). Zmienność genetyczna drzew kształtowana jest w dużym stopniu przez unikalne cechy ich biologii takie jak: długowieczność i znaczna produkcja nasion, efektywny system dyspersji pyłku i/lub nasion, niski poziom rekrutacji siewek i wynikający z niej niewielki odsetek potomstwa osiągający dojrzałość, znaczna plastyczność fenotypowa i potencjał adaptacyjny, zapewniający przeżywalność w zmiennych warunkach środowiskowych w trakcie ich długiego życia (Petit i Hampe 2006).

Czynnikiem o wzrastającym w ostatnich latach natężeniu, wpływającym na poziom zmienności genetycznej obserwowany u gatunków drzew leśnych, jest presja antropogeniczna. Wpływ ten w wielu przypadkach jest jednokierunkowy i skutkuje redukcją zmienności genetycznej populacji i obniżeniem ich zdolności adaptacyjnych. Do najpoważniejszych zagrożeń związanych z działalnością człowieka należą: niszczenie siedlisk i ich fragmentacja, zanieczyszczania środowiska, niezrównoważone użytkowanie zasobami leśnymi oraz postępujące zmiany klimatyczne (van Mantgem i in., 2009; Shearman i in., 2012; Fettig i in., 2013; Riitters i in., 2016). W niektórych przypadkach, działalność człowieka bywa ukierunkowana na zwiększenie poziomu zmienności genetycznej, poprzez tworzenie stref kontaktu populacji o różnym pochodzeniu lub tworzenie sztucznych stref hybrydyzacyjnych pomiędzy spokrewnionymi i wcześniej izolowanymi gatunkami (Schaberg i in. 2008, Grabenstein i Taylor 2018). Zjawisko to niesie za sobą jednak ryzyko związane z rozbiciem korzystnych układów genowych zapewniających optima adaptacyjne (depresja outbredowa), a także ryzyko erozji genetycznej, szczególnie istotne w przypadku ochrony zasobów genetycznych naturalnie powstałych mieszańców (Allendorf i in. 2010, Allendorf i in. 2012, Stronen i Paquet 2013).

Na kształtowanie zmienności genetycznej drzew mają również wpływ procesy demograficzne, związane z historycznymi zmianami wielkości i rozmieszczenia populacji, poziomem przepływu genów w obrębie i pomiędzy populacjami danego gatunku, jak i procesy ewolucyjne, odpowiadające za powstawanie i utrzymywanie zmienności neutralnego tła genetycznego oraz rejonów genomowych podlegających procesom selekcji. (Hewitt 2004, Neale i Ingvarsson 2008, Sork 2015, Ellegren i Galtier 2016). Zmiany zasięgów gatunków związane z cyklami glacjalnymi Plejstocenu, odcisnęły wyraźne piętno na współczesnym rozmieszczeniu geograficznym i wzorach zmienności genetycznej populacji na półkuli północnej i są dobrze udokumentowane u wielu ważnych ekologicznie gatunków drzew leśnych (Hewitt 1999, Hewitt 2004, Stewart, Lister i in. 2010). Izolacja geograficzna i brak przepływu genów pomiędzy populacjami mogła prowadzić do ich zróżnicowania i postępującej dywergencji. Jednocześnie w strefach kontaktu uprzednio izolowanych lecz blisko spokrewnionych taksonów mogło dochodzić do międzygatunkowego przepływu genów. W ostatnich latach przybywa dowodów na istotną rolę hybrydyzacji w procesie kształtowania zmienności genetycznej wielu gatunków roślin, w tym drzew leśnych, a także roli tego zjawiska w procesie specjacji (Arnold 2007, Gao i in. 2012, Abbott i in. 2013, Schumer i in. 2014).

Zastosowanie markerów genetycznych umożliwia wnioskowanie o przeszłości ewolucyjnej danego gatunku, pozwala na **analizę przepływu genów i wzorców rozmieszczenia zmienności powstałych w efekcie migracji i zmian zasięgów populacji, a także wpływu admiksji i introgresji na strukturę genetyczną** wcześniej izolowanych populacji. Dzięki rozwojowi technologicznemu w tym nowoczesnych technik sekwencjonowania DNA, ocena zmienności genetycznej na poziomie między- i wewnątrzgatunkowym stała się znacznie łatwiejsza. Wzrastające zasoby genomowe pozwalają również na badania z zakresu genetyki i genomiki porównawczej, w których wykorzystuje się systemy badawcze obejmujące blisko spokrewnione gatunki, dzięki którym możliwe jest poszukiwanie sygnatur doboru naturalnego, identyfikacja miejsc podlegających selekcji w wyniku lokalnej adaptacji do określonych nisz środowiskowych czy wreszcie regionów związanych z zachowaniem integralności gatunkowej. Nadal jednak w przypadku wielu nie-modelowych roślin, zwłaszcza drzew szpikowych, odznaczających się genomami o znacznej złożoności strukturalnej i dużych rozmiarach (w zakresie od kilkunastu do kilkudziesięciu miliardów par zasad), ich badanie stanowi wyzwanie i stwarza pilną potrzebę rozwoju nowych narzędzi molekularnych do analiz polimorfizmu DNA (Petit i Vendramin 2007, Roy i in. 2010, Whitlock i in. 2010). Badania te są niezbędne do poszerzenia wiedzy na temat procesów ewolucyjnych kształtujących zmienność genetyczną populacji, a także do rozwoju efektywnych programów związanych z zarzadzaniem gospodarką leśną, hodowlą drzew oraz ochroną istniejących zasobów genetycznych, co jest szczególnie istotne w obliczu postępujących zmian klimatycznych (Possingham i in. 2001, Pautasso 2009).

Sosna błotna (*Pinus uliginosa* N.) jest gatunkiem o ograniczonym zasięgu w Europie Środkowo-Wschodniej, przy czym najlepiej opisane stanowiska tego gatunku zlokalizowane są w Polsce (Boratyński 1994, Boratyński, Boratyńska i in. 2003). Specjalizacja ekologiczna, niewielka liczebność populacji połączona z utratą siedlisk, związanych z osuszaniem torfowisk wysokich stanowi realne zagrożenie jej wyginięcia (Holt 1990; Weltzin i in. 2003; Peterson i in. 2005; Audrey i in. 2009). Pozycja taksonomiczna tej sosny pozostaje nadal w pełni nierozstrzygnięta. Z uwagi na znaczne podobieństwo cech morfologicznych do blisko spokrewnionych z nią gatunków: kosodrzewiny (*P. mugo* T.) i sosny hakowatej (*Pinus uncinata* R.) jest ona włączana najczęściej w obręb tzw. kompleksu gatunków *Pinus mugo*. Określenie wzajemnych relacji taksonomicznych w obrębie tego kompleksu ma istotne znaczenie w kontekście ochrony ich zasobów genetycznych, zwłaszcza w przypadku zagrożonej wyginięciem i objętej ochrona gatunkową sosny błotnej. Dotychczas historia ewolucyjna taksonów w obrębię tej grupy była słabo poznana, a same badania oparte były w dużej mierze na analizie cech morfologicznych igieł i szyszek, izoenzymów oraz niewielkich zestawów markerów molekularnych (Siedlewska 1995, Prus-Glowacki i in. 1998, Boratyńska i Boratyński 2007, Boratynska i Lewandowska 2009, Wachowiak and Prus-Glowacki 2009, Wachowiak i in. 2011). Wyniki tych prac wskazywały na pośredni charakter sosny błotnej względem sosny zwyczajnej i taksonów kompleksu *P. mugo*. Jednak brak markerów diagnostycznych o odpowiedniej rozdzielczości utrudniał precyzyjne ustalenie relacji filogenetycznych w ramach tej grupy. Tym samym dotychczasowe badania nie udzieliły jednoznacznej odpowiedzi na pytania, czy podobny poziom zmienności genetycznej taksonów wewnątrz tego kompleksu jest skutkiem niedawnej dywergencji i segregacji w populacjach ancestralnego polimorfizmu (wspólnego przodka), czy też wynikiem międzygatunkowego przepływu genów (Wachowiak i in. 2011, Wachowiak i in. 2018). Dodatkowo, pomimo umieszczenia sosny błotnej na liście Polskiej Czerwonej Księgi Roślin (Kaźmierczakowa i in. 2014) i ochrony prawnej, nie prowadzono do tej pory kompleksowej oceny wzajemnych relacji fitogeograficznych populacji oraz zmienności genetycznej tego gatunku, co jest istotne w kontekście oceny jej kondycji genetycznej, potencjału adaptacyjnego i ryzyka wyginięcia na skutek zmian środowiskowych. Stąd celem nadrzędnym prac badawczych w ramach mojego doktoratu było lepszego poznanie procesów ewolucyjnych i historii populacji sosny błotnej, w tym: **1) określenie struktury genetycznej i poziomu zmienności izolowanych populacji tego gatunku; 2) ustalenie relacji filogenetycznych w obrębie taksonów kompleksu *P. mugo;* 3) przetestowanie alternatywnych modeli specjacji sosny błotnej w odniesieniu do kompleksu *P. mugo* i**

blisko spokrewnionej sosny zwyczajnej (*Pinus sylvestris*). W ramach realizacji założonych celów badawczych weryfikacji poddano następujące hipotezy:

H1: Niski poziom zmienności genetycznej u sosny błotnej wywołany jest izolacją geograficzną i niewielką liczebnością populacji w odróżnieniu od taksonów referencyjnych charakteryzujących się większym zasięgiem geograficznym.

H2: Populacje sosny błotnej wykazują istotne zróżnicowanie genetyczne, świadczące o długotrwałej izolacji i odmiennej historii populacji.

H3: Zmienność genetyczna *P. uliginosa* potwierdza jej odrębność taksonomiczną względem taksonów referencyjnych.

H4: Sosna błotna jest przykładem taksonu powstałym na drodze specjacji przez hybrydyzację.

Do realizacji postawionych celów badawczych wykorzystałem dane dotyczące zmienności wewnątrz- i międzygatunkowej pozyskane przy użyciu szeregu markerów molekularnych pochodzących z genomów chloroplastowych, mitochondrialnych i jądrowych, charakteryzujących się odmiennymi mechanizmami dziedziczeni i dyspersji u sosen. W badaniach wykorzystałem osobniki pochodzące z 6 najlepiej opisanych w literaturze populacji sosny błotnej oraz szeregu populacji referencyjnych blisko spokrewnionych gatunków, w tym sosny zwyczajnej, kosodrzewiny, sosny hakowatej oraz sosny nadmorskiej (*Pinus pinaster*), użytej jako grupa zewnętrzna. Stosunkowo niedawny czas dywergencji pomiędzy sosnami z kompleksu *P. mugo* a sosną zwyczajną, datowany na 5 milionów lat temu, słabo wykształcone bariery rozrodcze i generalnie podobny poziom zmienność neutralnej przy jednoczesnych różnicach fenotypowych i ekologicznych, czyni z tych blisko spokrewnionych gatunków sosen dogodny model do testowania postawionych hipotez badawczych.

Pierwsza część mojej rozprawy doktorskiej odpowiada na pytanie jaki jest wpływ izolacji geograficznej na kształtowanie struktury populacji sosny błotnej (Łabiszak i in. 2019, *AoB Plants*). Obserwowane współcześnie wyspowe rozmieszczenie populacji tego gatunku może odzwierciedlać ich długotrwałą izolację w przeszłości lub też wynikać ze stosunkowo niedawnego zmniejszenia się pierwotnie dużo rozleglejszego jej zasięgu i spadku liczebności populacji. Te alternatywne scenariusze mają różny wpływ na strukturę genetyczną i poziom zmienności populacji sosny błotnej – populacje będą wykazywać odpowiednio znaczne zróżnicowanie genetyczne wynikające z silniejszego działania dryfu genetycznego w małych populacjach, lub też cechować się będą jednorodnym tłem genetycznym i wspólną historią (Frankham 1996, Frankham i in. 2010).

U gatunków wiatropylnych, markerami preferowanymi w badaniach struktury genetycznej populacji są markery mitochondrialnego DNA, dziedziczonego w linii matecznej i ulegającego dyspersji przez nasiona na relatywnie niewielkich odległościach względem markerów przenoszonych przez pyłek (Tóth i in. 2017). Jednak z uwagi na relatywnie niskie tempo mutacji punktowej genomu mitochondrialnego, obecność regionów powtarzalnego DNA (o wielkości dochodzącej do 30 kpz) oraz rozmiary porównywalne z genomami bakteryjnymi (4~5 Mpz), badania struktury genetycznej sosen były dotychczas znacznie ograniczone z powodu braku odpowiednich markerów (Soranzo, Alia i in. 2000, Smith 2016, Toth i in. 2017). Tym samym, kluczowym etapem pierwszej części moje pracy doktorskiej było opracowanie nowych markerów mitochondrialnego DNA na bazie polimorfizmów wykrytych w uprzednio zsekwencjonowanych fragmentach genomu mitochondrialnego sosen (Łabiszak i in. 2019, *AoB Plants*). Używając nowoopracowanego zestawu markerów wykryłem istnienie silnej i istotnej statystycznie struktury genetycznej w populacjach sosny błotnej. Intrygujące było zwłaszcza znaczne zróżnicowanie genetyczne, wyrażone dużym dystansem genetycznym populacji występujących w relatywnie bliskim sąsiedztwie (< 80 km). Pomimo niewielkiej liczebności populacji, poziom zmienności mitochondrialnego DNA, wyrażony min. liczbą haplotypów, w tym znaczną nadreprezentacją rzadkich haplotypów był najwyższy w populacjach sosny błotnej względem badanych gatunków z kompleksu *P. mugo*. Uzyskane dane wskazują na możliwą długotrwałą izolację jako jedną z przyczyn znacznego zróżnicowania jej populacji oraz prawdopodobny przepływ genów z populacji innych gatunków sosen. Na ślad hybrydyzacji wskazuje współwystępowanie pewnych haplotypów zarówno u sosny błotnej, jak i kosodrzewiny, a we współczesnych strefach kontaktu tych gatunków obserwowane są przypadki powstawania mieszańców międzygatunkowych (Łabiszak i in. 2019, *AoB Plants*).

W drugiej części pracy doktorskiej badałem wpływ czynników demograficznych na poziom zmienności genetycznej sosny błotnej (Łabiszak i in. 2020, *Journal of Systematics and* Evolution). Badania z zakresu genetyki populacyjnej i genetyki konserwatorskiej skupiają dużą uwagę na procesach kształtujących poziom zmienności genetycznej małych i izolowanych populacji, poddanych silnej presji dryfu genetycznego. Prowadzi on do losowej utraty alleli, niezależnie od ich wpływ na dostosowanie osobników i szybszego spadku zmienności genetycznej na skutek utrwalania lub utraty określonych polimorfizmów. Jedną z konsekwencji niskiej liczebności populacji jest chów wsobny – polegający na krzyżowaniu się osobników blisko ze sobą spokrewnionych, co prowadzi do utraty dostosowania osobników i obniżenia potencjału adaptacyjnego populacji (Allendorf, Luikart i in. 2012, Frankham 2015). Wspomniane czynniki genetyczne, w połączeniu ze zmianami środowiskowymi i utratą siedlisk są szczególnie niebezpieczne dla zagrożonych gatunków, gdyż mogą prowadzić do cyklu sprzężeń zwrotnych („wiru wymierania”) znacznie zwiększających prawdopodobieństwo wyginięcia gatunku (Fagan i Holmes 2006, Frankham i in. 2010). Niemniej jednak, z uwagi na wyjątkowe cechy biologii drzew iglastych związane z długowiecznością, znacznym czasem trwania pokoleń połączonym z efektywnym systemem zapłodnienia krzyżowego, organizmy te są w pewnej mierze chronione przed negatywnymi konsekwencjami utraty zmienności genetycznej w wyniku fragmentacji siedlisk (Pautasso 2009, Lowe i in. 2015).

W celu zweryfikowania hipotezy o niskim poziom zmienności genetycznej u sosny błotnej wywołanej izolacją geograficzną i niewielką liczebnością populacji, w odróżnieniu od taksonów referencyjnych o znacznie bardziej rozległych współcześnie zasięgach, wykorzystałem markery mikrosatelitarne pochodzenia jądrowego (*n*SSR) i chloroplastowego (*cp*SSR) **(Łabiszak i in. 2020, *Journal of Systematics and* Evolution)**. Wyniki analizy danych *n*SSR wykazały porównywalny poziom zmienności genetycznej *P. uliginosa* względem pozostałych gatunków sosen oraz brak śladu chowu wsobnego mierzonego współczynnikiem F_{IS} (Wright 1922, Wright 1933). Niemniej, dane *cp*SSR wskazały na istotną redukcję zmienności genetycznej sosny błotnej, wyrażoną zarówno najniższą liczbą haplotypów *cp*DNA (w tym haplotypów specyficznych gatunkowo), jak i najniższym poziomem ich zróżnicowania w porównaniu do gatunków referencyjnych, a także danych uzyskanych dla innych gatunków sosen. Dodatkowo, rozmieszczenie identycznych haplotypów i alleli *cp*SSR u osobników w poszczególnych populacjach wskazywało na niewielką liczbę efektywnych donorów pyłku, świadczącą o wpływie krzyżowań między blisko spokrewnionymi osobnikami, co jest spójne z obserwacjami o niewielkiej cenzusowej, jak również oszacowanej efektywnej wielkości populacji sosny błotnej (Łabiszak i in. 2020, *Journal of Systematics and Evolution*).

W dalszej części pracy, przy użyciu metod modelowania procesów demograficznych z wykorzystaniem podejścia opartego na statystyce Bayesowkiej (ang. *Approximate Bayesian Computation*), a także przy użyciu danych paleoklimatycznych i modelowaniu zmiany nisz środowiskowych, odpowiedziałem na pytanie o prawdopodobne scenariusze historyczne, które doprowadziły do współczesnego wyspowego rozmieszczenia sony błotnej i miały wpływ na jej zmienność genetyczną. Model zakładający drastyczny efekt genetycznego wąskiego gardła (ang. *genetic bottleneck*), datowany na okres ostatniego zlodowacenia (26,400 – 33,000 lat temu), okazał się najbardziej prawdopodobnym wyjaśnieniem obserwowanej współcześnie zmienności genetycznej tego gatunku. Wniosek ten poparty został wynikami modelowania nisz środowiskowych, z których wynika znaczne zmniejszenie zasięgu sosny błotnej w okresie następującym po ostatnim maksimum glacjalnym (ang. *Last Glacial Maxiumum*) (Łabiszak i in. 2020, *Journal of Systematics and Evolution*).

Ostatnia część mojej pracy doktorskiej prezentuje wyniki analiz filogenetycznych taksonów kompleksu *P. mugo* oraz testowania alternatywnych modeli specjacji sosny błotnej (Łabiszak i Wachowiak, 2021, *Forests*). W ostatnich latach wzrasta zainteresowanie biologów ewolucyjnych zjawiskiem hybrydyzacji, zwłaszcza roli tego zjawiska w generowanie zmienności genetycznej podlegającej selekcji i powstawaniu nowych gatunków hybrydowych (Grant i Grant 2002, Baack i Rieseberg 2007, Schumer i in. 2014, Ru i in. 2018). Liczne modele teoretyczne uwzględniają obecnie rolę przepływu genów i introgresji w procesie specjacji (Nosil 2008, Nosil i in. 2009, Feder i in. 2012, Martin i in. 2013). Częsty brak kompletnych barier rozrodczych pomiędzy powstającymi gatunkami sprawia, że ustalenie ich historii ewolucyjnej bywa problematyczne, a z uwagi na liczne podobieństwa morfologiczne – gatunki te włączane są w obręb tzw. kompleksów gatunków. W rodzaju *Pinus* występują liczne przykłady takich kompleksów, m.in. u sosny alepskiej (*P. halpensis*), sosny wiotkiej (*P. kesiya*), sosny wydmowej (*P. contorta)* i sosny banksa *(P. banksiana*) (Bucci, Anzidei i in. 1998, Yeh i in. 2007, Businský i in. 2014). Dodatkowo, dobrze udokumentowaną specjację przez hybrydyzację wykryto w tym rodzaju dla *P. densata* z płaskowyżu Tybetańskiego (Gao i in. 2012). Dotychczasowe badania historii ewolucyjnej w obrębie kompleksu *P. mugo* skupiały się przed wszystkim na ustaleniu historii specjacji sosny błotnej, a w literaturze pojawiały się hipotezy upatrujące jej powstanie jako marginalnej populacji sosny hakowatej, bądź gatunku hybrydowego powstałego na skutek krzyżowania się sosny hakowatej i kosodrzewiny lub kosodrzewiny i sosny zwyczajnej (Krzakowa 1984, Lewandowski i in. 2000, Boratyńska i Boratyński 2007, Businský i Kirschner 2010, Wachowiak, Palme i in. 2011). Do tej pory jednak znaczenie procesu hybrydyzacji w aspekcie relacji filogenetycznych sosny błotnej względem jej blisko spokrewnionych taksonów pozostawało niewyjaśnione.

Dane sekwencyjne dla 48 genów jądrowych (niewykazujących śladów działania doboru), pozyskane dla 4 badanych gatunków sosen oraz sosny nadmorskiej (*P. pinaster*) użytej jako grupa zewnętrzna, zostały wykorzystane do odtworzenia ich filogenezy. W tym celu przeprowadzono analizę filogenetyczną metodą największej wiarygodności (ang. *Maximum Likelihood*) zarówno na poziomie osobniczym, z wykorzystaniem połączonych sekwencji wszystkich genów, jak również z odtworzeniem drzewa gatunkowego, na podstawie analizy koalescencyjnej poszczególnych genów, w której dopuszczone zostały wydarzenie o niedychotomicznym charakterze w przebiegu dywergencji. Przenalizowane zostały także dane dotyczące wzorców zmienności i różnicowania międzygatunkowego (min. obecność utrwalonych i dzielonych przez gatunki polimorfizmów), w tym także poziomu dywergencji względem grupy zewnętrznej. By odpowiedzieć na pytanie, jaki model specjacji doprowadził do powstanie sosny błotnej, zwłaszcza w kontekście wcześniejszych hipotez o jej hybrydowym pochodzeniu wykorzystano podejście koalescencyjne, testując zgodność danych empirycznych z danymi pochodzącymi z symulacji komputerowych, wygenerowanych przy założeniach demograficznych w ramach każdego z 16 testowanych modeli specjacji. Uzyskane dane wskazują na brak czysto hybrydowego pochodzenia *P. uliginosa*, jako utrwalonego mieszańca międzygatunkowego pozostałych taksonów kompleksu *P. mugo* bądź przedstawicieli tego kompleksu i *P. sylvestris*. Historia ewolucyjna w tej grupie sosen ma charakter retikularny, z asymetrycznym przepływem genów pomiędzy taksonami już po ich dywergencji. Po wyodrębnieniu się wspólnego przodka sosen z kompleksu *P. mugo* około 5.9 milionów lat temu, dalsze zróżnicowanie w ramach tej grupy przebiegało relatywnie szybko, z wydzieleniem się sosny błotnej ok. 4 miliony lat temu i ostatecznie podziałem na współczesne linie *P. mugo* i *P. uncinata* ok. 2 miliony lat temu (Łabiszak i Wachowiak, 2021, *Forests*).

Podsumowanie

Badania w ramach mojej pracy doktorskiej pozwoliły na rozpoznanie procesów ewolucyjnych i demograficznych kształtujących zmienność w naturalnych populacjach badanych drzew leśnych, określenie wpływu izolacji geograficznej i hybrydyzacji na dotychczas słabo poznaną ewolucję rodzaju *Pinus*, jakościową i ilościową ocenę zmienności genetycznej sosny błotnej a także dostarczyły danych o strukturze i demografii, niezbędnych do opracowanie strategii ochrony naturalnych pól genowych populacji tego gatunku. Za najbardziej prawdopodobną hipotezę wyjaśniającą zróżnicowanie populacji sosny błotnej należy uznać długotrwałą, prawdopodobnie poprzedzającą ostatnie zlodowacenie izolację i wielokrotne zmiany wielkości populacji, wynikające z przejściowego charakteru torfowisk i związku tych siedlisk z cyklem glacjalnym epoki Plejstocenu **(Łabiszak i in. 2019, *AoB***

***Plants*, Łabiszak i in. 2020, *Journal of Systematics and Evolution*)**. Jej zmienność genetyczna była kształtowana z jednej strony przez dryf genetyczny i chów wsobny, z drugiej zaś przez przepływ genów z blisko spokrewnionych taksonów, co odzwierciedlają oszacowania oparte na markerach o różnym pochodzeniu. Obserwowana i prognozowana utrata siedlisk, obniżona produkcja nasion i w konsekwencji słaba odnowa naturalna populacji tego gatunku wymaga zdecydowanych działań z zakresu ochrony jego zasobów genetycznych **(Łabiszak i in. 2020, *Journal of Systematics and Evolution*)**. Gatunek ten powstał przez dywergencje w obrębie kompleksu *P. mugo*. Historia ewolucyjna tej grupy jest w znacznej mierze kształtowana przez międzygatunkowy przepływ genów (również z sosną zwyczajną) a relacje filogenetyczne pomiędzy taksonami mają charakter retikularny **(Łabiszak i Wachowiak, 2021, *Forests*)**. Z uwagi na stosunkowo niedawną dywergencję i podobne tło genetyczne, taksony z kompleksu *P. mugo* są współcześnie trudne do jednoznacznej identyfikacji za pomocą markerów neutralnych. Jednocześnie, biorąc pod uwagę ich odrębność ekologiczną, a także różnice fenotypowe, sosny te stanowią dogodny obiekt do badań genetycznych podstaw ich zmienności adaptacyjnej i poszukiwania rejonów genomowych związanych z utrzymywaniem integralności gatunkowej.

References

Spis literatury

Abbott, R., D. Albach, S. Ansell, J. W. Arntzen, S. J. E. Baird, N. Bierne, J. Boughman, A. Brelsford et. al (2013). "Hybridization and speciation." Journal of Evolutionary Biology **26**(2): 229-246.

Allendorf, F. W., P. A. Hohenlohe and G. Luikart (2010). "Genomics and the future of conservation genetics." Nature Reviews Genetics **11**(10): 697-709.

Allendorf, F. W., G. H. Luikart and S. N. Aitken (2012). Conservation and the Genetics of Populations, Wiley.

Arnold, M. L. (2007). "Evolution Through Genetic Exchange." Evolution through Genetic Exchange: 1-252.

Audrey, C., L. L. Hsiang and P. Andreas (2009). "Are specialists at risk under environmental change? Neoecological, paleoecological and phylogenetic approaches." Ecology Letters **12**(8): 849-863.

Baack, E. J. and L. H. Rieseberg (2007). "A genomic view of introgression and hybrid speciation." Current Opinion in Genetics & Development **17**(6): 513-518.

Boratynska, K. and D. Lewandowska (2009). "Differences among three populations of Pinus uliginosa and their relation to P. sylvestris as expressed by the needle characters." Dendrobiology **61**: 37-46.

Boratyńska, K. and A. Boratyński (2007). "Taxonomic differences among closely related pines *Pinus sylvestris*, *P. mugo*, *P. uncinata*, *P. rotundata* and *P. uliginosa* as revealed in needle sclerenchyma cells." Flora **202**(7): 555-569.

Boratyński, A. (1994). "Protected and rare trees and shrubs from the Polish part of Sudety Mts. and its foothills. 7. Pinus mugo Turra and P. uliginosa Neumann." Arboretum Kórnickie **39**: 63-85.

Boratyński, A., K. Boratyńska, A. Lewandowski, Z. Gołąb and P. Kiciński (2003). "Evidence of the possibility of natural reciprocal crosses between Pinus sylvestris and P. uliginosa based on the phenology of reproductive organs." Flora - Morphology, Distribution, Functional Ecology of Plants **198**(5): 377-388.

Bucci, G., M. Anzidei, A. Madaghiele and G. G. Vendramin (1998). "Detection of haplotypic variation and natural hybridization in halepensis-complex pine species using chloroplast simple sequence repeat (SSR) markers." Molecular Ecology **7**(12): 1633-1643.

Businský, R., T. Frantík and P. Vít (2014). "Morphological evaluation of the Pinus kesiya complex (Pinaceae)." Plant Systematics and Evolution **300**(2): 273-285.

Businský, R. and J. Kirschner (2010). "Pinus mugo and P. Uncinata as Parents of Hybrids A Taxonomic and Nomenclatural Survey." Phyton; annales rei botanicae **50**: 27–57.

Ellegren, H. and N. Galtier (2016). "Determinants of genetic diversity." Nature Reviews Genetics **17**(7): 422-433.

Fagan, W. F. and E. E. Holmes (2006). "Quantifying the extinction vortex." Ecology Letters **9**(1): 51-60.

FAO (2018) "The State of the World's Forests 2018 - Forest pathways to sustainable development.".

Feder, J. L., S. P. Egan and P. Nosil (2012). "The genomics of speciation-with-gene-flow." Trends in Genetics **28**(7): 342-350.

Fettig, C. J., M. L. Reid, B. J. Bentz, S. Sevanto, D. L. Spittlehouse and T. L. Wang (2013). "Changing Climates, Changing Forests: A Western North American Perspective." Journal of Forestry **111**(3): 214-228.

Frankham, R. (1996). "Relationship of Genetic Variation to Population Size in Wildlife." Conservation Biology **10**(6): 1500-1508.

Frankham, R. (2015). "Genetic rescue of small inbred populations: meta-analysis reveals large and consistent benefits of gene flow." Molecular Ecology **24**(11): 2610-2618.

Frankham, R., J. D. Ballou and D. A. Briscoe (2010). Introduction to conservation genetics. Cambridge, UK ; New York, Cambridge University Press.

Gao, J., B. S. Wang, I. F. Mao, P. Ingvarsson, Q. Y. Zeng and X. R. Wang (2012). "Demography and speciation history of the homoploid hybrid pine Pinus densata on the Tibetan Plateau." Molecular Ecology **21**(19): 4811-4827.

Grabenstein, K. C. and S. A. Taylor (2018). "Breaking Barriers: Causes, Consequences, and Experimental Utility of Human-Mediated Hybridization." Trends in Ecology & Evolution **33**(3): 198-212.

Grant, P. R. and B. R. Grant (2002). "Unpredictable Evolution in a 30-Year Study of Darwin's Finches." Science **296**(5568): 707-711.

Hewitt, G. M. (1999). "Post-glacial re-colonization of European biota." Biological Journal of the Linnean Society **68**(1-2): 87-112.

Hewitt, G. M. (2004). "Genetic consequences of climatic oscillations in the Quaternary." Philos Trans R Soc Lond B Biol Sci **359**(1442): 183-195; discussion 195.

Holt, R. D. (1990). "The microevolutionary consequences of climate change." Trends Ecol Evol **5**(9): 311-315.

Krzakowa, M., Naganowska, B., Bobowicz, M.A. (1984). "Investigations on taxonomic status of Pinus uliginosa Neumann." Bulletin de la Société des Amis des Sciences et des Lettres de Poznań, serié D **24**: 87-96.

Lewandowski, A., A. Boratynski and L. Mejnartowicz (2000). "Allozyme investigations on the genetic differentiation between closely related pines - Pinus sylvestris, *P. mugo*, *P. uncinata*, and *P. uliginosa* (Pinaceae)." Plant Systematics and Evolution **221**(1-2): 15-24.

Kaźmierczakowa, R., Zarzycki, K., Mirek, Z. (2014). „Polska Czerwona Księga Roślin : paprotniki i rośliny kwiatowe" [Polish Red Data Book of Plants: Pteridophytes and Flowering Plants]. Instytut Ochrony Przyrody Polskiej Akademii Nauk.

Lowe, A. J., S. Cavers, D. Boshier, M. F. Breed and P. M. Hollingsworth (2015). "The resilience of forest fragmentation genetics--no longer a paradox--we were just looking in the wrong place." Heredity **115**(2): 97-99.

Łabiszak, B., J. Zaborowska and W. Wachowiak (2019). "Patterns of mtDNA variation reveal complex evolutionary history of relict and endangered peat bog pine (Pinus uliginosa)." AoB PLANTS **11**(2).

Łabiszak, B., J. Zaborowska, B. Wójkiewicz and W. Wachowiak (2020) "Molecular and paleo-climatic data uncover the impact of an ancient bottleneck on the demographic history and contemporary genetic structure of endangered Pinus uliginosa." Journal of Systematics and Evolution .

Martin, S. H., K. K. Dasmahapatra, N. J. Nadeau, C. Salazar, J. R. Walters, F. Simpson, M. Blaxter, A. Manica, J. Mallet and C. D. Jiggins (2013). "Genome-wide evidence for speciation with gene flow in Heliconius butterflies." Genome research **23**(11): 1817-1828.

Neale, D. B. and P. K. Ingvarsson (2008). "Population, quantitative and comparative genomics of adaptation in forest trees." Curr Opin Plant Biol **11**(2): 149-155.

Nosil, P. (2008). "Speciation with gene flow could be common." Molecular Ecology **17**(9): 2103-2106.

Nosil, P., L. J. Harmon and O. Seehausen (2009). "Ecological explanations for (incomplete) speciation." Trends Ecol Evol **24**(3): 145-156.

Pautasso, M. (2009). "Geographical genetics and the conservation of forest trees." Perspectives in Plant Ecology, Evolution and Systematics **11**(3): 157-189.

Peterson, A. T., Tian, H., Martínez-Meyer, E., Soberón, J., Sánchez-Cordero, V., Huntley, B (2005). Modeling distributional shifts of individual species and biomes. Climate Change and Biodiversity. Michigan, USA, Yale University Press**:** 211-228.

Petit, R. J. and A. Hampe (2006). "Some Evolutionary Consequences of Being a Tree." Annual Review of Ecology, Evolution, and Systematics **37**(1): 187-214.

Petit, R. J. and G. G. Vendramin (2007). "Plant phylogeography based on organelle genes: an introduction." Phylogeography of Southern European Refugia: 23-97.

Possingham, H., S. Andelman, B. Noon, S. Tromblak and H. Pulliam (2001). "Making smart conservation decisions. In Conservation Biology: Research Priorities for the Next Decade (Soule, M. E., and G. H. Orians)." Island Press, Washington, DC: 225-244.

Prus-Glowacki, W., E. Bujas and H. Ratynska (1998). "Taxonomic position of Pinus uliginosa Neumann as related to other taxa of Pinus mugo complex." Acta Societatis Botanicorum Poloniae **67**(3-4): 269-274.

Riitters, K., J. Wickham, J. K. Costanza and P. Vogt (2016). "A global evaluation of forest interior area dynamics using tree cover data from 2000 to 2012." Landscape Ecology **31**(1): 137-148.

Roy, S., A. Tyagi, V. Shukla, A. Kumar, U. M. Singh, L. B. Chaudhary, B. Datt, S. K. Bag, P. K. Singh, N. K. Nair, T. Husain and R. Tuli (2010). "Universal Plant DNA Barcode Loci May Not Work in Complex Groups: A Case Study with Indian Berberis Species." PLOS ONE **5**(10): e13674.

Ru, D., Y. Sun, D. Wang, Y. Chen, T. Wang, Q. Hu, R. J. Abbott and J. Liu (2018). "Population genomic analysis reveals that homoploid hybrid speciation can be a lengthy process." Molecular Ecology **27**(23): 4875-4887.

Schaberg, P. G., D. H. DeHayes, G. J. Hawley and S. E. Nijensohn (2008). "Anthropogenic alterations of genetic diversity within tree populations: Implications for forest ecosystem resilience." Forest Ecology and Management **256**(5): 855-862.

Schumer, M., G. G. Rosenthal and P. Andolfatto (2014). "How common is homoploid hybrid speciation?" Evolution **68**(6): 1553-1560.

Shearman, P., J. Bryan and W. F. Laurance (2012). "Are we approaching 'peak timber' in the tropics?" Biological Conservation **151**(1): 17-21.

Siedlewska, A., Prus-Głowacki, W. (1995). "Genetic-Structure and Taxonomic Position of Pinus-Uliginosa Neumann Population from Wielkie-Torfowisko-Batorowskie in Stolowe Mts (Locus-Classicus)." Acta Societatis Botanicorum Poloniae **64**(1): 51-58.

Smith, D. R. (2016). "The past, present and future of mitochondrial genomics: have we sequenced enough mtDNAs?" Briefings in Functional Genomics **15**(1): 47-54.

Soranzo, N., R. Alia, J. Provan and W. Powell (2000). "Patterns of variation at a mitochondrial sequence-tagged-site locus provides new insights into the postglacial history of

European Pinus sylvestris populations." Mol Ecol **9**.

Sork, V. L. (2015). "Gene flow and natural selection shape spatial patterns of genes in tree populations: implications for evolutionary processes and applications." Evolutionary applications **9**(1): 291-310.

Stewart, J. R., A. M. Lister, I. Barnes and L. Dalén (2010). "Refugia revisited: individualistic responses of species in space and time." Proc Biol Sci **277**(1682): 661-671.

Stronen, A. V. and P. C. Paquet (2013). "Perspectives on the conservation of wild hybrids." Biological Conservation **167**: 390-395.

Toth, E. G., Z. A. Kobolkuti, A. Pedryc and M. Hohn (2017). "Evolutionary history and phylogeography of Scots pine (Pinus sylvestris L.) in Europe based on molecular markers." Journal of Forestry Research **28**(4): 637-651.

Tóth, E. G., G. G. Vendramin, F. Bagnoli, K. Cseke and M. Höhn (2017). "High genetic diversity and distinct origin of recently fragmented Scots pine (Pinus sylvestris L.) populations along the Carpathians and the Pannonian Basin." Tree Genetics & Genomes **13**(2).

van Mantgem, P. J., N. L. Stephenson, J. C. Byrne, L. D. Daniels, J. F. Franklin, P. Z. Fule, M. E. Harmon, A. J. Larson, J. M. Smith, A. H. Taylor and T. T. Veblen (2009). "Widespread Increase of Tree Mortality Rates in the Western United States." Science **323**(5913): 521-524.

Wachowiak, W., A. E. Palme and O. Savolainen (2011). "Speciation history of three closely related pines Pinus mugo (T.), P. uliginosa (N.) and P. sylvestris (L.)." Molecular Ecology **20**(8): 1729-1743.

Wachowiak, W. and W. Prus-Glowacki (2009). "Different patterns of genetic structure of relict and isolated populations of endangered peat-bog pine (Pinus uliginosa Neumann)." Journal of Applied Genetics **50**(4): 329-339.

Wachowiak, W., J. Zaborowska, B. Labiszak, A. Perry, G. M. Zucca, S. C. Gonzalez-Martinez and S. Cavers (2018). "Molecular signatures of divergence and selection in closely related pine taxa." Tree Genetics & Genomes **14**(6).

Weltzin, J. F., S. D. Bridgham, J. Pastor, J. Chen and C. Harth (2003). "Potential effects of warming and drying on peatland plant community composition." Global Change Biology **9**(2): 141-151

Whitlock, B. A., A. M. Hale and P. A. Groff (2010). "Intraspecific Inversions Pose a Challenge for the trnH-psbA Plant DNA Barcode." PLOS ONE **5**(7): e11533.

Wright, S. (1922). "Coefficients of Inbreeding and Relationship." The American

Naturalist **56**(645): 330-338.

Wright, S. (1933). "Inbreeding and Homozygosis." Proceedings of the National Academy of Sciences of the United States of America **19**(4): 411-420.

Yeh, F., T. Ye and R.-C. Yang (2007). "Multilocus structure in the Pinus contorta - Pinus banksiana complex." Canadian Journal of Botany **85**: 774-784.

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2. **Łabiszak B.,** Zaborowska J., Wójkiewicz B., Wachowiak W. (2020) Molecular and paleo-climatic data uncover impact of ancient bottleneck on demographic history and contemporary genetic structure of endangered *Pinus uliginosa. Journal of Systematics and Evolution,* doi: 10.1111/jse.12573
3. **Łabiszak B.,** Wachowiak W. (2021) Molecular signatures of reticulate evolution within the complex of European pine taxa. *Forests*, 12, 489, doi:10.3390/f12040489

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Studies

Patterns of *mt*DNA variation reveal complex evolutionary history of relict and endangered peat bog pine (*Pinus uliginosa*)

Bartosz Łabiszak[1,*], Julia Zaborowska[1] and Witold Wachowiak[1,2]

[1]Institute of Environmental Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznań, Poland
[2]Institute of Dendrology, Polish Academy of Sciences, Parkowa 5, 62-035 Kórnik, Poland

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Abstract. Estimates of genetic differentiation at intra- and interspecific level are often hindered by the lack of suitable molecular markers. Low phylogeographic resolution limits development of appropriate conservation strategies especially in case of endangered forest tree species with small and disjunct distribution. In this study, we assessed fine-scale genetic structure of relict and endangered peat bog pine (*Pinus uliginosa*) and two other closely related European pine species (*Pinus mugo* and *Pinus uncinata*) using a set of 15 newly developed maternally inherited and seed-mediated mitochondrial DNA (*mt*DNA) markers and two previously known polymorphic *mt*DNA regions (*nad1*, *nad7*). Three main groups, corresponding in general to three investigated species were revealed in the haplotype network analysis. However, only *P. uncinata* was clearly distinct at all levels of analysis, whereas great genetic similarity and haplotype sharing was observed between *P. uliginosa* and *P. mugo*. Strong phylogeographic structure was found in *P. uliginosa* that showed high differentiation at relatively short geographical distance among populations and the existence of mitochondrial lineages of different evolutionary history. Hybridization with other pine species has likely contributed to genetic differentiation of *P. uliginosa* as indicated by contemporary distribution of *mt*DNA haplotypes. The research emphasizes the importance of accurate assessments of genetic structure of endangered species with complex evolutionary history for development of efficient conservation strategies.

Keywords: Endangered species; genetic structure; molecular markers; phylogeography; pines.

Introduction

Assessments of eco-evolutionary mechanisms that shape genetic structure of populations are of key importance to understand the influence of past and ongoing environmental changes on plant ecosystems. In recent years, molecular markers greatly improved our ability to assess genetic differentiation at within and among species level. However, due to genome complexity and limited access to suitable genomic resources, phylogenetic investigations remain still challenging especially in many non-model plant species (Petit and Vendramin 2007; Roy *et al.* 2010; Whitlock *et al.* 2010). Assessments of species boundaries and their underlying population structure are needed not only to improve taxonomic knowledge, but also to properly guide decision-making in conservation of endangered tree species (Newton *et al.* 1999).

*Corresponding author's e-mail address: bartosz.labiszak@amu.edu.pl

European hard pine taxa contain several species intensively studied due to their ecological and social value including representatives of the *Pinus mugo* complex (Wang *et al.* 1999; Gernandt *et al.* 2005; Eckert and Hall 2006). It contains closely related taxa, some undergoing severe population decline and being hard to delimit in an unambiguous way due to low resolution of available biometric and molecular markers (Christensen 1987; Hamernik and Musil 2007). One of the most intriguing representatives of the complex is the peat bog pine (*Pinus uliginosa*). It is a single-stemmed tree up to 20 m in height, inhabiting humid and nutrient-sparse bog environments in lowlands. Originally it has been described from two sites in Central Sudetes, Poland (Neumann 1837; Wimmer 1837), and at present only a few isolated stands are known in Poland, Germany and Ukraine (Boratyński 1994). The species strict ecological specialization together with restricted, island-like range poses a high extinction risk, especially in face of warmer and drier climate that severely affects peatland plant communities (Holt 1990; Weltzin *et al.* 2003; Peterson *et al.* 2005; Audrey *et al.* 2009). In Poland, where the majority of peat bog pine populations are located, rapid decline of trees was observed in recent years. Consequently, in some populations no more than 100 specimens of peat bog pine have been left (Danielewicz and Zieliński 2000) and this taxon is considered as highly endangered and protected, at least on national scale (Polish Plants Red Book).

Interestingly, almost 100 years after it was first described, taxonomic position of this species is not fully resolved. Research to date has focused mostly on peat bog pine evolutionary history and processes shaping its genetic structure, especially in the context of the species protection. Nonetheless, these studies were mainly based on morphological features of needles and cones (Boratynska and Boratynski 2007; Boratynska and Lewandowska 2009) and on isoenzymes (Siedlewska and Prus-Głowacki 1995; Prus-Glowacki *et al.* 1998; Wachowiak and Prus-Glowacki 2009), and they were often restricted to single population and/or individuals. Studies based on morphological data place peat bog pine together with other closely related pine species from the *P. mugo* complex including dwarf mountain pine (*P. mugo*) from mountain regions of Central and Western Europe and mountain pine (*Pinus uncinata*) from Iberian Peninsula (Christensen 1987; Hamernik and Musil 2007). However, the taxa exhibit also some similarity at biometric and biochemical traits to *Pinus sylvestris* (Boratynska and Boratynski 2007) and close relationship between these taxa is reflected in phylogeny of the genus (Grotkopp *et al.* 2004; Gernandt *et al.* 2005). Shared characteristics at some traits led the authors to hypothesis that *P. uliginosa* might be a marginal population of *P. uncinata* (Krzakowa *et al.* 1984) or possibly ancient, stabilized hybrid between *P. mugo* and *P. sylvestris* (Lewandowski *et al.* 2000; Boratynska and Boratynski 2007). Some indication of relatively recent divergence of peat bog pine from other taxa from the *P. mugo* complex was found at sequence variation at nuclear genes (Wachowiak *et al.* 2011); however, the exact genetic relationship between the taxa is not conclusive.

To date, efforts to describe a range-wide phylogeographic structure for peat bog pine were limited (Heuertz *et al.* 2010; Dzialuk *et al.* 2017). This may be in part attributed to insufficient number and low resolution of molecular markers developed for the pine complex. In case of forest tree species, cytoplasmic DNA markers that are haploid and transmitted uniparentally through pollen or seeds are of particular interest for population history studies. In wind-pollinated species such as pines, mitochondrial DNA (*mt*DNA) markers, maternally inherited and dispersed by seeds on short distances, are especially valuable as they best reflect past demographic changes and longer retain patterns of demographic structure (Toth *et al.* 2017). Although *mt*DNA variation was commonly used in previous population history studies in forest tree species, the obtained resolution was very weak due to low number of available markers described for European pines (Soranzo *et al.* 2000; Cheddadi *et al.* 2006; Naydenov *et al.* 2007). Difficulties in finding new *mt*DNA markers result mostly from large size of plant mitochondria, their complex structure with numerous repeated regions and generally low rate of sequence evolution (Guo *et al.* 2016; Smith 2016). However, recent advances in sequencing technologies allowed development of novel genomic resources in non-model plant, including descriptions of a large fragment of mitochondrial genome in pines (Donnelly *et al.* 2017). Based on the polymorphisms found in the regions we developed a large set of new *mt*DNA markers that proved to be useful in population genetic studies of closely related pine species.

Here, we present the results of first large-scale study on genetic structure of relict and endangered peat bog pine with the application of newly developed *mt*DNA markers. Using a set of peat bog pine populations and a collection of a reference samples of closely related taxa we: (i) looked at the population structure of the remaining stands of the peat bog pine, (ii) assessed levels of *mt*DNA variation in *P. uliginosa* populations to infer past population history processes, (iii) examined genetic relationship of *P. uliginosa* as compared to other pine species in reference to earlier hypothesis. Based on our findings we suggest potential conservation strategies for preservation of genetic resources of the endangered peat bog pine.

Materials and Methods

Sampling and marker development

Five populations of *P. uliginosa* were sampled together with 13 reference populations including 7 *P. mugo* and 6 *P. uncinata* stands sampled across the European ranges of the taxa. There are no other pines closely related to the studied taxa that occur in the sympatry of the analysed populations. Sample size ranged from 8 to 40 trees per population, resulting in a total of 384 individuals analysed (Fig. 1; Table 1). Genomic DNA was extracted from needle tissues using DNeasy Plant Mini Kit (Qiagen), following standard manufacturer protocol. In order to assess genetic structure and relationships between investigated taxa we developed a large-scale, cost-effective genotyping method of individuals at multiple loci using polymorphic *mt*DNA regions described in Donnelly *et al.* (2017). Initially, a set of approximately 30 regions were screened in Nebcutter V.2.0 (Vincze *et al.* 2003) in order to find suitable Single Nucleotide Polymorphism (SNPs) for Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) analysis. PCR amplification of 15 polymorphic regions was carried out in a total volume of 15 µL containing 15 ng of template DNA, 10 µM of each dNTP, 0.2 µM each of forward and reverse primers, 0.15 U *Taq* DNA polymerase, 1× BSA, 1.5 µM of $MgCl_2$ and 1× PCR buffer (Novazym). Standard amplification procedures were used with initial denaturation at 94 °C for 3 min followed by 35 cycles with 30 s denaturation at 94 °C, 30 s annealing at 60 °C for most loci and 1 min 30 s extension at 72 °C, and a final 5 min extension at 72 °C. The genotyping was done in all but one case using respective restriction enzyme and electrophoresis

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Figure 1. Geographic location of studied *Pinus uliginosa* populations (🟥) and reference stands of closely related pine species: *P. mugo* (▲) and *P. uncinata* (●). Distribution range of *P. mugo* and *P. uncinata* is marked with grey horizontal and crossed stripes, respectively. Population acronyms and exact location as in Table 1.

Table 1. Location, sample size, corresponding SAMOVA group and basic haplotype statistics of studied pine populations. *N*—number of individuals/number of individuals genotyped; H_n—number of haplotypes; H_d—haplotype diversity; H_s—number of singleton haplotypes.

Species/acronym	Population	Latitude N	Longitude E	Altitude (m)	*N*	H_n	H_d	H_s	SAMOVA group
P. uliginosa									
UL_POL_W	Poland, Sudety Mts., Low Silesian Pinewood, Węgliniec reserve	51°17′50″	15°14′20″	190	40/40	13	0.83	7	I
UL_GER_MI	Germany, Bavaria, Mittenwald	47°28′50″	11°16′27″	856	25/21	10	0.91	4	I
UL_POL_BAT	Poland, Sudety Mts., Batorów reserve	50°27′32″	16°23′01″	710	36/33	2	0.17	0	III
UL_POL_Z	Poland, Sudety Mts., Zieleniec reserve	50°20′54″	16°24′42″	755	30/27	18	0.96	13	III
UL_UKR_MS	Ukraine, Gorgany Mts., Mshana	48°40′33″	23°55′19″	830	12/12	4	0.74	1	IV
	All				143/133	40	0.91	22	–
P. mugo									
M_POL_SK	Poland, Sudety Mts., Śląskie Kamienie	50°46′35″	15°36′08″	1300	10/7	2	0.48	0	I
M_POL_DPS	Poland, Tatra Mts., Dolina Pięciu Stawów	49°13′09″	20°03′05″	1700	12/12	5	0.73	3	I
M_AUT_K	Austria, Karwendel Mts., Scharnitz	47°22′42″	11°17′45″	1400	22/22	5	0.71	2	I
M_UKR_MS	Ukraine, Gorgany Mts., Mshana	48°40′33″	23°55′19″	830	8/8	1	0.00	0	IV
M_ROU_E	Romania, Eastern Carpathians, Munti Rodnei	47°34′03″	24°48′00″	1720	22/19	4	0.30	3	I
M_BGR_P	Bulgria, Pirin Mts., Vikhren	41°46′07″	23°25′22″	2000	22/22	2	0.48	0	I
M_ITA_CA	Italy, Carnic Alps, Passo di Pramollo	46°32’45″	13°15′35″	1530	21/21	3	0.19	2	I
	All				117/111	16	0.87	7	–
P. uncinata									
UN_AND_VR	Andorra, Eastern Pyrenees, Vall de Ransol	42°35′02″	01°38′21″	2025	22/22	1	0.00	0	II
UN_AND_SM	Andorra, Eastern Pyrenees, San Miguel de Engolasters	42°31′28″	01°34′12″	2000	22/20	3	0.42	1	II
UN_ESP_LT	Spain, Western Pyrenees, La Trapa	42°41′19″	-00°32′12″	1720	22/22	2	0.37	0	II
UN_ESP_V	Spain, Sierra de Gudar, Valldelinares	40°28′49″	-00°41′51″	2000	22/20	2	0.42	0	II
UN_FRA_CDJ	France, Eastern Pyrenees, Col de Jau	42°39′19″	02°15′22″	1520	12/12	2	0.17	1	II
UN_FRA_CDC	France, Massif Central, Col de la Croix-Morand	45°36′00″	02°50′59″	1400	24/23	4	0.58	0	II
	All				124/119	5	0.53	1	–

of restriction products in 2 % agarose gel. List of all PCR primer pairs and restriction enzymes used in this study is given in **Supporting Information—Table S1**. Insertion/deletion (indel) polymorphism in PR34 region was genotyped using Sanger sequencing. The respective fragments were amplified as described above and PCR fragments were purified using Exonuclease I-Shrimp Alkaline Phosphatase enzymatic treatment. About 20 ng of PCR product was used as template in 10 μL sequencing reaction with the Big Dye Terminator DNA Sequencing Kit (Applied Biosystems). CodonCode Aligner (CodonCode Corporation) was used to edit and align sequences. Additionally, two previous *mt*DNA markers including *nad7* and *nad1* were genotyped according to methods described in Jaramillo-Correa *et al.* (2004) and Soranzo *et al.* (2000), respectively.

*mt*DNA haplotype analysis

Multilocus genotypes were assessed for each individual using all 17 markers. All except one marker (PR29) were found to be polymorphic in investigated species and thus 16 markers were used thereafter. Individuals with level of missing data ≥ 10 % were excluded from further analysis. Phylogenetically informative gaps (indels) in PR34, *nad1* and *nad7* were coded as single mutation events for analyses. The number of haplotypes (H_n) and haplotype diversity (H_d) were computed at species and population level using DnaSP v.5 (Librado and Rozas 2009). A median-joining network, illustrating phylogenetic relationship among *mt*DNA haplotypes, was constructed for all sequences with PopART (Bandelt *et al.* 1999). The geographic distribution of markers was assessed at the most frequent *mt*DNA haplotypes detected (i.e. those with frequency ≥ 1 %).

Population structure and differentiation

To show genetic relationships between populations and species, genetic distance based on all polymorphic *mt*DNA sites was calculated in MEGA 7 (Kumar *et al.* 2016) and used in principal coordinate analysis (PCoA) in GenAlEx 6.501 software (Peakall and Smouse 2006; Peakall and Smouse 2012). The genetic relationships between samples were also investigated using the unweighted pair group method with arithmetic mean (UPGMA) in MEGA 7.

The hierarchical analysis of spatial molecular variance in populations was conducted using SAMOVA 2.0 program (Dupanloup *et al.* 2002) in order to find *K* groups of maximally differentiated but geographically homogenous populations. The analysis was performed at *K* values ranging from 2 to 17. Genetic differentiation among groups identified by SAMOVA 2.0 was estimated using an analysis of molecular variance (AMOVA) implemented in Arlequin v.3.5.22 (Excoffier and Lischer 2010).

Additional measures of population differentiation (G_{ST}, N_{ST}) were calculated and compared to each other using a permutation test with 10 000 replicates in PermutCpSSR v.1.2.1 software (Pons and Petit 1996; Burban *et al.* 1999). The comparison between those estimates can elucidate presence of a formal phylogeographic structure in cases where N_{ST} value is higher than the G_{ST} value. Finally, isolation by distance hypothesis was verified by Mantel test using GenAlEx 6.501 software with 1000 random permutations of the relationship between genetic (based on N_{ST}) and geographic distance matrices.

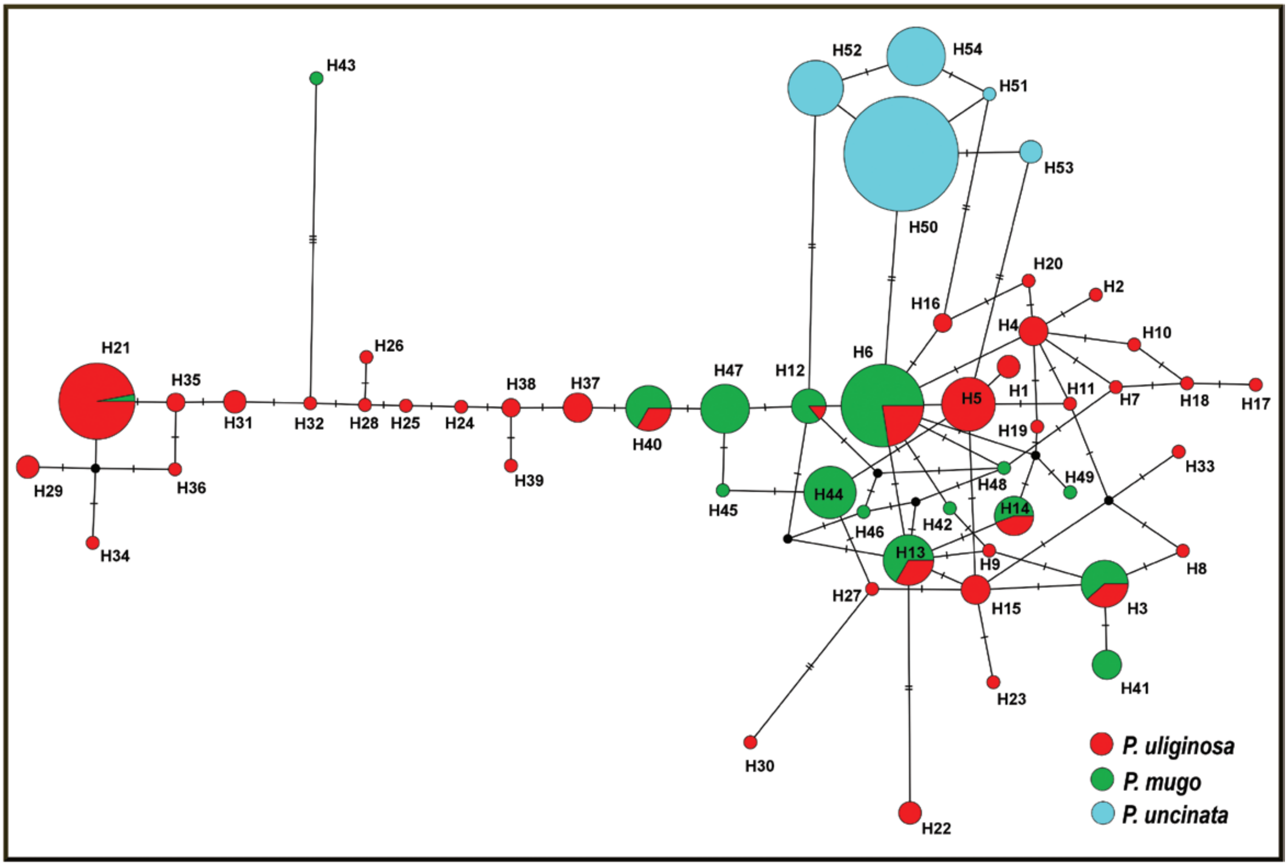

Figure 2. Median-joining network of haplotypes detected at 16 *mt*DNA regions in the taxa from the *Pinus mugo* complex. Sizes of the circles are proportional to haplotype frequencies, hatch marks represent numbers of nucleotide differences between them and shading indicates species.

Results

Based on 16 polymorphic *mt*DNA markers we were able to identify 54 novel haplotypes in 363 trees from three pine species (Fig. 2; **see Supporting Information—Table S2**). Overall, there was an abundance of minor frequency haplotypes with 37 haplotypes present in <1 % of all individuals (29 haplotypes were singletons and 8 were present only in 2–3 individuals). Particularly high number of singletons was found in *P. uliginosa*, especially in population UL_POL_Z (Zieleniec reserve), where an excess of rare haplotypes, with 13 singletons and highest value of haplotype diversity (H_d = 0.96), was observed (Table 1). Additionally, the highest number of haplotypes (H_n = 40) and average haplotype diversity (H_d = 0.91) were also detected in this species (Table 1). The average haplotype diversity was very similar for *P. mugo* (H_d = 0.87) but substantially lower for *P. uncinata* (H_d = 0.53). The three most common haplotypes were H50, H6 and H21 (Fig. 2). Haplotype H50 was exclusive to *P. uncinata* (except Spanish population from Valldelinares), H6 was almost fixed in *P. mugo* from Carnic Alps and occurred at low frequency in other dwarf mountain pine populations but was detected also in three peat bog pine populations (UL_POL_Z, UL_POL_W, UL_GER_MI) **[see Supporting Information—Table S3]**. Haplotype H21 was dominant in *P. uliginosa* from Batorów reserve, but it was also present in three individuals in adjacent population from Zieleniec reserve and interestingly in one *P. mugo* individual from the Tatra Mts. Similar sharing of haplotypes between *P. mugo* from Polish mountains (both Tatra and Sudety Mts.) and *P. uliginosa* from Węgliniec reserve was found at haplotype H3. Except the mentioned shared common haplotypes between individuals in different populations (i.e. haplotypes H3, H6, H13), some local variants were also found to co-occur in neighbouring populations of different taxa (UL_GER_MI and M_AUT_K shared two haplotypes; UL_UKR_MS and M_UKR_MS shared one haplotype) **[see Supporting Information—Table S3, Fig. S2]**. The pattern of median-joining haplotype network revealed three main groups which coincide in general with three investigated species (Fig. 2), although haplotype sharing was found between *P. uliginosa* and *P. mugo*. Unique haplotypes were found only in *P. uncinata*.

Presence of strong and significant phylogeographic structure was inferred from considerable genetic differentiation among populations ($N_{ST} > G_{ST}$; $P < 0.001$). Within species, population structure was observed in *P. uliginosa* and *P. mugo*, but not in *P. uncinata* (Table 2). After removing *P. uncinata* populations we still observed significantly greater N_{ST} than G_{ST} in the remaining populations based on PermutCpSSR analysis (data not shown).

Table 2. Genetic diversity estimates for *mt*DNA regions in *Pinus mugo* complex. H_T—total gene diversity; H_S—averaged gene diversity within populations; **significant at $P = 0.01$.

Species	H_T	H_S	N_{ST}	G_{ST}
P. uliginosa	0.98	0.72	0.605**	0.263
P. mugo	0.97	0.53	0.653**	0.457
P. uncinata	0.55	0.35	0.481	0.368
All	0.94	0.47	0.735**	0.505

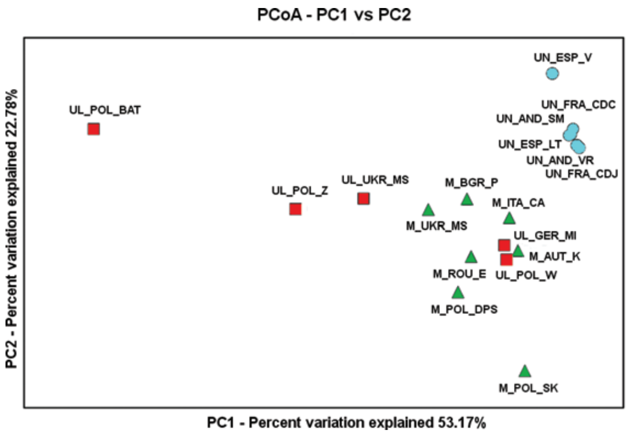

Figure 3. Results of PCoA based on average distances between studied populations calculated for a set of 16 *mt*DNA markers.

The evidence of population structure was further supported by results of the PCoA (Fig. 3). The majority of populations could be assigned to one of the three main clusters: (i) *P. mugo* together with *P. uliginosa* from Węgliniec and Mittenwald (UL_POL_W and UL_GER_MI); (ii) *P. uncinata* populations; (iii) *P. uliginosa*. However, two outlier populations including UL_POL_BAT and M_POL_SK showed distinct patterns of genetic variation and were isolated from other clusters. Similar relationships between the populations were observed in the UPGMA tree **[see Supporting Information—Fig. S3]**.

The result of SAMOVA at K = 2–17 is shown in **Supporting Information—Fig. S1**. The optimal number of groups, when the increment of Φ_{CT} was the largest, was four. The resulting SAMOVA groups did not exactly coincide with the taxa delineations but were similar to the pattern of genetic clusters indicated by the PCoA. The results show distinct character of *P. uncinata* populations (SAMOVA group II), similarity of two *P. uliginosa* and majority of *P. mugo* populations (SAMOVA group I), and unique character of the remaining *P. uliginosa* populations (SAMOVA groups III and IV) (Table 1). In the hierarchical AMOVA based on the division of populations into four groups, 60 % of the variation was due to differentiation between groups, while 24 % occured among populations within groups. Interestingly, the Mantel test showed statistically significant relationship between the genetic and geographic distances ($r = 0.54$, $P < 0.001$) suggesting presence of isolation by distance among populations. Nevertheless, when the three species were analysed separately, no statistically significant relationship was observed in any taxa ($P > 0.05$).

Discussion

High-resolution molecular markers are needed for fine-scale population structure assessments and proper testing of phylogeograpic hypothesis. Difficulties involved in finding such variable markers, comparable in resolving power to animal *mt*DNA, have been severe in phylogeography of plants, especially non-model species with limited genomic resources (Beheregaray 2008). Due to slow mutation rate in plant mitochondrial genome, only two *mt*DNA markers including variation at *nad1* and *nad7* regions were developed for closely related pines from *P. mugo* complex. However, resolution of those markers was too low to provide any clear patterns of the species differentiation and populations structure. The application of more variable chloroplast DNA (*cp*DNA) markers, inherited in pines in paternal line and distributed at large geographical distances by pollen, was limited for closely related pine species (Palmer 1992; Wang and Wang 2014; Toth *et al.* 2017). In case of peat bog pine, which was grouped due to some similarities at biometric traits and incomplete reproductive isolation into larger taxonomic unit of the *P. mugo* complex (Christensen 1987), assessment of its genetic relationship at interspecific level based on *cp*DNA markers was especially hard. For instance, it was not possible to discriminate *P. uliginosa* from *P. mugo* and *P. uncinata* using variation of chloroplast DNA barcode regions (Celiński *et al.* 2017). Consequently, due to slow evolution of cytoplasmic genomes and very limited number of the regions screened for polymorphism, it was difficult to find species-specific genetic differences between those taxa and properly assess their intraspecific differentiation.

In advance to earlier studies our data provide some evidence of genetic variation within studied pine complex. Screening of a large set of newly developed mitochondrial markers together with previously known polymorphisms at two *mt*DNA regions delivered 54 novel haplotypes in 18, range-wide sampled, populations of the three investigated species. The results have substantially increased resolution of previous taxonomic investigations and population structure assessments in this pine species complex. Although there was extensive sharing of haplotypes between *P. mugo* and *P. uliginosa*, we were able to find fixed differences at two markers (*nad1* and PR13) that differentiate *P. uncinata* from other taxa in the complex. Low haplotype diversity and presence of species-specific haplotypes show clear genetic differentiation of *P. uncinata* supporting earlier suggestions of limited interspecific gene flow and its ongoing divergence (Wachowiak *et al.* 2013). The results are also in line with earlier karyotype studies of distinct heterochromatin patterns between *P. mugo* and *P. uncinata* (Bogunic *et al.* 2011). There are many factors that could have impact on the pattern of neutral genetic diversity including: level of gene flow, past climatic fluctuation, realized ecological niche and distribution range. The relatively low level of genetic diversity in *P. uncinata* is consistent with two general predictions: (i) lower levels of genetic diversity are expected for species with smaller distribution ranges; (ii) mountain populations tend to have lower haplotype diversity due to their peripheral location along an increasingly harsh elevation gradient (Herrera and Bazaga 2008). The results of chloroplast DNA variation in *P. uncinata* support those expectations (Dzialuk *et al.* 2017). Additionally, we did not find sharing of mitotypes between *P. uncinata* and *P uliginosa*, as the latter was generally more similar to *P. mugo*. This could be attributed to limited gene flow due to greater geographical distance between *P. uncinata* and *P. ulginosa* as compared to *P. mugo* and *P. uliginosa*. Contemporary ranges of *P. mugo* and *P. uncinata* are mostly disjunct but, some populations of the taxa overlap in Western Alps and could potentially form a hybrid zone. However, haplotype sharing through interspecific gene exchange seems unlikely taking into account the *cp*SSR results showing that the alpine *P. uncinata* population from Pyrenees forms a separate group as compared to the neighbouring *P. mugo* populations (Dzialuk *et al.* 2017). Our results clearly reject hypothesis about *P. uliginosa* being a marginal population of *P. uncinata* (Krzakowa *et al*. 1984), and they do not support suggestion that *P. uliginosa* may result from hybridization between *P. mugo* and *P. uncinata* (Dzialuk *et al.* 2017).

Our results provide clear evidence that *P. uliginosa* has surprisingly strong population structure with striking genetic differentiation among populations. The data indicate existence of different mitochondrial lineages in *P. uliginosa* and show that population from its *locus classicus* from Batorów reserve is the most diverged population within this taxon. Significant differentiation between populations distributed at relatively short geographical distance could be explained by limited gene flow and long-lasting separation of populations inhabiting disjunctive stands throughout their evolutionary history. Signs of differentiation were previously indicated based on some biometric features of cones and needles (e.g. Boratynska and Lewandowska 2009; Boratynska *et al.* 2015) and biochemical markers (e.g. Lewandowski *et al.* 2002; Wachowiak and Prus-Głowacki 2009). Nevertheless, it seems rather unlikely that such differentiation could result recently from pure isolation and genetic drift due to slow mutation rate of *mt*DNA in pines and late time of the formation of most European peatlands. Those areas started forming no earlier than at the last glacial maximum (LGM) and reached its peak around 9 ky ago (Gajewski *et al.* 2001). Possibly the remaining *P. uliginosa* stands represent populations of different origin that diverged long before the last glacial period and recolonized the current distribution from multiple sources. The existence of such cryptic central and north European refugia was postulated for other pines and forest tree species (Stewart and Lister 2001; Tzedakis *et al.* 2013; Ruiz-González *et al.* 2013).

High within-species divergence of *P. uliginosa* could also result from independent hybrid origin of different parental populations. Natural hybridization is recently recognized as an important process shaping evolution in many animal and plant species and it is well documented in conifers (Mallet 2005; Gao *et al.* 2012; Sun *et al.* 2014). Ecological divergence and adaptation to specific environmental niches facilitate spread of hybrids, despite co-occurrence with their parental types (Gross and Rieseberg 2005). The results of controlled crosses indicate incomplete reproductive isolation within the investigated pine complex and also with *P. sylvestris*, suggesting that hybridization between these taxa was highly possible in contact zones and could have contributed to *P. uliginosa* gene pool (Lewandowski *et al.* 2000; Wachowiak *et al.* 2005). Our data provide evidence on high genetic similarity between *P. uliginosa* and *P. mugo*. Differentiation in *P. uliginosa* could have arisen as a result of hybridization in postglacial secondary contact zones between populations of different ancestry representing these two species. Some of the shared haplotypes (i.e. haplotype H6) are widespread and common in both taxa, and thus may represent ancestral haplotypes acquired in distant past and retained in both lineages. We also detected less frequent haplotypes shared locally between neighbouring populations, for example H14 (UL_GER_MI and M_AUT_K) and H40 (UL_UKR_MS and M_UKR_MS). Considering weak reproductive barriers, hybridization in contact zones with mitochondrial capture between those two species seems possible. The observed pattern of haplotype distribution may thus reflect different influences of past (haplotypes shared in many populations and over large distance) and more recent (haplotypes shared locally) hybridization events on contemporary haplotype variation in *P. uliginosa*. However, we cannot exclude retention of ancestral polymorphism in those taxa and therefore nuclear markers would be needed to fully test this hypothesis.

Hybridization could also be invoked as the casual factor shaping unexpectedly high haplotype diversity found within *P. uliginosa* population from Zieleniec reserve. This population is particularly interesting as it represents a contact zone of three pine species (*P. uliginosa*, *P. mugo*, *P. sylvestris*) in a diverse habitat of the peat bog complex and it contains viable hybrid trees (Wachowiak *et al.* 2016). Although our sampling was restricted to trees classified based on morphological features as *P. uliginosa*, accidental inclusion of hybrid trees with *P. uliginosa*-like phenotype in our data set cannot be excluded. Presence of such exceptional number of haplotypes in individuals from Zieleniec reserve could result from acquisition of different mitotypes from the species involved in hybridization events. However, given the sheer number of haplotypes (18 in 27 individuals), this process alone can hardly explain mitochondrial variation observed in this population. Alternatively, *mt*DNA recombination mediated by hybridization events seems possible. Hypothesis of homologous recombination promoted by occasional parental leakage and heteroplasmy of *mt*DNA was previously proposed to explain high *mt*DNA variation in hybrid zone of spruce species (Jaramillo-Correa and Bousquet 2005) and this phenomenon was observed also in other conifers (Semerikov and Lascoux 2003; Semerikova and Semerikov 2014). Although paternal leakage of the mitochondrial genome has previously been reported to occur in other *Pinus* species (Wagner *et al.* 1991), there are no reports describing this phenomenon in species from *P. mugo* complex. Further tests with dense sampling of individuals from the contact zone of those three taxa and individuals from controlled crosses would be needed to support the hypothesis of exceptional haplotype diversity of *P. uliginosa* from Zieleniec reserve.

Our data provide evidence of high genetic variation and complex evolutionary history of the remnant *P. uliginosa* populations. Such a complex population structure, involving putative past and/or ongoing hybridization events, demands thoughtful consideration while developing conservation strategies for the taxa. Although not all endangered tree species are affected in the same manner by similar threats (Pautasso 2009), it seems evident that all *P. uliginosa* stands deserve preservation throughout the species range considering high genetic diversity and high degree of differentiation amongst populations. Extinction due to the decrease of the primary habitat is among the biggest threats to the peat bog pine. Active protection of all of these rare stands, coupled with creating conditions for its natural regeneration seems urgent. The existing genotypes should be protected by creating the clone archives (e.g. in form of cryopreserved somatic embryos) (Choudhury *et al.* 2014). To maintain diversity and reduce the threat of inbreeding in small populations, some level of human-mediated admixture between these geographically distinct populations should also be permitted allowing for some genetic rescue, an increase in effective population size and greater additive genetic variation. On the other hand, contemporary threat by genetic erosion in some populations (e.g. Zieleniec reserve) requires special attention, and invokes challenging questions, regarding conservation status of natural hybrids (Allendorf *et al.* 2001; Wachowiak *et al.* 2005; Stronen and Paquet 2013).

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Contributions by the Authors

B.Ł. and W.W. conceived the study; B.Ł. and J.Z. obtained and analysed genetic data; B.Ł. led the writing with support of W.W. and J.Z., who read and contributed to the final version of the manuscript.

Conflict of Interest

None declared.

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Supporting Information

The following additional information is available in the online version of this article—

Table S1. Analysed loci and genotyping method.

Table S2. Major haplotypes and their frequency in the analysed taxa.

Table S3. Distribution of major haplotypes detected in studied pine taxa and populations.

Figure S1. Spatial analysis of molecular variance (SAMOVA).

Figure S2. Median-joining network of haplotypes detected at 16 mitochondrial DNA (*mt*DNA) regions in the taxa from the *Pinus mugo* complex.

Figure S3. Unweighted pair group method with arithmetic mean (UPGMA) phylogenetic tree of 18 studied pine populations.

Literature Cited

Allendorf FW, Leary RF, Spruell P, Wenburg JK. 2001. The problems with hybrids: setting conservation guidelines. *Trends in Ecology & Evolution* **16**:613–622.

Audrey C, Hsiang LL, Andreas P. 2009. Are specialists at risk under environmental change? Neoecological, paleoecological and phylogenetic approaches. *Ecology Letters* **12**:849–863.

Bandelt HJ, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**:37–48.

Beheregaray LB. 2008. Twenty years of phylogeography: the state of the field and the challenges for the Southern Hemisphere. *Molecular Ecology* **17**:3754–3774.

Bogunic F, Siljak-Yakovlev S, Muratovic E, Pustahija F, Medjedovic S. 2011. Molecular cytogenetics and flow cytometry reveal conserved genome organization in *Pinus mugo* and *P-uncinata*. *Annals of Forest Science* **68**:179–187.

Boratynska K, Boratynski A. 2007. Taxonomic differences among closely related pines *Pinus sylvestris*, *P-mugo*, *P-uncinata*, *P-rotundata* and *P-uliginosa* as revealed in needle sclerenchyma cells. *Flora* **202**:555–569.

Boratynska K, Jasinska AK, Boratynski A. 2015. Taxonomic and geographic differentiation of *Pinus mugo* complex on the needle characteristics. *Systematics and Biodiversity* **13**:901–915.

Boratynska K, Lewandowska D. 2009. Differences among three populations of *Pinus uliginosa* and their relation to *P. sylvestris* as expressed by the needle characters. *Dendrobiology* **61**:37–46.

Boratyński A. 1994. Protected and rare trees and shrubs from the Polish part of Sudety Mts. and its foothills. 7. *Pinus mugo* Turra and *P. uliginosa* Neumann. *Arboretum Kórnickie* **39**:63–85.

Burban C, Petit RJ, Carcreff E, Jactel H. 1999. Rangewide variation of the maritime pine bast scale *Matsucoccus feytaudi* Duc. (Homoptera: Matsucoccidae) in relation to the genetic structure of its host. *Molecular Ecology* **8**:1593–1602.

Celiński K, Kijak H, Wojnicka-Półtorak A, Buczkowska-Chmielewska K, Sokołowska J, Chudzińska E. 2017. Effectiveness of the DNA barcoding approach for closely related conifers discrimination: a case study of the *Pinus mugo* complex. *Comptes Rendus Biologies* **340**:339–348.

Cheddadi R, Vendramin GG, Litt T, François L, Kageyama M, Lorentz S, Laurent JM, de Beaulieu JL, Sadori L, Jost A, Lunt D. 2006. Imprints of glacial refugia in the modern genetic diversity of *Pinus sylvestris*. *Global Ecology and Biogeography* **15**:271–282.

Choudhury H, Kumaria S, Tandon P. 2014. *Pinus* biotechnology: progress and prospects. In: Ramawat KG, Mérillon JM, Ahuja MR. eds. *Tree biotechnology*. Boca Raton: CRC Press, 223–247.

Christensen Kl. 1987. Taxonomic revision of the *Pinus mugo* complex and *P. rhaetica* (*P. mugo sylvestris*) (Pinaceae). *Nordic Journal of Botany* **7**:383–408.

Danielewicz W, Zieliński J. 2000. Protection of the *Pinus uliginosa* Neumann on the area of the Low Silesian Pinewood. (in Polish). *Przegląd Przyrodniczy* **XI**:113–124.

Donnelly K, Cottrell J, Ennos RA, Vendramin GG, A'Hara S, King S, Perry A, Wachowiak W, Cavers S. 2017. Reconstructing the plant mitochondrial genome for marker discovery: a case study using *Pinus*. *Molecular Ecology Resources* **17**:943–954.

Dupanloup I, Schneider S, Excoffier L. 2002. A simulated annealing approach to define the genetic structure of populations. *Molecular Ecology* **11**:2571–2581.

Dzialuk A, Boratynska K, Romo A, Boratynski A. 2017. Taxonomic and geographic variation of the *Pinus mugo* complex on chloroplast microsatellite markers. *Systematics and Biodiversity* **15**:464–479.

Eckert AJ, Hall BD. 2006. Phylogeny, historical biogeography, and patterns of diversification for *Pinus* (Pinaceae): phylogenetic tests of fossil-based hypotheses. *Molecular Phylogenetics and Evolution* **40**:166–182.

Excoffier L, Lischer HE. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10**:564–567.

Gajewski K, Viau A, Sawada M, Atkinson D, Wilson S. 2001. Sphagnum peatland distribution in North America and Eurasia during the past 21,000 years. *Global Biogeochemical Cycles* **15**:297–310.

Gao J, Wang B, Mao JF, Ingvarsson P, Zeng QY, Wang XR. 2012. Demography and speciation history of the homoploid hybrid pine *Pinus densata* on the Tibetan Plateau. *Molecular Ecology* **21**:4811–4827.

Gernandt DS, Lopez GG, Garcia SO, Liston A. 2005. Phylogeny and classification of *Pinus*. *Taxon* **54**:29–42.

Grotkopp E, Rejmánek M, Sanderson MJ, Rost TL. 2004. Evolution of genome size in pines (*Pinus*) and its life-history correlates: supertree analyses. *Evolution* **58**:1705–1729.

Gross BL, Rieseberg LH. 2005. The ecological genetics of homoploid hybrid speciation. *The Journal of Heredity* **96**:241–252.

Guo W, Grewe F, Fan W, Young GJ, Knoop V, Palmer JD, Mower JP. 2016. Ginkgo and Welwitschia mitogenomes reveal extreme contrasts in gymnosperm mitochondrial evolution. *Molecular Biology and Evolution* **33**:1448–1460.

Hamernik J, Musil I. 2007. The *Pinus mugo* complex - its structuring and general overview of the used nomenclature. *Journal of Forest Science* **53**:253–266.

Herrera CM, Bazaga P. 2008. Adding a third dimension to the edge of a species' range: altitude and genetic structuring in mountainous landscapes. *Heredity* **100**:275–285.

Heuertz M, Teufel J, González-Martínez SC, Soto A, Fady B, Alía R, Vendramin GG. 2010. Geography determines genetic relationships between species of mountain pine (*Pinus mugo* complex) in western Europe. *Journal of Biogeography* **37**:541–556.

Holt RD. 1990. The microevolutionary consequences of climate change. *Trends in Ecology & Evolution* **5**:311–315.

Jaramillo-Correa JP, Beaulieu J, Bousquet J. 2004. Variation in mitochondrial DNA reveals multiple distant glacial refugia in black spruce (*Picea mariana*), a transcontinental North American conifer. *Molecular Ecology* **13**:2735–2747.

Jaramillo-Correa JP, Bousquet J. 2005. Mitochondrial genome recombination in the zone of contact between two hybridizing conifers. *Genetics* **171**:1951–1962.

Krzakowa M, Naganowska B, Bobowicz MA. 1984. Investigations on taxonomic status of *Pinus uliginosa* Neumann. *Bulletin de la Société des Amis des Sciences et des Lettres de Poznań, Serié D* **24**:87–96.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**:1870–1874.

Lewandowski A, Boratynski A, Mejnartowicz L. 2000. Allozyme investigations on the genetic differentiation between closely related pines - *Pinus sylvestris*, *P-mugo*, *P-uncinata*, and *P-uliginosa* (Pinaceae). *Plant Systematics and Evolution* **221**:15–24.

Lewandowski A, Smoćko J, Boratynska K, Boratynski A. 2002. Genetic differences between two Polish populations of *Pinus uliginosa*, compared to *P. sylvestris* and *P. mugo*. *Dendrobiology* **48**:51–57.

Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**:1451–1452.

Mallet J. 2005. Hybridization as an invasion of the genome. *Trends in Ecology & Evolution* **20**:229–237.

Naydenov K, Senneville S, Beaulieu J, Tremblay F, Bousquet J. 2007. Glacial vicariance in Eurasia: mitochondrial DNA evidence from Scots pine for a complex heritage involving genetically distinct refugia at mid-northern latitudes and in Asia Minor. *BMC Evolutionary Biology* **7**:233.

Neumann C. 1837. Über eine auf den Seefeldern bei Reinerz u. einigen ähnlichen Gebirgsmooren der königl. Oberförsterei Karlsberg in der Graftschaft Glatz vorkommende noch unbeschriebene Form der Gattung *Pinus*. *Jahresbericht der Schlesischen Gesellschaft für Vaterländische Cultur* **11**:52–57.

Newton AC, Allnutt TR, Gillies AC, Lowe AJ, Ennos RA. 1999. Molecular phylogeography, intraspecific variation and the conservation of tree species. *Trends in Ecology & Evolution* **14**:140–145.

Palmer JD. 1992. Mitochondrial DNA in plant systematics: applications and limitations. In: Soltis PS, Soltis DE, Doyle JJ, eds. *Molecular systematics of plants*. Boston, MA: Springer US, 36–49.

Pautasso M. 2009. Geographical genetics and the conservation of forest trees. *Perspectives in Plant Ecology, Evolution and Systematics* **11**:157–189.

Peakall R, Smouse PE. 2006. GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**:288–295.

Peakall R, Smouse PE. 2012. GenAlEx 6.5: genetic analysis in excel. Population genetic software for teaching and research–an update. *Bioinformatics* **28**:2537–2539.

Peterson AT, Tian H, Martínez-Meyer E, Soberón J, Sánchez-Cordero V, Huntley B. 2005. Modeling distributional shifts of individual species and biomes. In: Thomas E. Lovejoy TE, Lee Jay Hannah LJ. eds. *Climate change and biodiversity*. New Haven, CT: Yale University Press, 211–228.

Petit RJ, Vendramin GG. 2007. Plant phylogeography based on organelle genes: an introduction. In: Weiss S, Ferrand N. eds. *Phylogeography of Southern European Refugia*. Dordrecht: Springer, 23–97.

Pons O, Petit RJ. 1996. Measuring and testing genetic differentiation with ordered versus unordered alleles. *Genetics* **144**:1237–1245.

Prus-Glowacki W, Bujas E, Ratynska H. 1998. Taxonomic position of *Pinus uliginosa* Neumann as related to other taxa of *Pinus mugo* complex. *Acta Societatis Botanicorum Poloniae* **67**:269–274.

Roy S, Tyagi A, Shukla V, Kumar A, Singh UM, Chaudhary LB, Datt B, Bag SK, Singh PK, Nair NK, Husain T, Tuli R. 2010. Universal plant DNA barcode loci may not work in complex groups: a case study with Indian berberis species. *PLoS One* **5**:e13674.

Ruiz-González A, Madeira MJ, Randi E, Abramov AV, Davoli F, Gómez-Moliner BJ. 2013. Phylogeography of the forest-dwelling European pine marten (*Martes martes*): new insights into cryptic northern glacial refugia. *Biological Journal of the Linnean Society* **109**:1–18.

Semerikov VL, Lascoux M. 2003. Nuclear and cytoplasmic variation within and between Eurasian *Larix* (Pinaceae) species. *American Journal of Botany* **90**:1113–1123.

Semerikova SA, Semerikov VL. 2014. Mitochondrial DNA variation and reticulate evolution of the genus *Abies*. *Russian Journal of Genetics* **50**:366–377.

Siedlewska A, Prus-Głowacki W. 1995. Genetic-structure and taxonomic position of *Pinus-uliginosa* Neumann population from Wielkie-Torfowisko-Batorowskie in Stolowe Mts (locus-classicus). *Acta Societatis Botanicorum Poloniae* **64**:51–58.

Smith DR. 2016. The past, present and future of mitochondrial genomics: have we sequenced enough mtDNAs? *Briefings in Functional Genomics* **15**:47–54.

Soranzo N, Alia R, Provan J, Powell W. 2000. Patterns of variation at a mitochondrial sequence-tagged-site locus provides new insights into the postglacial history of European *Pinus sylvestris* populations. *Molecular Ecology* **9**:1205–1211.

Stewart JR, Lister AM. 2001. Cryptic northern refugia and the origins of the modern biota. *Trends in Ecology & Evolution* **16**:608–613.

Stronen AV, Paquet PC. 2013. Perspectives on the conservation of wild hybrids. *Biological Conservation* **167**:390–395.

Sun Y, Abbott RJ, Li L, Li L, Zou J, Liu J. 2014. Evolutionary history of purple cone spruce (*Picea purpurea*) in the Qinghai-Tibet Plateau: homoploid hybrid origin and Pleistocene expansion. *Molecular Ecology* **23**:343–359.

Toth EG, Kobolkuti ZA, Pedryc A, Hohn M. 2017. Evolutionary history and phylogeography of Scots pine (*Pinus sylvestris* L.) in Europe based on molecular markers. *Journal of Forestry Research* **28**:637–651.

Tzedakis PC, Emerson BC, Hewitt GM. 2013. Cryptic or mystic? Glacial tree refugia in northern Europe. *Trends in Ecology & Evolution* **28**:696–704.

Vincze T, Posfai J, Roberts RJ. 2003. NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acids Research* **31**:3688–3691.

Wachowiak W, Boratynska K, Cavers S. 2013. Geographical patterns of nucleotide diversity and population differentiation in three closely related European pine species in the *Pinus mugo* complex. *Botanical Journal of the Linnean Society* **172**:225–238.

Wachowiak W, Celinski K, Prus-Glowacki W. 2005. Evidence of natural reciprocal hybridisation between *Pinus uliginosa* and *P-sylvestris* in the sympatric population of the species. *Flora* **200**:563–568.

Wachowiak W, Palmé AE, Savolainen O. 2011. Speciation history of three closely related pines *Pinus mugo* (T.), *P. uliginosa* (N.) and *P. sylvestris* (L.). *Molecular Ecology* **20**:1729–1743.

Wachowiak W, Prus-Glowacki W. 2009. Different patterns of genetic structure of relict and isolated populations of endangered peat-bog pine (*Pinus uliginosa* Neumann). *Journal of Applied Genetics* **50**:329–339.

Wachowiak W, Zukowska WB, Wojkiewicz B, Cavers S, Litkowiec M. 2016. Hybridization in contact zone between temperate European pine species. *Tree Genetics & Genomes* **12:**48.

Wagner DB, Dong J, Carlson MR, Yanchuk AD. 1991. Paternal leakage of mitochondrial DNA in *Pinus*. *Theoretical and Applied Genetics* **82**:510–514.

Wang B, Wang XR. 2014. Mitochondrial DNA capture and divergence in *Pinus* provide new insights into the evolution of the genus. *Molecular Phylogenetics and Evolution* **80**:20–30.

Wang XR, Tsumura Y, Yoshimaru H, Nagasaka K, Szmidt AE. 1999. Phylogenetic relationships of Eurasian pines (*Pinus*, Pinaceae) based on chloroplast rbcL, MATK, RPL20-RPS18 spacer, and TRNV intron sequences. *American Journal of Botany* **86**:1742–1753.

Weltzin JF, Bridgham SD, Pastor J, Chen J, Harth C. 2003. Potential effects of warming and drying on peatland plant community composition. *Global Change Biology* **9**:141–151.

Whitlock BA, Hale AM, Groff PA. 2010. Intraspecific inversions pose a challenge for the trnH-psbA plant DNA barcode. *PLoS One* **5**:e11533.

Wimmer F. 1837. Uber die Zapfen von *Pinus silvestris*, *pumilio und uliginosa.* Verhandlungender botanischen Section. Übersicht der Arbeiten über Veränddder Schlesischen Naturhistorische Geselschaft 1837–1840: 93–98.

Supplementary material

Supplementary Table S1. Analysed loci and genotyping method.

Marker name	**Locus discovery**	**PCR primers (forward, reverse)**	**Product size**	**Position in amplicon, bp**[a]	**Restriction enzyme**	**Incubation temperature**	**Polymorphism**	**No. bands upon digestion**
PR5	Donnelly et al. 2017: locus 3	ATTCCTGTGCTTGGTTGGGA GGCGCTTACCCACACACTTA	570	279	DraI	37°	G / T	1 / 2
PR7	Donnelly et al. 2017: locus 5	TGAGTTCGTTGACCGCGTAA TCAGGCGAGCTTGTGCTTTA	514	409	DraI	37°	A / C	2 / 1
PR13	Donnelly et al. 2017: locus 11	GATCGGGTCGGAGGCATAAT AGTTGAAGCAAGCCAGCAAG	369	246	MseI	65°	C / A	2 / 1
PR14	Donnelly et al. 2017: locus 12	TTTACGAAGCCCTTGGCGAT CTGAACCGGGTGTAGCCTTT	548	382	MseI	65°	G / T	1 / 2
PR15	Donnelly et al. 2017: locus 13	CATCCTCTCCTCTCGATGGC GCTTTTGGCTTGGTGCGAAT	358	155	BpiI	37°	G / T	2 / 1
PR19	Donnelly et al. 2017: locus 17	CGGAGCGAGGTGAAGAAACT GCGAGAAGCAGTAGTGGGTT	593	413	DraI	37°	G / T	1 / 2
PR20	Donnelly - not publ.	GTTCCTACGATCCAGCCAGG ACCATGGATTCTTCGGACGG	382	173	MvaI	37°	C / A	3 / 2
PR21	Donnelly et al. 2017: locus 18	TCCGATGATGAGGTGGAGGT AGTTGAAGGCAGGAAGGTCG	522	421	HincII	37°	G / T	2 / 1
PR24	Donnelly et al. 2017: locus 19	TGCATTCTGGCTGGCTTTCT GGCGTCGATAGACTCGGTTT	434	272	BshNI	37°	T / G	1 / 2
PR25	Donnelly et al. 2017: locus 20	GGCATGTCCGCTATGGAAGT AGGCTCCGGAAGTACCTGT	398	123	BtsCI	55°	G / T	1 / 2
PR29	Donnelly et al. 2017: locus 22	GGTTGGTTGATCCATCCGGT CCGGCTTGGGTACGTCTTTT	558	226	BtsCI	55°	G [b]	1 / 2
PR30	Donnelly - not publ.	ACTTACATTGACCGGCGGAT CACACATCTAGGGCACAGGG	301	194	Alw26I	37°	G / T	1/ 2
PR31	Donnelly et al. 2017: locus 23	TGCGACCTGTGAATGGATGT CGGCGGTTCTAGCCTTGATT	558	293	VspI	37°	G / T	1 / 2
PR32	Donnelly - not publ.	ACCCTCCTTCAACTGATGCG CCTCAACCAACCGTCAGTCA	407	127	PfeI	37°	G / T	1 / 2
PR34	Donnelly - not publ.	GAACCCCCTCTTGCCTTGAT TTCGTGACGGTCCAATTCCA	398	176 -184	na	na	IN/DEL	na

[a] primers excluded, [b] monomorphic in this data set

Supplementary Table 2. Major haplotypes and their frequency in the analysed taxa.

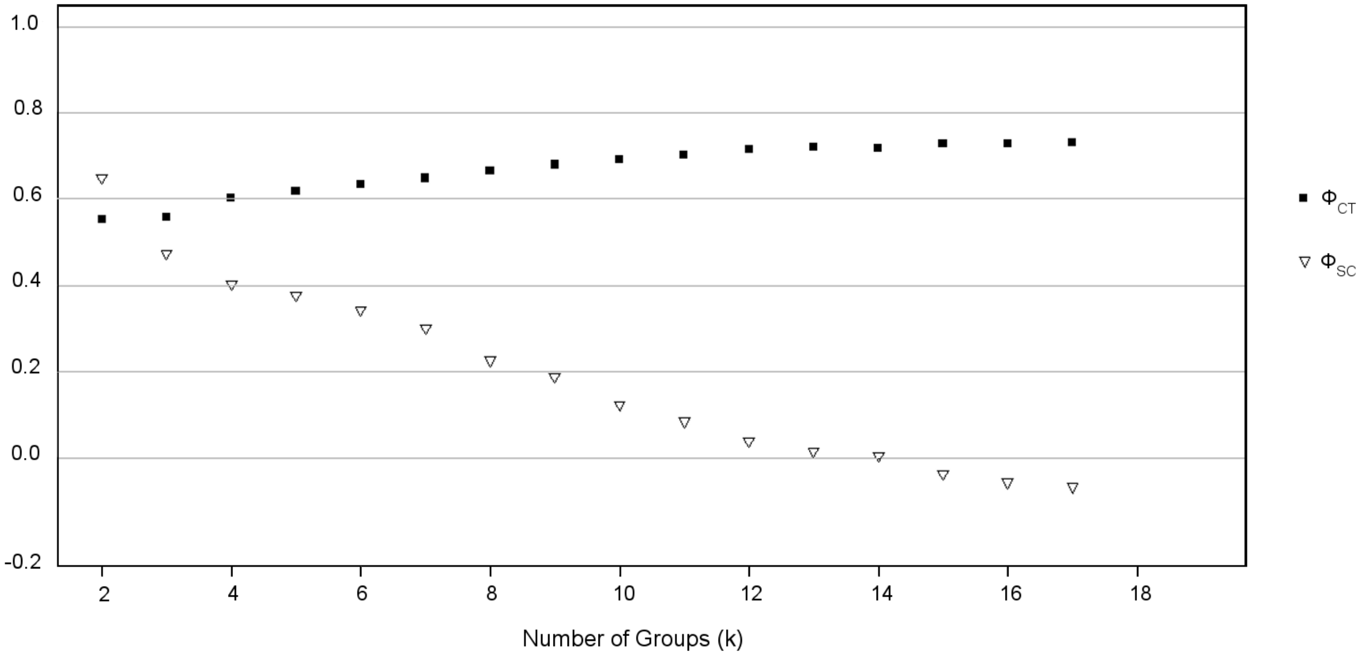
Marker	**PR5**	**PR7**	**PR13**	**PR14**	**PR15**	**PR19**	**PR20**	**PR21**	**PR24**	**PR25**	**PR30**	**PR31**	**PR32**	**PR34***	***nad1****	***nad7****	**Frequency [% (N of samples)]**			
																	ALL	**PUG**	**PM**	**PUN**
H3	G	A	C	G	G	T	C	G	T	G	G	G	G	IN	a	A	3.6 (13)	3.8 (5)	7.2 (8)	0.0 (0)
H4	T	C	C	G	G	G	C	G	T	G	G	G	G	DEL	a	A	1.4 (5)	3.8 (5)	0.0 (0)	0.0 (0)
H5	G	C	C	G	G	T	C	G	T	G	G	G	G	DEL	a	A	4.7 (17)	12.8 (17)	0.0 (0)	0.0 (0)
H6	G	C	C	G	G	G	C	G	T	G	G	G	G	DEL	a	A	11.0 (40)	6.8 (9)	27.9 (31)	0.0 (0)
H12	G	C	C	T	G	G	C	G	T	G	G	G	G	DEL	a	A	1.9 (7)	0.8 (1)	5.4 (6)	0.0 (0)
H13	G	A	C	G	G	G	C	G	T	G	G	G	G	DEL	a	A	4.1 (15)	3.8 (5)	9.0 (10)	0.0 (0)
H14	G	A	C	G	G	G	C	G	G	G	G	G	G	DEL	a	A	2.8 (10)	3.0 (4)	5.4 (6)	0.0 (0)
H15	G	A	C	G	G	T	C	G	T	G	G	G	G	DEL	a	A	1.4 (5)	3.8 (5)	0.0 (0)	0.0 (0)
H21	T	C	C	T	T	G	A	T	T	T	T	G	G	DEL	a	B	9.4 (34)	24.8 (33)	0.9 (1)	0.0 (0)
H37	G	C	C	T	G	G	C	G	T	T	G	G	T	DEL	a	B	1.4 (5)	3.8 (5)	0.0 (0)	0.0 (0)
H40	G	C	C	T	G	G	C	G	T	T	G	G	T	DEL	a	A	3.3 (12)	3.0 (4)	7.2 (8)	0.0 (0)
H41	G	A	C	G	G	T	C	G	T	G	G	G	T	IN	a	A	1.4 (5)	0.0 (0)	4.5 (5)	0.0 (0)
H44	G	C	C	G	G	T	C	G	T	T	G	G	G	DEL	a	A	4.4 (16)	0.0 (0)	14.4 (16)	0.0 (0)
H47	G	C	C	T	G	G	C	G	T	T	G	G	G	DEL	a	A	3.9 (14)	0.0 (0)	12.6 (14)	0.0 (0)
H50	G	C	A	G	G	G	C	G	T	G	G	G	G	DEL	b	A	21.2 (77)	0.0 (0)	0.0 (0)	64.7 (77)
H52	G	C	A	T	G	G	C	G	T	G	G	G	G	DEL	b	A	5.0 (18)	0.0 (0)	0.0 (0)	15.1 (18)
H54	G	C	A	T	G	G	C	G	T	G	G	T	G	DEL	b	A	5.5 (20)	0.0 (0)	0.0 (0)	16.8 (20)
																	86.2 (313)	**69.9 (93)**	**94.6 (105)**	**96.6 115)**
																Minor haplotype number	**37**	**28**	**6**	**3**
																Minor haplotype frequency	**13.8**	**30.1**	**5.4**	**3.4**

* PR34: IN – TCATCAATC, DEL– --A—AAGA*;* *nad1*: variants as in Naydenov et al. 2007; *nad7*: variants as in Soranzo et al. 2000;

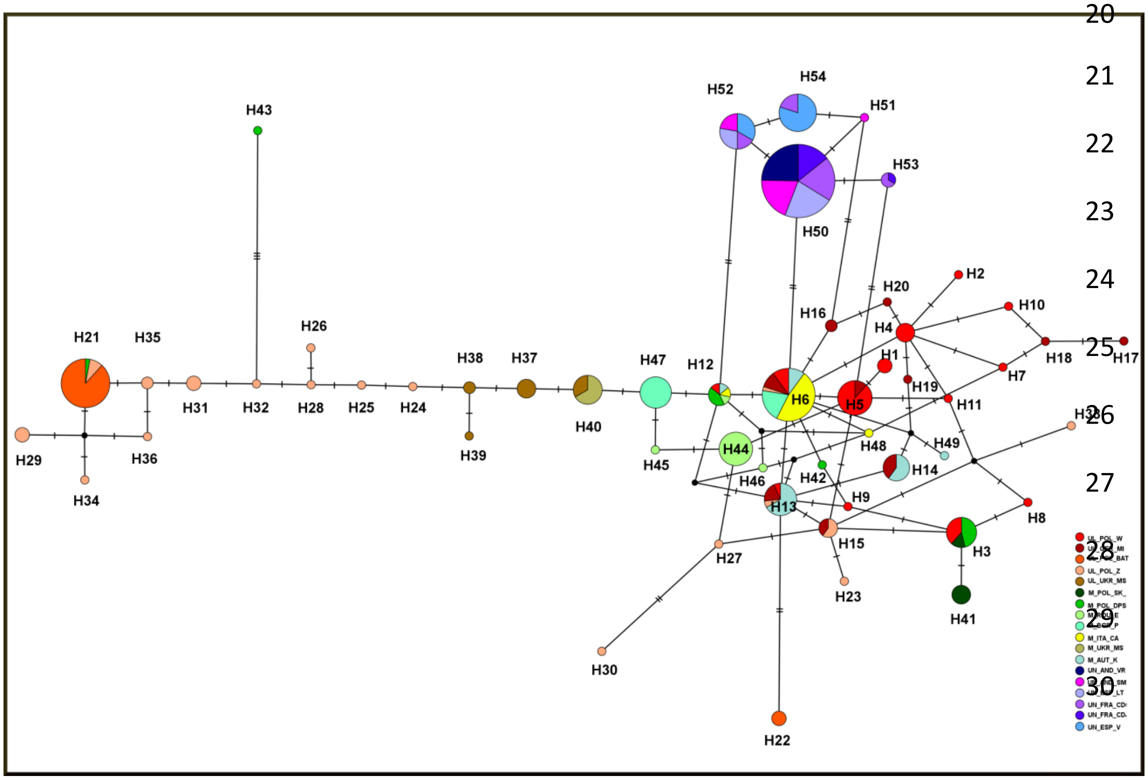
Table S3. Distribution of major haplotypes (present in at least 3 individuals) detected in studied pines taxa and populations.

Haplotype **Population**	H3	H4	H5	H6	H12	H13	H14	H15	H21	H37	H40	H41	H44	H47	H50	H52
UL_POL_W	x	x	x	x	x	x										
UL_GER_MI			x	x		x	x	x								
UL_POL_BAT									x							
UL_POL_Z				x		x			x							
UL_UKR_MS										x	x					
M_POL_SK	x											x				
M_POL_DPS	x				x				x							
M_AUT_K				x	x	x	x	x								
M_UKR_MS											x					
M_ROU_E					x								x			
M_BGR_P				x										x		
M_ITA_CA				x	x											
UN_AND_VR															x	x
UN_AND_SM															x	
UN_ESP_LT															x	x
UN_ESP_V																x
UN_FRA_CDJ															x	
UN_FRA_CDC															x	x
P. uliginosa	x	x	x	x	x	x	x	x	x	x	x					
P.mugo	x			x	x	x	x	x	x		x	x	x	x		
P. uncinata															x	x

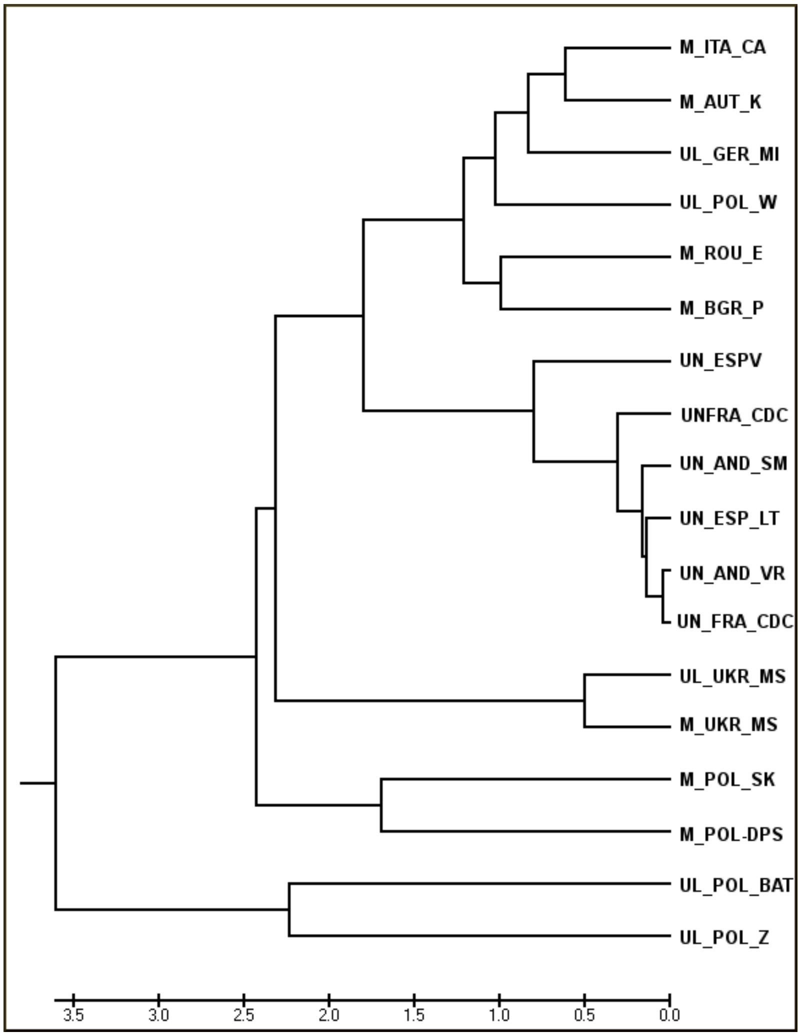
Supplementary Figure S1. Spatial analysis of molecular variance (SAMOVA). Results of the spatial analysis of molecular variance showing the genetic affinity between 18 populations of three different pine species. The most likely subdivisions of the whole distribution area consisted of four groups, when the increment of F_{CT} was the largest ($\Delta\Phi_{CT} = 0.04$).



Supplementary Figure S2. Median-joining network of haplotypes detected at 16 *mt*DNA regions in the taxa from the *P. mugo* complex. Size of the circles are proportional to haplotype frequencies, hatch marks represent numbers of nucleotide differences between them and shading indicates specific populations.



Supplementary Figure S3. UPGMA phylogenetic tree of 18 studied pine populations. The tree is constructed using genetic distances based on number of differences between all polymorphic *mt*DNA sites. The tree is drawn to scale with the branch lengths representing the evolutionary distances, the scale can be seen at the bottom of the tree.



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Research Article

Molecular and paleo-climatic data uncover the impact of an ancient bottleneck on the demographic history and contemporary genetic structure of endangered *Pinus uliginosa*

Bartosz Łabiszak[1]*, Julia Zaborowska[1], Błażej Wójkiewicz[2], and Witold Wachowiak[1,2]

[1]Institute of Environmental Biology, Adam Mickiewicz University, Uniwersytetu Poznanskiego 6, Poznan 61-614, Poland
[2]Institute of Dendrology, Polish Academy of Sciences, Parkowa 5, Kórnik 62-035, Poland
*Author for correspondence. E-mail: bartosz.labiszak@amu.edu.pl

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Abstract With the current rate of biodiversity loss, conservation management practices require a comprehensive understanding of eco-evolutionary relationships, history, and genetic structure of species. Assessments of genetic diversity are crucial, especially in rare, endemic, or threatened forest tree species with small and isolated populations, such as peat bog pine (*Pinus uliginosa* N.). Here, we used a novel approach, combining genetic diversity assessment, ecological niche modeling, and population demography inference to explore the complex history of a few remnant populations of this endangered pine. To asses the relative influence of isolation and fragmentation on genetic diversity in the taxonomic context, the patterns of genetic variation found in *P. uliginosa* were contrasted with those observed in its close relatives with much bigger distribution ranges and larger populations (*Pinus sylvestris*, *Pinus mugo*, and *Pinus uncinata*). We found a similar level of genetic diversity across the species at nuclear loci but contrasting patterns of variability distribution at chloroplast markers. We detected the signatures of an ancient genetic bottleneck dated at around 26 400 years ago, indicating a drastic reduction in the population size of *P. uligionosa* during the Last Glacial Maximum. In addition, we found substantial differentiation between current populations as a result of enhanced genetic drift during long-lasting isolation. The research suggests potential conservation management strategies for peat bog pine and emphasizes the importance of using complementary approaches for their successful development.

Key words: bottleneck, conservation management, ecological niche modeling, endangered species, phylogeographic modeling, pines, population isolation, population structure.

1 Introduction

Forest tree species are the foundation of ecosystems, which make up a vast majority of the world's terrestrial biodiversity (Petit & Hampe, 2006). The importance of ecosystem services provided by trees cannot be overstated (FAO, 2018). Loss of genetic diversity, as an outcome of numerous threats, including habitat destruction, fragmentation, pollution, environmental changes due to unsuitable use, and exploitation of land, may lead to particularly severe consequences for forest ecosystems (Van Mantgem et al., 2009; Shearman et al., 2012; Fettig et al., 2013; Riitters et al., 2016). This is because many forest tree species have a long lifespan, have limited dispersal ability, and slowly reach their reproductive maturity (Schaberg et al., 2008).

Decline in genetic diversity is especially dangerous for rare, endemic, or threatened tree taxa with small and isolated populations (Pautasso, 2009). These species are extremely vulnerable to falling into the so-called "extinction vortex"–a feedback cycle with gradually decreasing population size, a result of increased inbreeding and genetic drift – leading to reduced population fitness and further demographic instability (Frankham et al., 2010). Assessments of genetic variation and examination of demographic, ecological, and evolutionary mechanisms influencing the population structure may significantly improve our predictions of likely responses of tree species, in particular of the species consisting of small and isolated populations, to environmental changes. Studies based on neutral molecular markers may improve our ability to quantify patterns of genetic variation and serve as a tool for evaluating the extinction risk of taxa (Spielman et al., 2004; Jump et al., 2009).

The peat bog pine (*Pinus uliginosa*) is an example of endangered, geographically restricted, and ecologically specialized coniferous tree species. Its natural distribution area is limited to a few known isolated stands on bog environments in Poland, Germany, Czech Republic, and Ukraine (Neumann, 1837; Wimmer, 1837; Boratyński, 1994).

In recent years, especially in Poland, a severe decrease in the population size of the species has been observed, coupled with a lack of natural regeneration (Danielewicz & Zieliński, 2000). Major threats and concerns for *P. uliginosa* and other peatland plant communities in Central Europe are associated with drying out of the bog areas, caused by both human intervention (e.g., drainage) and increased duration or severity of droughts due to climatic changes (Holt, 1990; Weltzin et al., 2003; Turetsky et al., 2015). In addition, genetic factors, such as loss of genetic diversity, increased inbreeding, and decreased evolutionary potential for adaptation may also effectively elevate its extinction risk.

Previous studies on *P. uliginosa* were mostly focused on its complex evolutionary history and on the taxonomic relationships with its close relatives: *Pinus sylvestris* and pines from a broader group called *Pinus mugo* complex, including dwarf mountain pine (*P. mugo* Turra) and Pyrenean pine (*Pinus uncinata* Ramond) (Hamernik & Musil, 2007; Boratyńska & Lewandowska, 2009; Wachowiak & Prus-Glowacki, 2009; Łabiszak et al., 2019). These species diverged from their common ancestors about 5 million years ago (Wachowiak et al., 2011). Little is known, however, about the demographic history of pet-bog pine in contrast to the closely related, non-threatened species with an incomparably bigger distribution ranges and population sizes. Consequently, the relative influence of isolation, fragmentation, and population size fluctuations on contemporary levels of its genetic diversity are mostly unknown. It is also unclear how the interplay of these factors can affect the species extinction risk at present. These important questions can now be addressed due to the recent advances in biological and computational sciences, including availability of novel inferential methods and machine learning algorithms implemented, for instance, in Bayesian computation and ecological niche modeling (Tarca et al., 2007; Cornuet et al., 2008; Elith et al., 2011). These new research approaches have greatly improved our ability to utilize large datasets and have thus facilitated modeling of biological and evolutionary scenarios (Elith & Leathwick, 2009; Csilléry et al., 2010; Medley, 2010; Temunović et al., 2013; Koo et al., 2017).

Here, we used current genetic and paleo-environmental data to investigate the effects of past demographic events on the contemporary population structure and distribution of genetic diversity within and between the remaining stands of the peat bog pine. We combined population genetic methods, phylogeographic modeling within coalescent-based approximate Bayesian computation (ABC) framework, and ecological niche modeling to explore the population history of *P. uliginosa*. We investigated how geographic isolation and population size fluctuations affected the contemporary genetic structure of this species. Specifically, we (i) assessed the level of genetic diversity in remnant populations of peat bog pine and compared it with several reference taxa; (ii) established whether changes in population size (recent or more distant in the past) had left detectable signs of depleted genetic diversity in *P. uliginosa*; (iii) examined the presence of the population structure as a likely result of long isolation and independent evolutionary history of the remaining *P. uliginosa* populations. Our results provide new insights into the demographic history of peat bog pine, which may facilitate the development of conservation strategies and preservation of genetic resources of this endangered tree species.

2 Materials and Methods

2.1 Sampling and genotyping

Six known allopatric, natural populations of peat bog pine (138 individuals, Table 1) were used in the study. In addition, we examined 15 reference populations sampled across European ranges of three closely related pine species: Scots pine (*Pinus sylvestris*), dwarf mountain pine (*Pinus mugo*), and Pyrenean pine (*Pinus uncinata*) (Table 1). Sample sizes ranged from 14 to 40 trees per population (mean of 25 individuals), resulting in a total of 539 specimens analyzed. DNA samples were extracted from needle tissue, following the standard CTAB protocol (Dumolin et al., 1995). All samples were amplified and genotyped at 9 nuclear and 12 chloroplast microsatellite markers (nSSR and cpSSR hereafter). Specifically, we used nSSR loci: *psyl2*, *psyl25*, *psyl36*, *psyl42*, *psyl44*, *psyl57* (Sebastiani et al., 2012), *ptTX3025*, *ptTX4001*, and *ptTX4011* (Zhou et al., 2002); and cpSSR loci: *PCP1289*, *PCP26106*, *PCP30277*, *PCP36567*, *PCP41131*, *PCP45071*, *PCP87314*, *PCP102652* (Provan et al., 1998), *Pt15169*, *Pt26081*, *Pt30204*, and *Pt71936* (Vendramin et al., 1996). Genotyping methodology and raw nSSR and cpSSR data of *P. mugo* and *P. sylvestris* were derived from earlier studies (Wójkiewicz et al., 2016; Wójkiewicz & Wachowiak, 2016; Żukowska et al., 2017; Żukowska & Wachowiak, 2017). To avoid bias in assessing the population structure due to the presence of false homozygotes (Chapuis & Estoup, 2007), the frequency of null alleles in the dataset was assessed using Micro-Checker v. 2.2.3 (Van Oosterhout et al., 2004).

2.2 Genetic diversity and divergence

We employed the F_{ST}-based approach implemented in BayeScan v. 2.1 to test for potential selection at the loci (or closely linked regions) among populations. Further, to detect the loci that deviate from the Hardy–Weinberg equilibrium in all studied populations and species, we used the exact test based on the Markov Chain Monte Carlo (MCMC) algorithm with Bonferroni correction implemented in GENPOP v. 4.6 (Rousset, 2008). We also used this software to conduct a Bonferroni-corrected test for linkage disequilibrium (LD). Basic genetic diversity parameters were calculated in GenAlEx v. 6.5 (Peakall & Smouse, 2012) and Haplotype Analysis v. 1.05 software (Eliades, 2009) for nSSR and cpSSR data, respectively. Moreover, rarefied allelic richness (nA_R), private alleles rarefied richness (P_{AR}), observed heterozygosity (H_O), and expected heterozygosity (H_E) were estimated using FSTAT v. 2.9.4 (Goudet, 1995, 2001). Further, inbreeding coefficient (F_{IS}) with 95% confidence intervals (*CI*) on each estimate (999 bootstrap) was calculated in R package diveRsity (Keenan et al., 2013).

Genetic differentiation between the four pine taxa was assessed by permutation tests (10 000 permutations) in FSTAT for nA_R, H_O, H_E, and F_{IS}. In addition, to estimate the proportion of the overall genetic variation resulting from differentiation among the species, we calculated pairwise F_{ST} using both nSSR and cpSSR markers in Arlequin v. 3.5 (Excoffier & Lischer, 2010).

Table 1 Locations of 21 studied populations of four European pine taxa

Species	Acronym	Population	Latitude N	Longitude E	Altitude (m)
P. uliginosa					
	UL_W	Poland, Sudety Mts., Low Silesian Pinewood, Węgliniec	51°17′50″	15°14′20″	190
	UL_SW	Poland, Sudety Mts., Low Silesian Pinewood, Stary Węglowiec	51°19′00″	15°12′00″	185
	UL_MI	Germany, Bavaria, Mittenwald	47°28′50″	11°16′27″	856
	UL_BAT	Poland, Sudety Mts., Wielkie Torfowisko Batorowskie reserve	50°27′32″	16°23′01″	710
	UL_Z	Poland, Sudety Mts., Zieleniec reserve	50°20′54″	16°24′42″	755
	UL_MS	Ukraine, Gorgany Mts., Mshana	48°40′33″	23°55′19″	830
P. mugo					
	M_SUD	Poland, Sudety Mts., Kocioł Łomniczki	50°44′53″	15°44′37″	1020
	M_CA	Italy, Carnic Alps, Passo di Pramollo	46°32′45″	13°15′35″	1530
	M_CAR	Romania, Southern Carpathians, Muntii Bucegi	47°34′03″	24°48′00″	2070
	M_BM	Bosna and Hercegovina, Bjelasnica Mts., near Sarajewo	43°45′00″	18°13′08″	2120
P. uncinata					
	UN_WP	Spain, West Pyrenees, Belagua	43°00′00″	01°01′00″	1800
	UN_EPC	France, East Pyrenees, Col de Jau	42°39′19″	02°15′22″	1520
	UN_CP	Spain, Central Pyrenees, Pico de la Bonaiqua	42°39′48″	00°57′44″	2100
	UN_EPV	Spain, East Pyrenees, Vall de Nuria	42°20′45″	02°06′15″	2200
	UN_SG	Spain, Sierra de Gudar, Valldelinares	40°28′49″	−00°41′51″	2000
	UN_MC	France, Massif Central, Col de la Croix-Morand	45°36′00″	02°50′59″	1400
	UN_EPR	Andorra, East Pyrenees, Val de Ransol	42°35′02″	01°38′21″	2025
P. sylvestris					
	PS_DM	Serbia, Divčibare Mts.	44°06′00″	19°59′24″	977
	PS_FIN	Finland, Joutsa	64°41′24″	25°45′00″	50
	PS_SM	Andorra, St. Miguel d'Engolasters	42°31′28″	01°34′12″	1640
	PS_TM	Poland, Pieniny Mts.	49°25′12″	20°21′36″	750

2.3 Population structure

For illustration of the genetic relationships between *P. uliginosa* populations, we took advantage of two complementary approaches: (i) Principal coordinate analysis (PCoA), based on average Nei's genetic distances (Nei, 1973) for both types of markers, conducted in GenAlEx v. 6.5 software (Peakall & Smouse, 2012); and (ii) an individual-based Bayesian clustering method implemented in STRUCTURE v. 2.3.4 (Pritchard et al., 2000; Falush et al., 2007; Hubisz et al., 2009) using the nSSR dataset. In this analysis, we used the admixture model with no previous population information and correlated allele frequencies were assumed due to the shared ancestry. Twenty independent runs were performed for each K, from $K = 2$ to 8, with burn-in lengths of 500 000 and 750 000 iterations. To detect the number of genetic groups that best fit our data, both the likelihood estimate (Pritchard et al., 2000) and the Evanno method (Evanno et al., 2005) implemented in the software STRUCTURE HARVESTER (Earl & Vonholdt, 2012) were used. STRUCTURE plots were visualized using STRUCTURE PLOT v. 2.0 web application (Ramasamy et al., 2014). Furthermore, the Mantel test was also conducted to verify the hypothesis of isolation by distance (IBD) between populations. For this purpose, we used GenAlEx v. 6.5 software, with 1000 random permutations of the relationship between genetic differentiation, quantified as $F_{ST}/(1-F_{ST})$, and the corresponding geographical distance matrices between six *P. uliginosa* populations.

2.4 Demographic history

We used two different approaches to elucidate the demographic history of *P. uliginosa* and test whether past environmental changes coupled with potential population size variation have left detectable signatures of a genetic bottleneck.

In the first step, we employed the ABC framework in DIYABC v. 2.1.0 (Cornuet et al., 2014) to explore six plausible demographic history scenarios of *P. uliginosa* based on nSSR and cpSSR loci: Scenario 1 assumed constant population size; Scenarios 2 and 3 assumed population expansion (early and more recent, respectively); Scenarios 4 and 5 assumed population decline (early and more recent, respectively); and Scenario 6 assumed population bottleneck followed by re-expansion (Fig. 1). Following the results of STRUCTURE analysis, we considered *P. uliginosa* populations as a single unstructured group (see Section 3). Although a substantial number of complex scenarios can be simulated using ABC, we limited our analysis only to those six, as overfitting with numerous models could possibly result in poor parameter estimates (Bertorelle et al., 2010). We simulated 1×10^6 datasets with four single sample summary statistics (mean number of alleles, mean Nei's genetic diversity index, mean allele size variance, and mean Garza and Williamson's M) for each scenario using prior parameter settings presented in Table S3. The best supported scenario (with highest posterior probability) was chosen based on estimates of both direct and logistic regression analysis of the posterior

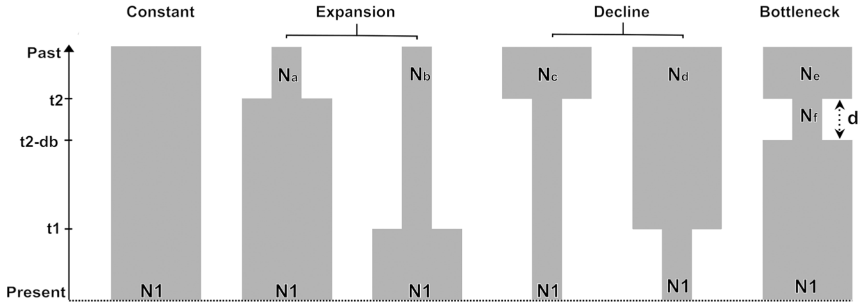

Fig. 1. Schematic representation of the six demographic scenarios tested by approximate Bayesian computation (ABC) for *Pinus uliginosa* based on nuclear and chloroplast microsatellite data (nSSR, cpSSR).

distribution of demographic parameters, and both Type I and Type II errors were estimated to evaluate the power of the model. Model checking was also performed using principal component analysis to evaluate whether the observed data fell within the range of simulated datasets. Finally, posterior parameter distribution was estimated for the best supported scenario. To avoid reporting time parameters in a number of generations (default output in DIYABC), we transformed them into calendar years (20–25 years), assuming the average generation time in those pines (Carlisle & Brown, 1968; Wachowiak et al., 2011).

As the second approach, we used the allelic frequency test, which took advantage of the transient deficiency in the number of alleles found in population during the recent bottleneck (i.e., less alleles than expected from the observed heterozygosity, assuming mutation-drift equilibrium), and carried out the analysis on nSSR markers using the software BOTTLENECK v. 1.2.02 (Cornuet & Luikart, 1996). We used recommended parameters accurate for most microsatellites under the two-phase model (TPM), which allows multiple-step mutations and is generally advised for markers with a mutation model different than strict step-wise mutation (SMM) (i.e., proportion of SMM in the TPM = 0.000 and a variance of the geometric distribution for TPM = 0.36) (Di Rienzo et al., 1994). We ran 1000 iterations of this analysis and used one-tailed Wilcoxon's sign-rank test to examine the significance of the heterozygosity excesses that might indicate a bottleneck.

We further explored the effects of a putative genetic bottleneck on population size by estimating the effective population sizes (N_e) for *P. uliginosa* populations using the molecular co-ancestry method of Nomura (2008) implemented in NeEstimator v. 2 (Do et al., 2014). We compared the outcomes to the reference taxa of *P. sylvestris*, *P. mugo*, and *P. uncinata*, characterized by a much wider distribution ranges and bigger census population sizes.

2.5 Species distribution modeling

To further explore the demographic history of *P. uliginosa*, species distribution models (SDMs) were used as complementary and independent of any genetic approaches for tracking changes in the species distribution range. Due to the restricted and island-like character of the distribution range of *P. uliginosa*, georeferenced occurrences for this species were limited to 10 records: 6 sampling sites used for this study (Table 1) and 4 additional occurrences obtained from the Global Biodiversity Information Facility (GBIF, http://www.gbif.org). Environmental variables used to construct SDMs were obtained at 2.5 arcmin resolution from WorldClim (https://www.worldclim.org; Hijmans et al., 2005) and ENVIREM (Title & Bemmels, 2018) databases. The complete list of explanatory variables, after the exclusion of redundant ones based on the results of a pairwise Pearson correlation test (correlation coefficient <0.75) incorporated in ENMTools software (Warren et al., 2010), is presented in Table S5. SDMs were constructed using a machine-learning technique with the maximum entropy approach implemented in Maxent v. 3.4.1 (Dudik et al., 2004; Phillips et al., 2006, 2017) to track the potential distribution of *P. uliginosa* throughout time, from the Last Glacial Maximum (LGM; ca. 21.5 ka) and Mid Holocene (ca. 6 ka) to the present day. After assessing the relative contribution of variables to the overall Maxent model performance, habitat suitability maps for each time-point were constructed using ArcGIS software (ESRI, 2012) and niche overlaps were measured using Schoener's D statistic (Schoener, 1968) implemented in ENMTools.

3 Results

3.1 Genetic diversity

Evidence for the presence of null alleles was found in three loci. However, as their frequencies did not exceed the threshold (0.2) over which null alleles can result in a significant underestimate of expected heterozygosity (Chapuis & Estoup, 2007; Belletti et al., 2012), they were kept and used in further analysis. The nuclear microsatellites appeared to be polymorphic in most populations, with a total of 77 alleles observed (Table S1), and the number of alleles per locus ranged from 3 to 16. The mean observed heterozygosity ($H_O = 0.392$) for these markers was slightly lower than the mean expected heterozygosity ($H_E = 0.412$). We did not detect any loci that showed departure from neutrality in our dataset. Deviations from the Hardy–Weinberg equilibrium were detected at some loci, based on skewed alleles frequency spectra (Table S1) but none of the loci showed any consistent pattern of deviation from HWE across all the populations. Correspondingly, there is no evidence for linkage disequilibrium at any loci. Therefore, all loci were kept and used in the subsequent analysis. At nine nuclear microsatellite loci, we detected three populations (UL_BAT, UN_WP and PS_TM) with F_{IS} values that significantly indicated inbreeding (95% *CIs* did not overlap 0). However, at the species level, the values of F_{IS} were low and not significantly different from each other (*P*-values >0.05) (Table 2). Rarefied allelic richness (nA_R) was similar across studied populations, with values ranging from 2.36 to 3.93, based on a minimum number of 12 diploid individuals. We detected significant differences in allelic richness between species (*P*-values <0.05) and both allelic richness (nA_R) and private allelic richness (P_{AR}) were lower in *Pinus uliginosa*, as compared to other examined species (Table 2). Estimates of genetic diversity based on nSSR H_O within each of the species were similar across all populations, as indicated by the lack of significant differences between genetic clusters after 10 000 permutations (*P*-values >0.05) (Fig. 2; Table 2). Interestingly, both the highest ($H_O = 0.486$) and the lowest ($H_O = 0.270$) observed heterozygosity values were detected in populations of *P. uliginosa* (populations UL_SW from Poland and UL_MS from Ukraine, respectively).

Nine chloroplast loci proved to be polymorphic in all analyzed populations, with a total of 100 alleles detected (Table S1), and the number of alleles per locus ranged from 3 to 18. The total number of haplotypes recorded was 345, with an abundance of both private haplotypes (89% of all) and singleton haplotypes (81% of all). Only 32 haplotypes, hereafter called major haplotypes, were present in more than two individuals (Table S2). The number of private haplotypes within populations ranged from 2 to 42, and haplotype diversity (H_d) ranged between 0.696 and 1. Population UN_SG from Spain was characterized by the lowest haplotype diversity; however overall, at the species level, the lowest haplotype diversity was found in *P. uliginosa* (Fig. 2; Table 2). Major haplotypes appeared to be mostly species-specific (although, haplotype sharing was found in four cases) and almost exclusively found in *P. uliginosa* and *P. uncinata* populations, except H245, recorded in *P. sylvestris*, and H33, found in *P. mugo*. Interestingly, among all studied species, populations of *P. uliginosa* were characterized by the

Table 2 Genetic estimators for nSSR and cpSSR for each population and taxa

	nSSR								cpSSR					
Population	N	A	nA_E	nA_R	P_{AR}	H_O	H_E	F_{IS} (95% CI)	Ah	cpA_E	A_P	cpA_R	H_d	D^2sh
UL_W	30	3.78	2.06	3.11	0.01	0.395	0.406	0.006 (−0.062; 0.144)	13	10.94	11	9.65	0.959	25.408
UL_SW	24	3.67	2.45	3.39	0.09	0.486	0.486	−0.043 (−0.170; 0.163)	10	5.54	2	6.28	0.855	7.808
UL_MI	20	3.67	2.28	3.44	0.02	0.422	0.457	0.048 (−0.073; 0.172)	19	16.00	7	11.20	0.978	5.511
UL_BAT	31	3.67	1.78	3.02	0.05	0.373	0.390	0.052 (0.001; 0.189)	10	5.63	3	6.15	0.851	5.668
UL_Z	19	3.78	2.13	3.42	0.01	0.381	0.420	0.065 (−0.083; 0.071)	18	16.67	13	12.04	0.989	7.187
UL_MS	14	2.44	1.53	2.36	0	0.257	0.270	0.026 (−0.074; 0.114)	3	2.97	2	2.00	0.714	19.176
M_SUD	30	3.11	1.59	2.70	0.04	0.363	0.336	0.004 (−0.141; 0.074)	27	25.00	25	12.37	0.993	13.595
M_CA	25	3.11	2.04	2.95	0	0.400	0.425	0.026 (−0.065; 0.077)	25	25.00	25	13.00	1.000	13.156
M_CAR	25	3.67	2.13	3.28	0.03	0.360	0.422	0.218 (−0.025; 0.307)	23	21.55	23	12.39	0.993	12.863
M_BM	25	4.44	2.10	3.81	0.26	0.427	0.469	0.064 (−0.039; 0.126)	25	25.00	25	13.00	1.000	11.671
UN_WP	24	3.67	2.19	3.22	0	0.363	0.403	0.051 (0.029; 0.191)	18	13.71	9	10.66	0.967	7.751
UN_EPC	24	3.44	2.00	3.13	0	0.382	0.394	0.025 (−0.057; 0.127)	20	16.94	9	11.53	0.982	6.738
UN_CP	24	3.89	2.10	3.39	0.12	0.407	0.408	−0.017 (−0.102; 0.119)	18	13.71	7	10.66	0.967	8.083
UN_EPV	24	3.78	1.97	3.34	0.11	0.366	0.395	0.020 (−0.117; 0.058)	21	16.00	9	11.65	0.978	8.810
UN_SG	24	3.56	2.08	3.15	0.01	0.398	0.403	−0.015 (−0.051; 0.066	5	3.00	2	3.11	0.696	20.805
UN_MC	24	4.00	2.45	3.49	0.01	0.450	0.450	−0.009 (−0.146; 0.063)	20	16.94	9	11.53	0.982	5.492
UN_EPR	24	3.56	2.06	3.12	0.06	0.324	0.397	0.103 (−0.094; 0.039)	18	13.54	12	9.75	0.957	9.239
PS_DM	26	5.22	2.31	3.93	0.61	0.462	0.432	−0.070 (−0.039; 0.126)	23	21.13	21	12.16	0.991	4.380
PS_FIN	25	4.89	2.06	3.83	0.15	0.449	0.428	−0.012 (−0.159; −0.011)	25	25.00	21	13.00	1.000	4.387
PS_SM	32	4.44	2.14	3.53	0.06	0.378	0.423	0.096 (−0.118; 0.051)	31	30.12	30	12.82	0.998	6.622
PS_TM	45	5.11	2.29	3.75	0.13	0.398	0.452	0.125 (0.004; 0.161)	43	41.33	42	12.82	0.998	5.772
P. uliginosa	138	5.222	2.253	3.124*	0.20	0.396	0.459	0.066 (−0.089; 0.083)	57	24.291	51	47.72	0.966	13.835
P. mugo	105	4.778	2.059	3.183*	0.35	0.386	0.460	0.075 (−0.068; 0.146)	100	95.870	98	99.00	0.999	13.486
P. uncinata	168	5.333	2.211	3.263*	0.29	0.384	0.426	0.077 (−0.077; 0.079)	77	28.567	72	54.741	0.971	9.415
P. sylvestris	128	7.333	2.374	3.762*	0.73	0.416	0.451	0.062 (−0.062; 0.098)	118	109.227	118	97.160	0.999	5.616

Allelic richness nA_R was rarefied for 12 individuals at the population level and for 38 individuals at the species level. Genetic parameters for nSSR: *A*, mean number of alleles per locus; F_{IS}, inbreeding coefficient; H_E, mean expected heterozygosity; H_O, mean observed heterozygosity; *N*, number of samples; nA_E, mean effective number of alleles per locus; nA_R, rarefied allelic richness; P_{AR}, private alleles rarefied richness, and cpSSR: *Ah*, mean number of haplotypes; A_P, number of private haplotypes; cpA_R, haplotype richness; cpA_E, mean effective number of haplotypes; H_d, haplotype diversity; D^2sh, mean genetic distance of individuals within population; *Statistically significant differences at $P \leq 0.05$.

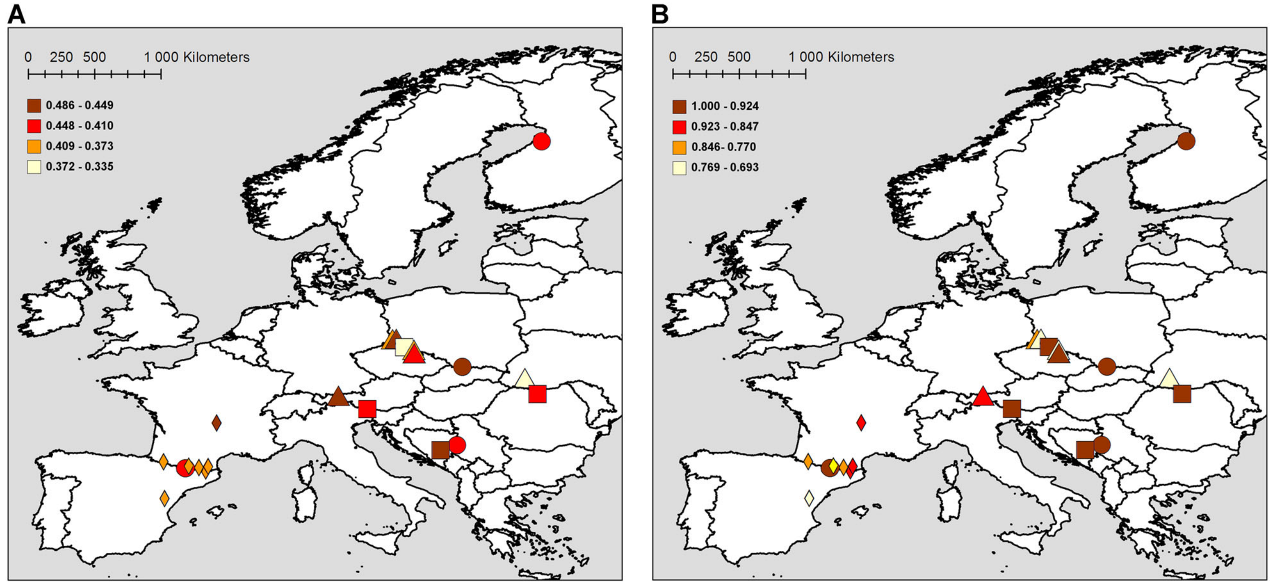

Fig. 2. Diversity estimated using different markers: (**A**) nuclear (nSSR diversity), (**B**) chloroplast (cpSSR diversity), for European populations of four pine species: *Pinus uliginosa* (▲), *P. mugo* (■), *P. uncinata* (◆), and *P. sylvestris* (●). Color intensity indicate level of diversity measured as: Haplotype diversity and expected heterozygosity (**A** and **B**, respectively). Map was created using ArcMap v10.1.

highest proportion of individuals possessing the major haplotypes (Table S2).

Significant differences between taxa were found using pairwise F_{ST} estimates of genetic differentiation. The lowest differentiation occurred among *P. uligionsa* and both *P. mugo* and *P. uncinata*, whereas *Pinus sylvestris* was the most diverged from other taxa (greatest F_{ST} values for both nSSR and cpSSR) (Table 3A).

3.2 Population structure within *P. uliginosa*

The Bayesian assignment of samples by the STRUCTURE revealed no significant clustering for *P. uliginosa* populations (Fig. 4A). The number of clusters indicated by peak in ΔK value was 2 ($K = 2$) (Figs. 4B, 4C). However, visual inspection of STRUCTURE plot suggests that this result may be rather an artifact that reflects the tendency of this procedure to force clustering (setting $K = 2$) even when the real structure is lacking (Janes et al., 2017).

Nevertheless, some indication of the population structure in peat bog pine was supported by the PCoA analysis using both cpSSR and nSSR (Figs. 3A, 3B). Generally, populations were assigned to two clusters: (i) UL_W and UL_SW; (ii) UL_MI, UL_BAT, UL_Z. Only population UL_MS showed a distinct pattern of genetic variation and was isolated from both clusters. Moreover, population UL_MS was highly differentiated from others based on pairwise F_{ST} (Table 3B). Overall, genetic differentiation between pairs of populations was substantial in *P. uliginosa*, in some cases, being even greater than the levels reported for species pairs (see Tables 3A, 3B).

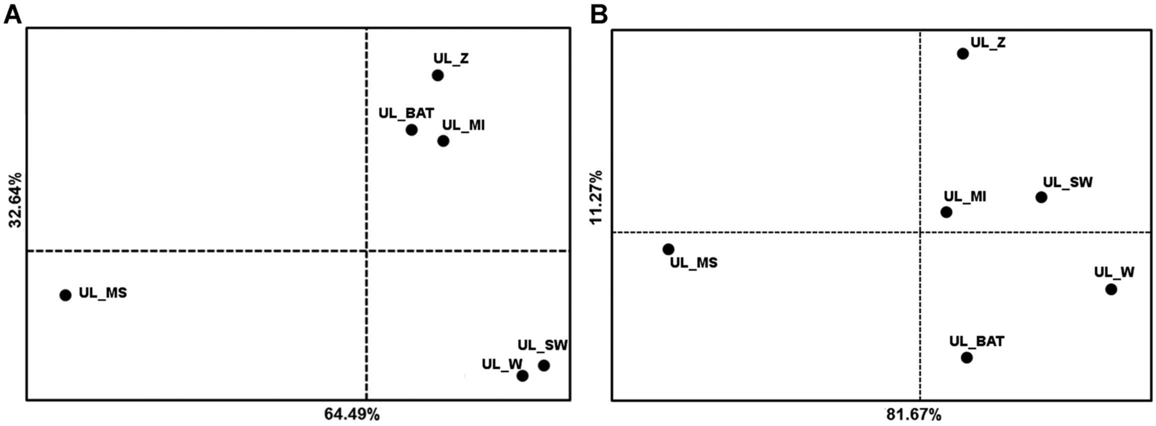

Fig. 3. Results of PCoA based on average Nei's distances between studied populations of *Pinus uliginosa* calculated for: **A**, set of 9 nuclear microsatellite markers; **B**, set of 12 chloroplast microsatellite markers.

Table 3 Pairwise F_{ST} estimated at 9 nuclear and 12 chloroplast microsatellites (below and above the diagonal, respectively); ** significant at $P = 0.01$: **A** in four pine species, **B** in *Pinus uliginosa* populations

A

	P. mugo	*P. sylvestris*	*P. uncinata*	*P. uliginosa*
P. mugo	–	0.386**	0.140**	0.068**
P. sylvestris	0.261**	–	0.463**	0.411**
P. uncinata	0.120**	0.139**	–	0.066**
P. uliginosa	0.053**	0.184**	0.056**	–

B

	UL_BAT	UL_W	UL_Z	UL_MI	UL_MS	UL_SW
UL_BAT	–	0.154**	0.173**	0.010	0.261**	0.110**
UL_W	0.058**	–	0.296**	0.131**	0.304**	0.007
UL_Z	0.103**	0.104**	–	0.148**	0.277**	0.250**
UL_MI	0.078**	0.075**	0.062**	–	0.254**	0.100**
UL_MS	0.186**	0.274**	0.192**	0.149**	–	0.295**
UL_SW	0.047**	0.032**	0.044**	0.042**	0.205**	–

The results of the Mantel test were not statistically significant, but we found quite a strong relationship between the genetic and geographic distance for both types of markers ($R^2 = 0.42$, $P = 0.086$ and $R^2 = 0.41$, $P = 0.09$ for nSSR and cpSSR, respectively) (Fig. S1). However, when the most genetically distinct population UL_MS from Ukraine was removed, there was no correlation between the genetic and geographic distance (not shown).

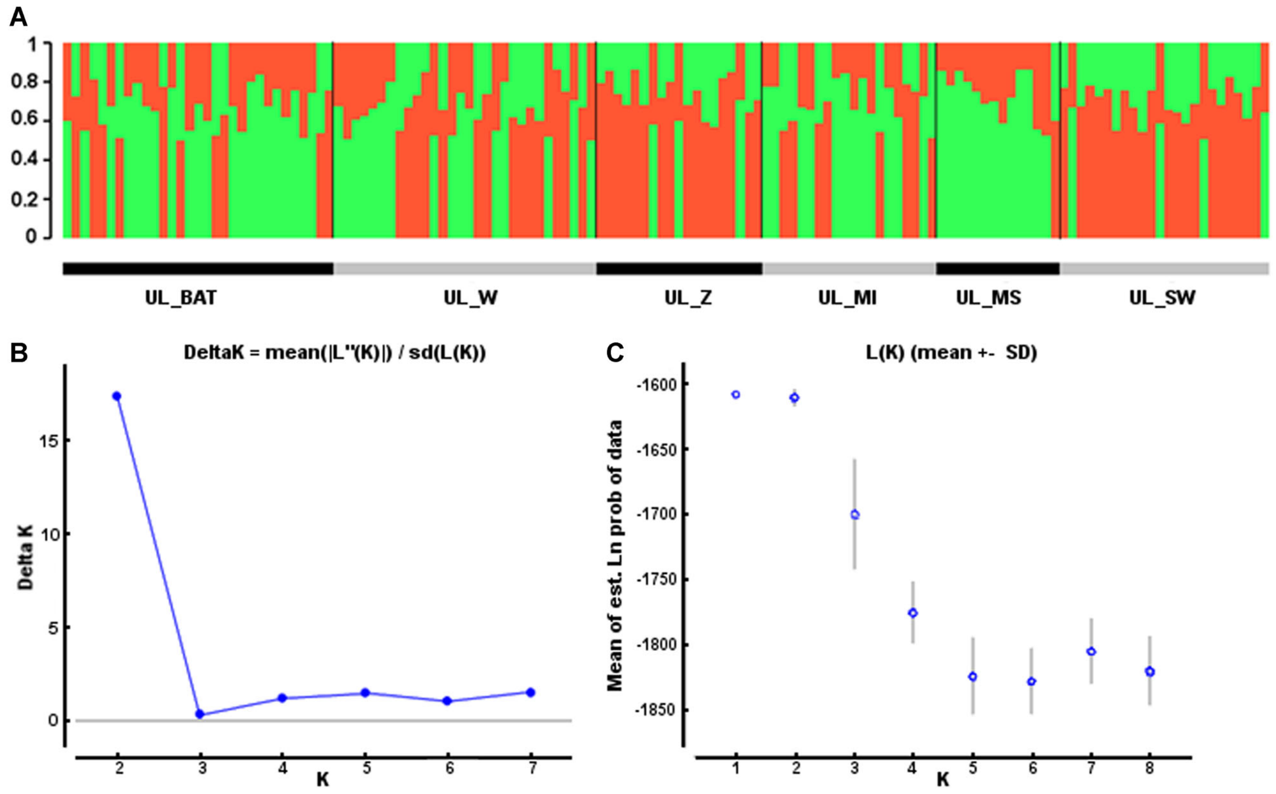

Fig. 4. **A**, STRUCTURE plot for *Pinus uliginosa*, based on nSSR markers for best supported number of genetic clusters ($K = 2$). The colors represent proportional assignment of individuals to different clusters. **B**, Δ*K* values of 20 runs that assumed $K = 2$–8 and **C**, log-likelihood value (mean ± SD) of each *K* value ($K = 2$–8) based on 20 runs. Analyses were implemented in STRUCTURE v2.2.3 (Pritchard et al., 2000); genetic structure graph (**A**) was drawn using the program Structure Plot V2.0 (Ramasamy et al., 2014) and the Δ*K* plot (**B**) and the log probability plot (**C**) were generated in Structure Harvester (Earl & Vonholdt, 2012).

Table 4 Estimates of posterior distributions of parameters obtained from the approximate Bayesian computation (ABC) for the best Scenario 6 for the demographic history of *Pinus uliginosa*

Parameter	Mean (a; b)	Median (a; b)	Mode (a; b)	5% (a; b)	95% (a; b)
Ne	6990	7420	8530	2820	9710
Nf	442	317	215	133	1220
N1	3450	3070	1500	691	7330
t2	25 600; 32 000	26 400; 33 000	30 600; 38 250	10 280; 12 850	38 600; 48 250
db	7580; 9475	7700; 9625	8680; 10 850	1708; 2135	13 000; 16 250
mean μmic_1	9.47×10^{-4}	7.60×10^{-4}	4.70×10^{-4}	1.73×10^{-4}	2.44×10^{-3}
mean p_1	3.09×10^{-1}	3.22×10^{-1}	4.00×10^{-1}	1.69×10^{-1}	3.99×10^{-1}
mean μmic_2	2.52×10^{-4}	2.65×10^{-3}	4.00×10^{-3}	7.61×10^{-4}	3.90×10^{-3}
mean p_2	1.38×10^{-1}	1.23×10^{-1}	1.00×10^{-1}	1.00×10^{-1}	2.32×10^{-1}

Estimation is based on 1% of the closest simulated datasets and the logit transformation of parameters was used. Time parameters (t2 and db [years]) were scaled by an assumed generation time of 20 (a) and 25 (b) years, respectively.

3.3 Demographic history of *P. uliginosa*

We investigated the demographic history and signatures of genetic bottleneck in *P. uliginosa* using two different approaches. Evidence for the bottleneck episode in *P. uliginosa* history came from the results of model-based inference using ABC. Among six different plausible demographic scenarios (see Fig. 1) tested in our DIYABC simulations, Scenario 6 (with bottleneck history) was the most likely one (Fig. S3). The degree of confidence in the best scenario was high, with low error rates in both Type I and Type II (Table S4). Prior and posterior distribution density curves for the best supported scenario, and model checking, are reported in Figs. S4 and S5, respectively. Estimated bottleneck had begun around 1320 generations ago and lasted around 385 generations. Following our initial assumption regarding average generation time of peat bog pine (20–25 years), the estimates correspond to the bottleneck that started about 26 400–33 000 and ended 18 700–23 375 years ago. The median value of possible effective population size of *P. uliginosa* prior to bottleneck event was estimated at 7420 individuals. After a strong population decline ($N_e = 317$), it is now estimated at 3070 individuals (Table 4). These N_e estimates fit well with the one revealed based on the LD method implemented in NeEstimator v. 2, where the sum of N_e across all *P. uliginosa* populations amounts to 2372 individuals. It is also worth noting that in our calculations, *P. uliginosa* is characterized by the lowest current mean effective population size as compared to the reference taxa. Furthermore, three out of six populations in this species have N_e below 50, the accepted threshold for minimum viable population (MVP) (Franklin, 1980) (Figs. 5, S2). The allelic frequency test in BOTTLENECK v 1.2.02 provided evidence of past bottleneck events in some, but not all *P. uliginosa* populations ($P < 0.01$). However, when populations were

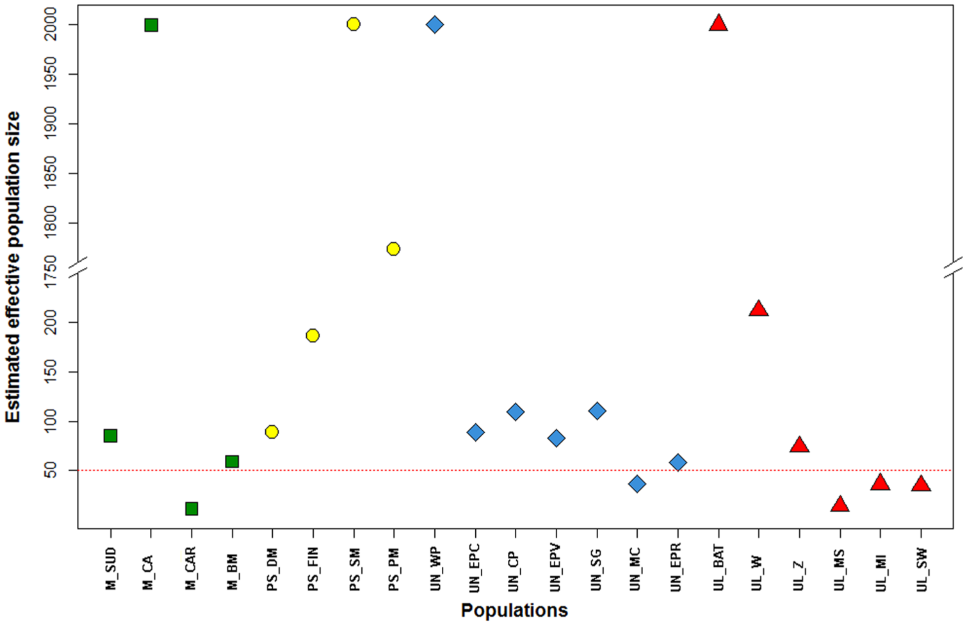

Fig. 5. Estimated effective sizes of 21 studied pine populations, calculated using LD method in NeEstimator v. 2. The red hatched line indicates $N_e = 50$, threshold for the minimum viable population (MVP) proposed by Franklin (1980).

analyzed together at species level, slightly lower but still statistically significant evidence for the bottleneck was detected ($P < 0.05$). Interestingly, a similar bottleneck signature was also observed in three reference species, with analysis power directly proportional to the species effective population size.

3.4 Species distribution modeling

Despite the limited number of occurrences (only 10 sites), our Maxent model showed good performance and was able to generate robust prediction of contemporary and potential past distribution of *P. uliginosa* (Fig. 6). Averaged areas under the ROC curve (AUC) ranged between 0.937 and 0.946, indicating that our models differed greatly from random expectation. The occurrence of peat bog pine is strongly affected by water availability, as revealed by the highest relative contribution to the model performance of environmental variables: aridity (29%) and pluvio_q (26%) (Table S5). The predicted current distribution of *P. uliginosa* clearly reflects the sampled population range but may also indicate a previously unknown location of this species. Results of niche overlap obtained using Schoener's D statistic (not shown) show no differences between the predicted present day and Mid-Holocene distributions, but their substantial differentiation during LGM. The LGM model showed considerable range contraction relative to the present day, with low habitat suitability (<0.50) for majority of eastern and central European parts of the current range. Moderate to strong habitat suitability (0.50–0.90) was found in some parts of Fennoscandia, but given the extent of ice sheet during this period (Mangerud et al., 2004) and the absence of this factor as an environmental variable used in our model, these observations are most likely false-positives.

4 Discussion

4.1 Genetic diversity and population structure

In this study, we investigated the genetic diversity and population structure of remnant stands of endangered peat bog pine (*Pinus uliginosa*) in comparison to much more widely distributed three closely related pine species. In case of small and fragmented populations, we could expect a low level of neutral genetic variation, mostly influenced by the impact of genetic drift and inbreeding (Frankham, 1996). In addition, there are known examples of reduced heterozygosity within small populations among species with differing abundances as compared to expectations under neutral theory (Spielman et al., 2004). Surprisingly, in peat bog pine we found a complex pattern of genetic variation and incongruence between the diversity assessments based on nuclear and chloroplast markers.

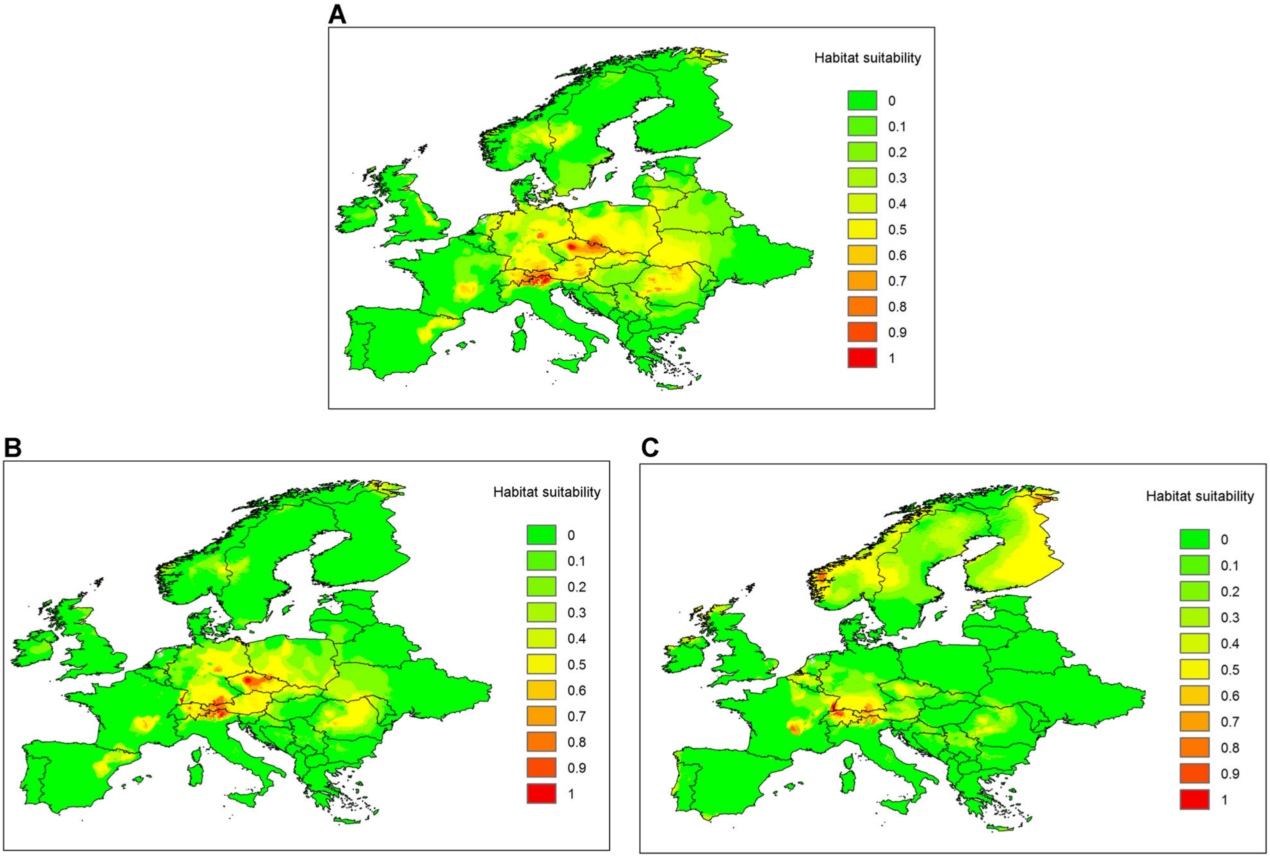

Fig. 6. Species distribution models for *Pinus uliginosa* based on ecological niche modeling using Maxent. Predicted habitat suitability (color scale: green, habitat not suitable; red, maximum habitat suitability) are shown for climates of: **A**, the current time period; **B**, Mid-Holocene (ca. 6 ka); **C**, the Last Glacial Maximum LGM (ca. 21.5 ka).

The level of genetic diversity at the nSSR markers, examined for the first time in peat bog pine, is in general comparable to those of the reference species (Wójkiewicz et al., 2016; Hebda et al., 2017; Żukowska & Wachowiak, 2017). Out of all measures of genetic diversity, only mean allelic richness was significantly lower ($P < 0.05$) in *P. uliginosa* compared to other species ($nA_R = 3.124$ vs. 3.183, 3.263, and 3.762 in *P. mugo*, *P. uncinata*, and *P. sylvestris*, respectively). It is worth noting, however, that this result was strongly driven by unusually low genetic variation found in population UL_MS from Ukraine. Its distinct character at biometric traits and low level of variation at mtDNA was reported earlier (Boratyńska et al., 2010; Łabiszak et al., 2019). Furthermore, contrary to predictions, we failed to detect signatures of inbreeding using nSRR data in any of the *P. uliginosa* populations based on formal statistical tests.

However, in strike contrast to the results from nuclear markers, our cpSSR dataset suggests serious reduction of genetic diversity in peat bog pine. First, this pine exhibited the lowest number of haplotypes, private haplotypes, and haplotype diversity among all taxa investigated in our study. This variation at chloroplast DNA markers is also lower compared to other pine species studied in Europe (Gomez et al., 2005; Afzal-Rafii & Dodd, 2007; Dzialuk et al., 2017). Second, *P. uliginosa* was characterized by the highest proportion of major haplotypes among all species and their distribution among populations, which indicates a small number of effective pollen donors. In highly outcrossing, anemophilous species, such as pines, we expect to observe high haplotype diversity, which is evident in case of *P. sylvestris* and *P. mugo,* but not *P. uliginosa*. A possible explanation of this phenomenon in peat bog pine is inbreeding due to limited access to reproductive partners, given the very low observed census and effective population sizes estimated for this taxon. The observed reduced diversity, with alleviated numbers of identical alleles and haplotypes present in populations could be also a result of homoplasy in cpSSR microsatellites. However, such a scenario would require a drastically higher mutation rate in *P. uligionsa,* compared to its congeners, which seems highly unlikely.

The contrasting patterns of genetic diversity found in our study could be attributed to the differences in inheritance and dispersal modes of organelle and nuclear genomes, reflecting dissimilar patterns despite common demographic history. In general, organelle genomes have two times smaller effective population sizes than the nuclear ones, being, therefore, more strongly affected by genetic drift (Wright, 1938). Given the generally higher levels of standing genetic variation in the nuclear genomes of trees (Hamrick, 2004; Alberto et al., 2013), it is feasible that the signatures of bottleneck were only detectable in the chloroplast genome. The effect of fragmentation and isolation on genetic diversity of trees turns out recently to be highly context-dependent, sometimes leading to contradictory responses, as is evident in case of several endangered tree species with different demographic histories (Pautasso, 2009; Lowe et al., 2015). Our results reaffirm that genetic diversity patterns vary between markers of different genomic origin. It is therefore important to use multiple marker types in conservation genetics assessments, as conclusions based on single type of markers can be misleading and should be interpreted with caution.

In general, forest trees, and pines in particular, are known to maintain a low level of genetic differentiation among populations due to their usually large population sizes and effective long-distance gene flow (Petit & Hampe, 2006). However, long periods of geographical isolation are likely to enhance genetic differentiation, especially in small populations affected by genetic drift (Pannell & Dorken, 2006). Indeed, we found some evidence of population structure and stronger genetic differentiation among populations of *P. uliginosa* compared to *P. sylvestris* and *P. uncinata*. Our mean F_{ST} values were 2–3 times larger for both types of markers in *P. uliginosa,* even excluding the highly differentiated population from Ukraine. Similarly, a high level of differentiation was found in *P. mugo* among populations inhabiting different mountain ranges, including the Sudetes, Alps, and Carpathians (Heuertz et al., 2010; Dzialuk et al., 2017; Żukowska et al., 2017), which can reflect the past fragmentation and long reproductive isolation between the stands (Dzialuk et al., 2017).

4.2 Demographic history

Dramatic changes in Pleistocene climate with several glacial-interglacial transitions have had profound influence on the demographic and evolutionary history of species of the Northern Hemisphere, including forest trees (Hewitt, 2000; Davis & Shaw, 2001). The response of many temperate tree species to climate cooling during glaciation was a range shift toward lower latitudes, sometimes leading to severe range reduction and loss of genetic diversity as a consequence. The results of our coalescent-based demographic analyses (DIYABC) indicate that the contemporary level of genetic diversity in *P. uliginosa* was shaped by an episode of ancient genetic bottleneck dated at around 26 400–33 000 years ago, which corresponds well with the beginning of the LGM, around 26 500 years ago (Clark et al., 2009). A similar timing of the bottleneck was also reported for other tree species, including pines (Naydenov et al., 2011; Xu et al., 2017; Fan et al., 2018), indicating that climatic changes during this period indeed had played a crucial role in shaping the evolutionary history of forest tree species.

In agreement with the DIYABC results discussed above, SDM provided insights into the demographic history of *P. uliginosa*, presenting a genetic bottleneck in the spatial context. Substantial range contraction during LGM predicted by Maxent could be attributed to a much colder and dryer climate during this period, which resulted in fewer available habitats for peat bog pine (Otto-Bliesner et al., 2006). The transient character of peat bogs could be related to glacial-interglacial cycles, where the onset of a continental ice sheet during the glaciation phase limits the extent of peatland area, and after warming and melting of the ice, these areas start to recover. Such a scenario is reflected in our SDM model for Mid Holocene, where range expansions since LGM were suggested. It should be stated that despite being widely used, SDMs have some limitations, particularly in case of species with a low number of known occurrence sites, as the accuracy of these models is highly correlated with the number of records used to create predictions. However, for species with narrow ecological niche, like *P. uliginosa,* the minimum sample size to make reliable niche prediction is much lower, than in the case of widespread species, and could be as low as 10 (Pearson et al., 2006; Van Proosdij et al., 2016).

One possible scenario for peat bog pine history, suggested by our data, is that it might represent a relict species, with populations isolated long before LGM and specialized to transient peat bog habitat, which had undergone through multiple fluctuation in population size during its Pleistocene history. Taking into consideration the geographical proximity between most of *P. uliginosa* populations, significant differentiation between them and the lack of a simple pattern of IBD, they together may indicate an ancient divergence and long-lasting isolation, possibly predating the Last Glacial Maximum. The possible relict character of *P. uliginosa* was recently discussed based on analysis of mitochondrial markers (Łabiszak et al., 2019).

In addition, some evidence of molecular basis of specialization and adaptation of peat bog pine to its unique niche was found in a recent study on signatures of selection in closely related pines (Wachowiak et al., 2018). Furthermore, results of DIYABC analysis suggest, that the estimated effective size of the ancestral population of *P. uliginosa,* prior bottleneck (7420 individuals) is much smaller than reported estimates for ancestral populations of other conifers (Heuertz et al., 2006; Pyhäjärvi et al., 2007). Large effective population sizes are prominent characteristics of forest tree species and only a few endemic, relict species exist (Petit & Hampe, 2006). Although we were unable to detect bottleneck episodes predating LGM with our data, higher resolutions nuclear markers could possibly increase temporal accuracy of demographic inference and help to further test our hypothesis.

4.3 Conservation implication

Our study emphasizes the importance of combining different types of molecular markers and multiple methods when assessing the genetic diversity of species with a complex evolutionary history. Incongruence between assessment of contemporary levels of genetic diversity based on nuclear and chloroplast markers clearly emphasizes that single marker inference could be misleading in such cases. Such comprehensive understanding of the history and population structure of species is needed to adequately identify its protection status and to implement appropriate management practices (Possingham et al., 2001). This is now more important than ever, given that current species extinction rates vastly exceed the average background rates (Ceballos et al., 2009, 2015; Urban, 2015). Considering the limited distribution range, small populations with signatures of inbreeding, high genetic differentiation due to genetic drift, and possible fitness loss, *P. uliginosa* is at high risk of extinction. Genetic rescue in form of an augmented gene flow between remnant peat bog populations seems necessary in order to prevent it from further negative influence of inbreeding and genetic drift. Recent meta-analysis by Frankham (2015) provided an argument for outcrossing small inbred populations, as this procedure has beneficial effects in 92.9% of cases measured as substantial increases in fitness. In addition, a strong in situ conservation program is required to preserve the unique habitat of *P. uliginosa* from further degradation. Future projections provided by niche modeling of threatened species greatly improve extinction risk assessment and signalize niche shifts as a response to climate change. However, due to the lack of data on future climate in ENVIREM database, we could not follow the same methodology, as in case of past and present projections. Nevertheless, results from present day niche predictions are also useful in conservation context and could serve as guidelines for detection of previously unknown populations and/or potential suitable habitats for this species, which might be used as possible introduction or restitution sites (Raxworthy et al., 2003; Bourg et al., 2005; Pearson et al., 2006). In recent years, applications of SDMs in biological studies have greatly aided conservation planning as evident by increasing number of research that utilize those methods (Araujo & Williams, 2000; Ferrier et al., 2002; Temunović et al., 2013; Koo et al., 2017). Given the poor natural regeneration observed in *P. uliginosa* stands (Danielewicz & Zieliński, 2000), we suggest also implementation of ex situ conservation methods, such as seed collection and propagation of seedlings in nurseries, to improve seedling survival and maintenance of existing genetic resources.

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References

Afzal-Rafii Z, Dodd RS. 2007. Chloroplast DNA supports a hypothesis of glacial refugia over postglacial recolonization in disjunct populations of black pine (*Pinus nigra*) in western Europe. *Molecular Ecology* 16: 723–736.

Alberto FJ, Aitken SN, Alia R, Gonzalez-Martinez SC, Hanninen H, Kremer A, Lefevre F, Lenormand T, Yeaman S, Whetten R, Savolainen O. 2013. Potential for evolutionary responses to climate change—Evidence from tree populations. *Global Change Biology* 19: 1645–1661.

Araujo MB, Williams PH. 2000. Selecting areas for species persistence using occurrence data. *Biological Conservation* 96: 331–345.

Belletti P, Ferrazzini D, Piotti A, Monteleone I, Ducci F. 2012. Genetic variation and divergence in Scots pine (*Pinus sylvestris* L.) within its natural range in Italy. *European Journal of Forest Research* 131: 1127–1138.

Bertorelle G, Benazzo A, Mona S. 2010. ABC as a flexible framework to estimate demography over space and time: Some cons, many pros. *Molecular Ecology* 19: 2609–2625.

Boratyńska K, Lewandowska D. 2009. Differences among three populations of *Pinus uliginosa* and their relation to *P. sylvestris* as expressed by the needle characters. *Dendrobiology* 61: 37–46.

Boratyńska K, Sulikowska A, Iakushenko DM, Jasińska AK, Sobierajska K. 2010. Tree-like pines on the Mshana peat bog in the Gorgany Mountains: A trace of *Pinus uliginosa* migration in the East Carpathians? *Acta Societatis Botanicorum Poloniae* 79: 129–137.

Boratyński A. 1994. Protected and rare trees and shrubs from the Polish part of Sudety Mts. and its foothills. 7. *Pinus mugo* Turra and *P. uliginosa* Neumann. *Arboretum Kórnickie* 39: 63–85.

Bourg NA, McShea WJ, Gill DE. 2005. Putting a cart before the search: Successful habitat prediction for a rare forest herb. *Ecology* 86: 2793–2804.

Carlisle A, Brown AHF. 1968. *Pinus sylvestris* L. *Journal of Ecology* 56: 269–307.

Ceballos G, Ehrlich PR, Barnosky AD, García A, Pringle RM, Palmer TM. 2015. Accelerated modern human-induced species losses: Entering the sixth mass extinction. *Science Advances* 1: e1400253.

Ceballos G, García A, Ehrlich P. 2009. The sixth extinction crisis loss of animal populations and species. *Journal of Cosmology* 8: 1821–1831.

Chapuis MP, Estoup A. 2007. Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution* 24: 621–631.

Clark PU, Dyke AS, Shakun JD, Carlson AE, Clark J, Wohlfarth B, Mitrovica JX, Hostetler SW, McCabe AM. 2009. The Last Glacial Maximum. *Science* 325: 710–714.

Cornuet JM, Luikart G. 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* 144: 2001–2014.

Cornuet JM, Pudlo P, Veyssier J, Dehne-Garcia A, Gautier M, Leblois R, Marin JM, Estoup A. 2014. DIYABC v2.0: A software to make approximate Bayesian computation inferences about population history using single nucleotide polymorphism, DNA sequence and microsatellite data. *Bioinformatics* 30: 1187–1189.

Cornuet JM, Santos F, Beaumont MA, Robert CP, Marin JM, Balding DJ, Guillemaud T, Estoup A. 2008. Inferring population history with DIY ABC: a user-friendly approach to approximate Bayesian computation. *Bioinformatics* 24: 2713–2719.

Csilléry K, Blum MGB, Gaggiotti OE, François O. 2010. Approximate Bayesian computation (ABC) in practice. *Trends in Ecology & Evolution* 25: 410–418.

Danielewicz W, Zieliński J. 2000. Protection of the *Pinus uliginosa* Neumann on the area of the Low Silesian Pinewood. *Przegląd Przyrodniczy* 11: 113–124.

Davis MB, Shaw RG. 2001. Range shifts and adaptive responses to Quaternary climate change. *Science* 292: 673–679.

Di Rienzo A, Peterson AC, Garza JC, Valdes AM, Slatkin M, Freimer NB. 1994. Mutational processes of simple-sequence repeat loci in human populations. *Proceedings of the National Academy of Sciences USA* 91: 3166–3170.

Do C, Waples RS, Peel D, Macbeth GM, Tillett BJ, Ovenden JR. 2014. NeEstimator v2: Re-implementation of software for the estimation of contemporary effective population size (Ne) from genetic data. *Molecular Ecology Resources* 14: 209–214.

Dudik M, Phillips SJ, Schapire RE. 2004. Performance guarantees for regularized maximum entropy density estimation. In: Shawe-Taylor J, Singer Y eds. *Learning Theory*. Proceedings of the 17th Annual Conference on Learning Theory, Banff, 1–4 July, 2004. Berlin: Springer. 472–486.

Dumolin S, Demesure B, Petit RJ. 1995. Inheritance of chloroplast and mitochondrial genomes in pedunculate oak investigated with an efficient PCR method. *Theorethical and Applied Genetics* 91: 1253–1256.

Dzialuk A, Boratyńska K, Romo A, Boratyński A. 2017. Taxonomic and geographic variation of the *Pinus mugo* complex on chloroplast microsatellite markers. *Systematics and Biodiversity* 15: 464–479.

Earl DA, Vonholdt BM. 2012. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources* 4: 359–361.

Eliades NG. 2009. HAPLOTYPE ANALYSIS: Software for analysis of haplotype data [online]. Available from http://www.uni-goettingen.de/en/134935.html [accessed 15 January 2020].

Elith J, Leathwick JR. 2009. Species distribution models: Ecological explanation and prediction across space and time. *Annual Review in Ecology, Evolution, and Systematics* 40: 677–697.

Elith J, Phillips SJ, Hastie T, Dudík M, Chee YE, Yates CJ. 2011. A statistical explanation of MaxEnt for ecologists. *Diversity and Distributions* 17: 43–57.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Molecular Ecology* 14: 2611–2620.

Excoffier L, Lischer HEL. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10: 564–567.

Falush D, Stephens M, Pritchard JK. 2007. Inference of population structure using multilocus genotype data: Dominant markers and null alleles. *Molecular Ecology Notes* 7: 574–578.

Fan LQ, Zheng HL, Milne RI, Zhang L, Mao KS. 2018. Strong population bottleneck and repeated demographic expansions of *Populus adenopoda* (Salicaceae) in subtropical China. *Annals of Botany* 121: 665–679.

Ferrier S, Watson G, Pearce J, Drielsma M. 2002. Extended statistical approaches to modelling spatial pattern in biodiversity in northeast New South Wales. I. Species-level modelling. *Biodiversity and Conservation* 11: 2275–2307.

Fettig CJ, Reid ML, Bentz BJ, Sevanto S, Spittlehouse DL, Wang TL. 2013. Changing climates, changing forests: A western North American perspective. *Journal of Forestry* 111: 214–228.

Food and Agriculture Organisation of the United Nations. 2018. The state of the world's forests 2018—Forest pathways to sustainable development [online]. Available from www.fao.org/3/ca0188en/ca0188en.pdf [accessed 15 January 2020].

Frankham R. 1996. Relationship of genetic variation to population size in wildlife. *Conservation Biology* 10: 1500–1508.

Frankham R. 2015. Genetic rescue of small inbred populations: Meta-analysis reveals large and consistent benefits of gene flow. *Molecular Ecology* 24: 2610–2618.

Frankham R, Ballou JD, Briscoe DA. 2010. *Introduction to conservation genetics*. Cambridge: Cambridge University Press.

Franklin IR. 1980. Evolutionary changes in small populations. In: Soulé ME, Wilcox BM eds. *Conservation biology: An evolutionary-ecological perspective*. Sunderland: Sinauer Associates. 135–149.

GBIF.org. 2019. GBIF Occurrence Download. doi: 10.15468/dl.mmv2pr.

Gomez A, Vendramin GG, Gonzalez-Martinez SC, Alia R. 2005. Genetic diversity and differentiation of two Mediterranean pines (*Pinus halepensis* Mill. and *Pinus pinaster* Ait.) along a latitudinal cline using chloroplast microsatellite markers. *Diversity and Distributions* 11: 257–263.

Goudet J. 1995. FSTAT (Version 1.2): A computer program to calculate F-statistics. *Journal of Heredity* 86: 485–486.

Goudet J 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3) [online]. Available from http://www2.unil.ch/popgen/softwares/fstat.htm [accessed 15 January 2020]. Updated from Goudet (1995).

Hamernik J, Musil I. 2007. The *Pinus mugo* complex – its structuring and general overview of the used nomenclature. *Journal of Forest Science* 53: 253–266.

Hamrick JL. 2004. Response of forest trees to global environmental changes. *Forest Ecology and Management* 197: 323–335.

Hebda A, Wójkiewicz B, Wachowiak W. 2017. Genetic characteristics of *Scots pine* in Poland and reference populations based on nuclear and chloroplast microsatellite markers. *Silva Fennica* 51: 1721.

Heuertz M, De Paoli E, Kallman T, Larsson H, Jurman I, Morgante M, Lascoux M, Gyllenstrand N. 2006. Multilocus patterns of nucleotide diversity, linkage disequilibrium and demographic history of Norway spruce [*Picea abies* (L.) Karst]. *Genetics* 174: 2095–2105.

Heuertz M, Teufel J, González-Martínez SC, Soto A, Fady B, Alía R, Vendramin GG. 2010. Geography determines genetic relationships between species of mountain pine (*Pinus mugo* complex) in western Europe. *Journal of Biogeography* 37: 541–556.

Hewitt G. 2000. The genetic legacy of the Quaternary ice ages. *Nature* 405: 907–913.

Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A. 2005. Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology* 25: 1965–1978.

Holt RD. 1990. The microevolutionary consequences of climate change. *Trends in Ecology & Evolution* 5: 311–315.

Hubisz MJ, Falush D, Stephens M, Pritchard JK. 2009. Inferring weak population structure with the assistance of sample group information. *Molecular Ecology Resources* 9: 1322–1332.

Janes JK, Miller JM, Dupuis JR, Malenfant RM, Gorrell JC, Cullingham CI, Andrew RL. 2017. The K=2 conundrum. *Molecular Ecology* 26: 3594–3602.

Jump AS, Marchant R, Penuelas J. 2009. Environmental change and the option value of genetic diversity. *Trends in Plant Science* 14: 51–58.

Keenan K, McGinnity P, Cross TF, Crozier WW, Prodohl PA. 2013. DiveRsity: An R package for the estimation and exploration of population genetics parameters and their associated errors. *Methods in Ecology and Evolution* 4: 782–788.

Koo KA, Park SU, Kong WS, Hong S, Jang I, Seo C. 2017. Potential climate change effects on tree distributions in the Korean Peninsula: Understanding model & climate uncertainties. *Ecological Modelling* 353: 17–27.

Łabiszak B, Zaborowska J, Wachowiak W. 2019. Patterns of mtDNA variation reveal complex evolutionary history of relict and endangered peat bog pine (*Pinus uliginosa*). *AoB Plants* 11: plz015.

Lowe AJ, Cavers S, Boshier D, Breed MF, Hollingsworth PM. 2015. The resilience of forest fragmentation genetics—no longer a paradox—we were just looking in the wrong place. *Heredity* 115: 97–99.

Mangerud J, Jakobsson M, Alexanderson H, Astakhov V, Clarke GKC, Henriksen M, Hjort C, Krinner G, Lunkka JP, Moller P, Murray A, Nikolskaya O, Saarnisto M, Svendsen JI. 2004. Ice-dammed lakes and rerouting of the drainage of northern Eurasia during the Last Glaciation. *Quaternary Science Reviews* 23: 1313–1332.

Medley KA. 2010. Niche shifts during the global invasion of the Asian tiger mosquito, *Aedes albopictus* Skuse (Culicidae), revealed by reciprocal distribution models. *Global Ecology and Biogeography* 19: 122–133.

Naydenov KD, Naydenov MK, Tremblay F, Alexandrov A, Aubin-Fournier LD. 2011. Patterns of genetic diversity that result from bottlenecks in Scots Pine and the implications for local genetic conservation and management practices in Bulgaria. *New Forests* 42: 179–193.

Nei M. 1973. Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences USA* 70: 3321–3323.

Neumann C. 1837. Über eine auf den Seefeldern bei Reinerz u. einigen ähnlichen Gebirgsmooren der königl. Oberförsterei Karlsberg in der Graftschaft Glatz vorkommende noch unbeschriebene Form der Gattung *Pinus*. *Jahresbericht der Schlesischer Gesellschaft* 11: 52–57.

Nomura T. 2008. Estimation of effective number of breeders from molecular coancestry of single cohort sample. *Evolutionary Applications* 1: 462–474.

Otto-Bliesner BL, Brady EC, Clauzet G, Tomas R, Levis S, Kothavala Z. 2006. Last Glacial Maximum and Holocene climate in CCSM3. *Journal of Climate* 19: 2526–2544.

Pannell JR, Dorken ME. 2006. Colonisation as a common denominator in plant metapopulations and range expansions: Effects on genetic diversity and sexual systems. *Landscape Ecology* 21: 837–848.

Pautasso M. 2009. Geographical genetics and the conservation of forest trees. *Perspectives in Plant Ecology, Evolution and Systematics* 11: 157–189.

Peakall R, Smouse PE. 2012. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* 28: 2537–2539.

Pearson RG, Raxworthy CJ, Nakamura M, Townsend Peterson A. 2006. Predicting species distributions from small numbers of occurrence records: A test case using cryptic geckos in Madagascar. *Journal of Biogeography* 34: 102–117.

Petit RJ, Hampe A. 2006. Some evolutionary consequences of being a tree. *Annual Review of Ecology, Evolution, and Systematics* 37: 187–214.

Phillips SJ, Anderson RP, Dudik M, Schapire RE, Blair ME. 2017. Opening the black box: An open-source release of Maxent. *Ecography* 40: 887–893.

Phillips SJ, Anderson RP, Schapire RE. 2006. Maximum entropy modeling of species geographic distributions. *Ecological Modelling* 190: 231–259.

Possingham H, Andelman S, Noon B, Tromblak S, Pulliam H. 2001. Making smart conservation decisions. In: Soule ME, Orians GH eds. *Conservation biology: Research priorities for the next decade*. Washington: Island Press. 225–244.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155: 945–959.

Provan J, Soranzo N, Wilson NJ, McNicol JW, Forrest GI, Cottrell J, Powell W. 1998. Gene-pool variation in Caledonian and European Scots pine (*Pinus sylvestris* L.) revealed by chloroplast simple-sequence repeats. *Proceedings of the Royal Society B: Biological Sciences* 265: 1697–1705.

Pyhäjärvi T, Garcia-Gil MR, Knürr T, Mikkonen M, Wachowiak W, Savolainen O. 2007. Demographic history has influenced nucleotide diversity in European *Pinus sylvestris* Populations. *Genetics* 177: 1713–1724.

Ramasamy RK, Ramasamy S, Bindroo BB, Naik VG. 2014. STRUCTURE PLOT: A program for drawing elegant STRUCTURE bar plots in user friendly interface. *SpringerPlus* 3: 431.

Raxworthy CJ, Martinez-Meyer E, Horning N, Nussbaum RA, Schneider GE, Ortega-Huerta MA, Townsend Peterson A. 2003. Predicting distributions of known and unknown reptile species in Madagascar. *Nature* 426: 837–841.

Riitters K, Wickham J, Costanza JK, Vogt P. 2016. A global evaluation of forest interior area dynamics using tree cover data from 2000 to 2012. *Landscape Ecology* 31: 137–148.

Rousset F. 2008. GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

Schaberg PG, DeHayes DH, Hawley GJ, Nijensohn SE. 2008. Anthropogenic alterations of genetic diversity within tree populations: Implications for forest ecosystem resilience. *Forest Ecology and Management* 256: 855–862.

Schoener TW. 1968. Anolis lizards of Bimini: Resource partitioning in a complex fauna. *Ecology* 49: 704–726.

Sebastiani F, Pinzauti F, Kujala ST, González-Martínez SC, Vendramin GG. 2012. Novel polymorphic nuclear microsatellite markers for *Pinus sylvestris* L. *Conservation Genetics Resources* 4: 231–234.

Shearman P, Bryan J, Laurance WF. 2012. Are we approaching 'peak timber' in the tropics? *Biological Conservation* 151: 17–21.

Spielman D, Brook B, Frankham R. 2004. Most species are not driven to extinction before genetic factors impact them. *Proceedings of the National Academy of Sciences USA* 101: 15261–15264.

Tarca AL, Carey VJ, Chen XW, Romero R, Drăghici S. 2007. Machine learning and its applications to biology. *PLOS Computational Biology* 3: e116.

Temunović M, Frascaria-Lacoste N, Franjić J, Satovic Z, Fernández-Manjarrés JF. 2013. Identifying refugia from climate change using coupled ecological and genetic data in a transitional Mediterranean-temperate tree species. *Molecular Ecology* 22: 2128–2142.

Title PO, Bemmels JB. 2018. ENVIREM: An expanded set of bioclimatic and topographic variables increases flexibility and improves performance of ecological niche modeling. *Ecography* 41: 291–307.

Turetsky MR, Benscoter B, Page S, Rein G, van der Werf GR, Watts A. 2015. Global vulnerability of peatlands to fire and carbon loss. *Nature Geoscience* 8: 11–14.

Urban MC. 2015. Accelerating extinction risk from climate change. *Science* 348: 571–573.

Van Mantgem PJ, Stephenson NL, Byrne JC, Daniels LD, Franklin JF, Fule PZ, Harmon ME, Larson AJ, Smith JM, Taylor AH, Veblen TT. 2009. Widespread increase of tree mortality rates in the western United States. *Science* 323: 521–524.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P. 2004. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4: 535–538.

Van Proosdij ASJ, Sosef MSM, Wieringa JJ, Raes N. 2016. Minimum required number of specimen records to develop accurate species distribution models. *Ecography* 39: 542–552.

Vendramin GG, Lelli L, Rossi P, Morgante M. 1996. A set of primers for the amplification of 20 chloroplast microsatellites in Pinaceae. *Molecular Ecology* 5: 595–598.

Wachowiak W, Prus-Glowacki W. 2009. Different patterns of genetic structure of relict and isolated populations of endangered peat-bog pine (*Pinus uliginosa* Neumann). *Journal of Applied Genetics* 50: 329–339.

Wachowiak W, Salmela MJ, Ennos RA, Iason G, Cavers S. 2011. High genetic diversity at the extreme range edge: Nucleotide variation at nuclear loci in Scots pine (*Pinus sylvestris* L.) in Scotland. *Heredity* 106: 775–787.

Wachowiak W, Zaborowska J, Łabiszak B, Perry A, Zucca GM, Gonzalez-Martinez SC, Cavers S. 2018. Molecular signatures of divergence and selection in closely related pine taxa. *Tree Genetics & Genomes* 14: 83.

Warren DL, Glor RE, Turelli M. 2010. ENMTools: A toolbox for comparative studies of environmental niche models. *Ecography* 33: 607–611.

Weltzin JF, Bridgham SD, Pastor J, Chen J, Harth C. 2003. Potential effects of warming and drying on peatland plant community composition. *Global Change Biology* 9: 141–151.

Wimmer F. 1837. Über die Zapfen von *Pinus silvestris*, *pumilio* und *uliginosa*. Verhandlungen der botanischen Section. *Übersicht der Arbeiten über Veränddder Schlesischen Naturhistorische Gesel-schaft* 1837–1840: 93–98.

Wójkiewicz B, Litkowiec M, Wachowiak W. 2016. Contrasting patterns of genetic variation in core and peripheral populations of highly outcrossing and wind pollinated forest tree species. *AoB Plants* 8: plw054.

Wójkiewicz B, Wachowiak W. 2016. Substructuring of Scots pine in Europe based on polymorphism at chloroplast microsatellite loci. *Flora* 220: 142–149.

Wright S. 1938. Size of population and breeding structure in relation to evolution. *Science* 87: 430–431.

Xu TT, Wang Q, Olson MS, Li ZH, Miao N, Mao KS. 2017. Allopatric divergence, demographic history, and conservation implications of an endangered conifer *Cupressus chengiana* in the eastern Qinghai-Tibet Plateau. *Tree Genetics & Genomes* 13: 100.

Zhou Y, Bui T, Auckland LD, Williams CG. 2002. Undermethylated DNA as a source of microsatellites from a conifer genome. *Genome* 45: 91–99.

Żukowska W, Boratyńska K, Wachowiak W. 2017. Comparison of range-wide chloroplast microsatellite and needle trait variation patterns in *Pinus mugo* Turra (dwarf mountain pine). *iForest - Biogeosciences and Forestry* 10: 250–258.

Żukowska WB, Wachowiak W. 2017. Nuclear microsatellite markers reveal the low genetic structure of *Pinus mugo* Turra (dwarf mountain pine) populations in Europe. *Plant Systematics and Evolution* 303: 641–651.

Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.12573/suppinfo:

Table S1. Basic characteristic of the nine nuclear and twelve chloroplast microsatellite loci used in this study: *N* – number of alleles per locus, *R* – range of allele size, *A* – mean number of alleles per locus, A_e – mean effective number of alleles per locus, H_O – mean observed heterozygosity, H_E – mean expected heterozygosity, F_{IS} – mean fixation index, *uh* – unbiased allelic diversity, ns – non significant, *statistically significant at $P \leq 0.05$.

Table S2. Distribution of 32 major chloroplast haplotypes (present in at least 3 individuals) detected in studied pines populations. Cells with non-zero counts are bolded for ease of visual interpretation. Bold linens separate populations belonging to different species. *indicates haplotypes shared between two species, **indicates haplotypes shared between three species.

Table S3. Prior distribution for all parameters used in the DIYABC simulations. Models based on nSSR and cpSSR data of *P. uliginosa* were simulated; for each model, six different scenarios (1 to 6) were tested (see Fig. 1 for schematic illustration of scenarios in each model). Population size parameters are in units of population effective size (N_e), while time parameters (including bottleneck duration) are in units of generations.

Table S4. Estimates of type I and type II error probabilities for the six scenarios in DIYABC based on simulated datasets. The most likely scenario with the highest posterior probability was chosen (shaded in gray).

Table S5. Species distribution model (SDM) parameters and performance for *P. uliginosa*. Climatic variables are the predictor variables retained in the final model, and the percent contribution of each variable to the SDM and the permutation importance were assessed by Maxent (Phillips et al., 2004, 2006, 2017) at three different times: A - the current time period, B - Mid- Holocene (ca. 6 ka), C- the Last Glacial Maximum LGM (ca. 21.5 ka). Model performance was assessed for three scenarios separately based on AUC (area under the curve) criterion (0.937, 0.946 and 0.942 for a, b, and c respectively).

Fig. S1. Results of Mantel test showing the correlation between pairwise genetic distance ($F_{ST}/(1–F_{ST})$ and geographical distance for the six populations of *P. uliginosa* based on (A) 9 nuclear, (B) 12 chloroplast microsatellites. The isolation-by-distance was assessed with GenAIEx version 6.0 (Peakall & Smouse, 2006).

Fig. S2. Mean estimated effective population sizes in four pine species. Boxplot: central value—mean, upper/lower hinges—1st and 3rd quartile, whiskers—extreme values.

Fig. S3. Plots showing fitness of six tested demographic scenarios, based on direct estimates and logistic regression, simulated in DIYABC. For the setting of parameters in each scenario, see Table S4; for the estimation of each parameter for the best-fit scenario, see Table 5.

Fig. S4. Prior and posterior distributions density curves calculated under scenario 6 for *P. uliginosa* in DIYABC. Times are not scaled.

Fig. S5. Model checking evaluation for the best supported demographic scenario 6. PCA in the space of summary statistics, showing datasets simulated from the prior distribution of the parameters (black open circles), from the posterior predictive distribution (black filled circles), as well as the observed dataset (yellow circle). Plots show combination of the first three principal components, which cumulatively represent 84% of the total variation.

SUPPLEMENTARY DATA

Molecular and paleo-climatic data uncover impact of ancient bottleneck on demographic history and contemporary genetic structure of endangered pine *Pinus uliginosa*.

Łabiszak B., Zaborowska J., Wójkiewicz B., Wachowiak W.

Table S1. Basic characteristic of the nine nuclear and twelve chloroplast microsatellite loci used in this study: N – number of alleles per locus, R – range of allele size, A – mean number of alleles per locus, A_e – mean effective number of alleles per locus, H_O – mean observed heterozygosity, H_E– mean expected heterozygosity, F_{is} – mean fixation index, uh – unbiased allelic diversity , ns – non significant, * - statistically significant at after Bonferroni correction.

nSSR									cpSSR					
Locus	**N**	**R**	**A**	**A_E**	**H_O**	**H_E**	**F_{is}**	**Deviation from HWE**	**Locus**	**N**	**R**	**A**	**A_E**	**uh**
psyl2	7	184-214	2.714	1.453	0.277	0.295	0.031	ns	PCP1289	6	106-111	2.714	1.401	0.270
psyl25[2]	3	213-216	1.667	1.141	0.103	0.097	-0.052	ns	PCP26106[1]	5	145-149	3.333	2.288	0.504
psyl36[3]	6	248-263	2.476	1.178	0.113	0.132	0.111	ns	PCP30277	15	115-140	4.714	3.848	0.687
psyl42	7	168-220	4.095	2.896	0.646	0.633	-0.024	ns	PCP36567[4]	4	109-112	2.190	1.730	0.284
psyl44	7	151-178	2.667	1.689	0.385	0.365	-0.055	ns	PCP41131	18	138-159	4.095	2.882	0.546
psyl57	9	180-207	5.286	2.828	0.602	0.618	0.022	ns	PCP45071	10	146-155	3.429	2.078	0.443
ptTX3025[1]	9	203-299	4.143	1.572	0.291	0.318	0.084	*	PCP87314	5	112-116	3.667	2.900	0.584
ptTX4001	16	201-233	5.857	2.776	0.586	0.601	0.032	ns	PCP102652[5]	3	114-116	1.476	1.120	0.075
ptTX4011	13	244-284	5.762	3.215	0.528	0.654	0.168	*	Pt15169	10	121-130	3.714	2.931	0.595
-	-	-	-	-	-	-	-	-	Pt26081	5	109-113	3.190	2.238	0.499
-	-	-	-	-	-	-	-	-	Pt30204	9	141-149	4.905	3.843	0.690
-	-	-	-	-	-	-	-	-	Pt71936	10	145-154	3.714	2.730	0.270

[1] Locus monomorphic for population: U; [2] Locus monomorphic for populations: S, A, P, H, HV, FX, AN, U; [3] Locus monomorphic for populations: H, FV, HII , UW, U; [4] locus monomorphic for populations: FV, HII, Z, AN; [5] locus monomorphic for populations: F, H, FV, HV, HX, FX, UB, UW, M, AN, U, SW (Table 1.)

Table S2. Distribution of 32 major chloroplast haplotypes (present in at least 3 individuals) detected in studied pines populations. Cells with non-zero counts are bolded for ease of visual interpretation. Bold linens separate populations belonging to different species. * - indicates haplotypes shared between two species, **- indicates haplotypes shared between three species. Populations acronyms as in Table 1.

		# of occurrences in populations																				
Haplotype	**# of occurrences overall**	**M1**	**M2**	**M3**	**M4**	**S**	**F**	**A**	**P**	**H**	**FV**	**HV**	**HX**	**HII**	**FX**	**AN**	**UB**	**UW**	**Z**	**M**	**U**	**SW**
H. 153	18	0	0	0	0	0	0	0	0	**4**	**3**	**4**	**1**	**3**	**1**	**2**	0	0	0	0	0	0
H. 73	17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**10**	0	0	0	**7**
H. 164 *	13	0	0	0	0	0	0	0	0	**1**	**2**	**2**	0	0	**1**	**3**	**2**	0	**1**	**1**	0	0
H. 170	13	0	0	0	0	0	0	0	0	**2**	0	**1**	0	**10**	0	0	0	0	0	0	0	0
H. 160	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**5**	0	**1**	0	**6**
H. 16	11	0	0	0	0	0	0	0	0	**1**	0	0	**1**	9	0	0	0	0	0	0	0	0
H. 72	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**2**	**1**	0	0	**5**	0
H. 28	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	**3**	0	0	0	**3**
H. 5	6	0	0	0	0	0	0	0	0	**1**	**2**	0	**1**	0	**1**	**1**	0	0	0	0	0	0
H. 38	6	0	0	0	0	0	0	0	0	**1**	0	**1**	**4**	0	0	0	0	0	0	0	0	0
H. 15	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**3**	0	0	0	**2**
H. 60	5	0	0	0	0	0	0	0	0	**1**	0	0	**1**	0	**3**	0	0	0	0	0	0	0
H. 69	5	0	0	0	0	0	0	0	0	0	**1**	0	**1**	0	**2**	**1**	0	0	0	0	0	0
H. 204	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**5**	0
H. 75	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**4**	0	0	0	0	0
H. 91	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**2**	0	0	**2**	0	0
H. 114	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**4**	0	0	0	0	0
H. 173	4	0	0	0	0	0	0	0	0	**1**	**1**	0	0	0	0	**2**	0	0	0	0	0	0
H. 175	4	0	0	0	0	0	0	0	0	0	**1**	0	0	0	**2**	**1**	0	0	0	0	0	0
H. 205	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**4**	0
H. 241	4	0	0	0	0	0	0	0	0	0	0	**2**	**1**	0	0	**1**	0	0	0	0	0	0
H. 23	3	0	0	0	0	0	0	0	0	**1**	**1**	0	**1**	0	0	0	0	0	0	0	0	0
H. 33 **	3	**1**	0	0	0	0	0	0	0	0	0	0	0	0	**1**	0	0	0	0	0	0	**1**
H. 90 *	3	0	0	0	0	0	0	0	0	0	0	**1**	0	0	0	0	0	**2**	0	0	0	0
H. 134	3	0	0	0	0	0	0	0	0	0	0	0	**1**	0	**1**	**1**	0	0	0	0	0	0
H. 149	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	0	**1**	0	**1**
H. 152	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**3**	0	0	0	0	0
H. 163	3	0	0	0	0	0	0	0	0	0	**1**	**1**	0	0	0	**1**	0	0	0	0	0	0
H. 177 *	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	**1**	0	0	0	0	**1**
H. 200	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**3**	0	0	0	0
H. 213	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**3**	0	0	0
H. 245	3	0	0	0	0	**2**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sum of major haplotype		1	0	0	0	2	1	0	0	13	12	12	12	22	12	14	19	28	4	5	14	21
Proportion of major haplotypes		0.03	0.00	0.00	0.00	0.08	0.04	0.00	0.00	0.54	0.50	0.50	0.50	0.92	0.50	0.58	0.61	0.93	0.21	0.25	1.00	0.88

Table S3. Prior distribution for all parameters used in the DIYABC simulations. Models based on nSSR and cpSSR data of *P. uliginosa* were simulated; for each model, six different scenarios (1 to 6) were tested (see Fig. 1 for schematic illustration of scenarios in each model). Population size parameters are in units of population effective size (Ne), while time parameters (including bottleneck duration) are in units of generations.

Priors for the demographic parameters	
N1	UN~[10 - 10000]
Na	UN~[10 - 10000]
Nb	UN~[10 - 10000]
Nc	UN~[10 - 10000]
Nd	UN~[10 - 10000]
Ne	UN~[10 - 10000]
Nf	UN~[10 - 10000]
t1	UN~[10 - 500]
t2	UN~[10 - 2000]
db	UN~[10 - 700]
Constraint on parameter	N1 > Na, N1 > Nb, N1 < Nc, N1 < Nd, Ne > Nf,
Priors for the mutation model for SSR (9 nSSR, 12 cpSSR)	
MEAN - μ	UN~[1 x 10^{-4} - 4 x 10^{-3}]
GAM - μ	GA~[1 x 10^{-5}, 1 x 10^{-2}, 2]
MEAN - P	UN~[0.1, 0.4]
GAM - P	GA~[1 x 10^{-2} , 9 x 10^{-1}, 2]

Table S4. Estimates of type I and type II error probabilities for the six scenarios in DIYABC based on simulated datasets. The most likely scenario with the highest posterior probability was chosen (shaded in gray).

True scenario used for simulation	**1**	**2**	**3**	**4**	**5**	**6**	
	Type II error						Type I error
1	-	0.237	0.245	0.267	0.223	0.271	0.389
2	0.422	-	0.442	0.162	0.235	0.201	0. 364
3	0.456	0.415	-	0.242	0.237	0.243	0.300
4	0.409	0.224	0.205	-	0.534	0.341	0.241
5	0.432	0.301	0.242	0.312	-	0.215	0.362
6	0.381	0.238	0.333	0.490	0.394	-	0.209
Mean	0.420	0.283	0.293	0.302	0.3246	0.254	-

Table S5. Species distribution model (SDM) parameters and performance for *Pinus* uliginosa. Climatic variables are the predictor variables retained in the final model, and the percent contribution of each variable to the SDM and the permutation importance were assessed by Maxent (Phillips et al., 2004, 2006, 2017) at three different times: A- the current time period, B- Mid- Holocene (ca. 6 ka), C- the Last Glacial Maximum LGM (ca. 21.5 ka). Model performance was assessed for three scenarios separately based on AUC (area under the curve) criterion (0.937, 0.946 and 0.942 for A, B, and C respectively).

(a) Current time

Climatic variable	**Abbreviation**	**Percent contribution**	**Permutation importance**
aridity index	aridity	29.5	47.6
Emberger's pluviothermic quotient	pluvio_q	21.2	13.5
potential evapotranspiration variability	pet_var	14.3	1.5
potential evapotranspiration of the driest quarter	pet_dry	11.6	11.5
precipitation seasonality (BIO15)	prec_var	11	9.5
temperature seasonality (BIO4)	temp_s	5.7	7.6
temperature annual range (BIO7)	temp_range	5	6.8
potential evapotranspiration of the coldest quarter	pet_cold	0.9	2
precipitation of coldest quarter (BIO19)	prec_cold	0.8	0
(b) Mid Holocene			
aridity index	aridity	27.9	18.3
Emberger's pluviothermic quotient	pluvio_q	26.4	61.3
potential evapotranspiration of the driest quarter	pet_dry	17.4	2
potential evapotranspiration variability	pet_var	12.1	1
precipitation seasonality (BIO15)	prec_var	10.1	5.9
temperature annual range (BIO7)	temp_range	4.1	5
precipitation of coldest quarter (BIO19)	prec_cold	0.9	0.1
temperature seasonality (BIO4)	temp_s	0.8	6
potential evapotranspiration of the coldest quarter	pet_cold	0.3	0.4
(c) Last Glacial Maximum			
aridity index	aridity	29	40.1
Emberger's pluviothermic quotient	pluvio_q	25.1	19.7
potential evapotranspiration of the driest quarter	pet_dry	15	7.2
potential evapotranspiration variability	pet_var	12.5	0.7
precipitation seasonality (BIO15)	prec_var	9.7	4.1
temperature seasonality (BIO4)	temp_s	3.5	16.6
temperature annual range (BIO7)	temp_range	2.9	8.6
precipitation of coldest quarter (BIO19)	prec_cold	1.3	2
potential evapotranspiration of the coldest quarter	pet_cold	0.9	0.9

Figure S1. Results of Mantel test showing the correlation between pairwise genetic distance ($F_{ST}/(1 - F_{ST})$) and geographical distance for the six populations of *P. uliginosa* based on (A) 9 nuclear, (B) 12 chloroplast microsatellites. The isolation-by-distance was assessed with GenAIEx version 6.0 (Peakall & Smouse 2006).

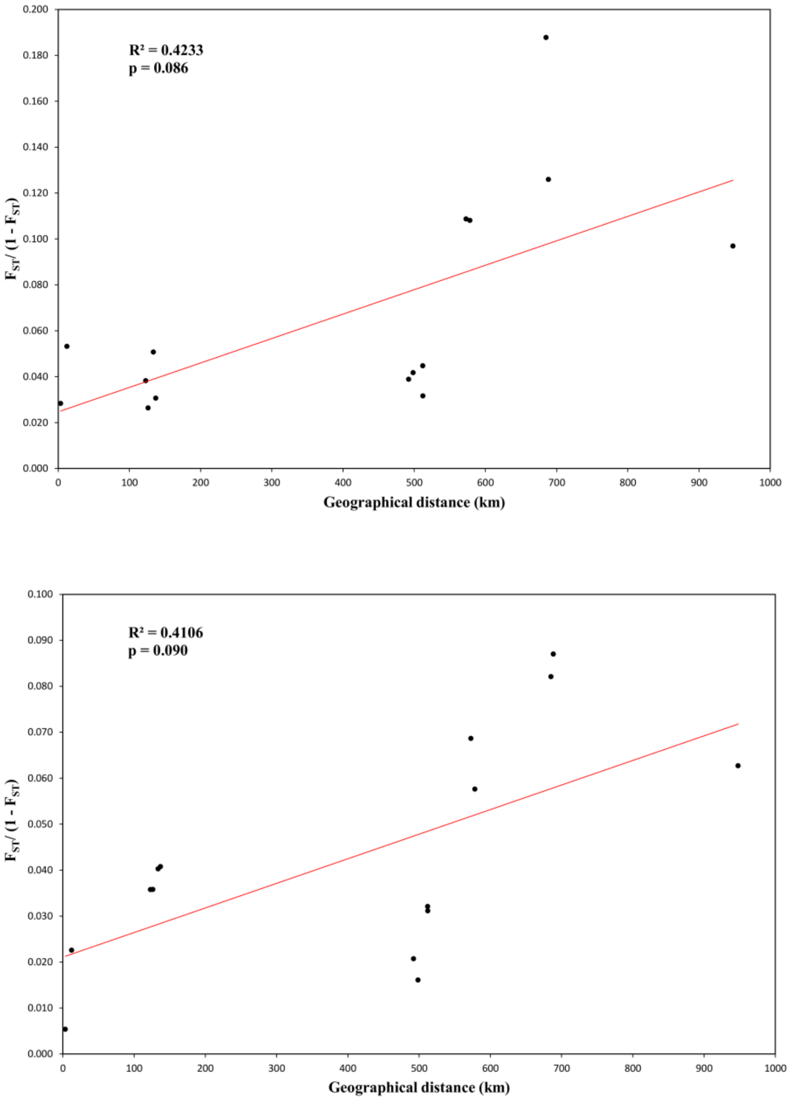


Figure S2. Mean estimated effective population sizes in four pine species. Boxplot: central value—mean, upper/lower hinges—1st and 3rd quartile, whiskers—extreme values.

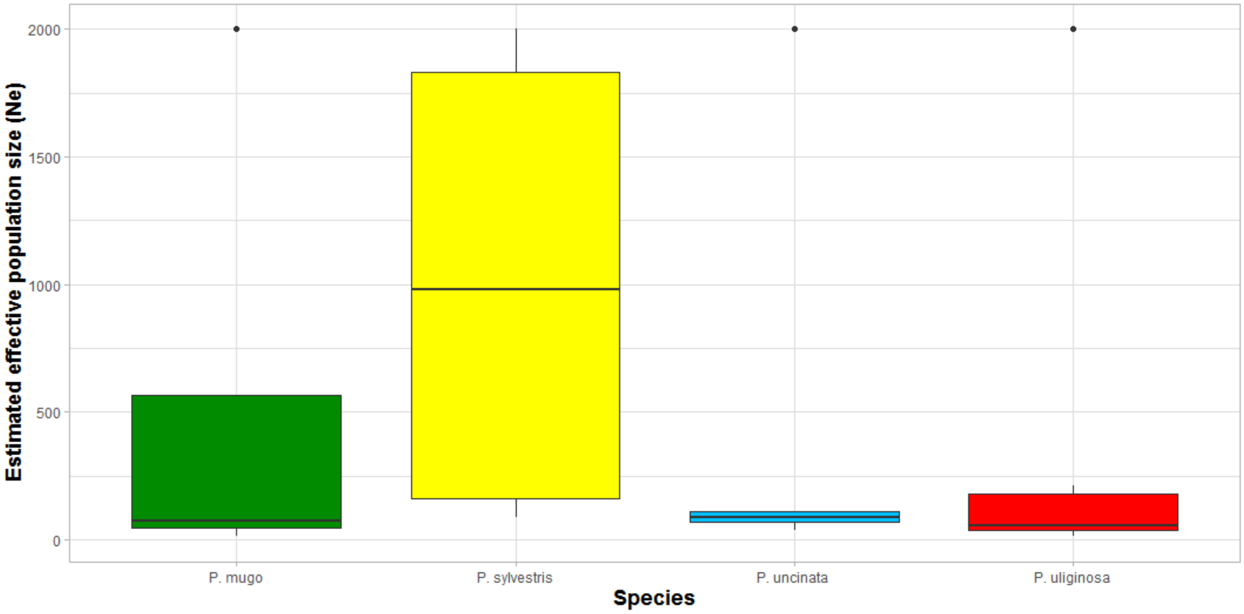


Figure S3. Plots showing fitness of six tested demographic scenarios, based on direct estimates and logistic regression, simulated in DIYABC. For the setting of parameters in each scenario, see Table S4; for the estimation of each parameter for the best-fit scenario, see Table 5.

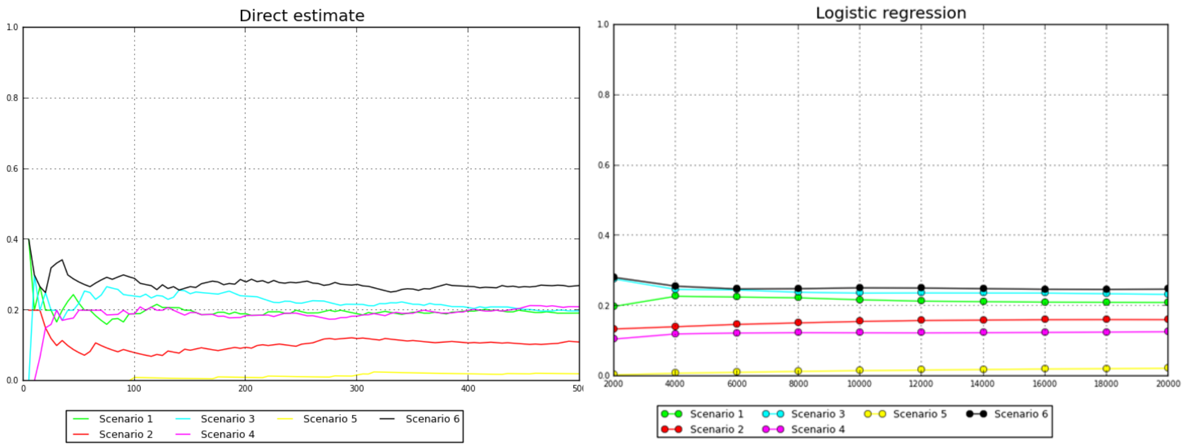


Figure S4. Prior and posterior distributions density curves calculated under scenario 6 for *P. uliginosa* in DIYABC. Times are not scaled.

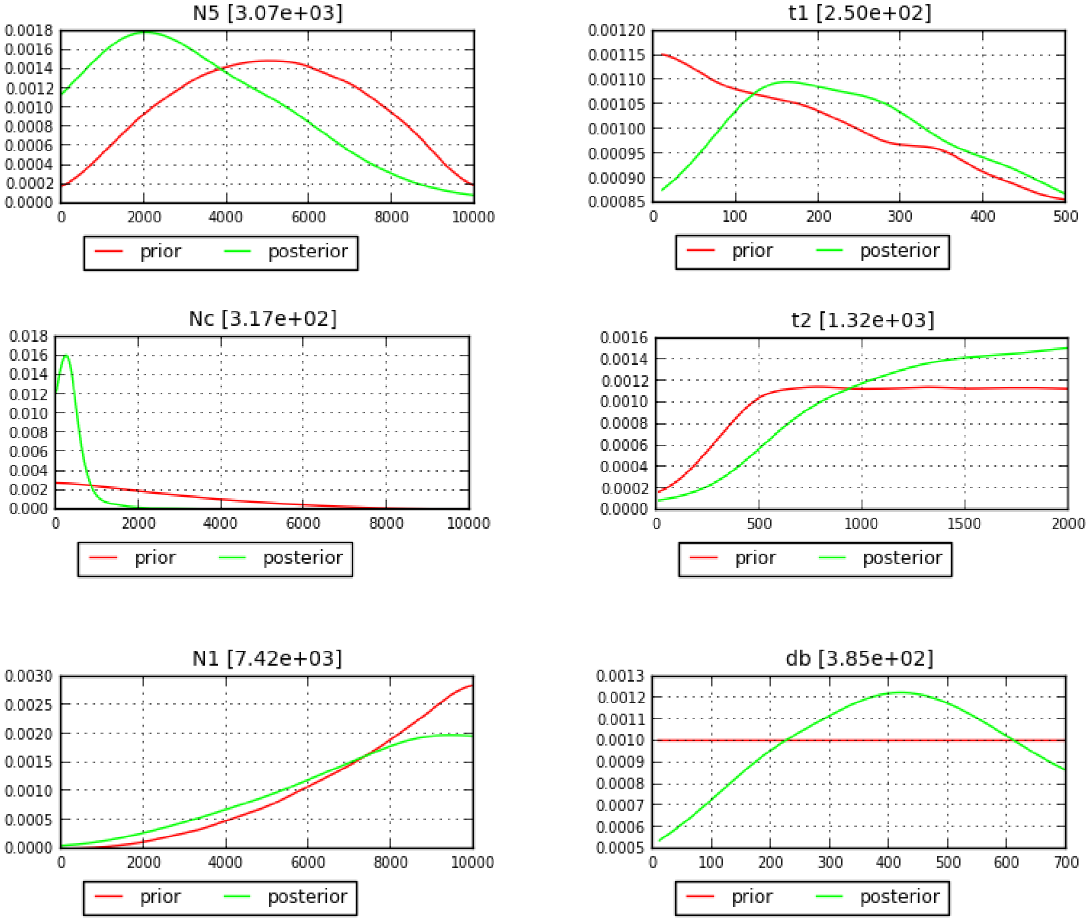
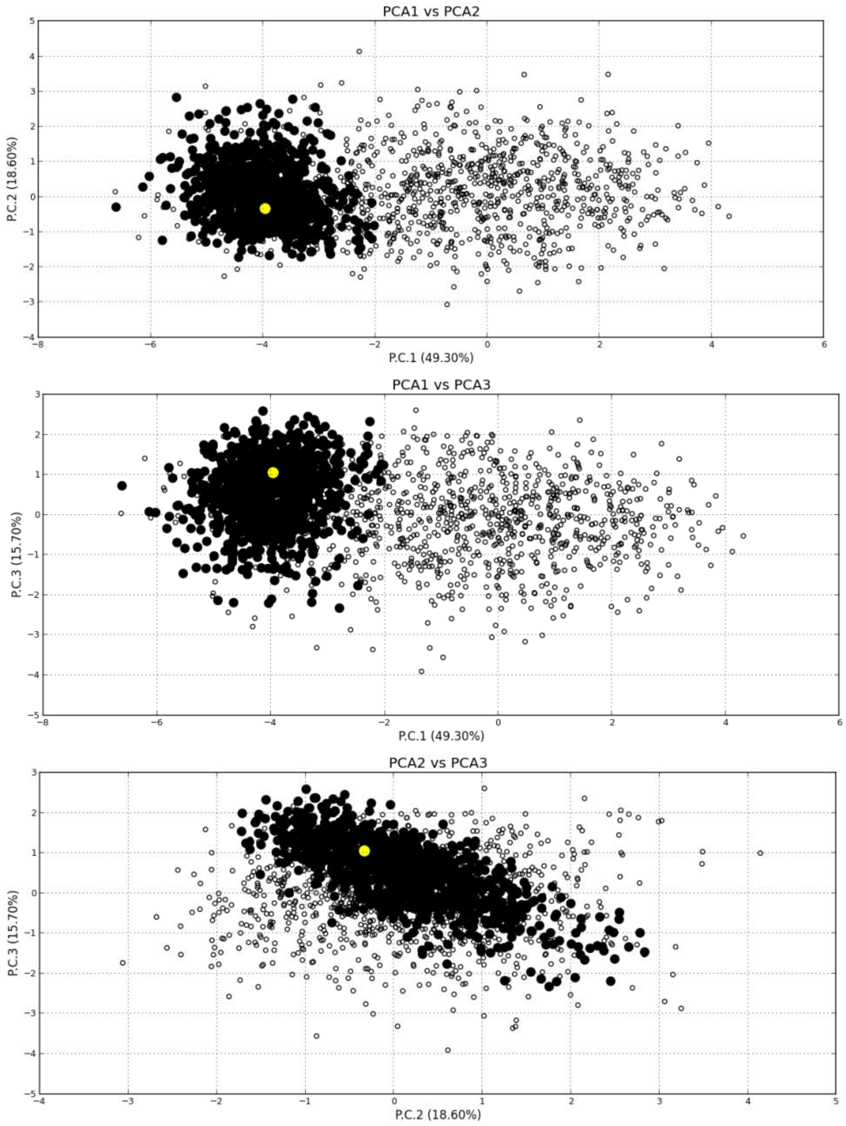


Figure S5. Model checking evaluation for the best supported demographic scenario 6. PCA in the space of summary statistics, showing datasets simulated from the prior distribution of the parameters (black open circles), from the posterior predictive distribution (black filled circles), as well as the observed dataset (yellow circle). Plots show combination of the first three principal components, which cumulatively represent 84% of the total variation.



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<u>Łabiszak B.,</u> Wachowiak W. (2021) Molecular signatures of reticulate evolution within the complex of European pine taxa. Forests, 12, 489, doi:10.3390/f12040489

forests

Article

Molecular Signatures of Reticulate Evolution within the Complex of European Pine Taxa

Bartosz Łabiszak [1],* and Witold Wachowiak [1,2]

[1] Institute of Environmental Biology, Adam Mickiewicz University in Poznań, Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland; witold.wachowiak@amu.edu.pl
[2] Institute of Dendrology, Polish Academy of Sciences, Parkowa 5, 62-035 Kórnik, Poland
* Correspondence: bartosz.labiszak@amu.edu.pl; Tel.: +48-61-829-574-747

Abstract: Speciation mechanisms, including the role of interspecific gene flow and introgression in the emergence of new species, are the major focus of evolutionary studies. Inference of taxonomic relationship between closely related species may be challenged by past hybridization events, but at the same time, it may provide new knowledge about mechanisms responsible for the maintenance of species integrity despite interspecific gene flow. Here, using nucleotide sequence variation and utilizing a coalescent modeling framework, we tested the role of hybridization and introgression in the evolutionary history of closely related pine taxa from the *Pinus mugo* complex and *P. sylvestris*. We compared the patterns of polymorphism and divergence between taxa and found a great overlap of neutral variation within the *P. mugo* complex. Our phylogeny reconstruction indicated multiple instances of reticulation events in the past, suggesting an important role of interspecific gene flow in the species divergence. The best-fitting model revealed *P. mugo* and *P. uncinata* as sister species with basal *P. uliginosa* and asymmetric migration between all investigated species after their divergence. The magnitude of interspecies gene flow differed greatly, and it was consistently stronger from representatives of *P. mugo* complex to *P. sylvestris* than in the opposite direction. The results indicate the prominent role of reticulation evolution in those forest trees and provide a genetic framework to study species integrity maintained by selection and local adaptation.

Keywords: coalescent analysis; hybridization; phylogeny; pines; speciation; species complex; interspecific gene flow

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1. Introduction

Since Darwin's original work, the origin of species and mechanisms of speciation has been a major focus of evolutionary biology. However, in recent years the understanding of these processes has shifted from a simple divergence model driven by the long-lasting isolation with a gradual accumulation of reproductive isolation between two lineages and now encompasses a wide range of complex scenarios within the speciation continuum framework [1,2]. Within this framework, speciation is understood as a process with no fixed endpoints and a lack of clear boundaries between each stage. This perspective poses a challenge to species delineation, especially when secondary contact via gene flow between emerging evolutionary lineages is facilitated at different times [3]. A growing body of evidence indicates that speciation can occur despite the homogenizing effect imposed by interspecific gene flow [4–7], and hybridization is now regarded as an important force shaping the genetic diversity of species that can lead to the emergence of new species [8–13]. Despite hybridization itself being a very widespread phenomenon, the emergence of a new hybrid lineage is usually very rare. Because emerging hybrid individuals are initially rare and must compete with well-adapted parental species, they must either establish reproductive isolation and a unique ecological niche or backcross to one of the parental species and share a niche, to survive. Nevertheless, the reproductive isolation from parental species is one of the fundamental criteria of homoploid hybrid speciation (HHS) proposed by Schumer [8]; however, the relationship between hybridization and reproductive isolation is often hard to find, especially for species with long generation times, such as pines. Therefore, studies of closely related species can inform us about the different stages of the complex speciation continuum and the production of novel genetic diversity of potential adaptive importance [14].

Incomplete reproductive barriers during speciation facilitate the exchange of large genomic regions or preferential introgression of loci between two taxa, and the time since divergence influences the level of shared polymorphisms between them [5,15,16]. Differences in both magnitude and timeframe of secondary contact between diverging taxa can lead to contrasting evolutionary outcomes: from highly divergent species with varying proportions of admixed genomes to hybrid swarms with multiple intermediate forms present. Additionally, species boundaries can be blurred by the lack of morphological or ecological differences between taxa [17,18]. Consequently, investigation of the relationship among closely related species may prove difficult, and such species are often grouped together in taxonomically challenging species complexes. Species complexes were reported in diverse groups of taxa [6,19–22], and they are well studied in plants, including forest trees [23–26]. Nowadays, the application of molecular markers and coalescent ancestry modeling with phylogenetic analyses can greatly improve our ability to resolve evolutionary relationships in such challenging groups of taxa [27–29]. Such research may provide new insights into speciation and mechanisms that maintain species integrity despite gene flow, as our understanding of these processes is still incomplete [30].

In this study, we aimed to disentangle the phylogenetic relationships of such a complex group of closely related European hard pine taxa from the *Pinus mugo* complex. Several species are recognized in this pine complex, including dwarf mountain pine (*Pinus mugo* Turra), peat bog pine (*Pinus uliginosa* G.E.Neumann), and mountain pine (*Pinus uncinata* Ramond ex DC) (see reference [31] for detailed taxonomic descriptions). *P. mugo* is a polycromic shrub or small tree up to 5 m, native to the subalpine zones of European mountain ranges up to 2700 m above sea level and forming dense carpets on the ground [32]. *P. uliginosa* is a single-stemmed tree up to 20 m height, growing in small and isolated populations on peat bogs in lowland areas of Central Europe. *P. uncinata* is a typical erect tree up to 25 m tall and occurs naturally in Alps and Pyrenees at altitudes between 600 and 1600 m above sea level. It shares many morphological features with *P. mugo*, except for tree habit and some characteristics of cones [32]. Pines from the *P. mugo* complex are closely related to Scots pine (*Pinus sylvestris* L.), which has the largest distribution of all pines, mostly lowland, and forms forests of great ecological importance and economic value in Europe and Asia. Due to their relatively recent divergence, weak reproductive barriers, and similar genetic variation at neutral loci but at the same time phenotypical and ecological differentiation, the *P. mugo* complex pines are especially suitable for speciation, hybridization, and local adaptation studies [33–37].

Earlier reports that addressed the genetic relationships between species were focused mainly on the alternative speciation hypothesis of the origin of *P. uliginosa* from Central Europe, considered either as a marginal population of *P. uncinata*, a hybrid between *P. mugo* and *P. uncinata*, and/or *P. mugo* and *P. sylvestris* [35,38] or an example of ancient homoploid hybrid between the later taxa [36,39,40]. However, those studies were based on small sets of molecular markers, lacking detailed phylogenetic analysis, and thus were inconclusive about the divergence history of Scots pine and taxa from the *P. mugo* complex.

Therefore, the main objective of the study was to investigate the evolutionary relationships within the *P. mugo* complex and its close relative *P. sylvestris*. Clear species delineation is needed in this group to better understand the species divergence history at the genomic level that will help us to search regions under selection that maintain species integrity and local adaptation despite ongoing and historical gene flow [41]. Furthermore, as some members of the pine complex are endangered, the exact assessment of the extinction risk may heavily rely on a proper understanding of species phylogeny [42–44]. In particular, we conducted coalescent ancestry modeling and phylogenetic analysis using nucleotide polymorphism data across multiple nuclear loci to (1) examine the alternative scenarios of species origin within the *P. mugo* complex; (2) explore the role of hybridization and putative reticulation events in the history of this group; (3) delineate species boundaries within the *P. mugo* complex pines.

2. Materials and Methods

2.1. Sampling and Genotyping

A total of 122 individuals of four pine species and additionally 10 specimens from the outgroup *P. pinaster* were used in this study (Table S1). Each species was represented by 30 individuals except for *P. uliginosa* ($n = 32$). Seeds were collected from allopatric stands of the species from different populations across its core range. For this study, we sampled *de novo* 8 *P. uliginosa* and 10 *P. pinaster* individuals (seeds were obtained from the PUG3 population from the Batorów reserve and from the collection of INIA Forest Research Center in Spain, respectively) and used raw sequence data derived from earlier studies [45]. Genomic DNA was extracted from haploid megagametophytes from germinated seeds using a DNeasy Plant Mini Kit (Qiagen, Germany).

A subset of 48 genes from 79 analyzed by Wachowiak et al. [46] with no signatures of selection detected therein was selected and sequenced (Table S2). PCR amplifications of the nuclear regions were carried out in a total volume of 15μL containing 15 ng of haploid template DNA, 10 μM of each dNTP, 0.2 μM each of forward and reverse primers, 0.15 U Taq DNA polymerase, 1 × BSA, 1.5 μM of $MgCl_2$ and 1 × PCR buffer (Novazym, Poland). Standard amplification procedures were used with an initial denaturation at 94 °C for 3 min followed by 35 cycles with 30 s denaturation at 94 °C, 30 s annealing at 60 °C for most loci and 1 min 30 s extension at 72 °C, and a final 5 min extension at 72 °C. PCR fragments were purified using Exonuclease I-Shrimp Alkaline Phosphatase enzymatic treatment. About 20 ng of PCR product was used as a template in 10 μL sequencing reactions with the Big Dye Terminator DNA Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and run commercially (Genomed, Poland). CodonCode Aligner (Codon Code Corporation, Centerville, MA, USA) was used to edit and align sequences. Concatenated sequences of all genes were created in DnaSP v.6 [46]

2.2. Genetic Diversity and Structure

We looked at the overall pattern of genetic variation at within and between species levels and calculated the following descriptive statistics of DNA polymorphism for each species at each nuclear loci and averaged across all loci using DnaSP v.6: nucleotide diversity (π), Tajima's D [47], silent divergence to *P. pinaster* (K), haplotype diversity (H_d) and a minimum number of recombination events (R) [48]. To investigate the level of divergence between the studied species, we calculated both locus by locus and global F_{ST} measures [49]. Negative values were reassigned to zero during the mean locus-wide F_{ST} calculation. In addition, net between-species divergences per site (D_{net}) were calculated using SITES 1.1 [50]. Shared polymorphic sites among species could indicate recent divergence, hybrid origin, or gene exchange after speciation. Thus, we recorded the number of polymorphic sites and their distribution for each nuclear locus within and among species, classifying polymorphic sites as either polymorphisms shared between species or fixed differences between species. We visualized all data using the ggpubr package in R [51,52]

Next, to identify evolutionary clusters across the four pine species, we performed principal component analysis (PCA) in R package ggfotify [53]. Then, Bayesian clustering implemented in STRUCTURE 2.3.4 was performed to further visualize the genetic structure in our dataset [54–56]. STRUCTURE was run with an admixture model, no prior population information, and correlated allele frequencies in two variants: with and without *P. pinaster* as an outgroup, to gain more insight into the fine structure of pines from the *P. mugo* complex. For each variant, twenty independent runs were performed for the number of clusters (K) from 2 to 10, with burn-in lengths of 200,000, followed by 300,000 Markov chain Monte Carlo (MCMC) iterations. To detect the most likely number of genetic clusters in our data, both the likelihood estimate [54] and the Evanno method [57] were used. Both likelihood value computation and STRUCTURE plot visualization were performed using the pophelper package in R [58].

2.3. Phylogenetic Analyses

To resolve the phylogenetic relationships within the taxa of the *P. mugo* complex and between them and *P. sylvestris*, we used two methods. Firstly, we conducted maximum-likelihood (ML) analysis on a concatenated sequence set composed of 48 nuclear loci to reconstruct the phylogeny of the studied species on an individual level. *Pinus pinaster* was used as an outgroup in all phylogenetic analyses to root trees. Following the evaluation of nucleotide evolutionary models in jModelTest v2.1.7 [59], the ML tree was constructed in RAxML v.8.1.20 [60] using the best-fit model GTRGAMMAI with 1000 bootstrap replicates.

Secondly, to test whether reticulation events were present in the evolutionary history of the studied species, we performed PhyloNet analysis. Phylogenies per gene were constructed using RAxML with 100 rapid bootstraps under the GTRGAMMA substitution model. Each consensus gene topology was recorded, and the number of resulting phylogenies showing different topologies was counted by hand. All bootstrap trees for each gene were used as an input for PHYLONET v.3.8.2 [61,62] after conversion to the required input file with a custom Phyton script. Maximum pseudolikelihood (MPL) in a coalescent framework was used to infer integrated species trees (using the command InferNetwork_MPL). The analysis involved 10 runs for each gene to ensure finding the best network and allowing for up to 4 reticulation events. This method is robust to gene flow, it is computably efficient, and the results are as accurate as in the case of the maximum likelihood one [63].

To further explore the possibility of gene flow between studied pines after their divergence, we used a four-taxon D statistic test [64]. The test compares two patterns of frequency of ancestral and derived alleles in ingroups and outgroups (so-called ABBA and BABA patterns) under the assumption of equal frequencies of ABBA and BABA topologies (D statistic = 0) given the stochastic lineage sorting. Thus, this test is useful in tracking gene flow between species and can help distinguish incomplete lineage sorting from hybridization or admixtures. In the case of hybrid origin of *P. uliginosa* we should expect the D value to be significantly different from 0 in two topologies ((X_1,U), X_2) and ((X_2,U), X_1), where X_1 and X_2 represent the putative parental species. We also explored other possible introgression scenarios and chose a combination of 12 different topologies to perform ABBA–BABA test and D statistic estimation using the HybridCheck R package [65].

2.4. Testing Speciation Models Using Coalescent Simulations

We used fastsimcoal2 [66,67] to test the fit of our data to different predefined speciation models using coalescent simulations. Multi-site frequency spectra (MSFS) for four pine species were created using Arlequin v.3.5 [68] and used as summary statistics to estimate demographic parameters under an ABC framework. Overall, 16 speciation models were tested, representing different topologies within the *P. mugo* complex with *P. sylvestris* as an outgroup. They differed in the allowed levels of migration between species after their divergence, namely: models 1–4 represented possible dichotomous and polytomous topologies between *P. mugo*, *P. uliginosa*, and *P. uncinata* with no migration allowed. Models 5–6 were classic homoploid hybrid speciation (HHS) models of *P. uliginosa* with different putative parental species, and no migration (*P. sylvestris* and ancestor of *P. uncinata* and *P. mugo* vs. *P. mugo* and *P. uncinata*). Models 7–14 had the same topologies as 1–4 but with different, asymmetric migration matrix allowed: between all species within the *P. mugo* complex and between *P. sylvestris* and their common ancestor (models 7–10) or between all four species after their divergence (models 11–14). Models 15–16 had the same topologies as 5–6 but included migration between all four species (Figure S1).

For each model, we ran 1,000,000 coalescent simulations to approximate the expected MSFS and calculate the associated log-likelihood. A maximum likelihood parameter estimate was obtained from 50 independent runs with 40 cycles of ECM algorithm in each run. In each model, the highest likelihood run (i.e., with the best fitting parameter estimates) was selected using the fsc-selectbestrun.sh script [69], and the best model was chosen using the calculateAIC.R script [70] based on Akaike information criterion (AIC), to account for numbers of parameters in each model. As used in other conifers, the mutation rate was set to a robust rate of 4.01×10^{-8}, and we assumed a generation time of 25 years [12,36,71,72]. To construct 95% confidence intervals (CI), 100 parametric bootstraps with 50 independent runs in each were run, and the parameter estimates of the best-run files of all bootstrapping replicates were calculated with the R package boot [73].

3. Results

3.1. Genetic Diversity and Population Structure

The nuclear dataset was comprised of 48 nuclear loci with no signatures of selection and a mean length of 404 bp (range: 213–720 bp), and 794 SNPs identified in four pines. The loci were found to be selectively neutral in an earlier study [45], and there was no indication of skew in the allelic frequency spectra across the genes in our dataset. Among all species, *P. uliginosa* was characterized by the highest number of polymorphic sites, singleton polymorphic sites, averaged nucleotide diversity, averaged divergence to outgroup, and haplotype diversity (Table 1).

Table 1. Summary statistic at 48 nuclear loci in four pine species.

Species	n	L (bp)	P	S	π	D	R	Ks	H_d (SD)
P. uliginosa	29.3	19,414	441	155	0.005245	−0.28	39	1.864	0.689 (0.066)
P. mugo	29.3	19,414	365	154	0.004039	−0.37	8	1.501	0.601 (0.071)
P. uncinata	29.6	19,414	363	113	0.004546	−0.14	8	1.656	0.623 (0.066)
P. sylvestris	27.8	19,414	367	128	0.004576	−0.16	14	1.682	0.598 (0.073)

n: average number of sequences analyzed per locus; L: total length of the sequence in base pairs excluding indels; P: total number of polymorphic sites; S: total number of singleton mutations; π: average nucleotide diversity; D: Tajima's D statistic; R: average number of recombination events; Ks: average pairwise divergence per site to the outgroup *P. pinaster* at all loci; Hd: haplotype diversity (SD standard deviation), none of the D values were statistically significant.

However, in general, the distribution of those statistics across all genes was very similar in those pines (Table S3), and the overall level of variability was much alike. Consistent with the low divergence, we found no fixed differences between the studied taxa (only in comparison with *P. pinaster* such fixed SNPs were found), and the number of shared polymorphisms between species was similar across all genes (Figures S3 and S4). In addition, both average net divergence and global F_{ST} were lowest within species from the *P. mugo* complex (with *P. uliginosa* being more similar to *P. mugo* and *P. uncinata*, than the latter two to each other). There was a 3–4 fold higher difference between them and *P. sylvestris* with the highest F_{ST} and D_{net} found between *P. sylvestris* and *P. mugo* (Table 2, Figures 1 and S4). Additionally, all studied pines shared similar patterns of F_{ST} distribution in pairwise comparison with *P. pinaster* (Figure S2).

Table 2. Summary statistics for F_{ST} and net divergence between species.

Species Pair	$F_{ST} \pm$ SD	$D_{\text{net}} \pm$ SD
P. uliginosa vs. *P. mugo*	0.068 ± 0.088	0.00032 ± 0.00042
P. uliginosa vs. *P. uncinata*	0.056 ± 0.067	0.00032 ± 0.00046
P. uliginosa vs. *P. sylvestris*	0.167 ± 0.155	0.00108 ± 0.00109
P. mugo vs. *P. uncinata*	0.088 ± 0.104	0.00054 ± 0.00087
P. sylvestris vs. *P. mugo*	0.260 ± 0.197	0.00181 ± 0.00203
P. sylvestris vs. *P. uncinata*	0.142 ± 0.138	0.00084 ± 0.00110

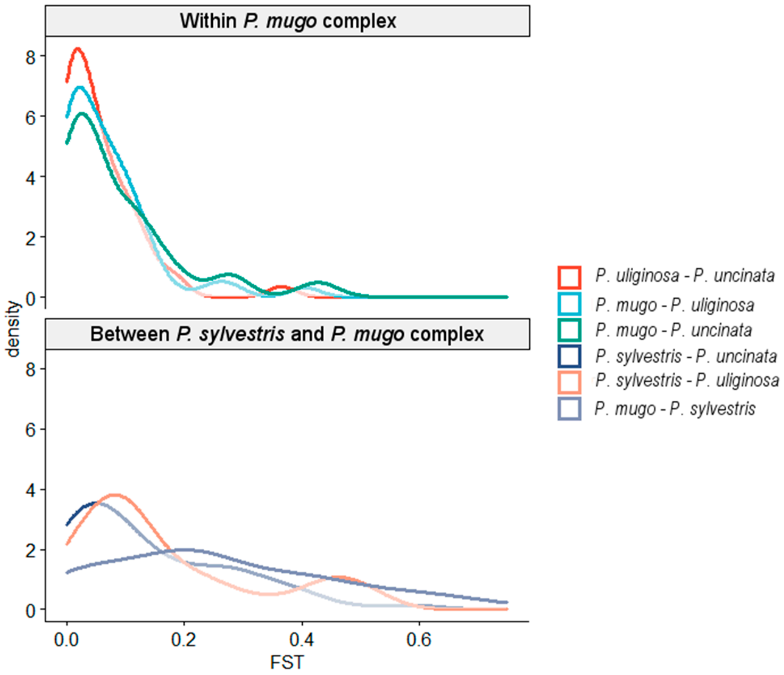

Figure 1. Distribution of genetic differentiation (F_{ST}) between species pairs based on all variable sites in a set of 48 nuclear loci.

PCA analysis could only clearly identify a distinct species-specific cluster in the case of *P. sylvestris* (and *P. pinaster* when it was included as an outgroup), with individuals from species within the *P. mugo* complex forming mostly overlapping clusters with different levels of homogeneity (*P. uncinata* and *P. uliginosa* were the most heterogeneous ones) and the first two principal components explaining 19.81% (21.24%) and 9.04% (8.48%), respectively (Figures 2 and S5).

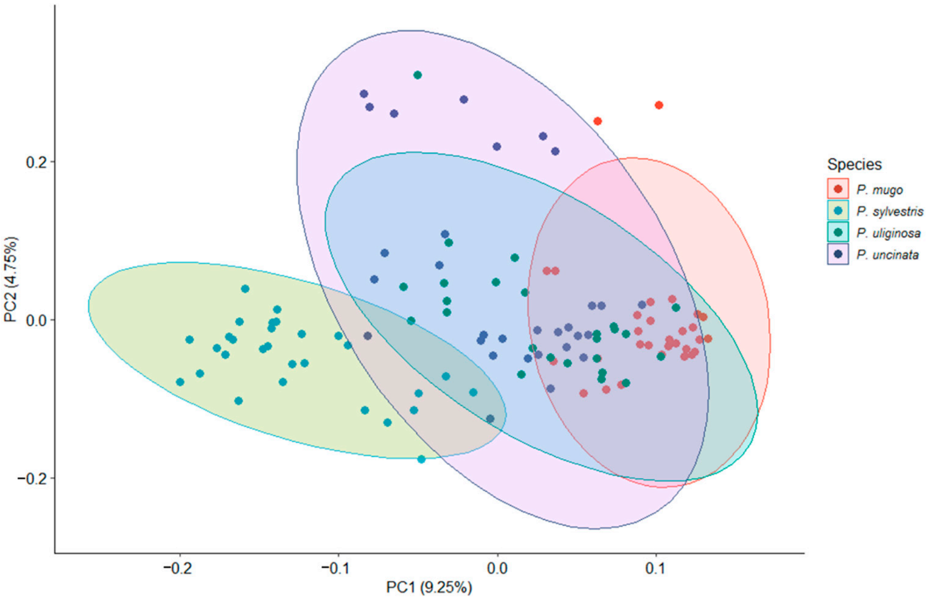

Figure 2. Results of principal component analysis (PCA) showing the differentiation of species by the first two principal axes.

Similarly, STRUCTURE results (with or without the outgroup *P. pinaster* included) revealed a close genetic relationship between *P. mugo*, *P. uliginosa*, and *P. uncinata* with the best-supported number of genetic clusters (K = 3) with outgroup and (K = 2) when

P. pinaster was excluded from the analysis (Figures 3a,b and S6). When *P. pinaster* was included, it formed its own cluster, while three taxa from the *P. mugo* complex were grouped together and were clearly delineated from the cluster composed of *P. sylvestris* individuals. The resolution of genetic structure within species from the *P. mugo* complex did not improved when *P. pinaster* was excluded and the two main clusters reflected *P. sylvestris* vs. *P. mugo* complex division with no signatures of further substructure. The contrast between *P. mugo* and *P. uliginosa*/*P. uncinata* was more evident, as the latter species shared a greater proportion of their genetic composition with *P. sylvestris* (Figure 3).

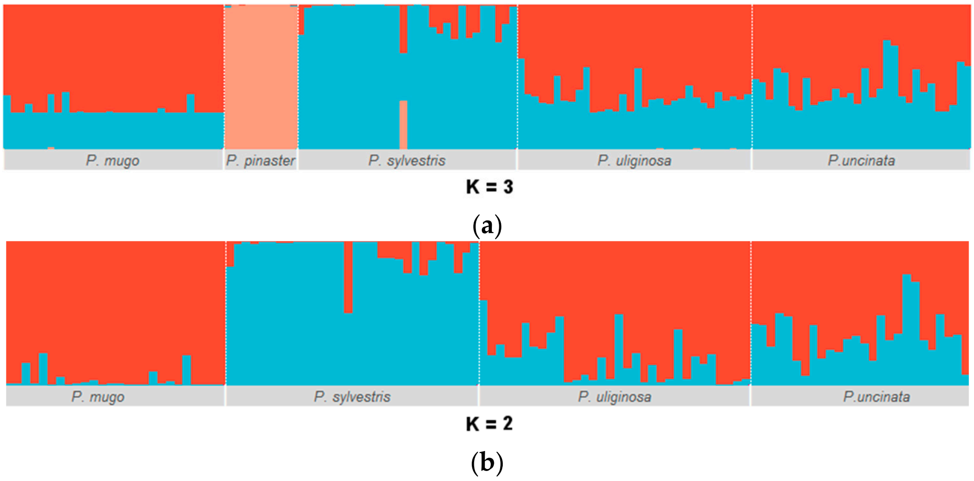

Figure 3. Results of STRUCTURE analysis between studied pine species with (**a**) and without (**b**) *P. pinaster* as outgroup species. The best number of genetic clusters K = 3 and K = 2 (with and without the outgroup, respectively) was indicated by the results of likelihood estimates and the Evano method.

3.2. Reticulate Phylogeny of Four Pines

The results of our phylogenetic ML analysis provided additional insight into the complex evolutionary history of the studied pine species and the possibility of non-bifurcating speciation events in their past. Three main clades could be identified in the phylogenetic tree obtained from 19,414 bp concatenated sequences from 48 nuclear genes. However, individuals from the same species form a monophyletic group only for the outgroup *P. pinaster*. The second clade was composed predominantly of *P. sylvestris* (with four *P. uncinata* and one *P. uliginosa* specimens), and the remaining *P. sylvetris* individuals were grouped together with the species from the *P. mugo* complex (Figure S7).

Additionally, contrasting topologies were also recorded from individual consensus gene trees. Overall, high numbers of individual topologies (10 out of 15 possible unrooted topologies for five species) were reconstructed across 48 genes. The three most frequent topologies (~60% in total) are shown in Figure 4a–c. The first two are similar in respect to the position of the basal clades (*P. pinaster* followed by *P. sylvestris*) but differ in relationships within the *P. mugo* complex: indicating either *P. mugo* and *P. uncinata* or *P. uliginosa* and *P. uncinata* as pairs of most closely related species. Surprisingly, the third topology places *P. sylvestris* as the innermost clade with *P. uncinata* and *P. uliginosa* followed by *P. mugo* at the base of the tree with *P. pinaster* as the outgroup. The corresponding PhyloNet species tree indicated the presence of at least three reticulation events in the evolutionary history of the studied pines, not limited only to the origins of *P. uliginosa*, but also involving other members of the *P. mugo* complex and *P. sylvestris* as well (Figure 4d). Finally, significant gene flow between members of the *P. mugo* complex and between them and *P. sylvestris* was found using the ABBA–BABA test (Table 3).

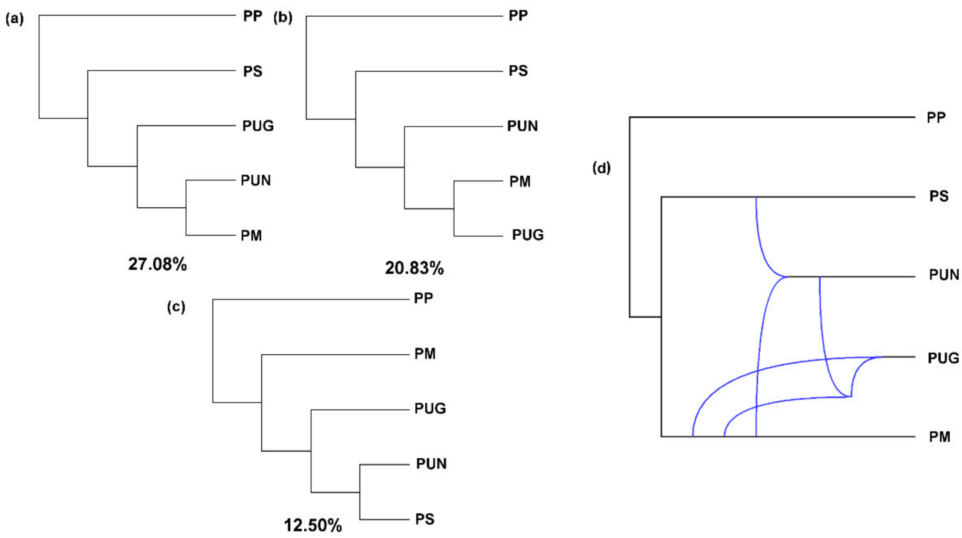

Figure 4. Genetic relationships between *P. uliginosa* (PUG), *P. mugo* (PM), *P. uncinata* (PUN), and *P. sylvestris* (PS) with *P. pinaster* (PP) as the outgroup. Three most frequent gene topologies obtained from maximum pseudo-likelihood analysis for a set of 48 gene trees are shown (**a**–**c**); (**d**) Maximum pseudolikelihood (MPL) bootstrap network generated by PhyloNet with up to four reticulations allowed. Reticulation events are drawn in blue.

Table 3. Results of the ABBA–BABA test.

P1	P2	P3	A	D	*p*	Z
PM	PUG	PUN	PP	0.601	ns	1.073
PM	PUN	PUG	PP	0.161	ns	1.172
PUN	**PUG**	**PM**	**PP**	**0.487**	**<0.0001**	**3.304**
PM	PS	PUN	PP	−0.035	ns	−0.128
PUN	**PS**	**PM**	**PP**	**0.288**	**<0.05**	**1.990**
PUN	PM	PS	PP	0.320	ns	1.67
PM	**PS**	**PUG**	**PP**	**0.516**	**<0.0001**	**4.521**
PM	**PUG**	**PS**	**PP**	**0.641**	**<0.0001**	**5.057**
PM	PUG	PUN	PS	0.603	ns	1.073
PM	PUN	PUG	PS	0.183	ns	1.172
PUN	**PUG**	**PM**	**PS**	**0.472**	**<0.0001**	**3.304**

Patterson's D value for introgression between taxa with Z score and significance values. Acronyms for species: PUG: *P. uliginosa*; PM: *P. mugo*; PUN: *P. uncinata*; PS: *P. sylvestris*; PP: *P. pinaster*. Topologies with statistically significant values of D are bolded.

3.3. Alternative Speciation Models

Among the 16 possible speciation models tested, model 12 (Figure 5) with dichotomous divergence within the *P. mugo* complex was chosen as the best fitting to our data, based on the lowest values of AIC (Supporting Table S4).

In this model, *P. mugo* and *P. uncinata* were sister species with basal *P. uliginosa* and asymmetric migration between all four species after their divergence. However, it is worth noting that the second-best model with relatively small ΔAIC was model 14 with an unresolved polytomous topology within the *P. mugo* complex after their split from *P. sylvestris* and migration between all species after the divergence (Figure S1, Table S4). The estimated parameters for the best model suggest that the common ancestors of the species from the *P. mugo* complex split from the *P. sylvestris* ~5.9 Ma (5–8.5 Ma, 95% CI), and further divergence within the complex occurred ~4Ma (3.9–5 Ma) with the origin of *P. uliginosa* and most recent divergence of *P. mugo* from *P. uncinata* ~2 Ma (1.5–2.4 Ma; Table 4). Under this scenario, the current effective population sizes for *P. mugo*, *P. uliginosa*, *P. uncinata*, and *P. sylvestris* were estimated to be 406,282, 77,680, 78,879, and 554,232, respectively (Table 4). The results indicate asymmetric gene flow and introgression between all four species with migration rates from 3.28^{-10} to 4.91^{-5} per generation and the strongest gene flow in pairs: *P. mugo* vs. *P. uliginosa*, *P. uncinata* vs. *P. uliginosa* and *P. sylvestris* vs. *P. uliginosa* (Table 4).

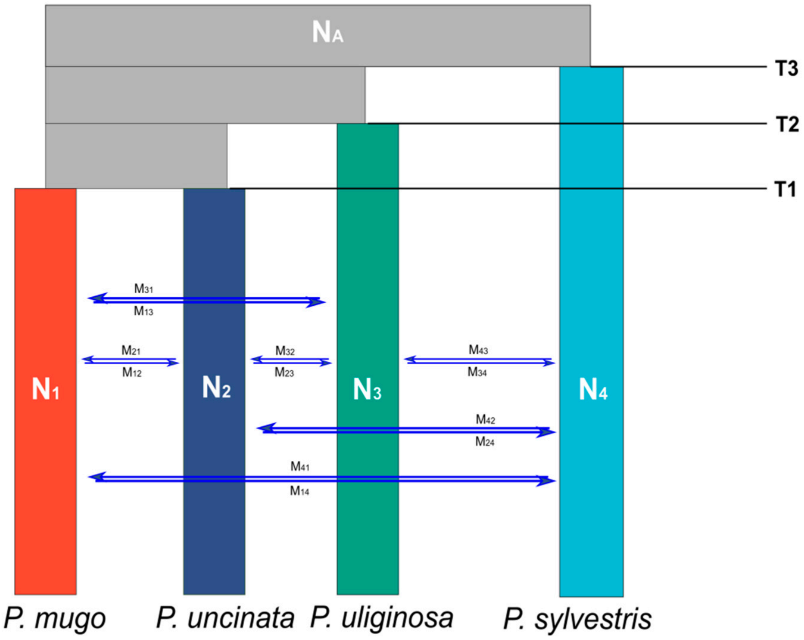

Figure 5. Schematic representation of the best fit model (12) inferred by fastsimcoal2. Detailed demographic parameters for this model are presented in Table 4. Parameters acronyms: N_A: ancestral population effective size; N_1:N_4: effective population sizes for the four studied pines (*P. mugo*, *P. uliginosa*, *P. uncinata*, and *P. sylvestris*, respectively); T_1:T_3: time for three divergence events in years; M: migration per generation after the divergence between pairs of species (arrows indicate migration direction).

Table 4. Maximum likelihood estimates and 95% confidence intervals of demographic parameters for the best supported model shown in Figure 5.

Parameters	Point Estimate	Lower Boundof 95% CI	Upper Boundof 95% CI
N_A	1,817,521	1,223,654	1,511,901
N_1	406,282	353,176	674,610
N_2	78,879	4839	88,690
N_3	77,680	5842	81,793
N_4	554,232	508,589	761,921
T_1	2,074,675	1,403,763	2,422,625
T_2	3,999,925	3,915,625	5,303,234
T_3	5,925,175	5,038,781	8,507,750
$M_{1\to2}$	3.28^{-10}	6.97^{-11}	9.83^{-9}
$M_{2\to1}$	1.26^{-9}	2.30^{-10}	7.83^{-8}
$M_{1\to3}$	8.11^{-8}	3.78^{-9}	4.00^{-7}
$M_{3\to1}$	4.91^{-5}	8.56^{-6}	1.41^{-4}
$M_{2\to3}$	1.24^{-7}	3.15^{-8}	2.00^{-7}
$M_{3\to2}$	3.36^{-5}	6.25^{-9}	8.75^{-4}
$M_{1\to4}$	1.62^{-6}	1.50^{-6}	1.76^{-6}
$M_{4\to1}$	1.55^{-7}	5.71^{-9}	5.39^{-6}
$M_{2\to4}$	2.94^{-5}	6.90^{-6}	1.06^{-5}
$M_{4\to2}$	1.55^{-7}	5.71^{-9}	5.39^{-6}
$M_{3\to4}$	6.03^{-6}	5.38^{-6}	9.47^{-6}
$M_{4\to3}$	1.55^{-7}	5.71^{-9}	5.39^{-6}

Parameters acronyms: N_A: ancestral population effective size; N_1:N_4: effective population sizes for four studied pines (*P. mugo*, *P. uliginosa*, *P. uncinata*, and *P. sylvestris*, respectively); T_1:T_3: time for three divergence events in years; M: migration per generation after the divergence between pairs of species (arrows indicate the migration direction). See Figure 5 for a model summary.

4. Discussion

4.1. Models of Speciation

The patterns of nucleotide polymorphism and the signatures of divergence between species, including clustering analysis, reflected the greater similarity of pines in the *P. mugo* complex and their slight distinctiveness from *P. sylvetsris*, than expected under the pure HHS model. However, as we demonstrate in our study, the speciation history of those pines did not conform to the strict bifurcating divergence but was heavily influenced by interspecific gene flow and reticulation events in the past. Out of 16 tested alternative evolutionary models of relationships between taxa, including the two most likely scenarios of homoploid hybrid speciation of *P. uliginosa*, the best fitting one indicated *P. mugo* and *P. uncinata* as a sister species with basal *P. uliginosa* and *P. sylvestris* as an outgroup. Furthermore, the most accurate model involved an asymmetric gene flow between all four species after their divergence (Table 4, Figure 5).

Our estimates of about 5 Ma divergence time between *P. sylvestris* and taxa from the *P. mugo* complex are in line with earlier reports [36]. Furthermore, we were able to estimate the time of two subsequent divergence events within the pine complex, which happened 4 Ma and 2 Ma, respectively. Initially, *P. uliginosa* split from the common ancestor of *P. uncinata* and *P. mugo*, and then those two pines further diverged, which led to the emergence of contemporary *P. uncinata* and *P. mugo* (Table 4). A short time since divergence could explain the lack of fixed differences between all studied pines, particularly the low divergence within taxa from the *P. mugo* complex and the generally high number of shared polymorphisms between them. Such similarity could also be explained by the time required for the reciprocal monophyly between diverging species - the greater the effective population size and the more time is necessary to observe it [74]. Given the generally large effective population sizes of the studied pine taxa, estimated here to be in range of 77,680–554,232, this time would be orders of magnitude greater than the mean divergence time between them (2 Ma). Our estimates of effective population sizes are analogous to the results of previous studies [36,75], with *P. sylvestris* and *P. mugo* characterized by the highest and *P. uncinata* and *P. uliginosa* by the lowest sizes. However, considerably lower estimates of effective population size were reported in a recent study of the demographic history of *P. uliginosa* [76]. The difference between those estimates is most likely caused by the number and type of loci (794 SNPs from nuclear genes vs. nine SSR and 12 cpSSR loci) used in each analysis and the fact that we provide estimates for the whole species but not individual populations.

4.2. Interspecific Gene Flow

Significant gene flow after divergence could further reduce the observed species' genetic differentiation [4,5,16]. Different tests used in our study confirmed that gene exchange had played a significant role in the evolutionary history of those pines (Table 3, Figures 4 and 5). Additionally, coalescent estimations helped us infer both the magnitude and relative timing of gene flow between species, which suggests that secondary contact was possible long after species divergence. Similar findings in different conifer systems confirm that reproductive barriers between congeners in this group are weak and reticulate speciation is not only possible but often influences patterns of species diversity in this genus [25,77,78]. Our data indicate that the pattern of introgression is not symmetric between taxa, and in general, stronger gene flow was estimated from representatives of the *P. mugo* complex to *P. sylvestris* than in the opposite direction. Earlier studies reported such asymmetric ongoing gene flow within present-day contact zones of *P. uliginosa*, *P. mugo*, and *P. sylvestris* [79–81]. Surprisingly, the strength of gene flow within the *P. mugo* complex is not consistent considering the genetic relatedness between the taxa-in fact, *P. mugo* and *P. uncinata* are characterized by the lowest reciprocal migration rates among all analyzed species. Those results may reflect their rapid divergence after the split, facilitated by the geographic isolation and contemporary disjoint distribution of sympatric populations found only in the Alps, with *P. uncinata* primarily located in the western and *P. mugo* in the eastern parts of the mountains. Patterns of mitochondrial DNA variation support this ongoing divergence, as both species, could be clearly delineated by mitochondrial markers, and *P. uncinata* harbors unique and fixed mitotypes [33]. Nevertheless, there is evidence for interspecific gene exchange between those pines in their contact zone in the Alps [82]. Considering the relatively strong signals of gene flow between *P. uncinata* and *P. uliginosa* and given their current allopatric ranges with the limited and fragmented distribution of the latter species, we hypothesize that it may reflect an ancient introgression between those pines. Although pollen records could infer past plant species distribution [83], palynological records only poorly distinguished taxa of the *P. mugo* complex from *P. sylvestris*, and further distinction within the complex is impossible [84].

Evidence of widespread introgression between studied pines is further supported by the particularly high number of inconsistencies of gene trees with species trees found in our dataset. This pattern was especially conspicuous for those gene topologies where *P. uncinata* and *P. sylvestris* were indicated as the most closely related species (Figure 4). Similar patterns could also arise as an effect of incomplete lineage sorting; however, it is less likely in our case, as the results of the ABBA–BABA test confirmed a significant excess of allelic patterns consistent with a history of introgression (Table 3). Introgression could also explain the pattern observed in individual-based phylogeny, where the three main clades were not species-specific and individuals from different species grouped together. It should also be noted that overall, the bootstrap support for most of the phylogenetic tree branches was low (<50) with highly supported nodes only in the case of outgroup *P. pinaster* main branch and some terminal nodes. Additionally, some specimens from two *P. sylvestris* populations (from Poland and Finland) were more closely related to individuals from *P. uliginosa* and *P. uncinata*, than those from conspecific populations (Figure S7). The wide distribution of *P. sylvestris* and its known long-distance migration associated with postglacial recolonization of Europe could facilitate overlap with other pine species and locally restricted gene flow. Previous studies reported similarities between Scots pine populations from Poland and Finland [85,86], reflecting their common phylogeographic history, and mitotype sharing between *P. uliginosa*, *P. mugo*, and *P. sylvestris* in their contact zones was also reported [33,80].

Although our dataset contained selectively neutral loci, such preferentially introgressed alleles could reveal the genomic location of regions of adaptive value that were predominantly targeted by introgression and linked to neutral variants [87]. Examples of such adaptive introgression in plant systems are emerging in recent years, especially in crop species and their wild relatives [88–91]. Thus, scans for signatures of selection in introgressed genomic regions could be valuable research targets in future studies.

4.3. Species Integrity within the P. mugo Complex

Due to introgression and hybridization between related species, the tree-like, bifurcating phylogeny is difficult or even impossible as widespread introgression across the genome will result in many genes with incongruent phylogenies. Thus, in case of frequent introgression, the maximum likelihood or most probable species tree from a series of genes may reflect proper relationships between taxa as a phylogeny consistent across the whole genome might not exist [92]. In systems like the pine taxa studied here, where successive divergence occurred relatively quickly, and the possibility of hybridization between both sister and no-sister species prevailed for long enough, we should rather seek phylogenetic webs or reticulate networks instead of a phylogenetic tree [92]. Nevertheless, despite interspecific gene flow, largely shared neutral polymorphism and reticulation events evident in the evolution of pines from the *P. mugo* complex and *P. sylvestris*, they maintain their distinct morphological and ecological features. The species can be recognized phenotypically and show patterns of local adaptations related to temperature, water availability, pathogen resistance, or photoperiod [93,94]. Such species integrity was found to be preserved due to natural selection in spite of gene flow and interspecific hybridization in other trees, such as oaks, poplars, and eucalyptuses [95]. Under the model of speciation with gene flow, a divergence between populations and species is considered mainly driven by directional selection in a few genomic regions, harboring genes associated with adaptation to different habitats, and it is accompanied by generally low levels of genetic differentiation at other loci in the genome [5]. Predictions regarding the heterogeneous genomic landscape of differentiation were recently confirmed in diverging populations of various taxa [96–99]. Ecological differences are evident within the *P. mugo* complex, including *P. uliginosa* adapted to peat-bog environments and *P. mugo* and *P. uncinata* adapted to mountain regions of Europe that reflects the species divergence in phenology and growth of young trees under common garden experiments [93]. However, the molecular basis of the species' adaptive variation to specific environmental variables is still mostly unknown.

Detailed inspections of species-specific niche envelopes within this complex are required to guide further studies of adaptively important traits associated with species divergence and maintenance of species integrity. The ability to conduct genome scans in search of loci under selection was restricted only to sets of candidate genes [45,100] as until recently, access to genomic resources was seriously imposed by the extremely large and complex genomes of pines [101,102]. However, the recent development of the transcriptome sequence of the species and the Affymetrix ~50 k SNPs array [103] overcomes the limitations of earlier studies, and genome-wide analyses are now feasible. Those studies should advance the search for genomic landscapes of divergence and selection to better understand the genetic basis of adaptation and speciation with interspecific gene flow.

5. Conclusions

Our study demonstrates the complex evolutionary history of the investigated taxa with strong patterns of reticulated rather than strictly bifurcating divergence as a result of speciation with a significant interspecific gene flow. Consequently, the taxa of *P. mugo* complex share much of the neutral genetic variation, different genes yield contrasting phylogenies, and the majority consensus tree could be the best approximation of species genetic relatedness. However, despite past hybridization and introgression, the species integrity is maintained through ecological and morphological differences, most likely due to selection at specific genomic regions and local adaptation to slightly disjunct environmental envelopes. Considering novel genomic resources and analytical tools recently developed for the investigated species, the pines could be useful to search for loci involved in the species phenotypic and ecological divergence.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/f12040489/s1, Figure S1: Schematic representation of the 16 demographic models tested in fastsimcoal2, Figure S2: Distribution of genetic differentiation (F_{ST}) in pairwise comparisons between four studied pines and an outgroup *P. pinaster* based on all variable sites in a set of 48 nuclear loci, Figure S3: Number of shared polymorphisms between studied species in pairwise comparisons across all of the 48 nuclear loci studied, Figure S4: Net between-species divergence per site: (A), and number of shared polymorphisms: (B) in pairwise comparisons averaged across 48 nuclear loci, Figure S5: The results of the principal component analysis (PCA) showing differentiation of species (*P. pinaster* included as an outgroup) by the first two principal axes, Figure S6: The results of likelihood estimate and Evanno method for STRUCTURE runs, Figure S7: Phylogenetic relationships (ML tree) of 132 samples of *P. uliginosa*, *P. mugo*, *P. uncinata* and *P. sylvestris* rooted with *P. pinaster* based on the concatenated sequence of 48 nuclear loci, Table S1: Location samples analysed, Table S2: Nuclear loci studied, Table S3: Summary statistics at each locus in four pine species, Table S4: Maximum likelihood and Akaike statistics for all 16 evolutionary models tested in fastsimcoal2.

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References

1. Hendry, A.P. Ecological speciation! Or the lack thereof? *Can. J. Fish. Aquat. Sci.* **2009**, *66*, 1383–1398. [CrossRef]
2. Mallet, J. Hybridization, ecological races and the nature of species: Empirical evidence for the ease of speciation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2008**, *363*, 2971–2986. [CrossRef]
3. Galtier, N. Delineating species in the speciation continuum: A proposal. *Evol. Appl.* **2019**, *12*, 657–663. [CrossRef] [PubMed]
4. Nosil, P. Speciation with gene flow could be common. *Mol. Ecol.* **2008**, *17*, 2103–2106. [CrossRef]
5. Feder, J.L.; Egan, S.P.; Nosil, P. The genomics of speciation-with-gene-flow. *Trends Genet.* **2012**, *28*, 342–350. [CrossRef]
6. Martin, S.H.; Dasmahapatra, K.K.; Nadeau, N.J.; Salazar, C.; Walters, J.R.; Simpson, F.; Blaxter, M.; Manica, A.; Mallet, J.; Jiggins, C.D. Genome-wide evidence for speciation with gene flow in *Heliconius* butterflies. *Genome Res.* **2013**, *23*, 1817–1828. [CrossRef]
7. Gailing, O.; Curtu, A.L. Interspecific gene flow and maintenance of species integrity in oaks. *Ann. For. Res.* **2014**, *57*, 5–18. [CrossRef]
8. Schumer, M.; Rosenthal, G.G.; Andolfatto, P. How common is homoploid hybrid speciation? *Evolution* **2014**, *68*, 1553–1560. [CrossRef] [PubMed]
9. Gao, J.; Wang, B.S.; Mao, I.F.; Ingvarsson, P.; Zeng, Q.Y.; Wang, X.R. Demography and speciation history of the homoploid hybrid pine *Pinus densata* on the Tibetan Plateau. *Mol. Ecol.* **2012**, *21*, 4811–4827. [CrossRef] [PubMed]
10. Arnold, M.L. *Evolution through Genetic Exchange*; Oxford University Press: Oxford, UK, 2007; pp. 1–252. [CrossRef]
11. Lamichhaney, S.; Han, F.; Webster, M.T.; Andersson, L.; Grant, B.R.; Grant, P.R. Rapid hybrid speciation in Darwin's finches. *Science* **2018**, *359*, 224–228. [CrossRef] [PubMed]
12. Ru, D.; Sun, Y.; Wang, D.; Chen, Y.; Wang, T.; Hu, Q.; Abbott, R.J.; Liu, J. Population genomic analysis reveals that homoploid hybrid speciation can be a lengthy process. *Mol. Ecol.* **2018**, *27*, 4875–4887. [CrossRef]
13. Mallet, J. Hybridization as an invasion of the genome. *Trends Ecol. Evol.* **2005**, *20*, 229–237. [CrossRef] [PubMed]
14. Nieto Feliner, G.; Álvarez, I.; Fuertes-Aguilar, J.; Heuertz, M.; Marques, I.; Moharrek, F.; Piñeiro, R.; Riina, R.; Rosselló, J.A.; Soltis, P.S.; et al. Is homoploid hybrid speciation that rare? An empiricist's view. *Heredity* **2017**, *118*, 513–516. [CrossRef]
15. Harrison, R.G.; Larson, E.L. Hybridization, introgression, and the nature of species boundaries. *J. Hered.* **2014**, *105*, 795–809. [CrossRef]
16. Nosil, P.; Funk, D.J.; Ortiz-Barrientos, D. Divergent selection and heterogeneous genomic divergence. *Mol. Ecol.* **2009**, *18*, 375–402. [CrossRef]
17. Bickford, D.; Lohman, D.J.; Sodhi, N.S.; Ng, P.K.L.; Meier, R.; Winker, K.; Ingram, K.K.; Das, I. Cryptic species as a window on diversity and conservation. *Trends Ecol. Evol.* **2007**, *22*, 148–155. [CrossRef]
18. Grant, P.R.; Grant, B.R. Unpredictable Evolution in a 30-Year Study of Darwin's Finches. *Science* **2002**, *296*, 707–711. [CrossRef] [PubMed]
19. O'Donnell, K.; Ward, T.J.; Robert, V.A.R.G.; Crous, P.W.; Geiser, D.M.; Kang, S. DNA sequence-based identification of *Fusarium*: Current status and future directions. *Phytoparasitica* **2015**, *43*, 583–595. [CrossRef]
20. Harbach, R.E. *Culex pipiens*: Species versus species complex taxonomic history and perspective. *J. Am. Mosq. Control Assoc.* **2012**, *28*, 10–23. [CrossRef]
21. Welch, M.E.; Rieseberg, L.H. Habitat divergence between a homoploid hybrid sunflower species, *Helianthus paradoxus* (Asteraceae), and its progenitors. *Am. J. Bot.* **2002**, *89*, 472–478. [CrossRef]
22. Crespo, M.; Azorín, M.; Mavrodiev, E. Can a rainbow consist of a single colour? A new comprehensive generic arrangement of the '*Iris sensu latissimo*' clade (Iridaceae), congruent with morphology and molecular data. *Phytotaxa* **2015**, *232*, 1–78. [CrossRef]
23. Bucci, G.; Anzidei, M.; Madaghiele, A.; Vendramin, G.G. Detection of haplotypic variation and natural hybridization in halepensis-complex pine species using chloroplast simple sequence repeat (SSR) markers. *Mol. Ecol.* **1998**, *7*, 1633–1643. [CrossRef]
24. Yeh, F.; Ye, T.; Yang, R.-C. Multilocus structure in the *Pinus contorta–Pinus banksiana* complex. *Can. J. Bot.* **2007**, *85*, 774–784. [CrossRef]
25. Sun, Y.; Abbott, R.J.; Lu, Z.; Mao, K.; Zhang, L.; Wang, X.; Ru, D.; Liu, J. Reticulate evolution within a spruce (*Picea*) species complex revealed by population genomic analysis. *Evolution* **2018**, *72*, 2669–2681. [CrossRef]
26. Moeller, M.; Gao, L.-M.; Mill, R.; Liu, J.; Zhang, D.-Q.; Poudel, R.; Li, D.-Z. A multidisciplinary approach reveals hidden taxonomic diversity in the morphologically challenging *Taxus wallichiana* complex. *Taxon* **2013**, *62*, 1161–1177. [CrossRef]

27. Pons, J.; Barraclough, T.G.; Gomez-Zurita, J.; Cardoso, A.; Duran, D.P.; Hazell, S.; Kamoun, S.; Sumlin, W.D.; Vogler, A.P. Sequence-Based Species Delimitation for the DNA Taxonomy of Undescribed Insects. *Syst. Biol.* **2006**, *55*, 595–609. [CrossRef] [PubMed]
28. Knowles, L.L.; Carstens, B.C. Delimiting Species without Monophyletic Gene Trees. *Syst. Biol.* **2007**, *56*, 887–895. [CrossRef] [PubMed]
29. Naciri, Y.; Linder, H.P. Species delimitation and relationships: The dance of the seven veils. *Taxon* **2015**, *64*, 3–16. [CrossRef]
30. Via, S. Natural selection in action during speciation. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 9939–9946. [CrossRef]
31. Christensen, K.L. Taxonomic revision of the *Pinus mugo* complex and *P. rhaetica* (*P. mugo sylvestris*) (*Pinaceae*). *Nord. J. Bot.* **1987**, *7*, 383–408. [CrossRef]
32. Farjon, A. *A Handbook of the World's Conifers (2 Vols.): Revised and Updated Edition*; Brill: Leiden, The Netherlands, 2010.
33. Łabiszak, B.; Zaborowska, J.; Wachowiak, W. Patterns of mtDNA variation reveal complex evolutionary history of relict and endangered peat bog pine (*Pinus uliginosa*). *AoB Plants* **2019**, *11*. [CrossRef]
34. Wachowiak, W.; Zukowska, W.B.; Wojkiewicz, B.; Cavers, S.; Litkowiec, M. Hybridization in contact zone between temperate European pine species. *Tree Genet. Genomes* **2016**, *12*. [CrossRef]
35. Businský, R.; Kirschner, J. *Pinus mugo* and *P. uncinata* as parents of hybrids a taxonomic and nomenclatural survey. *Phyton* **2010**, *50*, 27–57.
36. Wachowiak, W.; Palme, A.E.; Savolainen, O. Speciation history of three closely related pines *Pinus mugo* (T.), *P. uliginosa* (N.) and *P. sylvestris* (L.). *Mol. Ecol.* **2011**, *20*, 1729–1743. [CrossRef] [PubMed]
37. Dzialuk, A.; Boratyńska, K.; Romo, A.; Boratyński, A. Taxonomic and geographic variation of the *Pinus mugo* complex on chloroplast microsatellite markers. *Syst. Biodivers.* **2017**, *15*, 464–479. [CrossRef]
38. Krzakowa, M.; Naganowska, B.; Bobowicz, M.A. Investigations on taxonomic status of *Pinus uliginosa* Neumann. *Bull. Soc. Pol. Acad. Sci.* **1984**, *24*, 87–96.
39. Boratynska, K.; Boratynski, A. Taxonomic differences among closely related pines *Pinus sylvestris*, *P. mugo*, *P. uncinata*, *P. rotundata* and *P. uliginosa* as revealed in needle sclerenchyma cells. *Flora* **2007**, *202*, 555–569. [CrossRef]
40. Lewandowski, A.; Boratynski, A.; Mejnartowicz, L. Allozyme investigations on the genetic differentiation between closely related pines—*Pinus sylvestris*, *P. mugo*, *P. uncinata*, and *P. uliginosa* (*Pinaceae*). *Plant Syst. Evol.* **2000**, *221*, 15–24. [CrossRef]
41. Żukowska, W.B.; Wachowiak, W. Utility of closely related taxa for genetic studies of adaptive variation and speciation: Current state and perspectives in plants with focus on forest tree species. *J. Syst. Evol.* **2016**, *54*, 17–28. [CrossRef]
42. Newton, A.C.; Allnutt, T.R.; Gillies, A.C.M.; Lowe, A.J.; Ennos, R.A. Molecular phylogeography, intraspecific variation and the conservation of tree species. *Trends Ecol. Evol.* **1999**, *14*, 140–145. [CrossRef]
43. Frankham, R.; Ballou, J.D.; Briscoe, D.A. *Introduction to Conservation Genetics*, 2nd ed.; Cambridge University Press: Cambridge, UK; New York, NY, USA, 2010; 618p.
44. Allendorf, F.W.; Luikart, G.H.; Aitken, S.N. *Conservation and the Genetics of Populations*; Wiley: Hoboken, NJ, USA, 2012.
45. Wachowiak, W.; Zaborowska, J.; Labiszak, B.; Perry, A.; Zucca, G.M.; Gonzalez-Martinez, S.C.; Cavers, S. Molecular signatures of divergence and selection in closely related pine taxa. *Tree Genet. Genomes* **2018**, *14*, 83. [CrossRef] [PubMed]
46. Rozas, J.; Ferrer-Mata, A.; Sanchez-DelBarrio, J.C.; Guirao-Rico, S.; Librado, P.; Ramos-Onsins, S.E.; Sanchez-Gracia, A. DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. *Mol. Biol. Evol.* **2017**, *34*, 3299–3302. [CrossRef] [PubMed]
47. Tajima, F. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **1989**, *123*, 585–595. [CrossRef]
48. Hudson, R.R.; Kaplan, N.L. Statistical properties of the number of recombination events in the history of a sample of DNA sequences. *Genetics* **1985**, *111*, 147–164. [CrossRef]
49. Weir, B.S.; Cockerham, C.C. Estimating F-Statistics for the Analysis of Population Structure. *Evolution* **1984**, *38*, 1358–1370. [CrossRef]
50. Hey, J.; Wakeley, J. A coalescent estimator of the population recombination rate. *Genetics* **1997**, *145*, 833–846. [CrossRef] [PubMed]
51. Kassambara, A. Ggpubr: 'Ggplot2' Based Publication Ready Plots. Available online: https://CRAN.R-project.org/package=ggpubr (accessed on 15 January 2021).
52. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2020; Available online: https://www.R-project.org (accessed on 15 December 2020).
53. Tang, Y.; Horikoshi, M.; Li, W.X. ggfortify: Unified Interface to Visualize Statistical Results of Popular R Packages. *R J.* **2016**, *8*, 474–485. [CrossRef]
54. Pritchard, J.K.; Stephens, M.; Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* **2000**, *155*, 945–959.
55. Falush, D.; Stephens, M.; Pritchard, J.K. Inference of population structure using multilocus genotype data: Dominant markers and null alleles. *Mol. Ecol. Notes* **2007**, *7*, 574–578. [CrossRef]
56. Hubisz, M.J.; Falush, D.; Stephens, M.; Pritchard, J.K. Inferring weak population structure with the assistance of sample group information. *Mol. Ecol. Resour.* **2009**, *9*, 1322–1332. [CrossRef]
57. Evanno, G.; Regnaut, S.; Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol. Ecol.* **2005**, *14*, 2611–2620. [CrossRef]

58. Francis, R.M. pophelper: An R package and web app to analyse and visualize population structure. *Mol. Ecol. Resour.* **2017**, *17*, 27–32. [CrossRef]
59. Darriba, D.; Taboada, G.L.; Doallo, R.; Posada, D. jModelTest 2: More models, new heuristics and parallel computing. *Nat. Methods* **2012**, *9*, 772. [CrossRef]
60. Stamatakis, A. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **2014**, *30*, 1312–1313. [CrossRef] [PubMed]
61. Than, C.; Ruths, D.; Nakhleh, L. PhyloNet: A software package for analyzing and reconstructing reticulate evolutionary relationships. *BMC Bioinform.* **2008**, *9*, 322. [CrossRef] [PubMed]
62. Wen, D.; Yu, Y.; Zhu, J.; Nakhleh, L. Inferring Phylogenetic Networks Using PhyloNet. *Syst. Biol.* **2018**, *67*, 735–740. [CrossRef] [PubMed]
63. Elworth, R.; Ogilvie, H.; Zhu, J.; Nakhleh, L. Advances in Computational Methods for Phylogenetic Networks in the Presence of Hybridization. *Bioinform. Phylogenet.* **2019**, *29*, 317–360. [CrossRef]
64. Durand, E.Y.; Patterson, N.; Reich, D.; Slatkin, M. Testing for Ancient Admixture between Closely Related Populations. *Mol. Biol. Evol.* **2011**, *28*, 2239–2252. [CrossRef] [PubMed]
65. Ward, B.J.; van Oosterhout, C. hybridcheck: Software for the rapid detection, visualization and dating of recombinant regions in genome sequence data. *Mol. Ecol. Resour.* **2016**, *16*, 534–539. [CrossRef]
66. Excoffier, L.; Foll, M. fastsimcoal: A continuous-time coalescent simulator of genomic diversity under arbitrarily complex evolutionary scenarios. *Bioinformatics* **2011**, *27*, 1332–1334. [CrossRef]
67. Excoffier, L.; Dupanloup, I.; Huerta-Sanchez, E.; Sousa, V.C.; Foll, M. Robust demographic inference from genomic and SNP data. *PLoS Genet.* **2013**, *9*. [CrossRef]
68. Excoffier, L.; Lischer, H.E.L. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Resour.* **2010**, *10*, 564–567. [CrossRef]
69. Meier, J. Fsc-selectbestrun.sh. GitHub Repository. 2018. Available online: https://github.com/speciationgenomics/scripts/blob/master/fsc-selectbestrun.sh (accessed on 15 December 2020).
70. Meier, J. calculateAIC.R. GitHub Repository. 2018. Available online: https://github.com/speciationgenomics/scripts/blob/master/calculateAIC.sh (accessed on 15 December 2020).
71. Li, Y.; Stocks, M.; Hemmila, S.; Kallman, T.; Zhu, H.; Zhou, Y.; Chen, J.; Liu, J.; Lascoux, M. Demographic histories of four spruce (Picea) species of the Qinghai-Tibetan Plateau and neighboring areas inferred from multiple nuclear loci. *Mol. Biol. Evol.* **2010**, *27*, 1001–1014. [CrossRef] [PubMed]
72. De La Torre, A.R.; Li, Z.; Van de Peer, Y.; Ingvarsson, P.K. Contrasting rates of molecular evolution and patterns of selection among Gymnosperms and flowering plants. *Mol. Biol. Evol.* **2017**, *34*, 1363–1377. [CrossRef] [PubMed]
73. Davison, A.; Hinkley, D. Bootstrap Methods and Their Application. *J. Am. Stat. Assoc.* **1997**, *94*. [CrossRef]
74. Rosenberg, N.A. The shapes of neutral gene genealogies in two species: Probabilities of monophyly, paraphyly, and polyphyly in a coalescent model. *Evolution* **2003**, *57*, 1465–1477. [CrossRef] [PubMed]
75. Heuertz, M.; Teufel, J.; González-Martínez, S.C.; Soto, A.; Fady, B.; Alía, R.; Vendramin, G.G. Geography determines genetic relationships between species of mountain pine (*Pinus mugo* complex) in western Europe. *J. Biogeogr.* **2010**, *37*, 541–556. [CrossRef]
76. Łabiszak, B.; Zaborowska, J.; Wójkiewicz, B.; Wachowiak, W. Molecular and paleo-climatic data uncover the impact of an ancient bottleneck on the demographic history and contemporary genetic structure of endangered *Pinus uliginosa*. *J. Syst. Evol.* **2019**. [CrossRef]
77. Willyard, A.; Cronn, R.; Liston, A. Reticulate evolution and incomplete lineage sorting among the Ponderosa pines. *Mol. Phylogenet. Evol.* **2009**, *52*, 498–511. [CrossRef]
78. Li, J.; Milne, R.I.; Ru, D.; Miao, J.; Tao, W.; Zhang, L.; Xu, J.; Liu, J.; Mao, K. Allopatric divergence and hybridization within *Cupressus chengiana* (*Cupressaceae*), a threatened conifer in the northern Hengduan Mountains of western China. *Mol. Ecol.* **2020**, *29*, 1250–1266. [CrossRef]
79. Wachowiak, W.; Prus-Glowacki, W. Hybridisation processes in sympatric populations of pines *Pinus sylvestris* L., *P. mugo* Turra and *P. uliginosa* Neumann. *Plant Syst. Evol.* **2008**, *271*, 29–40. [CrossRef]
80. Sobierajska, K.; Wachowiak, W.; Zaborowska, J.; Labiszak, B.; Wojkiewicz, B.; Sekiewicz, M.; Jasinska, A.K.; Sekiewicz, K.; Boratynska, K.; Marcysiak, K.; et al. Genetic consequences of hybridization in relict isolated trees *Pinus sylvestris* and the *Pinus mugo* complex. *Forests* **2020**, *11*, 1086. [CrossRef]
81. Kormutak, A.; Galgoci, M.; Sukenikova, D.; Bolecek, P.; Libantova, J.; Gőmőry, D. Maternal inheritance of chloroplast DNA in *Pinus mugo* Turra: A case study of *Pinus mugo* × *Pinus sylvestris* crossing. *Plant Syst. Evol.* **2018**, *304*, 71–76. [CrossRef]
82. Zaborowska, J.; Łabiszak, B.; Wachowiak, W. Population history of European mountain pines *Pinus mugo* and *Pinus uncinata* revealed by mitochondrial DNA markers. *J. Syst. Evol.* **2019**. [CrossRef]
83. McLachlan, J.S.; Clark, J.S. Reconstructing historical ranges with fossil data at continental scales. *For. Ecol. Manag.* **2004**, *197*, 139–147. [CrossRef]
84. Willis, K.J.; van Andel, T.H. Trees or no trees? The Environments of central and eastern Europe during the last glaciation. *Quat. Sci. Rev.* **2004**, *23*. [CrossRef]
85. Wojkiewicz, B.; Litkowiec, M.; Wachowiak, W. Contrasting patterns of genetic variation in core and peripheral populations of highly outcrossing and wind pollinated forest tree species. *Aob Plants* **2016**, *8*. [CrossRef] [PubMed]

86. Hebda, A.; Wójkiewicz, B.; Wachowiak, W. Genetic characteristics of Scots pine in Poland and reference populations based on nuclear and chloroplast microsatellite markers. *Silva Fenn.* **2017**, *51*. [CrossRef]
87. Baack, E.J.; Rieseberg, L.H. A genomic view of introgression and hybrid speciation. *Curr. Opin. Genet. Dev.* **2007**, *17*, 513–518. [CrossRef]
88. Kwit, C.; Moon, H.S.; Warwick, S.I.; Stewart, C.N. Transgene introgression in crop relatives: Molecular evidence and mitigation strategies. *Trends Biotechnol.* **2011**, *29*, 284–293. [CrossRef]
89. Ellstrand, N.C.; Meirmans, P.; Rong, J.; Bartsch, D.; Ghosh, A.; de Jong, T.J.; Haccou, P.; Lu, B.-R.; Snow, A.A.; Neal Stewart, C.; et al. Introgression of crop alleles into wild or weedy populations. *Annu. Rev. Ecol. Evol. Syst.* **2013**, *44*, 325–345. [CrossRef]
90. Snow, A.A.; Pilson, D.; Rieseberg, L.H.; Paulsen, M.J.; Pleskac, N.; Reagon, M.R.; Wolf, D.E.; Selbo, S.M. A Bt transgene reduces herbivory and enhances fecundity in wild sunflowers. *Ecol. Appl.* **2003**, *13*, 279–286. [CrossRef]
91. Hufford, M.B.; Lubinksy, P.; Pyhäjärvi, T.; Devengenzo, M.T.; Ellstrand, N.C.; Ross-Ibarra, J. The genomic signature of crop-wild introgression in maize. *PLoS Genet.* **2013**, *9*, e1003477. [CrossRef]
92. Mallet, J.; Besansky, N.; Hahn, M.W. How reticulated are species? *Bioessays* **2016**, *38*, 140–149. [CrossRef]
93. Wachowiak, W.; Perry, A.; Donnelly, K.; Cavers, S. Early phenology and growth trait variation in closely related European pine species. *Ecol. Evol.* **2018**, *8*, 655–666. [CrossRef] [PubMed]
94. Perry, A.; Wachowiak, W.; Brown, A.V.; Ennos, R.A.; Cottrell, J.E.; Cavers, S. Substantial heritable variation for susceptibility to Dothistroma septosporum within populations of native British Scots pine (*Pinus sylvestris*). *Plant Pathol.* **2016**, *65*, 987–996. [CrossRef]
95. Ortiz-Barrientos, D.; Baack, E.J. Species integrity in trees. *Mol. Ecol.* **2014**, *23*, 4188–4191. [CrossRef] [PubMed]
96. Harr, B. Genomic islands of differentiation between house mouse subspecies. *Genome Res.* **2006**, *16*, 730–737. [CrossRef] [PubMed]
97. Renaut, S.; Grassa, C.J.; Yeaman, S.; Moyers, B.T.; Lai, Z.; Kane, N.C.; Bowers, J.E.; Burke, J.M.; Rieseberg, L.H. Genomic islands of divergence are not affected by geography of speciation in sunflowers. *Nat. Commun.* **2013**, *4*, 1827. [CrossRef]
98. Michel, A.P.; Sim, S.; Powell, T.H.; Taylor, M.S.; Nosil, P.; Feder, J.L. Widespread genomic divergence during sympatric speciation. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 9724–9729. [CrossRef] [PubMed]
99. Poelstra, J.W.; Vijay, N.; Bossu, C.M.; Lantz, H.; Ryll, B.; Muller, I.; Baglione, V.; Unneberg, P.; Wikelski, M.; Grabherr, M.G.; et al. The genomic landscape underlying phenotypic integrity in the face of gene flow in crows. *Science* **2014**, *344*, 1410–1414. [CrossRef] [PubMed]
100. Wachowiak, W.; Boratynska, K.; Cavers, S. Geographical patterns of nucleotide diversity and population differentiation in three closely related European pine species in the *Pinus mugo* complex. *Bot. J. Linn. Soc.* **2013**, *172*, 225–238. [CrossRef]
101. Neale, D.B.; Wegrzyn, J.L.; Stevens, K.A.; Zimin, A.V.; Puiu, D.; Crepeau, M.W.; Cardeno, C.; Koriabine, M.; Holtz-Morris, A.E.; Liechty, J.D.; et al. Decoding the massive genome of loblolly pine using haploid DNA and novel assembly strategies. *Genome Biol.* **2014**, *15*. [CrossRef] [PubMed]
102. Nystedt, B.; Street, N.R.; Wetterbom, A.; Zuccolo, A.; Lin, Y.C.; Scofield, D.G.; Vezzi, F.; Delhomme, N.; Giacomello, S.; Alexeyenko, A.; et al. The Norway spruce genome sequence and conifer genome evolution. *Nature* **2013**, *497*, 579–584. [CrossRef] [PubMed]
103. Perry, A.; Wachowiak, W.; Downing, A.; Talbot, R.; Cavers, S. Development of a single nucleotide polymorphism array for population genomic studies in four European pine species. *Mol. Ecol. Resour.* **2020**, *20*, 1697–1705. [CrossRef] [PubMed]

Table S1. Location of samples analysed. A total 132 individuals from 5 pine species were obtained (including 30 individuals of *Pinus mugo*, 32 of *P. uliginosa*, 30 *P. uncinata*, 30 of *P. sylvestris* and 10 of outgroup *P. pinaster*)

Species	Acr.	Location	Longitude	Latitude	N
P. mugo	PM5	Romania, Southern Carpathians	25°27’6”	45°25’55”	7[a]
	PM8	Montenegro, Durmitor Mts.	19°05’27”	43°09’33”	7[a]
	PM12	Austria, Karwendel Alps	11°17’45”	47°22’42”	6[a]
	PM14	Italy, Carnic Alps	13°08’50”	46°32’40”	4[a]
	PM16	Italy, Abruzzi	13°58’30”	41°46’20”	6[a]
P. uliginosa	PUG1	Poland, Węgliniec reserve	15°14’20”	51°17’50”	10[a]
	PUG2	Germany, Mitelwalde	11°16’27”	47°28’50”	9[a]
	PUG3	Poland, Batorów reserve	16°23’1”	50°27’32”	13[a,c]
P. uncinata	PUN17	Andora, Vall de Ransol	1°38’21”	42°35’02”	6 [a]
	PUN18	Andora, San Miguel de Engolasters	1°34’12”	42°31’28”	6 [a]
	PUN23	Spain, Castiello de Jaca	-0°32’12”	42°41’19”	6 [a]
	PUN24	Spain, Sierra de Gudar	0°41’51”	40°28’49”	6 [a]
	PUN28	France, Col de la Croix de Morano	-2°50’44”	45°35’58”	6 [a]
P. sylvestris	PS30	Scotland, Shieldaig	-5°38’24”	57° 30’35”	6[b]
	PS31	Scotland, Glen Tanar	-2°51'36”	57° 2’60”	6[b]
	PS37	Spain, Trevenque	-3°32’51”	37 05’47”	6[b]
	PS39	Finland, Punkaharju	61°45’33”	29° 23’21”	6[b]
	PS43	Poland, Jarocin	51°58’20”	17°28’40”	6[b]
P. pinaster	PP1	Spain, Oria	-2°17’42”	37°29’41”	1[c]
	PP2	Spain, San Cipriano	-7°26’21”	43°41’13”	1[c]
	PP3	Spain, Coca	-4 °31’48”	41°13’04”	1[c]
	PP4	Spain, Olba	0 °37’22”	40 °08’01”	1[c]
	PP5	France, Mimizan	-1 °14’56”	44 °11’56”	1[c]
	PP6	France, Bras Nord Var	5 °56’01”	43 °29’11”	1[c]
	PP7	France, Pineta	9 °22’17.5”	41 °40’39”	1[c]
	PP8	Portugal, Maria de Castelo Branco	7 °29’49”	39 °49’31”	1[c]
	PP9	Morocco, Ifran	5 °03’00”	33 °35’53”	1[c]
	PP10	Morocco, Zaouia d Ifrane	-5 °07’10”	33 °33’58”	1[c]

[a] data from Wachowiak *et al.* (2018), [b] data from Wachowiak et al. (unpublished), [c] sequenced in this study

Table S2. Nuclear loci studied.

Locus Acronym	PCR Primers (F-upper, and R-lower)	Gene function [Category[3]]	Base pairs screened [4].	Gene Bank Acc. Nr.
Pr1_1	[1]AGGAAGGAAGGGGAAAC [2]CAGGCTCCACTATATTG	putative beta-alanine ligase [M]	358	KC979156 - KC979177
Pr1_11	[1]GACCAGGCAAGGAAACAAAAG [2]TTGGCAATCGGTTGATGGGGAG	putative glucuronidase 3 [M]	720	KC979259 - KC979288
Pr1_12	[1]TCCCCATTCTCCAAAC [2]CCATCCAATCCTTCATC	NAD(P)-linked oxidoreductase-like protein [M]	434	KC979289 - KC979325
Pr1_14	[1]CTGTATGGCGTTCTTC [2]ACTGGGCGTCAAGTTTC	phospholipase A1-Igamma [M]	353	KC979348 - KC979373
Pr1_15	[1]CATTATTATCCAAGGGCGAG [2]GAGGCTTTGAGTCACCGTTAC	mitogen-activated protein-kinase [ST]	592	KC979374 - KC979410
Pr1_17	[1]TGGGTTGTCATCTGTGG [2]TGAGTTGCTGTGAGAGG	glutamate transporter [T]	319	KC979424 - KC979435
Pr1_18	[1]AAGCGACTCAAAAGGGG [2]TCGGCTGTATTGTCTC	alpha 1,3-glucosidase [M]	436	KC979436 - KC979460
Pr1_19	[1]CCGTATGCAAAGCATTTC [2]ACCTGATCGTGTTGTG	Glycosyltransferase [M]	327	KC979461-KC979482
Pr1_21	[1]GGGTGCATGTTTCATCCACAG [2]GCAGCAGCAAAAGCATTTGAAG	histone H3 K4-specific-methyltransferase [M]	363	KC979483-KC979505
Pr1_22	[1]TGAAGGGAGAGGACTAC [2]ACCCAGAAACACAAAGAGGAAAC	hypothetical protein [UN]	277	KC979506-KC979528
Pr1_24	[1]ATGGATATTCTCCATGATGCAC [2]ATGGTCGTCTTTTTGCTTC	putative S-adenosylmethionine-dependent protein [ST]	262	KC979529-KC979537
Pr1_26	[1]CCCATTTTAGCAAACCC [2]GAAGTGAAGATGAGCATAAG	putative pre-mRNA branch site-protein p14 [E]	386	KC979538 - KC979603
Pr1_29	[1]CCATTGGTGTGTCTTCTC [2]AATACCCTTTCAAGGCAAGCATATC	short-chain dehydrogenase Protein [M]	242	KC979622 - KC979653
Pr1_36	[1]GCGTTCATCATCTCAAGCC [2]CTAATCTCTCTTATTGTCATCTCCACC	transcribed locus [UN]	387	KC979674 - KC979696
Pr1_43	[1]GGACATTGTACTGTTGG [2]GGGTAAATGGAAAGAGTATTGG	beta-galactosidase [M]	591	KC979722 - KC979747
Pr1_45	[1]GAAATAGTCCTCTTCCTTTG [2]GGCTGCTTTGGATTATATTG	O-fucosyltransferase-like protein [M]	310	KC979748 - KC979765
Pr1_47	[1]GTAAATCTTCTTGCCTCTTCATCC [2]TATGCTCAACATACAGTACC	glycoprotein glucosyltransferase[M]	542	KC979786 - KC979800
Pr1_48	[1]ACCAATGCACATGCCAC [2]TATTACATCACTCCACCTTC	transport protein [T]	383	KC979801- KC979827
Pr2_7	[1]CAAAACCCTTTGAGCAC [2]GAGAACTTCTTCCATTCC	transcribed locus [UN]	410	KC979875 - KC979887
Pr2_11	[1]ACAGCAGCGATTCAAC [2]AACACCTCTTCCTCGTC	3-ketoacyl-CoA synthase 6-like[M]	216	KC979904 - KC979919
Pr2_16	[1]TCACTTGGCAGAAGAC [2]GAGAGATTCTTTGGAGAC	glutamyl-tRNA reductase [M]	384	KC979936 - KC979946

Pr2_17	^{1}GCATTAGTCTGTCTGTTC ^{2}GTGTTTCTAGGGCAATC	putative polyol transporter [T]	390	KC979947 - KC979961
Pr2_20	^{1}TCGAAGACAAGCTCTG ^{2}GACGACGATAAATGCTAC	GDP-dissociation inhibitor family-Protein [T]	380	KC979962 - KC979984
Pr2_23	^{1}GCCCAAATGGTTATACATAACACTC ^{2}CCATTCCATCGGCACAGTCATC	Transcribed locus [UN]	337	KC979985 - KC980011
Pr2_25	^{1}TCCCTGAAATCAAATCCCAC ^{2}AACCCAGCAATCTGAGCAAAGAAAAAC	hypothetical protein [UN]	403	KC980012 - KC980024
Pr2_28	^{1}CCTCCCATCATTCTTTCTTCC ^{2}GAATTGCAGCCCTTGCACAAGAC	basic leucine zipper transcription-factor-like protein [E]	484	KC980025 - KC980041
Pr2_29	^{1}GGCTTTGAACACCCTCAAAAATAC ^{2}TAAGGACATCAATACCAGTTTGCTCAG	hypothetical protein [UN]	333	KC980042 - KC980058
Pr2_30	^{1}CACTTGTCATCTGCTC ^{2}CTTGGAAGGATAGAATCTG	U-box domain-containing [ST] protein	362	KC980059 - KC980073
Pr2_32	^{1}GAATAGAAATAGAGTGCGATGG ^{2}AAAAATGATGGCTGCGTGGAGG	hypothetical protein [UN]	366	KC980074 - KC980091
Pr2_34	^{1}CATTTCCAAGAGAAGACGAC ^{2}TTGCCAACTCCACTCCCTAC	ATP/ADP transporter [T]	362	KC980092 - KC980103
Pr2_35	^{1}ACCCACAAATTGCCAG ^{2}GCCGTGATTATCGAAGAG	DEAD-box ATP-dependent RNA- helicase [M]	420	KC980104 - KC980122
Pr2_38	^{1}CCATCATACAACTATCCAC ^{2}ACAGAGAATAATGGGGCAC	hypothetical protein [UN]	451	KC980123 - KC980143
Pr2_41	^{1}GAAAAGGATCAAATTGTGGG ^{2}GCTAACATTGGCTGTGG	F-box protein GID2 [ST]	372	KC980144 - KC980160
Pr2_45	^{1}AACCGTCCTGATGAGCCTTG ^{2}CAGCCTTTCTTACAGACAC	hypothetical protein [UN]	399	KC980189 - KC980218
Pr2_47	^{1}TTCATAAAGCCCCCCATCC ^{2}TCTGATTTCAAAGTCGCC	hexokinase 1 [ST]	552	KC980219 - KC980234
Pr2_48	^{1}GCTATGCGTTACTTGG ^{2}TGAGTTGAGCTGCTTG	S-methyl-5-thioribose kinase [ST]	713	KC980235 - KC980278
ccoaomt	GCAGCAGAAGTGAAGGCTCAGA TCTTTCCATCATCGGGCAATG	Caffeoyl CoA O-methyltransferase [d] [M]	381	KC980357 - KC980372
rps10	CACCCAGAAATTGATGTTCCAAATC CCAGCCTTRTCACCAAATTCTCCAG	ribosomal protein S10 [a] [M]	355	KC980507 - KC980518
hp927	^{1}GCAATGAGGGATTGAATTAC ^{2}TTGGAAGAATACAAGGCAGG	hypothetical protein [UN]	360	KC980519 - KC980542
Pr4-5	^{1}CATCTCCTTCAAACCTCTTATTTCC ^{2}GATGCTTGAACATGATCCC	calcium dependent proteokinase [e] [ST]	357	KC980680 - KC980695
Pr4-10	^{1}CATTGCCTACGATTTCC ^{2}CTTTTGAGATGAACCAGAC	mys transcription factor [e] [E]	358	KC980696 - KC980702
Pr4-11	^{1}CCTTCTATTTGAATCCCTTG ^{2}CATAGTAACAGCCTACAG	scl1 protein [e] [E]	410	KC980703 - KC980724
Pr4-12	^{1}CTGCTCAAGTGAAAGG ^{2}CTGATTGTGGATTCTGTG	proton myo-inositol transporter [e] [T]	530	KC980725 - KC980745
Pr4-18	^{1}AGAGAGGGAATTGGTTGAG	myblike DNA-binding protein [e] [E]	321	KC980763 - KC980769

	[2]AAGGAAAGAAAAAGTCTGCTGATGG			
Pr4-21	[1]ACATGGTGTTTGGCAGG [2]AATGAGGAGGGTGGTAGAG	Receptor protein kinase [e] [ST]	357	KC980786 - KC980810
Pr4-27	[1]TAGCAGACGGTATTCACACAGTCC [2]CCACAACCACCTTGCATCATTATTT	putative auxin induced - transcription factor [e] [E]	403	KC980811 - KC980829
Pr4-34	[1]ACCCTGTATCGATGGGTATGGAGAT [2]TTTCATGTGGTTTGTTGGTACAGAACCTGCAATCA	transcription factor bHLH62-like- gene [e] [E]	360	KC980830 - KC980841
Pr4-38	[1]TTATTTACATCCAACAGCGCCATTT [2]GAAAGTATGGATTGCCAACTTGCAC	SET-B-like gene [e] [M]	512	KC980842 - KC980863
Pr4-41	[1]TGCAAGCTGTAAGGTAAAACCCTCAT [2]CAACATCAAAACTGAAACCACCAGTC	ethylene responsive element-binding protein-like gene [e] [ST]	591	KC980864 - KC980906

[1,2] - vector sequence (1=GTAAAACGACGGCCAGT and 2=CAGGAAACAGCTATGACC) was present as a part of PCR primers used for amplification of the loci studied; [3] – E-gene expression regulation; M-metabolisms; ST-signal transduction; T-transport; UN-unknown; [4]-average across all samples. DNA regions described in: [a] Palmé et al. 2008; [b] Pyhäjärvi et al., 2007; [c] Wachowiak et al. 2009; [d] Eveno et al., 2007; [e] Ersoz et al. 2010, [f] Donnelly et al. 2016.

Table S3. Summary statistic at each locus in four pine species.

						Nucleotide polymorphisms					
Locus	**Species**	**N**	**L (bp)**	**P**	**S**	**p_{total}**	***D***	***R***	**Ks**	**H_d**	**SD**
ccoamt	*P. uliginosa*	31	381	11	6	0.00401	-1.423	1	1.392	0.643	0.088
	P. mugo	29	381	6	3	0.00244	-1.124	0	0.844	0.547	0.096
	P. uncinata	29	381	6	4	0.00202	-1.43	0	0.696	0.424	0.111
	P. sylvestris	29	381	6	2	0.00295	-0.762	0	1.018	0.254	0.100
hp927	*P. uliginosa*	31	360	7	2	0.00562	0.45	2	0.702	0.847	0.042
	P. mugo	30	360	5	2	0.00335	-0.124	0	0.951	0.683	0.049
	P. uncinata	30	360	7	3	0.00531	0.236	0	0.515	0.625	0.076
	P. sylvestris	28	360	11	2	0.00815	0.123	0	0.684	0.841	0.039
PR_1_1	*P. uliginosa*	26	358	4	1	0.00264	-0.264	0	0.79226	0.698	0.062
	P. mugo	30	358	5	3	0.00292	-0.467	0	0.89655	0.653	0.069
	P. uncinata	30	358	7	4	0.00396	-0.582	0	1.21576	0.706	0.064
	P. sylvestris	28	358	6	1	0.00263	-1.129	0	0.7991	0.619	0.098
PR_1_11	*P. uliginosa*	21	720	22	5	0.00959	0.44801	1	5.71167	0.848	0.054
	P. mugo	30	720	18	4	0.00298	**-1.83234**	0	2.34726	0.51	0.109
	P. uncinata	30	720	20	2	0.00932	1.10422	2	5.8256	0.8	0.056
	P. sylvestris	27	720	18	0	0.00964	1.6588	3	6.85705	0.821	0.038
PR_1_12	*P. uliginosa*	32	434	15	7	0.00823	-0.36001	1	2.85327	0.893	0.037
	P. mugo	30	434	16	7	0.00805	-0.6537	0	2.78294	0.92	0.024
	P. uncinata	30	434	7	2	0.00601	0.85965	0	2.08267	0.763	0.039
	P. sylvestris	29	434	12	8	0.00293	**-1.93035**	0	1.29887	0.377	0.115
PR_1_14	*P. uliginosa*	28	353	9	2	0.00461	-0.34752	0	2.04938	0.69	0.068
	P. mugo	29	353	8	3	0.00661	0.44777	0	2.28571	0.722	0.07
	P. uncinata	27	353	10	3	0.00712	-0.10203	0	2.43269	0.627	0.094
	P. sylvestris	29	353	7	2	0.00581	0.4459	1	2.04202	0.786	0.05
PR_1_15	*P. uliginosa*	24	592	18	6	0.00538	-1.2321	0	2.92977	0.442	0.124
	P. mugo	28	592	26	17	0.00699	-1.4823	0	3.93333	0.635	0.104
	P. uncinata	30	592	16	13	0.00212	**-2.34263**	0	1.17457	0.469	0.114
	P. sylvestris	27	592	13	1	0.00565	0.03948	0	3.10345	0.55	0.108
PR_1_17	*P. uliginosa*	32	319	13	2	0.01066	-0.29051	3	2.72214	0.861	0.050
	P. mugo	30	319	6	0	0.00581	0.63752	0	1.52854	0.825	0.041
	P. uncinata	29	319	6	3	0.00527	0.28628	0	1.39153	0.751	0.049
	P. sylvestris	29	319	2	0	0.0012	-0.50812	0	0.42816	0.192	0.09
PR_1_18	*P. uliginosa*	32	436	8	2	0.00489	0.18747	1	2.10138	0.835	0.045
	P. mugo	30	436	8	5	0.00368	-0.64815	0	1.62591	0.761	0.043
	P. uncinata	30	436	10	1	0.00536	-0.26255	0	2.28631	0.885	0.022
	P. sylvestris	29	436	9	2	0.00498	-0.18324	0	2.14286	0.732	0.072
PR_1_19	*P. uliginosa*	28	327	9	1	0.01018	1.38607	0	2.84662	0.786	0.046
	P. mugo	30	327	11	3	0.00993	0.54669	0	2.84995	0.809	0.043
	P. uncinata	30	327	8	1	0.00837	1.07752	0	2.37969	0.54	0.099
	P. sylvestris	29	327	7	1	0.00908	1.9854	1	2.56408	0.739	0.045
PR_1_21	*P. uliginosa*	31	363	10	4	0.00554	-1.14317	2	1.91978	0.871	0.039
	P. mugo	29	363	10	7	0.00405	-1.3638	0	1.55718	0.714	0.068
	P. uncinata	30	363	5	2	0.00358	-0.01497	0	1.40249	0.63	0.058
	P. sylvestris	27	363	7	4	0.00426	-0.47302	0	1.617	0.632	0.096
PR_1_22	*P. uliginosa*	22	277	14	8	0.01034	-0.91956	1	2.34603	0.732	0.072
	P. mugo	29	277	4	0	0.00628	1.8327	1	1.47104	0.697	0.04
	P. uncinata	30	277	8	1	0.00794	0.27175	0	1.84211	0.766	0.049
	P. sylvestris	28	277	10	1	0.01501	1.96915	0	3.33333	0.5	0.07
PR_1_24	*P. uliginosa*	26	262	5	2	0.00497	-0.01892	0	1.02546	0.535	0.096
	P. mugo	28	262	6	2	0.00421	-0.82607	0	0.88254	0.524	0.103
	P. uncinata	30	262	5	0	0.00372	-0.62122	0	0.79031	0.451	0.103
	P. sylvestris	28	262	4	1	0.00473	0.53505	0	0.99048	0.442	0.087
PR_1_26	*P. uliginosa*	25	386	18	5	0.01411	0.2655	4	4.32552	0.827	0.067
	P. mugo	28	386	28	15	0.01743	-0.24467	5	5.36931	0.923	0.035
	P. uncinata	23	386	23	8	0.01639	0.02691	3	4.81212	0.905	0.041
	P. sylvestris	29	386	29	8	0.0175	-0.34362	5	5.38492	0.889	0.033

PR_1_29	*P. uliginosa*	30	242	6	2	0.00686	0.27368	1	1.6907	0.611	0.088
	P. mugo	29	242	7	2	0.00491	-0.99482	0	1.26339	0.473	0.11
	P. uncinata	30	242	5	1	0.00575	0.27821	0	1.44619	0.343	0.097
	P. sylvestris	28	242	7	2	0.00903	0.64537	0	2.16726	0.638	0.061
PR_1_36	*P. uliginosa*	30	387	13	8	0.00493	-1.47236	1	2.12302	0.825	0.052
	P. mugo	29	387	7	2	0.00421	-0.3856	0	1.91667	0.759	0.047
	P. uncinata	30	387	12	7	0.00537	-1.30811	0	2.25536	0.839	0.038
	P. sylvestris	28	387	10	3	0.00525	-0.77939	0	2.24339	0.804	0.041
PR_1_43	*P. uliginosa*	28	591	11	2	0.00402	-0.52141	2	2.64915	0.87	0.036
	P. mugo	30	591	12	8	0.00328	-1.17787	0	2.29664	0.814	0.044
	P. uncinata	30	591	10	2	0.00421	-0.04623	0	2.72104	0.844	0.037
	P. sylvestris	29	591	10	5	0.00381	-0.36986	0	2.54699	0.685	0.065
PR_1_45	*P. uliginosa*	30	310	10	2	0.00995	0.02815	2	2.60153	0.818	0.060
	P. mugo	27	310	10	2	0.00726	-0.72263	0	1.93473	0.781	0.071
	P. uncinata	30	310	10	3	0.01038	0.47475	0	2.71073	0.809	0.043
	P. sylvestris	29	310	9	2	0.01049	0.84323	0	2.72449	0.786	0.04
PR_1_47	*P. uliginosa*	22	542	3	0	0.00165	0.14824	0	0.82653	0.506	0.106
	P. mugo	30	542	0	0	0	0	0	0.11111	0	0
	P. uncinata	30	542	4	1	0.00143	-0.60921	0	0.75287	0.522	0.091
	P. sylvestris	29	542	7	2	0.00375	0.41296	0	1.79592	0.709	0.059
PR_1_48	*P. uliginosa*	32	383	9	3	0.00628	-0.1055	1	2.4265	0.875	0.042
	P. mugo	28	383	8	3	0.00479	-0.33014	0	1.99148	0.772	0.057
	P. uncinata	30	383	6	2	0.00397	0.00946	0	1.73347	0.621	0.091
	P. sylvestris	29	383	20	13	0.00602	**-1.92302**	0	2.35135	0.569	0.106
PR_2_7	*P. uliginosa*	31	410	31	26	0.00677	**-2.52116**	0	3.23333	0.624	0.099
	P. mugo	30	410	1	0	0.00045	-0.40885	0	0.15747	0.186	0.088
	P. uncinata	30	410	1	0	0.00031	-0.76373	0	0.88177	0.129	0.079
	P. sylvestris	25	410	4	1	0.00262	0.03571	0	1.79444	0.457	0.105
PR_2_11	*P. uliginosa*	31	213	3	2	0.00143	-1.37016	0	0.32222	0.243	0.099
	P. mugo	28	213	3	2	0.00184	-1.16485	0	0.39731	0.323	0.108
	P. uncinata	30	213	2	1	0.00117	-1.02235	0	0.27389	0.246	0.098
	P. sylvestris	28	213	2	1	0.00229	-0.07831	0	0.48036	0.474	0.079
PR_2_16	*P. uliginosa*	31	384	9	0	0.00627	0.21331	1	1.96491	0.733	0.058
	P. mugo	30	384	11	3	0.00834	0.4934	0	2.59646	0.669	0.076
	P. uncinata	30	384	10	2	0.00526	-0.63174	0	1.6384	0.639	0.069
	P. sylvestris	27	384	2	0	0.00248	1.76461	0	0.75566	0.655	0.049
PR_2_17	*P. uliginosa*	32	390	3	1	0.00274	1.00411	0	0.92804	0.569	0.048
	P. mugo	30	390	4	1	0.00314	0.54829	0	1.04753	0.605	0.052
	P. uncinata	30	390	5	1	0.00344	0.17386	0	1.14259	0.667	0.063
	P. sylvestris	27	390	5	2	0.0018	-1.28947	0	0.61538	0.447	0.113
PR_2_20	*P. uliginosa*	30	380	4	2	0.00199	-0.65598	0	0.98719	0.395	0.100
	P. mugo	29	380	7	4	0.00363	-0.68198	1	1.52521	0.773	0.057
	P. uncinata	30	380	14	2	0.00621	-1.11634	0	2.35862	0.701	0.062
	P. sylvestris	29	380	7	3	0.00374	-0.60788	1	1.56303	0.635	0.077
PR_2_23	*P. uliginosa*	30	337	9	5	0.00504	-0.78372	0	1.71571	0.779	0.063
	P. mugo	28	337	6	1	0.00404	-0.33785	0	1.43965	0.733	0.066
	P. uncinata	30	337	8	0	0.00489	-0.56861	0	1.66782	0.733	0.079
	P. sylvestris	27	337	10	7	0.00454	-1.33449	1	1.57669	0.755	0.059
PR_2_25	*P. uliginosa*	31	403	7	2	0.00432	-0.02436	0	1.46911	0.804	0.044
	P. mugo	30	403	4	1	0.00276	0.25599	0	0.96681	0.563	0.072
	P. uncinata	30	403	5	2	0.00348	0.30306	1	1.1973	0.687	0.055
	P. sylvestris	27	403	5	2	0.00341	0.16435	1	1.1573	0.675	0.056
PR_2_28	*P. uliginosa*	31	403	7	2	0.00432	-0.02436	0	1.46911	0.804	0.044
	P. mugo	30	403	4	1	0.00276	0.25599	0	0.96681	0.563	0.072
	P. uncinata	30	403	5	2	0.00348	0.30306	1	1.1973	0.687	0.055
	P. sylvestris	27	403	5	2	0.00341	0.16435	1	1.1573	0.675	0.056
PR_2_29	*P. uliginosa*	30	333	7	3	0.00428	-0.5713	0	1.18774	0.57	0.092
	P. mugo	30	333	6	2	0.00242	-1.33328	0	0.67241	0.492	0.1
	P. uncinata	29	333	4	1	0.00402	0.81756	0	1.1102	0.463	0.080
	P. sylvestris	24	333	5	0	0.00408	0.02707	0	1.08116	0.576	0.097
PR_2_30	*P. uliginosa*	31	362	5	0	0.00493	1.15297	0	1.60928	0.753	0.032
	P. mugo	27	362	5	0	0.00351	-0.05742	0	1.19247	0.604	0.085

	P. uncinata	29	362	5	2	0.0031	-0.32432	0	1.08108	0.692	0.063
	P. sylvestris	27	362	4	1	0.00233	-0.49529	0	0.86279	0.55	0.085
PR_2_32	*P. uliginosa*	32	366	10	3	0.00608	-0.32609	1	1.9377	0.869	0.036
	P. mugo	29	366	5	2	0.00373	0.19747	0	1.27676	0.539	0.093
	P. uncinata	30	366	8	3	0.00541	-0.05679	0	1.74176	0.766	0.061
	P. sylvestris	25	366	6	1	0.00677	1.03887	0	2.06111	0.803	0.04
PR_2_34	*P. uliginosa*	29	362	3	2	0.00036	-1.44456	0	0.53151	0.129	0.079
	P. mugo	30	362	1	0	0.00036	-0.76373	0	0.2532	0.129	0.079
	P. uncinata	27	362	2	0	0.00192	0.71889	0	0.74279	0.604	0.066
	P. sylvestris	28	362	2	1	0.0013	-0.21831	0	0.54209	0.442	0.087
PR_2_35	*P. uliginosa*	32	420	9	2	0.00594	-0.00916	1	2.42459	0.756	0.070
	P. mugo	30	420	3	1	0.00165	-0.21724	0	0.97173	0.487	0.097
	P. uncinata	30	420	2	0	0.0009	-1.18883	0	0.71824	0.356	0.106
	P. sylvestris	29	420	5	2	0.00261	-0.39886	0	1.30291	0.606	0.081
PR_2_38	*P. uliginosa*	32	441	10	4	0.00552	0.00859	2	1.73005	0.724	0.074
	P. mugo	29	441	6	2	0.00402	0.52754	0	1.5551	0.643	0.054
	P. uncinata	29	441	5	0	0.00488	2.00776	0	1.45306	0.576	0.048
	P. sylvestris	28	441	8	1	0.00388	-0.46127	0	1.0915	0.601	0.061
PR_2_41	*P. uliginosa*	31	372	8	3	0.00413	-0.70313	0	1.28649	0.727	0.070
	P. mugo	30	372	7	4	0.00275	-1.24401	0	0.85249	0.586	0.098
	P. uncinata	30	372	8	3	0.0058	0.20604	0	1.79694	0.752	0.058
	P. sylvestris	29	372	4	0	0.00364	0.74237	0	1.87755	0.655	0.057
PR_2_45	*P. uliginosa*	23	399	5	1	0.00393	0.02788	0	1.2346	0.644	0.092
	P. mugo	30	399	7	4	0.00389	-0.67509	0	1.09074	0.586	0.098
	P. uncinata	30	399	5	1	0.00366	-0.44101	0	1.01089	0.736	0.056
	P. sylvestris	29	399	6	4	0.00328	-1.06068	0	0.89961	0.8	0.038
PR_2_47	*P. uliginosa*	26	552	4	0	0.00233	0.58288	0	1.47143	0.64	0.091
	P. mugo	29	552	3	0	0.00268	2.20539	0	1.63595	0.675	0.034
	P. uncinata	29	552	6	2	0.00389	1.13995	0	2.25346	0.663	0.054
	P. sylvestris	28	552	9	3	0.0045	0.20506	0	2.96543	0.685	0.083
PR_2_48	*P. uliginosa*	29	713	16	7	0.00409	-1.04631	1	2.22772	0.756	0.079
	P. mugo	29	713	23	11	0.00529	-1.3217	0	2.85592	0.906	0.03
	P. uncinata	29	713	21	6	0.00682	-0.40107	0	3.69475	0.899	0.033
	P. sylvestris	27	713	23	11	0.00766	-0.38989	0	4.0097	0.883	0.033
PR_4_5	*P. uliginosa*	32	357	6	2	0.00346	-0.47883	1	1.17191	0.637	0.076
	P. mugo	30	357	4	3	0.0016	-1.11781	0	0.6565	0.515	0.089
	P. uncinata	30	357	6	2	0.00359	-0.48865	0	1.18347	0.453	0.105
	P. sylvestris	29	357	4	1	0.00374	0.74237	0	1.22368	0.463	0.08
PR_4_10	*P. uliginosa*	31	358	2	0	0.00253	1.61377	1	0.7935	0.695	0.036
	P. mugo	30	358	2	1	0.00101	-1.22455	0	0.38908	0.352	0.103
	P. uncinata	30	358	2	1	0.00164	0.32082	0	0.5546	0.549	0.038
	P. sylvestris	27	358	1	1	0.00021	-1.15354	0	0.18018	0.074	0.067
PR_4_11	*P. uliginosa*	29	410	9	2	0.00494	-0.48618	0	1.61315	0.781	0.048
	P. mugo	30	410	9	1	0.00524	-0.2963	0	1.68027	0.653	0.085
	P. uncinata	30	410	9	2	0.00469	-0.59504	0	1.51648	0.766	0.053
	P. sylvestris	29	410	9	5	0.004	-0.99106	0	1.30729	0.685	0.09
PR_4_12	*P. uliginosa*	32	530	8	2	0.00463	0.7039	0	2.42695	0.704	0.050
	P. mugo	30	530	9	3	0.00501	0.5276	0	2.61638	0.763	0.052
	P. uncinata	30	530	6	0	0.00457	1.70738	0	2.3944	0.715	0.040
	P. sylvestris	29	530	6	6	0.00091	**-2.1762**	0	0.57834	0.261	0.106
PR_4_18	*P. uliginosa*	31	321	4	3	0.00147	-1.34255	0	0.54167	0.385	0.099
	P. mugo	30	321	1	1	0.00021	-1.147	0	0.23077	0.067	0.061
	P. uncinata	30	321	1	1	0.00021	-1.147	0	0.23077	0.067	0.061
	P. sylvestris	27	321	2	0	0.00156	-0.07121	0	0.57051	0.467	0.094
PR_4_21	*P. uliginosa*	31	357	22	6	0.01397	-0.59885	6	4.1664	0.92	0.031
	P. mugo	28	357	6	2	0.00435	0.02085	0	1.57115	0.765	0.054
	P. uncinata	30	357	7	1	0.00422	-0.43676	0	1.53487	0.805	0.041
	P. sylvestris	27	357	6	2	0.00377	-0.39901	0	1.41973	0.744	0.054
PR_4_34	*P. uliginosa*	29	360	3	0	0.00156	-0.62937	0	0.49421	0.502	0.099
	P. mugo	27	360	5	5	0.00103	**-2.00406**	0	0.34286	0.279	0.112
	P. uncinata	30	360	3	3	0.00056	-1.73178	0	0.21053	0.131	0.082
	P. sylvestris	29	360	2	0	0.00074	-0.98857	0	0.26255	0.256	0.102

PR_4_38	*P. uliginosa*	28	512	5	3	0.00097	-1.71968	0	0.41326	0.328	0.112
	P. mugo	30	512	6	4	0.00164	-1.30296	0	0.66724	0.543	0.1
	P. uncinata	30	512	6	5	0.00123	-1.68413	0	0.51552	0.446	0.101
	P. sylvestris	27	512	3	0	0.0011	-0.70335	0	0.45738	0.33	0.108
PR_4_41	*P. uliginosa*	28	591	13	2	0.00555	-0.06307	1	2.75573	0.862	0.047
	P. mugo	29	591	13	6	0.00493	-0.40084	0	2.48263	0.776	0.06
	P. uncinata	30	591	18	7	0.00742	-0.11928	0	3.65828	0.876	0.041
	P. sylvestris	28	591	16	11	0.00394	-1.4919	0	2.01911	0.907	0.034
rps10	*P. uliginosa*	31	355	4	0	0.00376	0.84416	1	1.34464	0.742	0.046
	P. mugo	29	355	3	1	0.0024	0.27469	1	1.10854	0.547	0.096
	P. uncinata	30	355	4	0	0.00382	0.88151	1	1.26188	0.761	0.044
	P. sylvestris	22	355	2	0	0.00246	1.34546	0	0.84127	0.593	0.085
Species average											
	P. uliginosa	29.3	19414	441	155	0.00525	-0.28	39	1.86431	0.68933	0.06642
	P. mugo	29.3	19414	365	154	0.00404	-0.37	8	1.50080	0.60092	0.07071
	P. uncinata	29.6	19414	363	113	0.00455	-0.14	8	1.65601	0.62260	0.06590
	P. sylvestris	27.8	19414	367	128	0.00458	-0.16	14	1.68221	0.59831	0.07269

N - average number of sequences analyzed per locus; L - total length of sequence in base pairs excluding indels; P - total number of polymorphic sites; S - total number of singleton mutations); π - average nucleotide diversity; D - Tajima's D statistic; R – average number of recombination events Ks - average pairwise divergence per site to the outgroup *P. pinaster* at all loci; Hd - haplotype diversity; SD - standard deviation of haplotype diversity. Tajimas's D values significant at $p < 0.05$ are bolded.

Table S4. Maximum likelihoods and Akaike statistics for all 16 evolutionary models tested in fastsimcoal2. (see Figure 5 for details of scenario)

Model	Δlikelihood	AIC	ΔAIC
S12	349.645	4239	0
S14	353.749	4252	13
S15	355.781	4267	28
S11	355.375	4269	30
S13	356.333	4270	31
S16	366.586	4319	80
S6	384.285	4376	138
S1	385.150	4378	140
S10	384.380	4385	146
S7	384.519	4391	153
S4	390.027	4395	156
S3	390.547	4403	164
S8	387.833	4405	166
S2	393.085	4415	176
S9	390.620	4418	179
S5	395.510	4428	189

Best model is indicated in grey (lowest value of AIC). See Figure for details of each scenario.

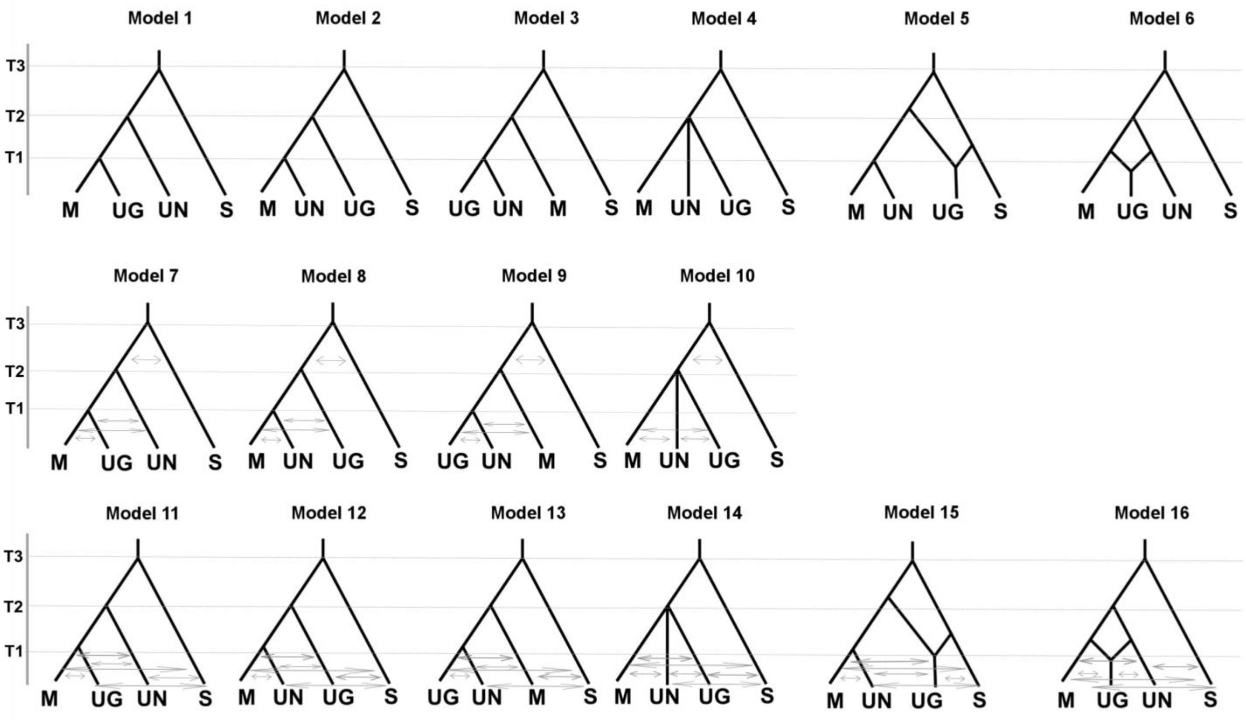

Figure S1 Schematic representation of the 16 demographic models tested in fastsimcoal2. Four models represent possible dichotomous and polytomous divergence between taxa from *P. mugo* complex after their split from *P. sylvestris* (models 1-4), two represent possible hybrid origin of *P. uliginosa* with different putative parental taxa (model 5-6), and the rest is variation of models 1-6 with different asymmetric migration matrix between species. Population divergence/hybridization times are indicated by T1-T3 parameters, and migration is indicated by arrows.

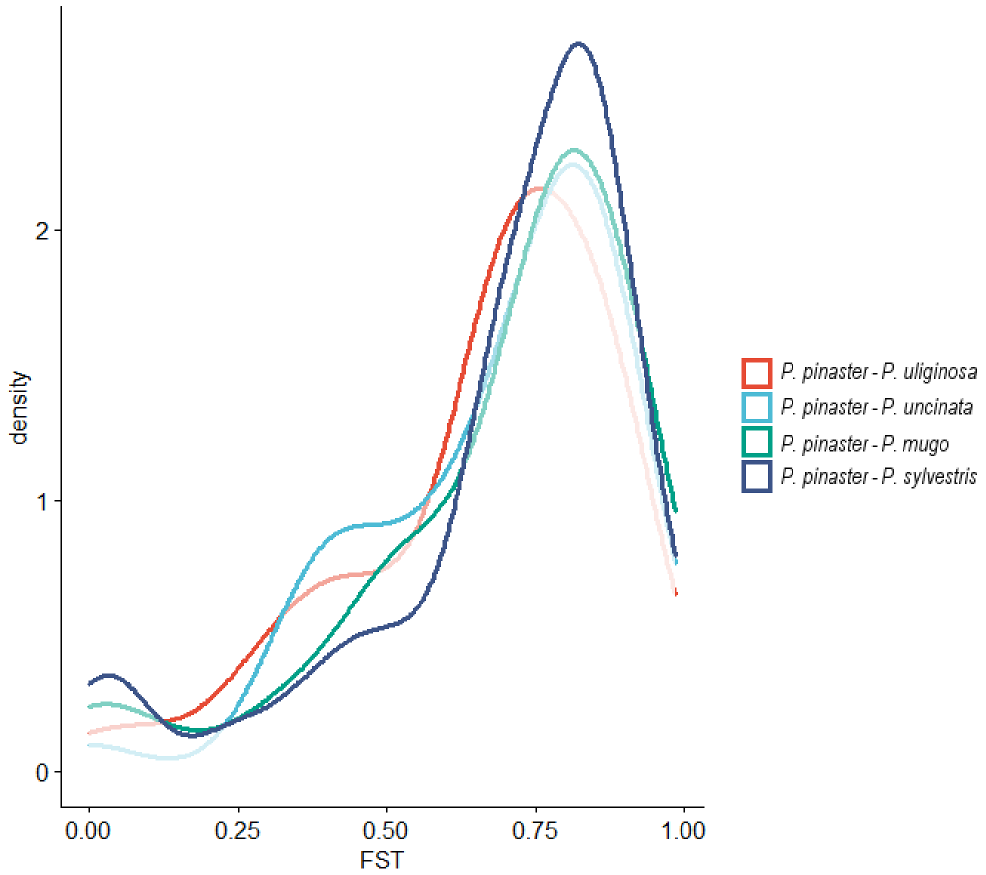

Figure S2. Distribution of genetic differentiation (*F_{ST}*) in pairwise comparisons between four studied pines and an outgroup *P. pinaster* based on all variable sites in a set of 48 nuclear loci.

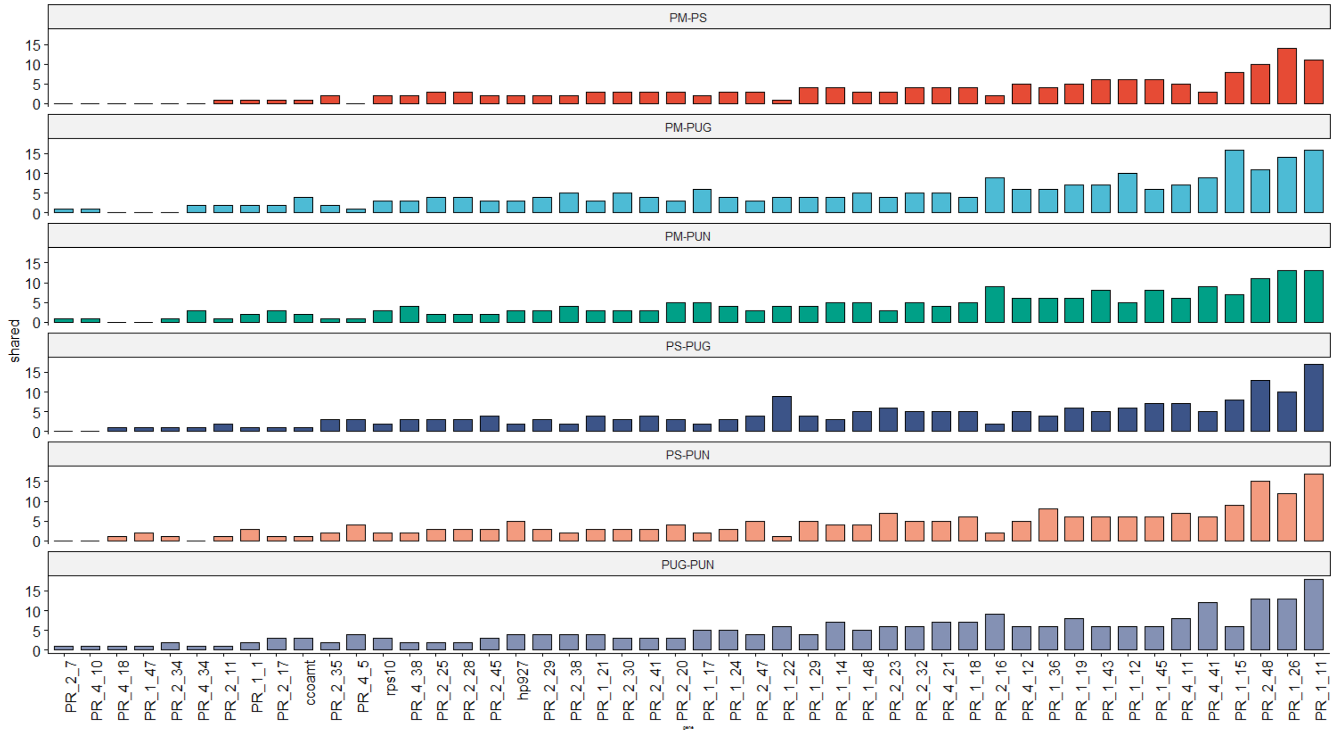

Figure S3. **Numbers of shared polymorphism between studied species in pairwise comparisons across all of the 48 nuclear loci studied.**

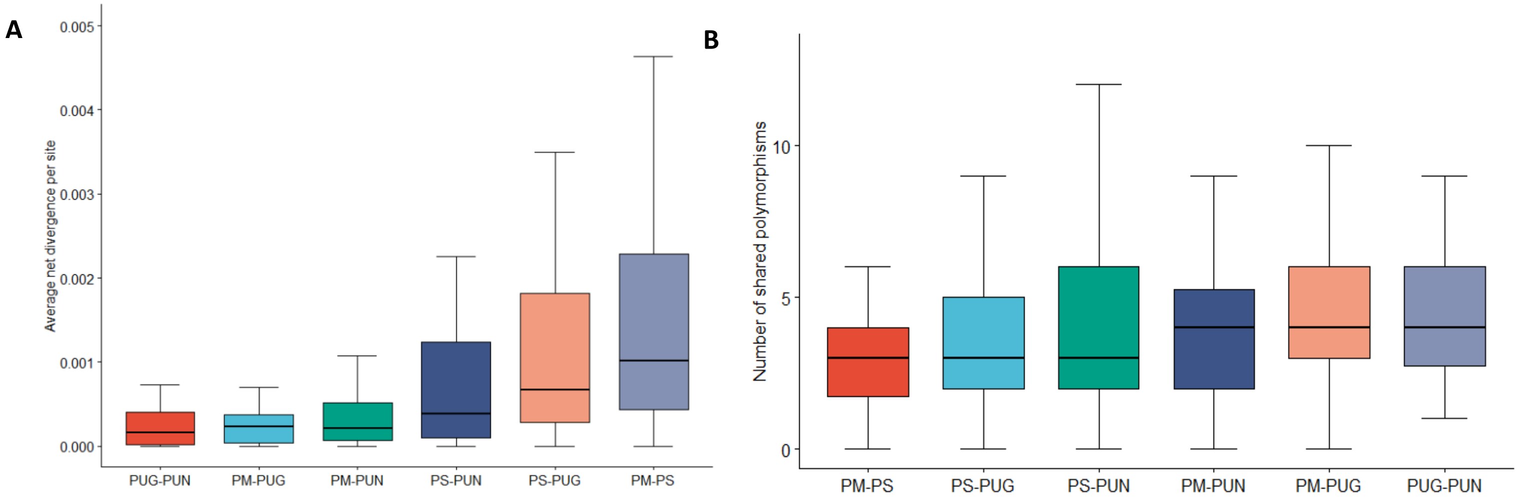

Figure S4. Net between-species divergence per site (A), and number of shared polymorphisms (B) in pairwise comparisons averaged across 48 nuclear loci.

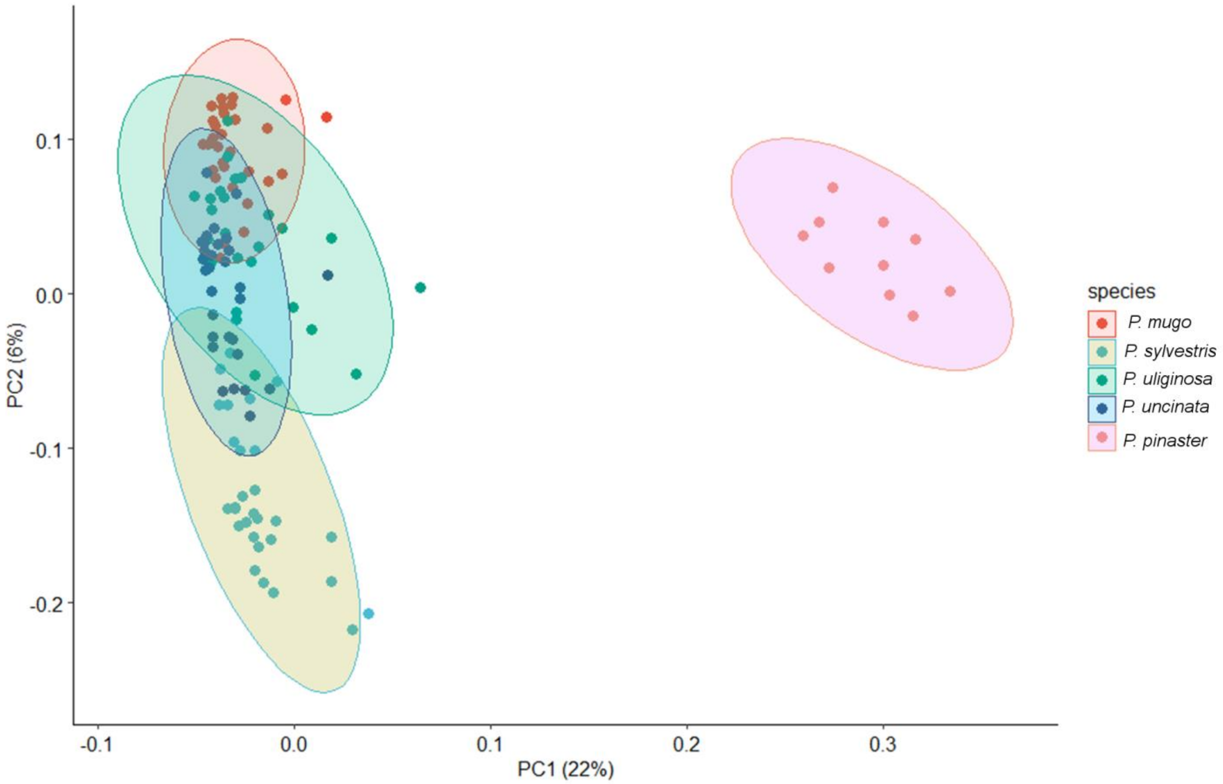

Figure S5. The results of the principal components analysis (PCA) showing differentiation of species (*P. pinaster* included as an outgroup) by the first two principal axes.

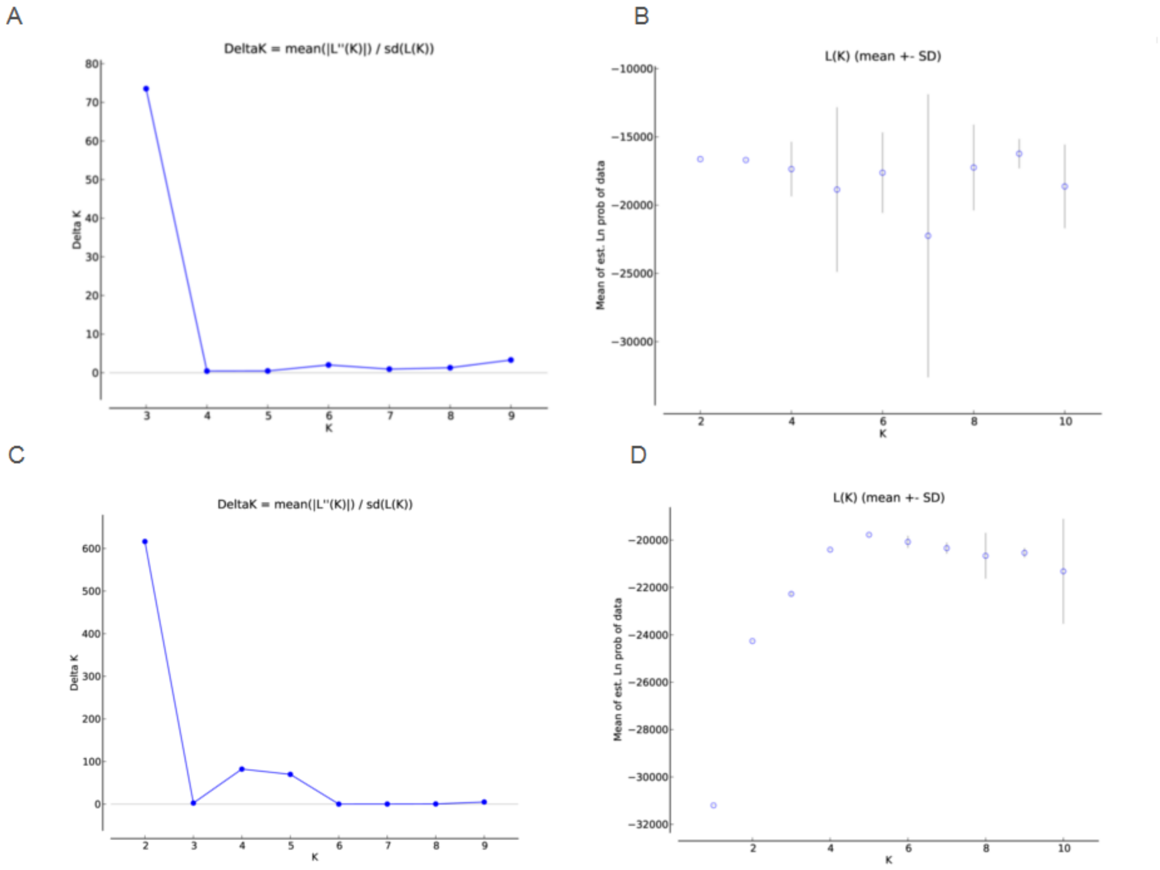

Figure S6. The results of likelihood estimate and Evanno method for STRUCTURE runs. results of likelihood estimate ΔK and the log probability Evanno method for STRUCTURE runs with *P. pinaster* (A, B); and without *P. pinaster* as an outgroup (C, D). For STRUCTURE results, see Figure 3.

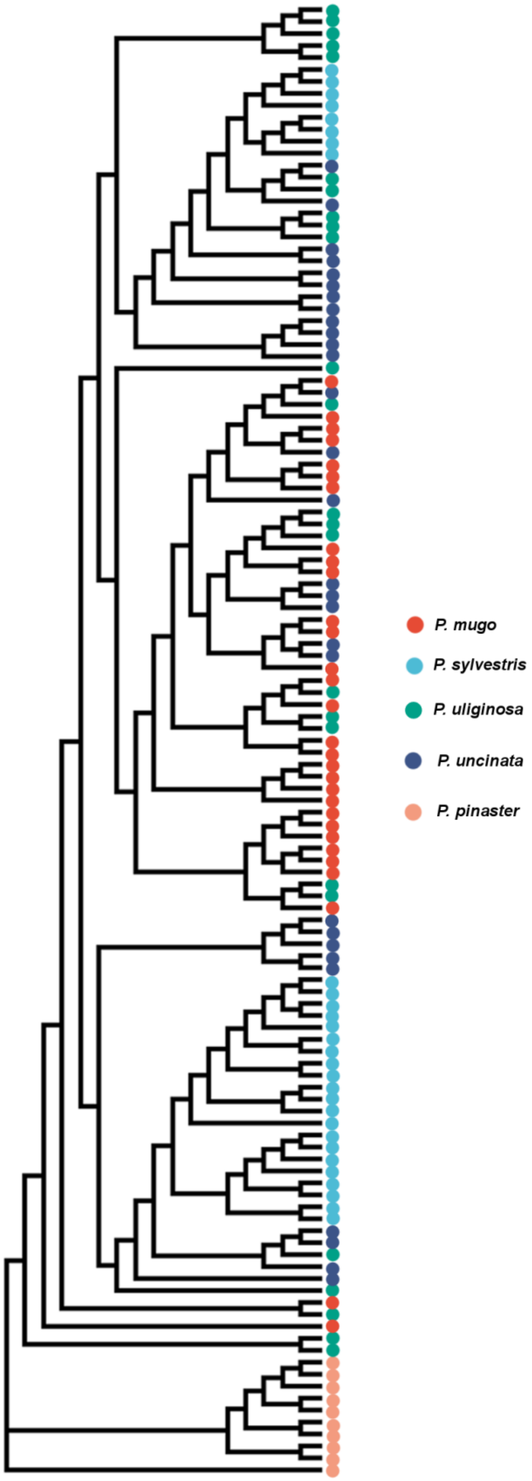

Figure S7 Phylogenetic relationships (ML tree) of 132 samples of *P. uliginosa*, *P. mugo*, *P. uncinata* and *P. sylvestris* rooted with *P. pinaster* based on the concatenated sequence of 48 nuclear loci. Due to the overall excess of low bootstrap values (< 50) bootstrap values were not shown to improve figure readability. Species color coded as in Figure 3 and Figure 5.

IV. Authorship statements

Oświadczenia określające wkład poszczególnych autorów w powstanie artykułów

Authorship statements of the PhD candidate

Oświadczenia określające wkład doktoranta w powstanie artykułów

1. **Łabiszak B.,** Zaborowska J., Wachowiak W. (2019) Patterns of mtDNA variation reveal complex evolutionary history of relict and endangered peat bog pine (*Pinus uliginosa*). *AoB Plants*, 11(2): plz015, doi:10.1093/aobpla/plz015

Niniejszym oświadczam, że mój wkład w powstanie powyższego artykułu polegał na: planowaniu badań, opracowaniu markerów mitochondrialnego DNA, pracach laboratoryjnych i genotypowaniu badanych osobników z wykorzystaniem nowoopracowanych markerów, analizie statystycznej i interpretacji danych, pisaniu i korekcie manuskryptu. W pracy jestem pierwszym autorem i pełnię rolę autora korespondencyjnego.
Mój całkowity wkład w pracę oceniam na 70%.

2. **Łabiszak B.,** Zaborowska J., Wójkiewicz B., Wachowiak W. (2020) Molecular and paleo-climatic data uncover impact of ancient bottleneck on demographic history and contemporary genetic structure of endangered *Pinus uliginosa*. *Journal of Systematics and Evolution*, doi: 10.1111/jse.12573

Niniejszym oświadczam, że mój wkład w powstanie powyższego artykułu polegał na: planowaniu badań, analizie statystycznej, analizie bioinformatyczej w zakresie filogenezy, historii demograficznej i modelowania nisz środowiskowych, interpretacji danych, pisaniu i korekcie manuskryptu. W pracy jestem pierwszym autorem i pełnię rolę autora korespondencyjnego.
Mój całkowity wkład w pracę oceniam na 70%.

3. **Łabiszak B.,** Wachowiak W. (2021). Molecular signatures of reticulate evolution within the complex of European pine taxa. *Forests*, 12, 489, doi:10.3390/f12040489

Niniejszym oświadczam, że mój wkład w powstanie powyższego artykułu polegał na: planowaniu badań, przeprowadzeniu prac laboratoryjnych związanych z izolacją materiału genetycznego, przygotowaniu reakcji PCR, oczyszczeniu i przygotowaniu prób do sekwencjonowania metodą Sangera, analizie bioinformatyczej danych sekwencyjnej, analizie danych z zakresu genetyki populacyjnej, filogenetyki, i biologii ewolucyjnej, interpretacji danych, pisaniu i korekcie manuskryptu. W pracy jestem pierwszym autorem i pełnię rolę autora korespondencyjnego.
Mój całkowity wkład w pracę oceniam na 80%.

Poznań, 16.04.2021

Łabiszak

Mgr Bartosz Łabiszak
Zakład Ekologii Roślin i Ochrony Środowiska
Instytut Biologii Środowiska
Wydział Biologii UAM w Poznaniu
bartosz.labiszak@amu.edu.pl

Authorship statements of co-authors of the article

Oświadczenia określające wkład współautorów w powstanie artykułu

Łabiszak B., Zaborowska J., Wachowiak W. (2019) Patterns of mtDNA variation reveal complex evolutionary history of relict and endangered peat bog pine (*Pinus uliginosa*). *AoB Plants*, 11(2): plz015, doi:10.1093/aobpla/plz015

Poznań, 16.04.2021

Mgr Julia Zaborowska
Zakład Ekologii Roślin i Ochrony Środowiska
Instytut Biologii Środowiska
Wydział Biologii UAM w Poznaniu
Uniwersytetu Poznańskiego 6, 61-614 Poznań
julzab1@amu.edu.pl

Oświadczenie określające wkład w powstanie artykułu

Niniejszym oświadczam, że jestem współautorem artykułu:

Łabiszak B., Zaborowska J., Wachowiak W. (2019) Patterns of mtDNA variation reveal complex evolutionary history of relict and endangered peat bog pine (*Pinus uliginosa*). AoB Plants, 11(2): plz015

Mój wkład w powstanie artykułu polegał na pomocy w opracowaniu markerów mitochondrialnego DNA i analizie danych z ich wykorzystaniem oraz przesłaniu uwag do maszynopisu publikacji przed wysłaniem jej do druku. Oceniam mój wkład na 10%.

Julia Zaborowska

Poznań, 16.04.2021

Prof. dr hab. Witold Wachowiak
Zakład Ekologii Roślin i Ochrony Środowiska
Instytut Biologii Środowiska
Wydział Biologii UAM w Poznaniu
Uniwersytetu Poznańskiego 6, 61-614 Poznań
witwac@amu.edu.pl

Oświadczenie określające wkład w powstanie artykułu

Niniejszym oświadczam, że jestem współautorem artykułu:

Łabiszak B., Zaborowska J., Wachowiak W. (2019) Patterns of mtDNA variation reveal complex evolutionary history of relict and endangered peat bog pine (*Pinus uliginosa*). AoB Plants, 11(2): plz015

Mój wkład w powstanie artykułu polegał na pomocy przy planowaniu badań i analizie wyników, a także współtworzeniu artykułu i jego korekcie przed wysłaniem do druku. Oceniam mój wkład na 20%.

Authorship statements of co-authors of the article

Oświadczenia określające wkład współautorów w powstanie artykułu

Łabiszak B., Zaborowska J., Wójkiewicz B., Wachowiak W. (2020) Molecular and paleo-climatic data uncover impact of ancient bottleneck on demographic history and contemporary genetic structure of endangered *Pinus uliginosa*. *Journal of Systematics and Evolution*, doi: 10.1111/jse.12573

Poznań, 16.04.2021

Mgr Julia Zaborowska
Zakład Ekologii Roślin i Ochrony Środowiska
Instytut Biologii Środowiska
Wydział Biologii UAM w Poznaniu
Uniwersytetu Poznańskiego 6, 61-614 Poznań
julzab1@amu.edu.pl

Oświadczenie określające wkład w powstanie artykułu

Niniejszym oświadczam, że jestem współautorem artykułu:

Łabiszak B., Zaborowska J., Wójkiewicz B., Wachowiak W. (2020) Molecular and paleo-climatic data uncover impact of ancient bottleneck on demographic history and contemporary genetic structure of endangered *Pinus uliginosa. Journal of Systematics and Evolution,* doi: 10.1111/jse.12573

Mój wkład w powstanie artykułu polegał na pomocy przy analizie danych oraz przesłaniu uwag do maszynopisu publikacji przed wysłaniem do druku. Oceniam mój wkład na 5%.

Julia Zaborowska

Poznań, 10.04.2021

Błażej Wójkiewicz
Zakład Genetyki i Interakcji Środowiskowych
Instytut Dendrologii
Polska Akademia Nauk
Parkowa 5, 62-035 Kórnik
bwojkiew@man.poznan.pl

Oświadczenie określające wkład w powstanie artykułu

Niniejszym oświadczam, że jestem współautorem artykułu:

Łabiszak B., Zaborowska J., Wójkiewicz B., Wachowiak W. (2020) Molecular and paleo-climatic data uncover impact of ancient bottleneck on demographic history and contemporary genetic structure of endangered *Pinus uliginosa*. *Journal of Systematics and Evolution*, doi: 10.1111/jse.12573

Mój wkład w powstanie artykułu polegał na wygenerowaniu danych mikrosatelitarnych badanych sosen oraz przesłaniu uwag do maszynopisu publikacji przed wysłaniem do druku. Oceniam mój wkład na 5%.

Błażej Wójkiewicz

Poznań, 16.04.2021

Prof. dr hab. Witold Wachowiak
Zakład Ekologii Roślin i Ochrony Środowiska
Instytut Biologii Środowiska
Wydział Biologii UAM w Poznaniu
Uniwersytetu Poznańskiego 6, 61-614 Poznań
witwac@amu.edu.pl

Oświadczenie określające wkład w powstanie artykułu

Niniejszym oświadczam, że jestem współautorem artykułu:

Łabiszak B., Zaborowska J., Wójkiewicz B., Wachowiak W. (2020) Molecular and paleo-climatic data uncover impact of ancient bottleneck on demographic history and contemporary genetic structure of endangered *Pinus uliginosa. Journal of Systematics and Evolution,* doi: 10.1111/jse.12573

Mój wkład w powstanie artykułu polegał na pomocy przy planowaniu badań i analizie wyników, a także współtworzeniu artykułu i jego korekcie przed wysłaniem do druku. Oceniam mój wkład na 20%.

Authorship statements of co-authors of the article

Oświadczenia określające wkład współautorów w powstanie artykułu

Łabiszak B., Wachowiak W. (2021) Molecular signatures of reticulate evolution within the complex of European pine taxa. *Forests*, 12, 489, doi:10.3390/f12040489

Poznań, 16.04.2021

Prof. dr hab. Witold Wachowiak
Zakład Ekologii Roślin i Ochrony Środowiska
Instytut Biologii Środowiska
Wydział Biologii UAM w Poznaniu
Uniwersytetu Poznańskiego 6, 61-614 Poznań
witwac@amu.edu.pl

Oświadczenie określające wkład w powstanie artykułu

Niniejszym oświadczam, że jestem współautorem artykułu:

Łabiszak B., Wachowiak W. (2021) Molecular signatures of reticulate evolution within the complex of European pine taxa. *Forests,* 12, 489. https://doi.org/10.3390/f12040489

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