UNIWERSYTET IM. A. MICKIEWICZA W POZNANIU
WYDZIAŁ BIOLOGII
ZAKŁAD EKOLOGII ROŚLIN I OCHRONY ŚRODOWISKA

ZMIENNOŚĆ GENETYCZNA STREF HYBRYDOWYCH I POPULACJI ALLOPATRYCZNYCH SPOKREWNIONYCH GATUNKÓW SOSEN (RODZAJ *PINUS*).

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POZNAŃ, 2025

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GENETIC DIVERSITY OF THE HYBRID ZONES AND ALLOPATRIC POPULATIONS OF CLOSELY RELATED PINE SPECIES (GENUS *PINUS*).

SEBASTIAN SZCZEPAŃSKI

DOCTORAL THESIS

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POZNAŃ, 2025

PODZIĘKOWANIA

Pragnę podziękować moim Promotorom — prof. dr hab. Witoldowi Wachowiakowi oraz dr. Bartoszowi Łabiszakowi — za nieocenione wsparcie, życzliwość oraz cierpliwość okazywaną na każdym etapie realizacji tej rozprawy. Tygodnie spędzone razem podczas pracy w terenie były dla mnie fascynującą przygodą, a wielogodzinne dyskusje na temat otrzymywanych wyników rozwinęły mnie zarówno naukowo, jak i intelektualnie.

Serdecznie dziękuję również pracownikom czterech parków narodowych: Parku Narodowego Gór Stołowych, Karkonoskiego Parku Narodowego, Tatrzańskiego Parku Narodowego oraz Babiogórskiego Parku Narodowego za umożliwienie przeprowadzenia badań terenowych oraz wsparcie w zbiorze prób. W szczególności Paniom Wioletcie Niemczyk i Dianie Mankowskiej-Jurek z Parku Narodowego Gór Stołowych oraz Panu Tomaszowi Zwijaczowi-Kozicy z Tatrzańskiego Parku Narodowego.

Dziękuję również Pani prof. UAM dr hab. Mirosławie Dabert oraz pracownikom Laboratorium Technik Biologii Molekularnej, w szczególności Pani mgr Małgorzacie Łośko, a także pracownikom Instytutu Dendrologii Polskiej Akademii Nauk, za techniczne wsparcie w realizacji badań.

WORKS INCLUDED IN THE DISSERTATION

The thesis consists of 4 chapters:

1. Szczepański S., Łabiszak B., Wachowiak W. Development of a SNaPshot assay for the genotyping of organellar SNPs in four closely related pines. *Dendrobiology*. 2023;90:76–85.
2. Szczepański S., Łabiszak B., Lasek M., Wachowiak W. Hybridization has localized effect on genetic variation in closely related pine species. *BMC Plant Biology*. 2024;24:1007.
3. Łabiszak B., Szczepański S., Wachowiak W. Repeatable genomic outcomes along the speciation continuum: insights from pine hybrid zones (genus *Pinus*). In review in *Molecular Ecology*. 2025.
4. Szczepański S., Łabiszak B., Wachowiak W. Molecular signatures of introgressive divergence and selection in contact zones of closely related pine species (*Pinus* genus). In review in *BMC Plant Biology*. 2025.

FUNDING

The main source of funding was Polish National Science Centre grant OPUS 20 2020/39/B/NZ9/00051 – “Zmienność genetyczna stref hybrydowych i populacji allopatrycznych spokrewnionych gatunków sosen – implikacje w badaniach procesów selekcji, różnicowania ekotypów i gospodarowania naturalnymi zasobami genomowymi”. Principal investigator of the project – prof. dr hab. Witold Wachowiak.

SUMMARY

This doctoral dissertation consists of four research articles describing genetic studies of allopatric and hybrid populations of Scots pine (*Pinus sylvestris* L.), dwarf mountain pine (*Pinus mugo* T.), and peat-bog pine (*Pinus uliginosa* N.). The main research focus was on three contact zones between *P. sylvestris* and *P. mugo*, where hybrid forms sometimes exhibit a phenotype resembling *P. uliginosa*. These zones included two populations located in the Central Sudetes: Błędne Skały in the Stołowe Mountains and the Torfowisko pod Zieleńcem peat bog in the Bystrzyckie Mountains, as well as the Bór na Czerwonem nature reserve in Nowy Targ, situated between the Gorce and Tatra Mountains. The reference group comprised a cross-section of natural *P. sylvestris* populations, both lowland and montane, within the Polish and broader European range. Reference populations of *P. mugo* included all Polish mountain ranges where this species occurs, as well as selected European sites. Reference material for *P. uliginosa* was collected from its *locus classicus* – the Wielkie Torfowisko Batorowskie peat bog – and two forest stands near Węgliniec. Sampling was conducted between 2021 and 2023.

The first article, published in *Dendrobiology* and entitled *Development of a SNaPshot assay for the genotyping of organellar SNPs in four closely related pines*, presents a newly developed genotyping method targeting a panel of 13 maternally inherited mitochondrial SNPs and a diagnostic chloroplast marker inherited paternally, which allows the identification of *P. sylvestris* versus *P. mugo* complex origin. The aim of this study was to improve and simplify existing genotyping protocols and validate them across 12 populations representing four pine species, including *P. sylvestris*, *P. mugo*, and *P. uliginosa*. The method demonstrated high accuracy and efficiency, enabling the identification of genotypes for 146 out of 147 tested individuals from the European range of the selected species. Twelve distinct mitochondrial haplotypes were identified, with the highest average diversity observed in *P. uliginosa* and *P. sylvestris*. The study also confirmed the specificity of the chloroplast diagnostic marker: all *P. sylvestris* individuals carried the "C" variant, whereas all individuals from the *P. mugo* complex carried the "A" variant at the *trnL-trnF* locus.

The second article, published in *BMC Plant Biology* and entitled *Hybridization has localized effect on genetic variation in closely related pine species*, describes an assessment of genetic variation in hybrid pine populations compared to allopatric reference populations, using 13 mitochondrial markers generated with the SNaPshot method and 14 nuclear microsatellite markers (nSSRs). Individuals from contact zones were classified into species groups based on nuclear genotypes (nSSRs), morphological features, and the diagnostic chloroplast DNA marker. Both nuclear and mitochondrial genetic variation were estimated using metrics such as allelic richness and haplotype richness—defined as the average number of alleles or haplotypes in a population normalized by the smallest population size. Additionally, a mitochondrial haplotype analysis was performed across multiple European populations to establish a database and network of observed variants. The results revealed no significant increase in genetic variation in the hybrid zones as would be expected; in fact, mitochondrial genome diversity was often reduced. No novel variants were found in the contact zones, which instead appeared to be homogenized to one or two haplotypes, commonly observed within the Polish range of

the studied species. Nearby allopatric populations appeared unaffected by the processes in hybrid populations, indicating that hybridization has a localized effect.

The third chapter corresponds to an article currently under review in *Molecular Ecology*, entitled *Repeatable genomic outcomes along the speciation continuum: insights from pine hybrid zones (genus Pinus)*. This study represents the first attempt to use genomic data (7,390 SNP markers) to assess genetic differentiation, population structure, and patterns of genetic ancestry in the examined contact zones. Although genetically pure *P. mugo* individuals were rare, ancestry from this species dominated in every sympatric population. Moreover, genetic diversity—estimated using thousands of markers located in functional genes—was higher in hybrid individuals than in the allopatric populations of the parental species.

The fourth and final chapter presents an article under review in *BMC Plant Biology*, entitled *Molecular signatures of introgressive divergence and selection in contact zones of closely related pine species (Pinus genus)*. It focuses on selection processes and genomic introgression occurring in hybrid zones. Individuals were grouped into genetic classes based on the proportion of their genomic ancestry, calculated from 7,390 nuclear SNPs. Three commonly used methods—pcadapt, OutFLANK, and BayeScan—were applied to identify loci under selection (outliers). Markers identified by each method were compared and only loci identified as outliers by at least two methods were selected. The aim of the study was to determine which genes were subject to selection within specific species classes in hybrid zones, relative to allopatric reference populations. The results revealed considerably stronger selective pressure in *P. sylvestris*-like hybrids compared to individuals with predominantly *P. mugo* ancestry. Furthermore, signs of selection were also found in numerous genes of *P. sylvestris* individuals occurring in contact zones that exhibited pure genetic ancestry, the typical phenotype of the species, and the diagnostic chloroplast marker variant.

In summary, this dissertation demonstrates that hybridization, gene flow, and selection play distinct yet complementary roles in shaping genetic variation in the contact zones of the studied pine species. While neutral markers point to homogenization of gene pools, functional *loci* reveal increased genetic diversity and differentiation between hybrids and genetically pure *P. sylvestris* individuals in sympatric populations. The prevalence of *P. mugo* genomic background among most hybrids and the low selective pressure acting on such individuals suggest adaptive introgression and better environmental fit of *P. mugo*-like hybrids. In contrast, pure *P. sylvestris* individuals from contact zones appear to be subject to selection linked to adaptation to environmental conditions that are challenging for typical representatives of the species. The findings presented in the four scientific articles comprising this dissertation contribute to our understanding of hybridization as both an adaptive mechanism and a driver of speciation. New insights into the genomics of forest tree hybrid zones hold importance not only for evolutionary biology, but also have practical implications for nature conservation, genetic resource management, and biodiversity preservation in forest ecosystems facing increasing environmental pressures.

STRESZCZENIE

Niniejsza praca doktorska składa się z czterech artykułów opisujących badania genetyczne allopatrycznych oraz mieszańcowych populacji sosny zwyczajnej (*Pinus sylvestris* L.), kosodrzewiny (*Pinus mugo* T.) oraz sosny błotnej (*Pinus uliginosa* N.). Głównym obiektem badań były trzy strefy kontaktu sosny zwyczajnej i kosodrzewiny, na których niekiedy formy hybrydowe przyjmują fenotyp przypominający sosnę błotną – były to dwie populacje zlokalizowane w Sudetach Środkowych: Błędne Skały w Górach Stołowych oraz Torfowisko pod Zieleńcem w Górach Bystrzyckich, a także Bór na Czerwonem w Nowym Targu, położony między Gorcami a Tatrami. Grupę referencyjną obejmował przekrój naturalnych populacji sosny zwyczajnej, zarówno górskich, jak i nizinnych, w zasięgu polskim oraz europejskim. Referencyjne populacje kosodrzewiny obejmowały wszystkie polskie pasma górskie, w których ten gatunek występuje, oraz wybrane stanowiska europejskie. Jako referencje dla sosny błotnej wykorzystano jej *locus classicus* – Wielkie Torfowisko Batorowskie – oraz dwa drzewostany zlokalizowane w okolicy Węglińca. Materiał zbierano w latach 2021–2023.

Pierwszy artykuł opublikowany w *Dendrobiology*, zatytułowany *Development of a SNaPshot assay for the genotyping of organellar SNPs in four closely related pines*, opisuje opracowaną metodę genotypowania panelu 13 dziedziczonych w linii matczynej mitochondrialnych polimorfizmów (SNP) oraz diagnostycznego markera chloroplastowego, dziedziczonego w linii ojcowskiej i pozwalającego określić pochodzenie danego osobnika od sosny zwyczajnej lub sosen z kompleksu *Pinus mugo*. Celem tej pracy było udoskonalenie i uproszczenie dotychczasowych metod genotypowania oraz ich walidacja na 12 populacjach czterech gatunków sosen w tym min. *P. sylvestris*, *P. mugo i P. uliginosa*. Metoda wykazała dużą dokładność i skuteczność, pozwalając na określenie genotypów dla 146 ze 147 badanych osobników z europejskiego zasięgu wybranych gatunków. W badanej próbie zidentyfikowano 12 różnych haplotypów mitochondrialnych, a ich najwyższą, uśrednioną różnorodność wykazano dla sosny błotnej i sosny zwyczajnej. Badania te potwierdziły również specyficzność chloroplastowego markera diagnostycznego – wszystkie sosny zwyczajne posiadały wariant „C”, natomiast wszystkie sosny z kompleksu *Pinus mugo* miały wariant „A” w locus *trnL-trnF*.

Drugi artykuł, opublikowany w *BMC Plant Biology*, pod tytułem *Hybridization has localized effect on genetic variation in closely related pine species*, opisuje oszacowanie zmienności genetycznej hybrydowych populacji sosen w odniesieniu do allopatrycznych populacji referencyjnych, na podstawie 13 markerów mitochondrialnych wygenerowanych za pomocą metody SNaPshot oraz 14 jądrowych markerów mikrosatelitarnych (nSSR). W tym celu osobniki zasiedlające strefy kontaktu podzielono na klasy gatunkowe, uwzględniając ich genetyczne pochodzenie (na podstawie genotypów nSSR), morfologię oraz wariant diagnostycznego markera chloroplastowego DNA. Oszacowano zarówno zmienność genomu jądrowego, jak i mitochondrialnego, przyjmując za jej miarę m.in. bogactwo alleliczne (*allelic richness*) i bogactwo haplotypowe (*haplotype richness*), czyli średnią liczbę alleli lub haplotypów w danej populacji, odniesioną do populacji o najmniejszej liczbie osobników. Przeprowadzono również analizę haplotypów mitochondrialnych dla wielu populacji w zasięgu europejskim, w celu stworzenia bazy i sieci występujących wariantów. Wyniki badań wskazały

na brak spodziewanego istotnego zwiększenia zmienności genetycznej w obrębie stref hybrydowych, a nawet wykazały jej zmniejszenie – szczególnie w przypadku genomu mitochondrialnego. Co więcej, w strefach kontaktu nie stwierdzono żadnych nowych wariantów, a populacje wydają się ujednolicać do jednego lub dwóch haplotypów, najczęściej spotykanych w polskim zasięgu występowania badanych gatunków. Okoliczne populacje allopatryczne nie wydają się być w żaden sposób dotknięte procesami zachodzącymi w populacjach hybrydowych, co wskazuje na lokalny efekt hybrydyzacji.

Trzeci rozdział stanowi artykuł w trakcie recenzji w czasopiśmie *Molecular Ecology*, zatytułowany *Repeatable genomic outcomes along the speciation continuum: insights from pine hybrid zones (genus Pinus)*. Badanie to było pierwszą próbą wykorzystania danych genomicznych (7390 markerów SNP) do oszacowania zróżnicowania genetycznego, struktury populacyjnej oraz wzorców pochodzenia genetycznego w analizowanych strefach kontaktu. Pomimo że zidentyfikowano jedynie nieliczne osobniki reprezentujące genetycznie czystą kosodrzewinę, pochodzenie od tego gatunku rodzicielskiego było dominujące w każdej populacji sympatrycznej. Co więcej, różnorodność genetyczna — oszacowana na podstawie tysięcy markerów zlokalizowanych w genach funkcjonalnych — była wyższa u mieszańców niż w populacjach allopatrycznych gatunków rodzicielskich.

Czwarty i ostatni rozdział stanowi artykuł *Molecular signatures of introgressive divergence and selection in contact zones of closely related pine species (Pinus genus)* będący w recenzji w *BMC Plant Biology*, dotyczący procesów selekcji i introgresji genomicznej zachodzących w strefach hybrydowych. Osobniki zostały podzielone na klasy genetyczne zgodnie z proporcjami ich genetycznego pochodzenia, obliczonymi na podstawie genotypów 7390 jądrowych markerów SNP. Do określenia loci podlegających selekcji, tzw. outlierów, zastosowano trzy powszechnie używane metody: pcadapt, OutFLANK oraz BayeScan. Zbiory markerów wybranych każdą metodą dla kilkunastu układów grup lub populacji porównano ze sobą i wybrano wyłącznie te, które zostały określone jako outliery przez co najmniej dwie z metod. Celem badań było określenie, które geny ulegają selekcji w wybranych klasach gatunkowych w strefach hybrydowych, w odniesieniu do referencyjnych populacji allopatrycznych danego gatunku. Badania wykazały znacznie większą presję selekcyjną obserwowaną w grupach hybrydów sosny zwyczajnej w porównaniu do osobników z przewagą genów kosodrzewiny. Co więcej, wykazano oznaki selekcji działającej również na wiele genów u osobników sosny zwyczajnej występujących w strefach kontaktu, posiadających czyste genetyczne pochodzenie od sosny zwyczajnej, typowy dla niej fenotyp oraz wariant diagnostycznego markera chloroplastowego.

Podsumowując, niniejsza rozprawa doktorska wykazuje, że hybrydyzacja, przepływ genów oraz selekcja odgrywają odmienne, lecz komplementarne role w kształtowaniu zmienności genetycznej w strefach kontaktu badanych gatunków sosen. Podczas gdy markery neutralne wskazują na ujednolicenie pul genowych, *loci* funkcjonalne ujawniają zwiększoną różnorodność genetyczną oraz zróżnicowanie między osobnikami hybrydowymi i czystymi osobnikami sosny zwyczajnej z populacji sympatrycznych. Przewaga genomu *P. mugo* wśród większości osobników hybrydowych oraz niska presja selekcyjna na takie mieszańce sugeruje występowanie adaptacyjnej introgresji oraz lepsze przystosowanie mieszańców kosodrzewiny. Natomiast czyste osobniki *P. sylvestris* ze stref kontaktu podlegają selekcji związanej z adaptacją do warunków środowiskowych, trudnych dla typowych przedstawicieli tego gatunku. Wyniki zaprezentowane w czterech publikacjach naukowych, będących rozdziałami tej rozprawy przyczynią się do pogłębienia wiedzy na temat hybrydyzacji jako mechanizmu adaptacji oraz czynnika napędzającego specjację. Nowa wiedza o genomice stref hybrydowych drzew ma znaczenie nie tylko dla biologii ewolucyjnej, lecz również niesie ze sobą istotne implikacje praktyczne dla ochrony przyrody, zarządzania zasobami genetycznymi oraz zachowania bioróżnorodności w ekosystemach leśnych poddawanych rosnącej presji środowiskowej.

GENERAL INTRODUCTION

Hybridization, the interbreeding of genetically distinct lineages, is increasingly recognized as a widespread and evolutionarily important process across the tree of life. Once considered rare or insignificant from an evolutionary perspective, hybridization is now known to influence patterns of genetic diversity, speciation, and adaptation in many groups, including plants, animals, and fungi (Abbott et al. 2013, Taylor and Larson 2019). When divergent lineages come into secondary contact, their interaction may result in a spectrum of outcomes—from transient gene flow with limited evolutionary consequence to extensive introgression that reshapes adaptive potential or even gives rise to new species. The ecological and genomic context in which hybridization occurs plays a central role in determining its evolutionary significance.

Hybrid zones, defined as geographic regions where differentiated taxa meet and produce hybrid offspring offer a unique lens through which to study the genomic architecture of divergence, the dynamics of introgression, and the potential for hybridization to generate adaptive diversity. Depending on ecological conditions and genomic compatibility, hybridization may promote local adaptation via the transfer of advantageous alleles (adaptive introgression), lead to the emergence of stabilized hybrid lineages, or result in the erosion of species boundaries through genetic swamping (Rieseberg and Carney 1998, Suarez-Gonzalez et al. 2018). These outcomes are often shaped by environmental heterogeneity, life history traits, and the genomic architecture of reproductive isolation. As such, expanding the study of hybrid zones in long-lived and ecologically dominant taxa—particularly forest trees—is essential for understanding how hybridization affects genetic structure, fitness, and species persistence. Trees provide an exceptional opportunity in this regard: their high fecundity, wide ecological distributions, and weak intrinsic barriers facilitate gene flow between species, often producing persistent hybrid populations. Hybridization has been observed in many forest tree genera including *Quercus*, *Picea*, *Populus*, and *Betula* and *Pinus* (Curtu et al. 2007, Tsuda et al. 2016, Zhang et al. 2023). Yet, despite the frequency of hybridization in forest trees, the evolutionary outcomes of these events remain poorly understood, particularly in terms of their repeatability across landscapes and environments. Are hybrid zones shaped by predictable genomic responses to similar selective pressures, or are they context-dependent and driven by local demographic and ecological histories? This question becomes especially relevant in trees, where hybridization often occurs across highly contrasting environments, that impose divergent selective regimes on hybrid genomes. Addressing these gaps is critical for understanding how hybridization contributes to adaptation and speciation in forest trees, where interspecific gene flow can influence key traits such as growth, environmental stress tolerance, and reproductive success—ultimately shaping complex genetic structures and driving adaptive variation within and among species.

Pines are a particularly valuable system for studying these questions. The genus *Pinus* includes more than 100 species distributed across the Northern Hemisphere, occupying a wide range of habitats from boreal forests to Mediterranean woodlands (Farjon 2018). Due to their widespread occurrence, ecological diversity, and economic importance, pines are not only critical to forest ecosystems but also serve as model organisms for studies of adaptation, speciation, and hybridization. A key species in this context is Scots pine (*Pinus sylvestris*), the most widespread conifer in Eurasia and a dominant component of forest ecosystems in Poland and much of northern and central Europe. Its broad distribution encompasses highly variable environmental conditions, and its populations exhibit local adaptation to climatic and edaphic gradients (Wachowiak and Prus-Głowacki 2009, Wójkiewicz et al. 2016). In regions where its range overlaps with other pine taxa, *P. sylvestris* forms contact zones with members of the *P. mugo* complex—particularly dwarf mountain pine (*P. mugo*) and peat-bog pine (*P. uliginosa*). These species of the *P. mugo* complex are typically restricted to subalpine and postglacial peatland habitats and exhibit more limited, fragmented distributions. *P. mugo* is common in high-altitude shrubby formations across the Alps and Carpathians, while *P. uliginosa* is a rare and endangered taxon found in isolated peat bogs, particularly in the Sudetes of Central Europe (Hamernik and Musil 2007, Boratyńska et al. 2015). Although morphologically and ecologically distinct, these taxa share a recent evolutionary origin and are only partially reproductively isolated (Wachowiak et al. 2011). This genetic proximity allows for extensive hybridization in areas of sympatry, where hybrid individuals often display a range of transgressive and intermediate phenotypes. In Poland, several well-characterized hybrid zones exist between *P. sylvestris*, *P. mugo*, and *P. uliginosa*, each embedded in contrasting environments such as montane rock outcrops and nutrient-poor peat bogs. These replicated contact zones present an exceptional opportunity to examine how hybridization plays out across ecological gradients, and to test whether the genomic outcomes of hybridization are repeatable across landscapes—or whether they are shaped by site-specific environmental and demographic factors.

Therefore the major goal of my study was to identify the evolutionary consequences of hybridization in Scots pine (*Pinus sylvestris*) and taxa distinguished in the *P. mugo* complex. Specifically, to: 1) assess the extent and genetic patterns of hybridization between *P. sylvestris, P. mugo,* and *P. uliginosa*; 2) look at signatures of interspecific gene flow at neutral and potentially adaptive loci; 3) revise the evolutionary origin and taxonomic status of *P. uliginosa,* 4) examine the impact of different environments and species composition on evolutionary outcomes of hybridization; 5) identify genomic regions under selection and test the role of adaptive introgression. To address the research objectives, the following hypotheses were tested:

Hypothesis 1 – Hybridization is spatially restricted to contact zones and interspecific gene flow does not affect the allopatric populations.
Hypothesis 2 – Hybrid zones serve as a source of new genetic variation.
Hypothesis 3 – Hybridization is a repeatable process across distinct contact zones.
Hypothesis 4 – Genomic data support the taxonomic position of *Pinus uliginosa* as a true parental taxon within the *P. mugo* complex.
Hypothesis 5 – Similar ecological conditions can drive parallel introgression and selection on shared genomic regions across independent hybrid zones.

Hypothesis 6 – Hybrid genotypes show signatures of introgressive divergence at the loci under selection compared to the distribution of nucleotide polymorphisms at the genes in the parental species.

To address the research questions and objectives, I used genome-wide SNP data derived from over 1,500 individuals sampled from multiple hybrid zones and allopatric reference populations. These data encompass both neutral and putatively adaptive genetic markers from nuclear and organellar genomes, allowing for a comprehensive assessment of spatial, genomic, and ecological patterns of divergence and introgression. I developed new assay for genotyping mitochondrial DNA polymorphisms, applied a suite of complementary analytical approaches, including population history, structure and admixture analysis, detection of signatures of natural selection and introgressive divergence analysis. These methods enabled precise assessment of interspecific gene flow patterns across the investigated hybrid zones and reference allopatric stands, detection of introgression patterns, identification of loci under selection, and evaluation of the repeatability and adaptive significance of hybridization in this system. Together, the results provide novel insight into how hybridization interacts with selection, environmental heterogeneity, and evolutionary history in long-lived, ecologically dominant tree species.

The first part of my PhD thesis was devoted to development of a new assay for genotyping of predefined mitochondrial DNA markers in the studied species (Szczepański et al. 2023). One of the most useful resources for historical and phylogeographic inference in conifers has been organellar DNA — mitochondrial (*mt*DNA) and chloroplast (*cp*DNA) — due to their uniparental inheritance and limited recombination. In pines, *cp*DNA is typically paternally inherited via pollen, whereas *mt*DNA is maternally inherited and dispersed through seeds (Jaramillo-Correa and Bousquet 2005). This asymmetry allows to distinguish pollen- and seed-mediated gene flow. While *cp*DNA is relatively small (~150 kbp), and has been widely sequenced for many *Pinus* species (Asaf et al. 2018, Yu et al. 2022), conifer *mt*DNA is substantially larger and structurally complex with low mutation rates and repetitive content thus making it more difficult to analyze (Smith 2015, Jackman et al. 2016).

Recent advances in sequencing technologies have improved the accessibility of *mt*DNA markers for population-scale studies. In particular, PCR-RFLP markers developed based on sequenced large fragments of *mt*DNA regions by Donnelly et al. 2017 were shown to be effective for population-level studies in the *Pinus mugo* complex (Łabiszak et al. 2019, Zaborowska et al. 2020). However, these PCR-RFLP methods remain time and labor-intensive, requiring separate amplification, digestion, and electrophoresis steps for each marker. In response to these limitation, I developed and implemented a cost-effective genotyping strategy using SNaPshot™ technology (Thermo Fisher) to enable simultaneous detection of multiple SNP and indel variants at organellar genomes of the species (Hu et al. 2016, Weiler et al. 2016). The developed SNP panel included 13 mitochondrial markers and one diagnostic chloroplast marker (*trnL-trnF*) capable of distinguishing paternal lineages between *P. sylvestris* and the *P. mugo* complex (Wachowiak et al. 2000). The markers were organized into two multiplex PCR reactions, with seven loci in each SNaPshot run. The method was validated on 147 individuals from 12 natural populations of four species – *P. sylvestris* and taxa from the *Pinus mugo* complex – including among others *P. mugo* T. and *P. uliginosa* N.

Compared to the PCR-RFLP method, the SNaPshot multiplexed approach was highly efficient, reliable, less laborious, more accurate, and approximately four times cheaper (Szczepański et al. 2024). It enabled a broad-scale research on mitochondrial markers for over 2,500 individuals, laying the foundation for the large-scale analyses described in the following chapter of this thesis.

The second part of my PhD thesis explores patterns of genetic diversity within hybrid zones and parental reference populations based on neutral variance (Szczepański et al. 2024). Understanding patterns of neutral genetic diversity is crucial in population genetics and evolutionary biology, as it provides a baseline for detecting the influence of demographic processes, gene flow, and natural selection. Neutral markers can reveal historical connectivity between populations, detect hidden structure, and serve as a reference for identifying adaptive loci. In conifers, insights into neutral variation are further enriched by examining both nuclear and organellar genomes, which are shaped by distinct evolutionary forces and inheritance patterns. In *Pinus*, nuclear DNA is biparentally inherited and dispersed by both pollen and seed while mitochondrial DNA (*mt*DNA) is maternally inherited and dispersed through seeds and chloroplast DNA (*cp*DNA) is typically paternally inherited via pollen (Jaramillo-Correa and Bousquet 2005). These contrasting dispersal mechanisms often result in cytonuclear discordance, where organellar and nuclear markers reveal different patterns of genetic structure and introgression (Arnold 1993, Naydenov et al. 2014, 2023, Marshall et al. 2021, DeRaad et al. 2023, Palacios et al. 2023). Such discrepancies can offer insights into sex-biased gene flow and historical population dynamics, particularly in hybrid zones.

An increase of genetic diversity would be expected as a consequence of interspecific gene flow, when recombination leads to formation of new variants resulting from mixing separate gene pools in the hybrid individuals (Ortíz-Barrientos et al. 2002, Latta et al. 2007). However, differences in gene flow of certain genomes can affect the diversity of hybrids. For example, the gene flow of mitochondrial genome in pines is significantly lower due to the limits of seed dispersal, compared to the pollen, which carries nuclear and plastid genomes. To explore these dynamics, I assessed genetic diversity in sympatric and allopatric populations of *P. sylvestris*, *P. mugo*, and *P. uliginosa* using two complementary datasets: previously described SNaPshot markers (13 mitochondrial and one plastid) and 14 nuclear Simple Sequence Repeat (SSR) loci analysed in three multiplex PCR reactions as described earlier (Żukowska and Wachowiak 2017).

Firstly, all the individuals from the hybrid zones were grouped into genetic classes, according to their ancestry percentages, calculated as individual ancestry coefficients in *LEA* R package (Frichot and François 2015), based on their genotypes obtained from nuclear SSRs. The individuals were grouped into "pure" *P. mugo* (>97% PM ancestry; also named as "parent *P. mugo*"), hybrid *P. mugo* (97%-60% PM ancestry), F1 (60-40% PM ancestry; first generation of hybrids), "pure" *P. sylvestris* (>97% PS ancestry; also named as "parent *P. sylvestris*), hybrid *P. sylvestris* (97-60% PS ancestry). The division was consistent in articles showed in chapters

II-IV of the thesis, although in some analyses in chapters II and III, the F1 was included in corresponding hybrid groups, based on their ancestry.

Allelic richness and haplotype richness were chosen as the best parameters to estimate the diversity of nuclear and mitochondrial genetic markers in studied sympatric and allopatric populations. Both those measures are standardized to the population/group with the lowest sample number. Although it was expected that the hybrid group would have the highest diversity, at least within the biparentally transmitted SSR markers, it was only slightly elevated compared to the reference populations – similar to the allelic richness of reference Scots pine populations and higher than reference dwarf mountain pines and peat-bog pines. The group with the highest diversity at nuclear loci was the parent *P. sylvestris* group – Scots pines growing in the contact zones. Surprisingly, the results obtained from mitochondrial data also showed that the parent *P. sylvestris* pines are the most diverse among sympatric groups, although all the groups of individuals from the hybrid zones had low mitochondrial genome variability and there were no significant differences between mitotype composition of species classes from specific contact zone. Moreover, no new mitotypes were found in hybrid zones and their composition was limited to one or two major haplotypes, the most common ones in Poland.

The analysis of individual ancestry coefficients showed that majority of reference populations are strictly classified to one of two genetic clusters, associated with the corresponding species. No signs of significant admixture in the allopatric populations supports the **Hypothesis 1**. The results show that not only the sympatric populations of pines do not produce new neutral genetic variability, especially in the mitochondrial genome, but they also act as its sink. No new mitotypes are generated in those zones and the variability of neutral nuclear genes do not differ significantly from reference parental species. These findings do not fully support the **Hypothesis 2**, at least in case of neutral genetic variation analyzed in this study. The preliminary assessments of ancestry of the studied samples indicated that the process of hybridization has similar outcomes in all studied sympatric populations, what partially supports the **Hypothesis 3**. This issue was fully addressed in SNP data analysis in the following chapters of the thesis.

In the third and fourth part of my PhD thesis I focused on the analysis of nuclear polymorphisms genotyped with the application of the Axiom Affymetrix SNPs genotyping array, to investigate the species hybrid zones in the broader context of a conceptual model of the speciation continuum that recognizes speciation as a gradual and often incomplete process (Shaw and Mullen 2014). In this view, lineages can diverge genetically and ecologically while still exchanging genes, and hybrid populations may persist for extended periods without complete reproductive isolation. Pines are very suitable organisms for exploring this continuum, as their long generation times, high gene flow, and ecological plasticity allow partial barriers to remain porous across evolutionary timescales (Mallet, 2007; Wachowiak et al., 2011). Understanding whether hybrid populations represent transient mixtures, stable hybrid swarms, or incipient species is essential to interpreting patterns of divergence and introgression in these systems.

All studied hybrid zones exhibited a set of consistent genomic patterns, fully supporting the **Hypothesis 3** that hybridization in *Pinus* is a repeatable process across geographically distinct contact zones. In each zone, majority of the individuals showed admixed ancestry, and hybrids were skewed towards *P. mugo* ancestry. Our analysis indicated presence of pure *P. sylvestris* across all contact zones, and presence of a few parental individuals of *P. mugo* at one site (Torfowisko pod Zieleńcem). We detected continuum of parental ancestries, but majority of hybrid individuals showed genomic composition of advanced backcross hybrids, and F1 hybrids were relatively rare. However, the majority of F1 hybrids carried *cp*DNA haplotypes derived from *P. mugo*, indicating a directional bias in gene flow—most likely due to *P. mugo* acting as the pollen donor in hybridization events. Such cytonuclear patterns were consistent across all zones and hybrid types, suggesting that this asymmetry is not stochastic but reflects a persistent, directional mechanism of hybrid formation. This repeated pattern across independent hybrid zones provides strong empirical support for **Hypothesis 3**. The genetic diversity of hybrids, especially F1's, was significantly higher than in pure individuals derived from allopatric populations, providing more conclusive results than the neutral markers and therefore supporting the **Hypothesis 2**. The patterns in genetic diversity of hybrid individuals, continuum of hybrid ancestries and transgressive phenotypes observed across all hybrid zones suggest, that the contact zones represent relatively old (many generations) instances of hybridization between the taxa.

In addition to repeatable patterns of hybridization, our genomic data shed light on the evolutionary status of *P. uliginosa*. Genome-wide SNP analyses revealed that *P. uliginosa* shares substantial ancestry with both *P. mugo* and *P. sylvestris,* and consistently occupies an intermediate genetic position in Błędne Skały and Torfowisko pod Zieleńcem contact zones. Patterns of admixture, as well as phylogenetic and principal component analyses, indicate that *P. uliginosa* is best understood not as a distinct taxon within the *P. mugo* complex, but rather as a stabilized hybrid lineage that has persisted through recurrent gene flow in sympatric zones. In reference to **Hypothesis 4** the results challenge recognition of *P. uliginosa* as a true parental species and reinforcing its hybrid origin (Łabiszak et al. 2025; submitted).

In the fourth part of my PhD thesis I specifically focused on patterns of introgressive divergence and selection in the pine contact zones (Szczepański et al. 2025, submitted). As the environmental niches within studied pine hybrid zones are not typical for any of parental species, it is expected that they experience strong selective pressure in order to adapt to this specific environment. It can either be achieved purely by selecting genotypes best fitted to those environmental properties (Stapley et al. 2010, Radwan and Babik 2012, Savolainen et al. 2013) or by a process of adaptive introgression, where hybrid lineage is better fitted than parental phenotypes (Hamilton and Miller 2016, Suarez-Gonzalez et al. 2018). There are multiple outlier detection methods to identify markers under selective pressure. In my research, three different approaches were employed – OutFLANK (Whitlock and Lotterhos 2015), BayeScan (Foll and Gaggiotti 2008) and pcadapt (Luu et al. 2017). OutFLANK and BayeScan are FST-based methods and pcadapt uses principal component analysis to determine which markers best discriminate two or more individuals/groups. Using at least two outlier detection methods and choosing only markers detected by both of them is a standard procedure in selection studies

(Di Santo et al. 2022, Muli et al. 2022, Fasanella et al. 2024, Łabiszak and Wachowiak 2024, Lasek et al. 2024). The strategy of selecting only the markers detected by two or three methods resulted in the set of 296 unique outliers, found across a number of different comparisons – grouped *P. mugo* hybrids and F1's versus reference *P. mugo* populations; grouped parent *P. sylvestris*, hybrid *P. sylvestris* and F1's versus reference *P. sylvestris* populations and the same scheme of comparisons, but divided for each hybrid zone. Annotation of those outliers resulted in 132 markers derived from transcriptome data and one candidate gene – dehydrin 9.

The annotated outliers were mainly associated with biological regulatory processes such as protein phosphorylation, proteolysis and transmembrane transport. Among them, especially interesting were the nonsynonymous mutations, resulting in change of the produced amino acids. Three examples of such SNPs for Scots pines are receptor-like protein 4 (RLP4; hybrid and parent *P. sylvestris*), dehydrin 9 (DHN9; hybrid *P. sylvestris*) and geraniol 8-hydroxylase (hybrid *P. sylvestris*). Although only general gene ontology terms were available for RLP4, its function was described in *Arabidopsis thaliana* - cell-wall-associated RLPs, RLP4 and RLP4-L1, which localize to cell edges, respond to changes in cell wall mechanics and are functionally linked to directional growth control (Elliott et al. 2024). DHN9 is an enzyme well-studied as a candidate gene for cold resistance in pines (Close 1997, Seppänen et al. 2000, Kalberer et al. 2006, Palmé et al. 2009). Geraniol 8-hydroxylase is an enzyme that catalyzes reaction producing 8-Hydroxygeraniol, described as a potential insect repellent (Wang et al. 2023).

The studied groups of individuals differed significantly in the number of outliers. Most of them were found in comparisons of hybrid *P. sylvestris* and parent *P. sylvestris* groups with reference Scots pines, indicating the most powerful selective pressure on trees with the dominance of *P. sylvestris* ancestry. In contrary, there were only few outliers detected for hybrid *P. mugo* groups. However, these groups represented the largest proportion of individuals within each sympatric population, suggesting that hybrids with a predominance of *P. mugo* ancestry may be better adapted to the specific environmental conditions of the hybrid zones. Although hybrid individuals with dominant *P. mugo* ancestry exhibited a relatively low number of outlier loci, some were nonsynonymous mutations located in functionally important genes. These included glutamine synthetase (GS1b), associated with water stress response and the maintenance of glutamate–glutamine balance for nitrogen transport in vascular cells (de la Torre et al. 2002, El-Khatib et al. 2004); bHLH transcription factor RHL1 – described as the best candidate for root hair development in barley (Gajewska et al. 2018) and rice (Ding et al. 2009, Moon et al. 2019).

Altogether, the results partially support the **Hypothesis 5**, as there are more shared outliers associated with introgression than those resulted solely from environmental adaptation. Although, the most of outliers were common in all hybrid zones, some of them, especially when comparing parental *P. sylvestris* groups, were unique for the specific sympatric population, suggesting environmental adaptation. Overall, outliers found in the hybrid groups had the tendency to be shared between the contact zones, especially in the case of *P. mugo*. These findings support also **Hypothesis H6** of introgressive divergence in the hybrids, as for the majority of hybrid groups, outliers were found and they were not scored within reference populations of corresponding parental species.

To sum up, by leveraging natural hybrid zones and combining organellar and nuclear data, the four chapters of this thesis presented in a form of research articles contributes to our understanding of hybridization as both a mechanism of adaptation and a driver of speciation. The results enhance our understanding the genomics of hybrid zones in forest tree species. These findings are not only relevant for evolutionary biology, but also carry practical implications for conservation, management of genetic resources and preservation of biodiversity in forest ecosystems under increasing ecological pressure.

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Molecular signatures of introgressive divergence and selection in contact zones of closely related pine species (*Pinus* genus)

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ABSTRACT

Background: Natural hybridization plays a key role in shaping genetic diversity, local adaptation, and the dynamics of speciation through interspecific gene flow. Hybrid zones serve as valuable natural systems for studying these processes. In this research, we used genotyping data at thousands of nuclear SNPs to investigate genomic outcomes of hybridization and selection across three contact zones of closely related pine species including Scots pine (*Pinus sylvestris* L.) and dwarf mountain pine (*P. mugo* T.). The reference allopatric stands of parental species were used to assess introgression dynamics.

Results: Individuals from the hybrid zones showed distinct genetic ancestry patterns and were assigned to groups including pure species, first-generation hybrids, and advanced backcrosses. Genotypes of the majority of hybrids were shifted towards *P. mugo* ancestry. Most outlier loci were shared across all sympatric populations, although some were specific to individual contact zones. The annotated outliers were mainly associated with regulatory biological processes related to phosphorylation, proteolysis, and transmembrane transport. Selection in contact zones depends on the genomic background of individuals and it was strongest in pure *P. sylvestris* and hybrids with a majority of *P. sylvestris* ancestry. The pattern suggests that it may be driven by adaptation to peat bog habitats situated outside the species' core ecological niche.

Conclusions: Our findings indicate strong selection acting on hybrids and pure *Pinus sylvestris* individuals across all studied hybrid zones. In contrast, the weaker signal of selection observed in individuals with *P. mugo* ancestry suggests that relict populations of this species, which historically spread across postglacial peat bogs, were pre-adapted to such environments. While several outlier loci were shared across different contact zones, others were uniquely associated with specific regions, indicating that local environmental pressures and introgressive divergence shape the genomic composition of the populations. These results highlight the role of hybridization in generating adaptive diversity and emphasize the evolutionary significance of hybrid zones in pines.

1. BACKGROUND

Natural hybridization is a significant evolutionary process that shapes the genetic architecture and adaptive potential of populations. It can promote speciation by generating novel genetic combinations that are exposed to natural selection [1]. The exchange of genetic material between species may lead to new phenotypic traits, some of which may confer fitness advantages under changing environmental conditions [2]. Understanding the dynamics of hybridization provides valuable insights into mechanisms of introgressive divergence, including how it generates novel evolutionary trajectories and how natural selection influences the fitness of hybrid individuals. This is particularly relevant for assessing the evolutionary potential and ecological resilience of plant species in a changing environment.

In recent years, numerous cases of hybridization have been documented in both animal [3–6] and plant systems [7–10]. Plants, in particular, show a high propensity for hybridization, driven by factors such as overlapping reproductive strategies, frequent polyploidy, clonal reproduction, and generally weaker reproductive barriers compared to animals [11]**.** Despite this, only a limited number of plant groups have been studied in detail in this context, including model species from *Helianthus* [12], *Iris* [13], *Mimulus* [14], and *Senecio* [15] genus. In plants, hybridization can lead to a range of outcomes—from transient gene exchange to the formation of stable hybrid lineages or even new species. These outcomes are often highly context-dependent, shaped by environmental heterogeneity, life history traits, and the genomic architecture of reproductive barriers. Thus, expanding the study of hybrid zones—especially in long-lived and ecologically dominant taxa such as forest trees—is essential for advancing our understanding of how hybridization influences genetic structure, fitness, and long-term population viability in plants. Forest trees, particularly closely related species, offer compelling systems for studying natural hybridization, as seen in oaks [16–19], poplars [20–22], and birches [23–25]. In conifers, hybridization has been documented for instance in spruces [26–28] firs [29, 30], junipers [31, 32], yews [33], and pines [34–39]. Trees are particularly well-suited for studying hybridization due to their long generation times, large effective population sizes, and extensive pollen and seed dispersal, which allow introgressed alleles to persist and spread across landscapes. Their broad ecological niches expose hybrid populations to diverse selection pressures, making them ideal for investigating how gene flow interacts with local adaptation and shapes evolutionary trajectories [40]. These processes are especially significant in forest ecosystems, where interspecific gene flow can influence traits related to growth, resistance to environmental stress, and reproductive success, resulting in complex genetic structures and adaptive variation.

Pines (*Pinus* spp.) are evergreen conifers of the *Pinaceae* family, widespread across the Northern Hemisphere [41] They exhibit diverse and dynamic patterns of hybridization facilitated by overlapping geographic distributions, similar reproductive biology, and shared ecological strategies [42–51]. Within this genus, Scots pine (*Pinus sylvestris* L.) and taxa from the *Pinus mugo* complex, including dwarf mountain pine (*P. mugo* Turra) represent a particularly interesting system for investigating taxonomic relationships, hybridization, and evolutionary processes. These taxa are primarily allopatric across Europe, occupying a broad range of habitats from alpine and subalpine zones to peat bogs and lowland forests [52]. Taxonomic relationships within the *Pinus mugo* complex are intricate, with ongoing debates regarding species delineation and genetic differentiation. Genetic and phylogeographic studies have revealed a complex history shaped by historical events, such as glaciations, and contemporary gene flow [50–56]. However, critical gaps remain in our understanding of the genetic interactions and adaptive processes shaping these closely related taxa.

The main objectives of this study were to investigate the role of interspecific gene flow and natural selection in shaping genetic diversity and evolutionary trajectories across several contact zones between *Pinus sylvestris* and *P. mugo*, each differing in environmental context. We applied high-throughput genotyping of thousands of nuclear SNP polymorphisms across more than 1,500 individuals sampled from both allopatric populations and hybrid zones. Specifically, we aimed to (i) characterize patterns of interspecific gene flow, (ii) assess the influence of hybridization on genetic structure in contrasting environments, and (iii) identify genomic regions that undergo introgression and loci under selection. We hypothesized that adaptive introgression facilitates the transfer of beneficial alleles between taxa, with positive selection acting on loci associated with local environmental adaptations. Additionally, we expected that similar environments would promote the selection on the same genetic variants, whereas distinct habitats would exert divergent selective pressures on different alleles. To our knowledge, this is one of the most extensive genomic investigations of hybridization in *Pinus*, integrating high-resolution nuclear SNP data across multiple contact zones and environmental contexts. This approach allows us to explore how ecological and geographic variation jointly shape patterns of gene flow and selection in hybridizing conifer taxa.

2. MATERIALS AND METHODS

2.1 Studied populations and sampling

A total of 1,558 trees were genotyped from 24 populations, comprising three hybrid zones and 21 reference populations. The reference populations included 12 Scots pine (*Pinus sylvestris*, PS) and 9 dwarf mountain pine (*P. mugo*, PM) populations (Fig. 1). The hybrid zones investigated in this study were located in southern and southwestern Poland and included Bór na Czerwonem (BC), Błędne Skały (BS), and Torfowisko pod Zieleńcem (TZ) (Fig. 2, Table S1). Bór na Czerwonem is a peat bog situated in the Orava–Nowy Targ Basin, near the Tatra Mountains. This site comprises co-occurring populations of *P. sylvestris*, *P. mugo,* and their natural hybrids. The Błędne Skały and Torfowisko pod Zieleńcem populations are located in the Stołowe and Bystrzyckie Mountains, respectively. These sites host individuals representing both taxa, as well as numerous phenotypically intermediate forms. The Błędne Skały population, estimated to be 7,000–8,000 years old, is found on the crests of Cretaceous sandstone formations [54] Torfowisko pod Zieleńcem is the largest of the hybrid zones studied, encompassing approximately 232 hectares of peat bog habitat, divided into three subpopulations by forest patches and access routes [53, 55, 56].

2.2 DNA extraction and SNPs genotyping

Field sampling (2021–2023) was conducted under permits issued by the Polish Ministry of Climate and Environment (DOP-WPN.61.116.2021.MGr; DOP-WOPPN.61.35.2022.WH) and the Polish State Forests (ZG.7021.2.2021). We morphologically classified individuals in the field as parental or hybrid types [53, 57]. Prof. Witold Wachowiak and Dr. Bartosz Łabiszak conducted the formal identification of the plant material. No voucher specimen was deposited as a few fresh needle samples were used only for DNA isolation. DNA was extracted using the Genomic Mini AX Plant Kit (A&A Biotechnology, Poland). DNA concentration was quantified using the Qubit 4 fluorometer with the Broad Range Assay Kit, and all samples were diluted to a working concentration of 40 ng/µl. Genotyping was performed using the Axiom_PineGAP SNP array which initially produced 49,829 SNPs, containing polymorphisms identified in transcriptome sequences and during the resequencing of candidate genes in the studied pine species [58]. We retained only high-quality markers classified as “Best and Recommended” ($QC \geq 90$, $DQC \geq 0.82$) in the Axiom Analysis Suite. Further filtering excluded SNPs with a minor allele frequency ($MAF < 0.05$) and those in linkage disequilibrium ($LD > 0.8$), resulting in 7,390 SNPs used for downstream analyses.

2.3 Genetic assignment of samples

To assess the distribution of genetic variance and identify the broad genetic structure of the studied pines within the hybrid zones we performed principal component analysis (PCA) using the *adegenet* R package [59, 60]. Next, we used Latent Factor Models implemented in the *LEA* R package [48] to identify a number of ancestral clusters and to classify individuals from the hybrid zones based on their ancestry coefficients into predefined genotypic classes. The number of ancestral clusters (K) was tested from $K = 1$ to 10, with 10 replicates per value, to determine cross-entropy. The graphical illustration of individual ancestry coefficients was plotted using POPHELPER Structure Web App v1.0.10 [61]. As the optimal K was 2 corresponding with *P. sylvestris* and *P. mugo* as ancestry (see Results), we classified our sampled trees based on the estimates of the ancestry coefficient expressed as Q-scores into parental species (*Pinus sylvestris*, *Pinus mugo*) and hybrids.

Individuals with ≥97% ancestry from one taxon were designated as “pure species” (PS and PM). Those with 40–60% ancestry from each parent were classified as putative F1 hybrids. The remaining individuals were assigned to either *P. sylvestris* or *P. mugo* hybrids based on their predominant genomic ancestry (referred hereafter as PS_H and PM_H, respectively). The spatial distribution of genetic classes was visualized using the *ggplot2* [49] and *ggmap* [50] packages in R [51], with Google Maps API tiles (zoom levels: BC = 16, BS = 17, TZ = 15). Data handling was performed using *dplyr* [62], *forcats* [63], *tidyr* [64], and *tidyverse* [65]. Figure layouts were refined using *ggpubr* [66] and *cowplot* [67].

2.4 Outliers analysis

Outliers detection was performed using three complementary approaches. Prior to analysis, SNP data were converted using the *dartR* package [68] to generate input formats compatible with pcadapt, OutFLANK, and BayeScan. The first method, pcadapt v4.3.3 [69], detects candidate loci under selection by assessing their association with principal components (PCs) of genetic variation, without relying on predefined population assignments. We tested values of K (the number of retained PCs) from 1 to 15 and selected K = 2 for final analysis, based on scree plot inspection and explained variance. Loci were ranked by their Mahalanobis distance from the center of the PC distribution, and statistical significance was assessed via false discovery rate (FDR) correction. The second approach, OutFLANK v0.2 [70], identifies outliers by modeling the distribution of F_{ST} values expected under neutrality. It trims the extreme tails of the empirical F_{ST} distribution to estimate the parameters of the null distribution, which is then used to identify loci with unusually high F_{ST} values indicative of divergent selection. This method is designed to be conservative, minimizing false positives, and does not require prior knowledge of population structure beyond population labels. The final method, BayeScan v2.1 [71], applies a Bayesian framework to estimate the posterior probability that each locus is under selection. It models allele frequencies using population-specific and locus-specific F_{ST} coefficients, distinguishing between selection and demographic effects. A reversible-jump MCMC algorithm is used to sample the parameter space, and loci with strong support for selection (based on posterior odds) are identified. We applied a prior odds ratio of 10, following standard recommendations for moderate stringency.

To control the false discovery rate across all three methods, only SNPs with q-values < 0.1—calculated using the *qvalue* package [72]—were retained as candidate outliers. Functional annotation of outlier loci was performed using the BLASTx algorithm [73] implemented in Diamond within the OmicsBox platform [74]. For SNPs derived from transcriptome datasets, we aligned full unigene sequences to improve annotation quality and retained only those SNPs located within the aligned regions. Functional classification included InterProScan domain analysis [75], Gene Ontology (GO) mapping [76, 77], and the merging of GO terms across annotation methods.

2.5 Ancestry informative markers, hybrid index, and genomic clines

To complement ancestry estimates obtained from LEA, we calculated hybrid indices for all individuals sampled from the contact zones using the *gghybrid* R package [78], which applies a Bayesian genomic cline model to estimate the proportion of ancestry derived from each parental species. Pure, allopatric *P. mugo* populations were defined as parental group S0, and pure *P. sylvestris* populations as parental group S1. The model was run under default parameters with 6,000 MCMC iterations and a burn-in of 2,000 and repeated three times to ensure consistency of parameter estimates. Convergence of MCMC chains was verified using the Gelman–Rubin diagnostic (N = 10,000), with values <1.1 interpreted as evidence of satisfactory convergence across independent runs.

Additionally, we identified ancestry-specific markers (AIMs) by comparing allele frequencies between allopatric reference populations of *P. sylvestris* and *P. mugo*. Markers were retained if the allele frequency was ≥0.97 in one species and ≤0.03 in the other, representing putatively fixed, diagnostic variants useful for tracing genomic ancestry and introgression. We then assessed the distribution of these AIMs in hybrid individuals to investigate potential biases in ancestry contributions. Specifically, we calculated the mean allele frequency of species-specific SNPs within hybrids to evaluate whether AIMs exhibited a consistent shift towards one parental species. This analysis was extended across multiple hybrid zones to assess spatial consistency in AIM distributions. To detect signatures of non-neutral introgression, we examined the frequency distribution of AIMs in hybrids with known hybrid indices. Deviations from expected frequencies under neutral models can signal the presence of reproductive barriers, local adaptation, or selective sweeps acting on introgressed alleles. By integrating outlier detection and allele frequency divergence, our approach aimed to identify genomic regions contributing to the maintenance of species boundaries or facilitating adaptive gene flow between *P. sylvestris* and *P. mugo* within hybrid zones.

3. RESULTS

3.1 Genetic composition of the hybrid zones

Principal Component Analysis (PCA) provided strong evidence that ancestry from *Pinus sylvestris* and *P. mugo* constitutes a major source of genetic variation in the studied populations. The analysis clearly separated allopatric reference populations of the two species along the first principal component (PC1), which explained 19.2% of the total genetic variance. Individuals from hybrid zones formed a genetic continuum between the parental clusters, reflecting varying degrees of admixture (Fig. 3). This was further corroborated by the species assignment analysis performed in *LEA*, which identified two main genetic groups (K = 2, Figures 4, S1) representing the ancestry of *P. sylvestris* and *P. mugo*. We observed mixed ancestry, as evidenced by Q-scores ranging from 0.03 to 0.97 among individuals in the contact zones, while individuals from allopatric populations were classified as pure *P. mugo* or *P. sylvestris.*

Across all contact zones, genotypes with a predominance of *Pinus mugo* ancestry were most common (Fig. 2), although only three individuals were classified as pure *P. mugo* (Q-scores >0.97), and all of these were found exclusively in the Torfowisko pod Zieleńcem (TZ) population. Individuals with 60–97% *P. mugo* ancestry predominated across all zones, with the highest number in the TZ population. In contrast, the Błędne Skały population contained the highest number of F1 hybrids, while Bór na Czerwonem harbored the greatest proportion of pure *P. sylvestris* individuals, highlighting considerable variation in genetic composition across sites.

3.2 Outliers detection

Outlier SNPs were detected in multiple comparisons among species and populations (Fig. S2, S3). Both OutFLANK and BayeScan identified fewer outliers compared to pcadapt, reflecting their more conservative thresholds (Fig. S4). A set of 29 SNPs was consistently identified as outliers across all three methods (Fig. S5, S5, S6). The highest number of shared outliers between any two methods (143 SNPs) was observed in comparisons between TZ F1 individuals and reference *P. sylvestris*, followed by comparisons between hybrid *P. sylvestris* individuals and the same reference group (108 SNPs; Fig. S6, S7). No common outlier SNPs were found in several comparisons: F1 individuals vs. reference *P. mugo,* hybrid *P. sylvestris* individuals from TZ vs. reference *P. sylvestris*; BC F1 individuals vs. reference *P. sylvestris,* and BC F1 individuals vs. reference *P. mugo*. The lowest number of outliers (5 common SNPs in both pcadapt and BayeScan analysis) was found between hybrid *P. mugo* vs. reference *P. mugo*.

Out of 296 unique outlier SNPs detected in at least two methods, 288 (~97.3%) were successfully aligned to reference transcriptome, with 218 associated with annotated proteins based on InterPro and Gene Ontology (GO). The final set included 133 SNPs located in coding regions of known function (Fig. 5). The comparison between hybrid *P. sylvestris* and its reference population revealed the greatest number of biologically and functionally annotated GO terms, followed by comparisons between F1 individuals from Torfowisko pod Zieleńcem and *P. sylvestris* (Fig. S8, S9, S10).

Some outlier loci were common for different group comparisons of reference and contact zones populations including several genes related to biological processes and signal transduction such as receptor-like protein, protein kinase, and genes related to cellular transport (Fig. 6, Table S2). All reference *P. sylvestris* had only one common outlier with the population of the species from contact zones (methionine aminopeptidase 2B) that was also found in comparison among reference *P. mugo* populations (Table S3).

Overall, *P. sylvestris* showed a broader and more complex landscape of genomic divergence under selection compared to *P. mugo*. The number of outlier loci detected in comparisons involving *P. sylvestris* was consistently higher (Table S2) than those involving *P. mugo* (Table S3), particularly in the Błędne Skały parental population (BS_PS). Interestingly, in this contact zone, we detected a notably high number of unique outlier loci (n = 66) in the hybrid group (PS_H) relative to the reference *P. sylvestris* populations. In contrast, only one or no unique outliers were identified in the corresponding hybrid groups from the Bór na Czerwonem (BC) and Torfowisko pod Zieleńcem (TZ), respectively. Unexpectedly, no outliers were shared between the TZ and BC peat bogs, despite their broadly similar habitat characteristics. The only instance of outlier sharing between PS_H was observed between BC and Błędne Skały (BS), where five loci overlapped (Fig. S11). Several outlier loci were consistently detected across different comparisons involving PS and hybrid groups. Those outliers were located in several genes of known functions including Receptor-like protein 4, Serine/threonine-protein kinases (D6PK, UCNL-like) and U-box domain-containing protein 34. Some loci were population-specific such as Small GTP-binding protein and ribonucleoside-diphosphate reductase, unique to Błędne Skały PS and NPGR2-like protein was exclusive to the Torfowisko pod Zieleńcem PS population.

In contrast, *P. mugo* and its introgressants had fewer outliers overall, a lower number of shared outliers across hybrid and parental comparisons, and several exclusive outliers found only in reference PM populations, not observed in contact zones or hybrid groups. We found no evidence of extensive outlier sharing between the Torfowisko pod Zieleńcem (TZ) and Bór na Czerwonem (BC) populations, with only three outliers shared between them. In contrast, both populations possessed a relatively higher number of unique outliers, with eight loci detected exclusively in each. In the Błędne Skały (BS) hybrid group, only a single unique outlier was identified, indicating limited divergence at outlier loci in this population. Several annotated outliers correspond to transcriptional regulators and metabolic transporters including bHLH transcription factor RHL1, auxin response factor 10, and serine/threonine-protein kinase VPS15 that play roles in development, signaling, and response to environmental stress. Other outliers included glutamine synthetase, adenine nucleotide transporter BT1, and molybdenum cofactor sulfurase imply metabolic differentiation, especially in nitrogen and energy pathways. A small number of loci were shared between hybrids and interspecific comparisons, such as the thyroid adenoma-associated protein homolog, identified in both F1 vs PS and PM vs PS comparisons. These shared outliers may reflect regions under selection in hybrid zones, potentially indicative of adaptive introgression or the presence of barriers to gene flow.

3.3 Ancestry informative markers, hybrid index and genomic clines

Hybrid index analysis confirmed the presence of highly admixed individuals in all contact zones, as initially detected by PCA and STRUCTURE-like clustering. (Fig. 7). Moreover, it ruled out alternative explanations—such as isolation by distance—that these methods alone cannot reliably distinguish [79]. Ancestry assignments were highly consistent across approaches, with a strong correlation between LEA admixture coefficients and hybrid index estimates (r = 0.99; Fig. S12), indicating the robustness of both methods for detecting hybrid ancestry. Additionally, hybrid index analysis reveals asymmetric introgression across zones. The Torfowisko pod Zieleńcem (TZ) contact zone shows the strongest shift toward *P. mugo* ancestry, while Błędne Skały (BS) also shifts in the same direction but less strongly. In contrast, the Bór na Czerwonem (BC) zone exhibits a more balanced admixture, with some hybrid groups (e.g., BC_H_PS) skewed slightly toward *P. sylvestris*. These differences highlight spatial variation in gene flow dynamics, with asymmetry toward *P. mugo* more pronounced in TZ, suggesting localized ecological or demographic influences.

We identified 29 ancestry-specific outlier loci, of which 15 were fixed or nearly fixed in *Pinus sylvestris* and 14 in *P. mugo* reference populations (Fig. 7; Table S4). Of these, 10 loci also overlapped with those identified as selection outliers, suggesting that a subset of ancestry-informative markers (AIMs) may be under directional selection. Notably, only three of these overlapping loci were successfully annotated: abscisic acid receptor PYL4, bHLH transcription factor RHL1, and thyroid adenoma-associated protein homolog—genes with putative roles in stress response, development, and reproductive regulation. Surprisingly, high frequencies of *P. sylvestris*-fixed alleles were observed across numerous loci in *P. mugo*-like hybrids (PM_H) from all contact zones, with this pattern being most pronounced in individuals from the Torfowisko pod Zieleńcem (TZ) population. Despite an overall genomic composition skewed toward *P. mugo,* hybrids in the TZ_H_PM group consistently exhibited elevated frequencies of PS-specific alleles. At several loci (e.g., 117420110, 117433680, 117434739), allele frequencies approached or exceeded 0.5, indicating substantial and potentially non-neutral introgression into a *P. mugo*-dominated background. Among these, only locus 117434739 was associated with a gene of known function—vacuolar protein sorting-associated protein 29, which may play a role in protein trafficking and cellular homeostasis.

In contrast, introgression of *P. mugo*-fixed alleles into *P. sylvestris*-like hybrids (PS_H) was limited. Several loci (e.g., 117420230, 117425397, 117431689, 117443799) consistently showed low frequencies of PM alleles across PM_H groups, particularly in the BS and BC contact zones, often not exceeding 0.25. Only in the TZ_H_PM group did these loci display elevated frequencies, broadly consistent with their overall hybrid index. Among these outliers, some were located in genes with known functional roles, including bHLH transcription factor RHL1 (117443799) and putative pumilio homolog 8, chloroplastic (117420230), both implicated in transcriptional regulation and post-transcriptional gene silencing.

4. DISCUSSION

4.1 Hybridization and population structure

Our results demonstrate that natural hybridization between *Pinus sylvestris* and members of the *P. mugo* complex is pervasive in the studied contact zones. All three sympatric populations exhibited a continuous spectrum of phenotypic forms, ranging from typical Scots pines, through hybrids representing a wide array of transgressive phenotypes, to typical shrub-like dwarf mountain pines. The most prevalent genetic class across all sympatric stands were hybrids, the majority of which showed predominant *P. mugo* ancestry. However, the genetic composition of individuals across the hybrid zones confirmed the presence of a full spectrum of genomes spanning from pure *P. sylvestris* to nearly pure *P. mugo*, with a continuum reflected in the smooth distribution of hybrid indices (i.e., proportions of *P. sylvestris* vs. *P. mugo* ancestry) (Fig. 2, 3, 4, 7).

Yet, despite the clear morphological resemblance of some individuals to *P. mugo*, the genomic ancestry spectrum did not fully mirror the phenotypic variation. A large number of individuals in the hybrid zones were genetically almost pure *P. sylvestris* as demonstrated also in PCA analysis, whereas virtually none were pure *P. mugo* (only three individuals across all sites exhibited >97% *P. mugo* ancestry). Although some possible F1 individuals were found, most of the trees resulted from backcrossing with one of the parental species or hybrids of different ancestry proportions. Moreover, trees classified as *P. mugo* hybrids from all hybrid zones always had less than 90% of the PM ancestry. Notably, we found no evidence of introgression in the genomes of parental taxa outside the hybrid zones that supports earlier observations that interspecific gene flow is restricted to pine contact zones [51]. As a more detailed analysis of hybrid zone formation and evolutionary dynamics in this system is presented elsewhere [80], we focus here on the molecular signatures of introgression and selection in hybrids.

4.2 Patterns of introgression

The genomic data from thousands of SNPs allowed us to characterize the patterns of introgression across the genome in these hybrid zones. We found that hybridization between *P. sylvestris* and *P. mugo* is highly asymmetrical, with a strong bias in hybrids with the majority of *P. mugo* ancestry. As assumed that most loci were subjected to introgression and can be freely exchanged between the hybridizing species – meaning that the probability of having a variant derived from a putative parent must be proportional to the hybrid index. Interestingly, we found also that some loci were introgressed more frequently than expected considering only the hybrid index of an individual. For example, we observed that a *P. sylvestris*-derived allele at a glutamine synthetase gene (GS1b), which is normally rare in allopatric *P. mugo* (frequency <10%), occurred at a much higher frequency in the *P. mugo*-like hybrids (up to ~80% in one zone). Glutamine synthetase is involved in nitrogen metabolism and has been linked to plant water-stress responses [81, 82]. The introgression and increase of the Scots pine variant in *P. mugo* hybrids suggests adaptive value—potentially helping dwarf pines cope with the waterlogged, nutrient-poor peat bog conditions, especially because this mutation is nonsynonymous. Similarly, a MED15a gene allele characteristic of Scots pine (and nearly fixed in allopatric *P. sylvestris*) was found to have introgressed into hybrid *P. mugo* individuals that showed a notable increase in the frequency of the Scots pine variant at this locus. MED15a encodes a transcriptional regulator of stress-response genes (including heat-shock proteins) and was previously noted to differ between northern and southern Scots pine populations [83–86]. Its introgression into dwarf pines in the hybrid zones (where the Scots pine allele was absent in pure *P. mugo* populations) again points to potential selective benefits conferred by *P. sylvestris* alleles. Another nonsynonymous mutation was in geraniol 8-hydroxylase, which is an enzyme that catalyses reaction producing 8-Hydroxygeraniol, which is described as a potential insect repellent [87]. The variant characteristic within the reference PM populations was introgressed into BS PS hybrids and TZ F1s. These cases exemplify adaptive introgression, where gene flow introduces alleles that improve fitness in the recipient species' environment.

Indeed, we found also examples of alleles that remain species-specific and resist introgression despite extensive hybridization towards *P. mugo*. One striking example is a nonsynonymous mutation in a basic helix-loop-helix (bHLH) transcription factor gene (putatively *RHL1*), which is fixed for alternate alleles in the parental species (one allele fixed in *P. mugo*, the other fixed in *P. sylvestris*). In the hybrid populations, the *P. mugo*-derived allele at this locus remained nearly fixed among dwarf pine hybrids, effectively acting as a barrier locus that maintains species differentiation. RHL1 is known to regulate root hair development in grasses [88–90], a trait potentially crucial for nutrient uptake in bog soils. The maintenance of the *P. mugo* allele exclusively in hybrids suggests that the *P. sylvestris* variant of this gene has likely a negative impact on fitness in the peat bog environment or in the genomic background of *P. mugo*. In general, such loci that remain unintrogressed (or show deficient introgression of the alternate allele) likely reflect selective constraints – they may underlie key species-specific adaptations or genetic incompatibilities. This pattern aligns with theoretical expectations of introgressive hybridization under selection, where neutral or advantageous alleles move freely between species, while maladaptive or incompatible alleles are purged from the opposite background [91, 92]. It also aligns with observations in other tree species hybrid zones (e.g., spruces and oaks), where asymmetric introgression and locus-specific barriers produce a patchwork genome structure [18, 27].

4.3 Asymmetric selection

Despite the asymmetrical gene flow, and high bias favoring the repeated formation of *P. mugo*-like hybrids across hybrid zones, we found, that natural selection imposes substantially stronger evolutionary constraints on *P. sylvestris* (and hybrids with a large *P. sylvestris* genomic component) compared to *P. mugo*. The results of genome scan analyses for loci under selection fully support this conclusion: we detected a much higher number of outlier SNPs in comparisons involving Scots pine genomes than in those involving dwarf mountain pine genomes. In particular, hybrid individuals genetically classified as *P. sylvestris* (i.e. those with majority of *P. sylvestris* ancestry) showed a large set of allele frequency outliers when compared to allopatric *P. sylvestris* reference populations (Fig. 6). Likewise, even "pure" *P. sylvestris* individuals growing inside the hybrid zones – which had no detectable *P. mugo* introgression as confirmed by their nuclear ancestry, morphology, and a diagnostic *cp*DNA marker [51] – exhibited numerous outlier loci distinguishing them from reference allopatric populations. By contrast, *P. mugo*-like hybrids (with majority *P. mugo* ancestry) yielded far fewer outlier loci in analogous comparisons to allopatric *P. mugo* references (Fig. S13). These patterns of a much broader and more complex landscape of genomic divergence under selection in *P. sylvestris* indicate that in the unfavorable conditions of peat bogs, individuals of Scots pine ancestry undergo stronger selection. It seems that their genomic background is less fit compared to that of dwarf mountain pines, which results in a lower number of hybrid individuals with dominant PS ancestry found in all hybrid zones. Peat bog environments lie at the edge of the ecological niche for *P. sylvestris*—a widespread generalist typically found on more mineral-rich soils at lower elevations [93, 94]. This aligns with our field observations within hybrid zones, where Scots pines were typically restricted to the drier margins of peat bogs and largely absent from the wettest, central areas. The strong selection signatures detected in *P. sylvestris* from these zones suggest that only genotypes with specific adaptive traits can establish and persist under such marginal conditions. These may include individuals carrying pre-adapted alleles from standing genetic variation within *P. sylvestris*, or hybrids inheriting adaptively introgressed alleles. Importantly, our data indicate that adaptation of *P. sylvestris* to the bog environment does not primarily rely on introgression from *P. mugo*. If survival were largely mediated through adaptive introgression, we would expect hybrids and backcrossed individuals to dominate, with pure *P. sylvestris* individuals being rare or absent. However, we identified several genetically pure (or nearly pure) Scots pines thriving within the contact zones. These individuals showed signals of divergent selection on their own genomic backgrounds—relative to allopatric *P. sylvestris*—rather than extensive *P. mugo* introgression. Among these pure individuals, we detected outlier SNPs in coding regions of genes such as MetAP2B (methionine aminopeptidase 2B), FAAH (fatty acid amide hydrolase isoform X2), and OMA1 (mitochondrial metalloendopeptidase), which are associated with protein maturation, lipid signaling, and mitochondrial function, respectively. These genes play key roles in abiotic stress response, energy regulation, and cellular homeostasis, suggesting that selection in peat bogs may act on standing genetic variation to promote physiological resilience in *P. sylvestris* [95–102].

However, hybrids with predominantly *P. sylvestris* ancestry showed some molecular signatures of adaptive introgression. These hybrids were rare across hybrid zones and far outnumbered by *P. mugo*-like individuals, suggesting strong selection against them. Notably, in these *P. sylvestris*-like hybrids, we observed fixed *P. mugo*-derived allelic variants at several loci, indicating that introgressed alleles were facilitating their persistence and may act as a genomic rescue mechanism in an otherwise less adapted genetic background. Several of these introgressed variants occurred in genes involved in environmental response and stress regulation, including RLP4 (cell wall integrity and directional growth), Expansin B1 (morphological plasticity under abiotic stress), and UCNL-like kinase (signal transduction). One of the most ecologically informative outliers and a nonsynonymous mutation was Dehydrin 9, a gene associated with cold and dehydration tolerance and previously identified as a candidate for climatic adaptation in conifers [103]. In *P. sylvestris*-like hybrids, the *P. mugo*-derived allele at this locus was often fixed or present in the heterozygous state. This strongly supports the hypothesis that adaptive introgression at stress-relevant loci contributes to the persistence of *P. sylvestris* genetic backgrounds in these hybrid zones under the adverse environmental conditions and nutrient limitations characteristic of peat bog ecosystems [104–107].

The genomic background of *P. mugo* appears more stable and is subjected to weaker selective filtering in such environments. This likely reflects *P. mugo*'s ecological specialization for harsh, nutrient-poor, and waterlogged conditions typical of the mountain and subalpine habitats [108]. For example, *P. mugo* is known for its higher tolerance to abiotic stressors such as low temperatures, poor soil aeration, and high water table levels—traits that are beneficial in peat bog ecosystems [109]. These adaptive traits appear to provide a consistent fitness advantage and are likely retained in *P. mugo* hybrids despite gene flow from *P. sylvestris*, helping maintain their ecological performance under local conditions. The historical context of these stands further supports such observations. Generally dwarf mountain pines ancestry and its *cp*DNA variants dominates in all three studied contact zones and most likely inhabited those areas before Scots pines. During the postglacial period, *P. sylvestris* migrated northward from southern refugia into northern Europe. This migration led *P. sylvestris* to advance into post-glacial peat bogs already occupied by established *P. mugo* populations in these habitats [80, 110]. Consequently, *P. mugo* underwent more generations of selection, enhancing its ecological advantage and optimizing its fitness in situ.

Several outliers found in a group of samples with a predominance of *P. mugo* ancestry included bHLH transcription factor RHL1, mediator of RNA polymerase II (Med15a), and serine/threonine-protein kinase VPS15, the latter one associated with altitudinal adaptation and autophagy pathways. We also found a few SNP that showed selection patterns between reference *P. mugo* stands that were derived from genes including subtilisin-like proteases (SBT2.2, SBT2.5), that is related to hormone activation and plant architecture and argonaute 1 (AGO1), a core gene for RNA silencing and post-transcriptional regulation.

Taken together these findings indicate, that *P. sylvestris* persists in these peat bog environments either as a pure taxon shaped by strong selection on standing genetic variation or as a hybrid lineage rescued by targeted introgression. In contrast, individuals of predominant *P. mugo* ancestry maintain high fitness across zones, likely due to their preadaptation of their ancestors to such harsh ecological conditions. Similar asymmetrical selection dynamics have been reported in other hybrid zones where one species is locally adapted and the other is a more generalist or a recent colonizer. For example, in *Populus* hybrid zones, native species often exhibit genomic stability, while introgressed genotypes from less-adapted species are selectively filtered [111]. In *Helianthus*, hybridization has facilitated ecological expansion only when adaptive traits were acquired from the locally adapted parent [112, 113]. Likewise, in hybrid zones of *Quercus* and *Pinus*, species pre-adapted to local environmental conditions have been shown to impose strong selective pressures on their hybridizing counterparts, resulting in directional or asymmetrical introgression [38, 114, 115]. These cases, like our findings, highlight that even within a shared physical habitat, hybridizing species may experience very different selective regimes depending on their ecological proximity to the local adaptive optimum.

4.4 Convergence evolutionary outcomes

Our comparative analysis of outlier loci across the zones reveals a notable degree of overlap in genetic targets of selection. A majority of outlier SNPs identified in one hybrid population were also detected as outliers in at least one other population, and several loci showed consistent signals of selection across all three zones. For example, a SNP in the RLP4 gene, encoding a receptor-like protein kinase associated with cell wall integrity and directional growth in *Arabidopsis*, was identified as an outlier in hybrids or local *P. sylvestris* across all sites. The recurrent selection on this gene suggests that modulation of cell wall properties may represent a common adaptive requirement in pine hybrids—potentially to withstand mechanical stress from snow, wind, or water saturation in open bog environments. Other genes including serine/threonine-protein kinases (D6PK, UCNL-like) and U-box domain-containing protein 34 were frequently detected across *P. sylvestris* and hybrids, suggesting roles in cell signalling, stress responses, and/or reproductive compatibility [116, 117]. Methionine aminopeptidase 2B (MetAP2), FAAH, and OMA1 were scored across multiple hybrid zones and they are linked to basic plant growth, energy metabolism, and stress tolerance. Similarly, an outlier SNP in Expansin B1, a gene involved in cell wall loosening and root growth, was detected in multiple zones. Expansins have been linked to enhanced root development and abiotic stress tolerance in various plant species [105–107], suggesting that the selection on this gene may promote rooting in loose, acidic peat substrates or enhance physiological resilience under environmental stress. [97]

Contrary to our initial expectation, we did not observe strong genetic distinctiveness in the Błędne Skały (BS) hybrid zone relative to the Torfowisko pod Zieleńcem (TZ) and Bór na Czerwonem (BC) zones. BS is the only site where hybrids occupy sandstone rock formations with a shallow soil layer, and it also shows a subtle shift toward *P. sylvestris* ancestry, pointing to a potentially stronger role of environmental selection in shaping hybrid composition. Although there was only one outlier purely unique for PS group from BS hybrid zone (putative clathrin assembly protein At2g25430, associated with salt stress [118, 119]), we detected many outlier loci present only in grouped PS hybrids and the PS hybrids from BS zone, such as described before expansin B1 and dehydrin 9, but also pfkB-like carbohydrate kinase family protein, associated with controlling of plant development [120]. However, the fact that most outlier loci were shared among multiple sites suggests that convergent selection pressures outweigh divergent, site-specific responses, reinforcing the idea of parallel evolution in these independently formed hybrid zones. This suggests that adaptation in contact zones populations in PS occurs through selection within the species with support of adaptive introgression from PM in some cases.

5. CONCLUSIONS

Our study demonstrates consistent and repeatable genomic patterns of hybridization between *Pinus sylvestris* and *P. mugo* across three distinct contact zones, despite differences in local climate and biogeographic history. All zones were dominated by hybrid individuals, with asymmetric introgression favoring *P. mugo* ancestry and stronger selection signatures observed in *P. sylvestris*. However, contrary to expectations of parallel adaptation in similar habitats, we detected only a limited number of shared outlier loci among zones, indicating that similar environments did not consistently select for the same genomic regions. Instead, local adaptation appears to involve distinct sets of loci in each hybrid zone, shaped by context-dependent selection and the unique genetic backgrounds of local populations. Certain genes, including RLP4, GS1b, Expansin B1, and Dehydrin 9, showed evidence of non-neutral introgression and may contribute to environmental stress tolerance in hybrids. In contrast, the genomic background of *P. mugo* remained comparatively stable, suggesting preadaptation to the ecological conditions of the contact zones. These results indicate that while the demographic and directional aspects of hybridization are repeatable, the genetic basis of adaptation in hybrid zones is largely zone-specific. This underscores the role of hybrid zones as dynamic systems where introgression and selection interact to generate locally unique evolutionary trajectories, rather than uniform responses across replicated environments.

6. DECLARATIONS

6.1 Ethics approval and consent to participate

The sampling was conducted based on permissions from Ministry of Climate and Environment (DOP-WPN.61.116.2021.MGr; DOP-WOPPN.61.35.2022.WH) and Polish State Forests (ZG.7021.2.2021). Clinical trial number: not applicable.

6.2 Consent for publication

Not applicable.

6.3 Availability of data and materials

The datasets generated and/or analysed during the current study are freely available in the open access Figshare repository: DOI: 10.6084/m9.figshare.29097569.

Prof. Witold Wachowiak and Dr. Bartosz Łabiszak conducted the formal identification of the plant material. No voucher specimen was deposited as the samples of a few fresh needles were used only for DNA isolation.

6.3 Competing interests

The authors declare that they have no competing interests.

6.4 Funding

The research was funded by the Polish National Science Centre (Grant Nos. 2020/39/B/NZ9/00051 and 2020/37/B/NZ9/01496).

6.5 Authors’ Contributions

SS: Sampling, Methodology, Software, Validation, Data acquisition, Formal analysis, Investigation, Writing – Original Draft, Visualization; **BŁ**: Conceptualization, Sampling, Methodology, Software, Formal analysis, Data Curation, Writing – Review & Editing, Visualization, Supervision; **WW:** Conceptualization, Sampling, Methodology, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

6.6 Acknowledgements

We thank Wioletta Niemczyk and Diana Mankowska-Jurek from Góry Stołowe National Park and Tomasz Zwijacz-Kozica from Tatra National Park for their help with sample collection. We also thank the employees of Tatra NP, Góry Stołowe NP, Babiogórski NP and Karkonoski NP, where the sampling was conducted.

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FIGURE CAPTIONS

Figure 1. Geographic distribution of the analyzed populations in Poland. Red circles indicate populations from Scots pine–dwarf mountain pine contact zones. Blue symbols represent reference populations of *Pinus sylvestris*, while yellow symbols denote reference stands of *P. mugo*. Numbers correspond to population codes listed in Supplementary Table S1.

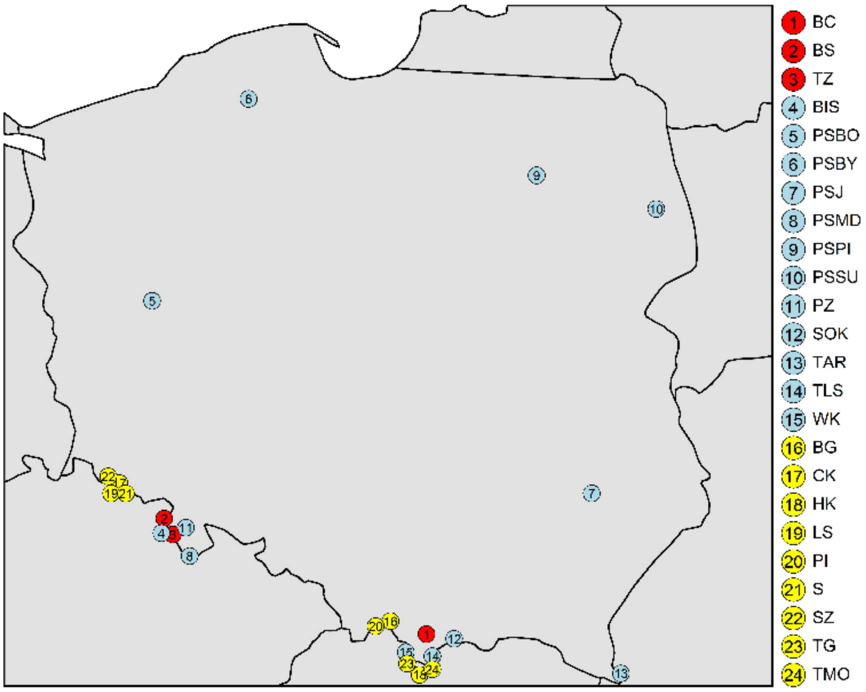


Figure 2. Spatial distribution and genetic classification of pine individuals across three hybrid zones: Bór na Czerwonem (BC), Błędne Skały (BS), and Torfowisko pod Zieleńcem (TZ). Maps show the geographic position of sampled individuals colored by species class as inferred from genetic clustering: pure *Pinus sylvestris* (light blue), hybrid *P. sylvestris* (dark blue), F1 hybrids (red), hybrid *P. mugo* (orange), and pure *P. mugo* (yellow). Bar plots indicate the number of individuals assigned to each class per site, illustrating differences in hybrid composition and taxonomic structure among zones.

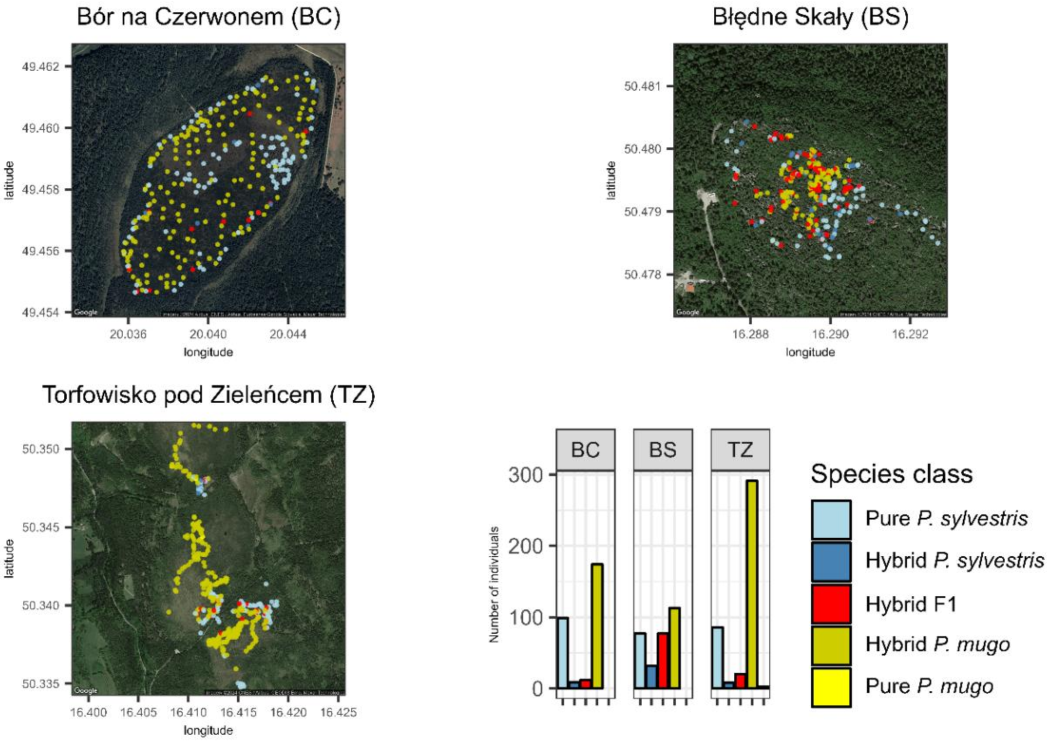


Figure 3. Principal component analysis (PCA) of genetic variation among sampled pine individuals based on nuclear SNP data. Colours indicate population origin: contact zones (BC, BS, TZ), reference *Pinus sylvestris* stands (shades of blue), and reference *P. mugo* populations (shades of yellow). Population codes correspond to sampling locations listed in Table S1**.**

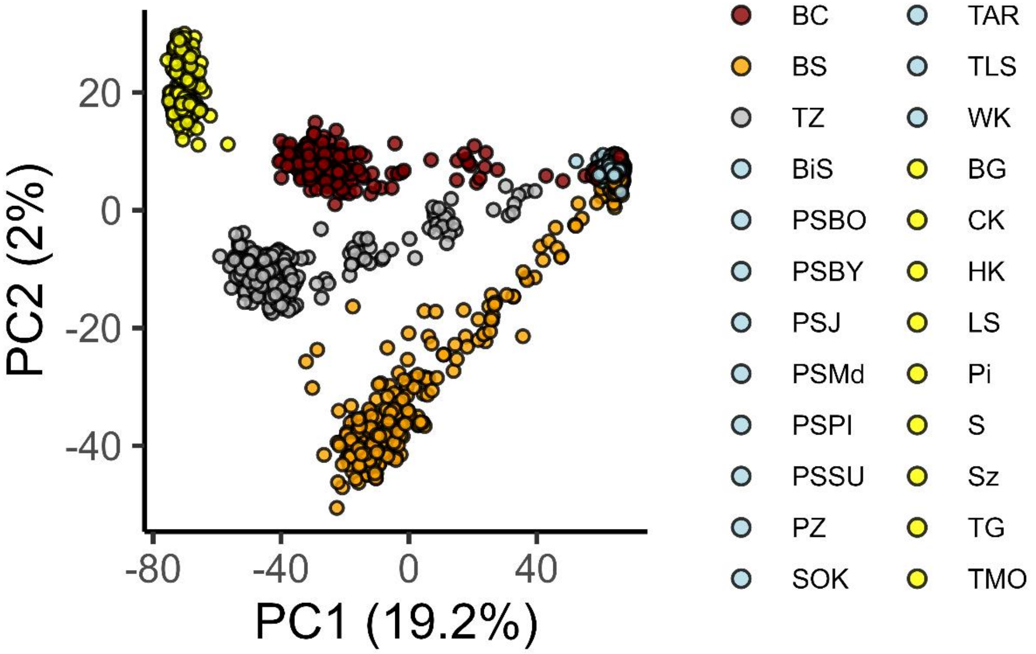


Figure 4. Individual ancestry proportions inferred using LEA based on nuclear SNP data (K = 2). Each vertical bar represents an individual, with assignment to one of two genetic clusters: *Pinus sylvestris* (light blue) and *P. mugo* (yellow). Individuals are grouped by population (labels below), with contact zone populations (BC, BS, TZ) positioned centrally. Populations are ordered to emphasize the transition in ancestry proportions across zones. Full population names are provided in Table S1.

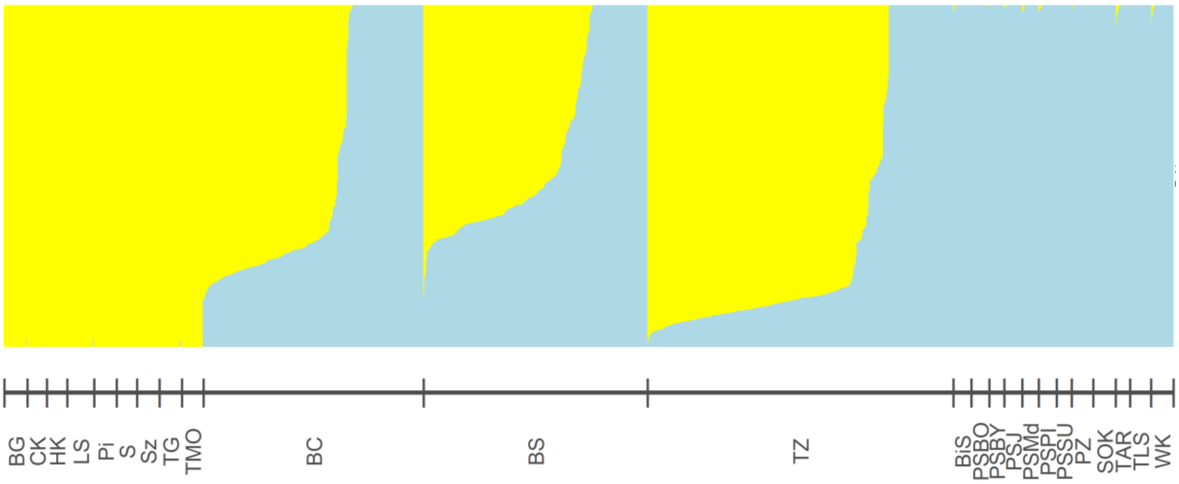


Figure 5. Gene Ontology (GO) term enrichment among outlier loci. Shown are GO categories associated with at least two candidate SNPs across all comparisons. Terms are grouped into three categories: Biological Processes, Molecular Functions, and Cellular Components. Bars represent the number of outlier SNPs associated with each term, highlighting functional pathways potentially involved in adaptive divergence.

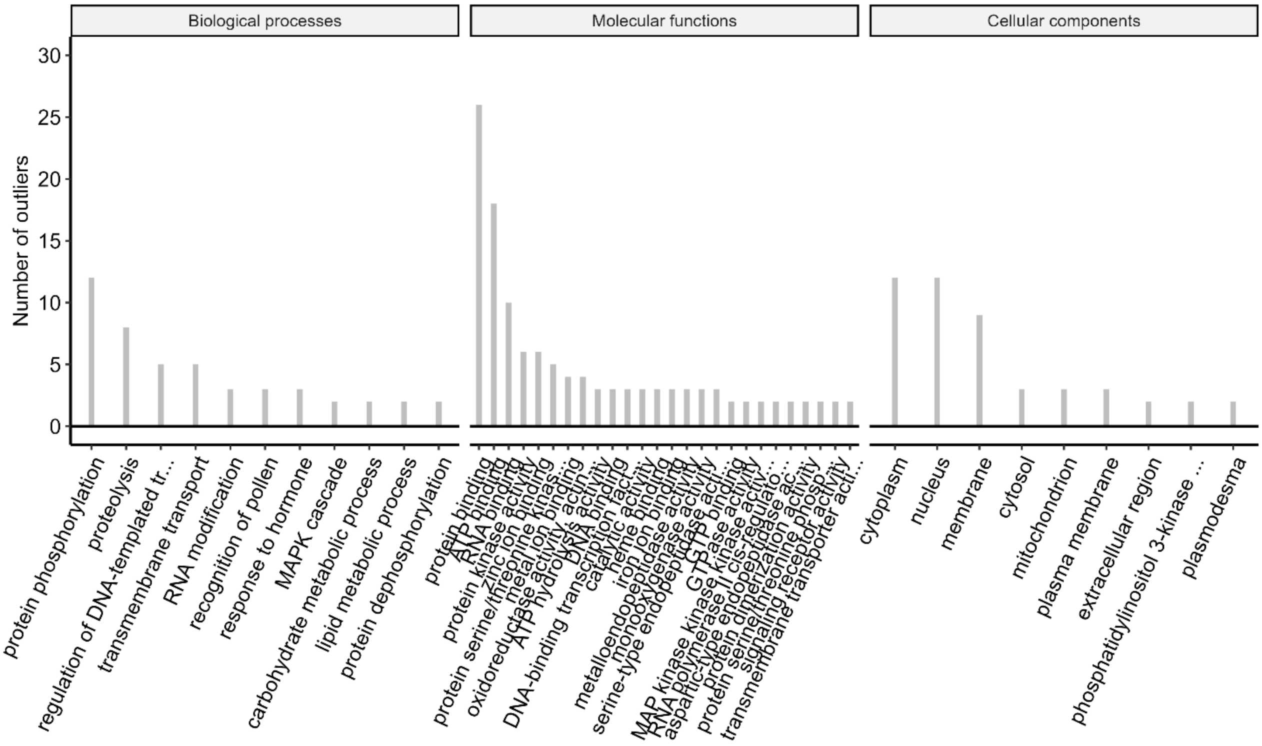


Figure 6. Overlap of outlier loci identified in pairwise comparisons involving hybrid classes, parental *Pinus sylvestris*, and reference populations. Left: Venn diagram summarizing shared and unique outliers detected in global comparisons. Right: Zone-specific comparisons for Bór na Czerwonem (BC), Błędne Skały (BS), and Torfowisko pod Zieleńcem (TZ) contact zones show the extent of overlap in outlier loci between hybrid, F1, and local parental classes versus reference *P. sylvestris*.

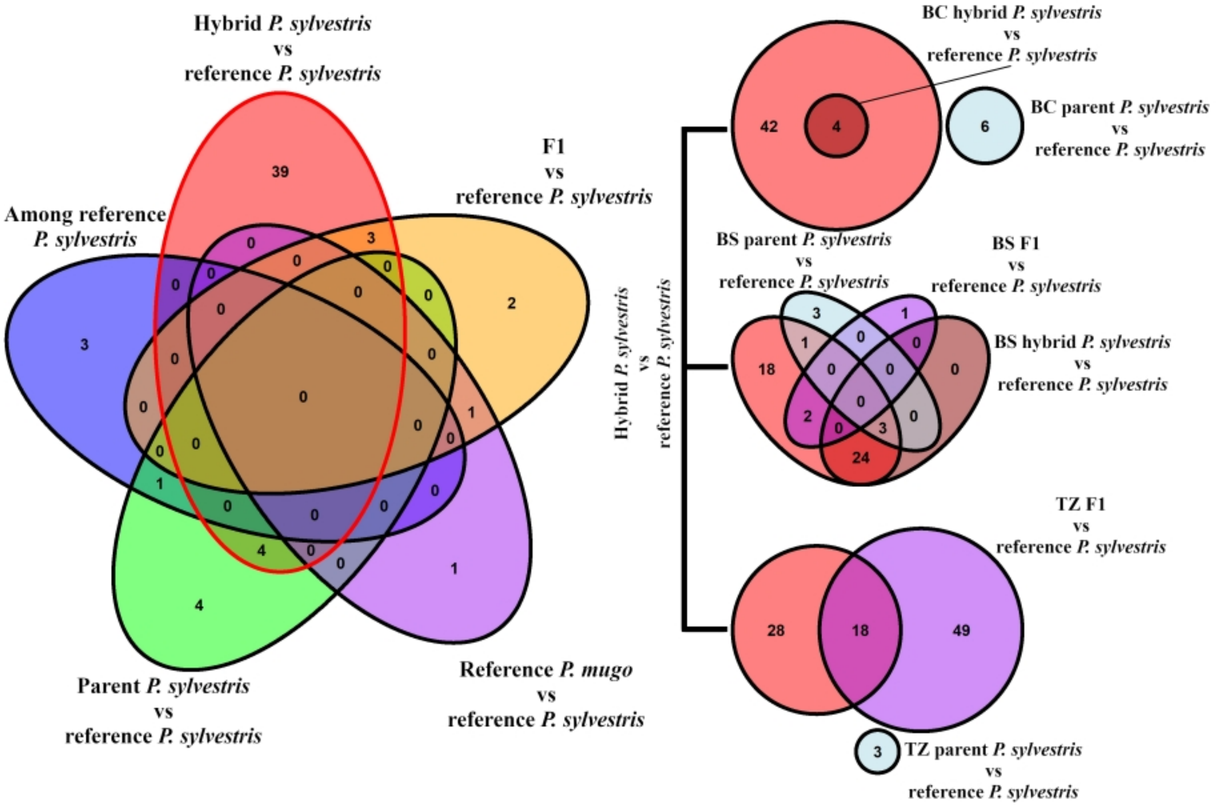


Figure 7. Hybrid Index calculated for all the studied sympatric populations (top) and frequencies of Ancestry Informative Markers (AIMs) in reference and hybrid populations. Description of populations as in Table S1. Colours correspond to pure *P. mugo* (yellow), hybrid PM (dark yellow), F1 (red), hybrid PS (dark blue) and pure *P. sylvestris* (light blue).

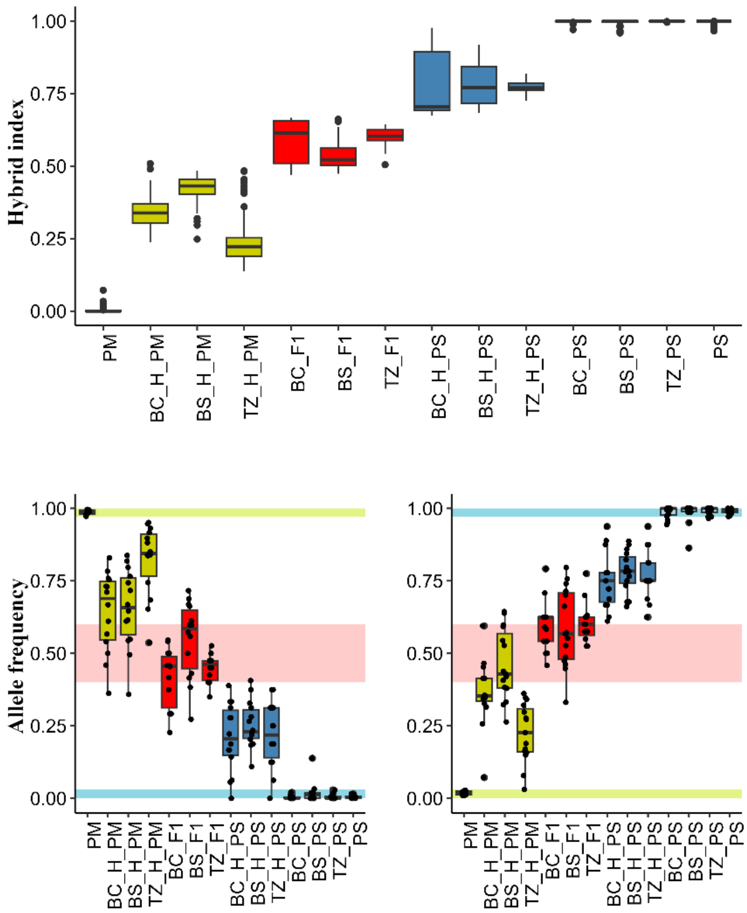


Table S1. Localization of four contact zones of studied pines and reference allopatric populations of parental taxa.

	Acronym	**N**	**Population/group**	**Longitude (E)**	**Latitude (N)**
Bór na Czerwonem contact zone	BC_H_PM	174	Poland, Bór na Czerwonem; hybrids *P. mugo*	20°2'23.64"	49°27'37.44"
	BC_F1	12	Poland, Bór na Czerwonem; F1	20°2'23.64"	49°27'37.44"
	BC_H_PS	9	Poland, Bór na Czerwonem; hybrids *P. sylvestris*	20°2'23.64"	49°27'37.44"
	BC_PS	77	Poland, Bór na Czerwonem; *P. sylvestris*	20°2'23.64"	49°27'37.44"
Błędne Skały contact zone	BS_H_PM	113	Poland, Błędne Skały; hybrids *P. mugo*	16°17'15.72"	50°28'49.44"
	BS_F1	77	Poland, Błędne Skały; F1	16°17'15.72"	50°28'49.44"
	BS_H_PS	32	Poland, Błędne Skały; hybrids *P. sylvestris*	16°17'15.72"	50°28'49.44"
	BS_PS	30	Poland, Błędne Skały; *P. sylvestris*	16°17'15.72"	50°28'49.44"
Torfowisko pod Zieleńcem contact zone	TZ_PM	3	Poland, Torfowisko pod Zieleńcem; *P. mugo*	16°24'45"	50°20'50.28"
	TZ_H_PM	291	Poland, Torfowisko pod Zieleńcem; hybrids *P. mugo*	16°24'45"	50°20'50.28"
	TZ_F1	20	Poland, Torfowisko pod Zieleńcem; F1	16°24'45"	50°20'50.28"
	TZ_H_PS	8	Poland, Torfowisko pod Zieleńcem; hybrids *P. sylvestris*	16°24'45"	50°20'50.28"
	TZ_PS	86	Poland, Torfowisko pod Zieleńcem; *P. sylvestris*	16°24'45"	50°20'50.28"
Allopatric reference *P. mugo*	BG	31	Poland, Babia Góra	19°31'50.88"	49°34'23.52"
	CK	26	Poland, Karkonosze Mountains, Czarny Kocioł	15°35'5.64"	50°47'18.6"
	HK	27	Poland, Tatra Mountains, Hala Kondratowa	19°56'48.48"	49°14'51"
	LS	36	Poland, Karkonosze Mountains, Łabski Szczyt	15°31'53.04"	50°47'26.52"
	PI	30	Poland, Pilsko	19°19'1.56"	49°31'44.4"
	S	27	Poland, Karkonosze Mountains, Śnieżka	15°44'24.72"	50°44'11.76"
	SZ	30	Poland, Karkonosze Mountains, Szrenica	15°30'54.36"	50°47'33"
	TG	30	Poland, Tatra Mountains, Grześ	19°45'59.76"	49°14'12.12"
	TMO	29	Poland, Tatra Mountains, Morskie Oko	20°4'4.8"	49°12'0.72"
Allopatric reference *P. sylvestris*	PZ	29	Poland, Polanica-Zdrój	16°29'51.72"	50°23'52.08"
	BIS	24	Poland, Stołowe Mountains, Białe Skały	16°21'38.96"	50°27'32.3"
	SOK	30	Poland, Pieniny Mountains, Sokolica	20°26'29.04"	49°25'6.24"
	TAR	19	Poland, Tarnawa	22°49'46.92"	49°6'45.72"
	TLS	28	Poland, Tatra Mountains, Łysa Skałka	20°6'48.96"	49°15'53.64"
	WK	30	Poland, Tatra Mountains, Koryciska Wielkie	19°48'30.24"	49°16'10.56"
	PSBY	20	Poland, Bytów	17°29'55.68"	54°10'39"
	PSBO	24	Poland, Bolewice	16°7'4.08"	52°23'50.28"
	PSMD	22	Poland, Międzylesie	16°39'17.64"	50°8'51.72"
	PSJ	24	Poland, Janów Lubelski	22°24'42.12"	50°42'0"
	PSPI	24	Poland, Pisz-Dziadki	21°37'21.36"	53°30'11.52"
	PSSU	20	Poland, Supraśl	23°20'6.72"	53°12'26.64"

N – number of individuals sampled

Table S2. Outliers scored for listed groups when compared to Scots pine reference populations

		Grouped comparisons					Individual hybrid zone comparisons						
SNP ID	Description	Among reference PS	Hybrid PS	Parent PS	Reference PM	F1	BC hybrid PS	BS hybrid PS	BC parent PS	BS parent PS	TZ parent PS	BS F1	TZ F1
117408287	receptor-like protein 4		X	X			X	X		X			
117426549	serine/threonine-protein kinase UCNL-like		X	X			X	X		X			
117422981	serine/threonine-protein kinase D6PK		X	X				X		X			
117442017	probable carboxylesterase 17		X				X	X					X
117399376	expansin B1		X					X					X
117404532	SWI/SNF complex subunit SWI3A isoform X2		X					X					X
117407503	probable chalcone--flavonone isomerase 3		X					X					X
117412986	geraniol 8-hydroxylase		X					X					X
117414888	ATP-dependent zinc metalloprotease FTSH 7, chloroplastic		X					X					X
117418131	probable BOI-related E3 ubiquitin-protein ligase 3		X					X					X
117423292	cytochrome P450 71AU50-like		X				X						X
117427863	hypothetical protein Mapa_017458		X					X					X
117430188	heavy metal-associated isoprenylated plant protein 39		X					X					X
117431263	Methionine aminopeptidase 2B	X		X							X		
117434579	THO complex subunit 1		X					X					X
117434919	AT-hook motif nuclear-localized protein 10		X			X		X					
117436073	aspartyl protease family protein 2-like		X					X					X
117441547	protein JOKA2		X					X					X
117443031	probable LRR receptor-like serine/threonine-protein kinase IRK		X			X						X	
117444983	ABC transporter B family member 15-like		X			X						X	
117396032	dehydrin 9		X					X					
117399143	protein NUCLEAR FUSION DEFECTIVE 4							X					X
117400305	vacuolar-sorting receptor 6		X					X					
117406566	NADH dehydrogenase [ubiquinone] iron-sulfur protein 1, mitochondrial		X					X					
117406818	UDP-galactose/UDP-glucose transporter 5		X					X					
117412399	E3 ubiquitin-protein ligase SHPRH isoform X1		X										X
117414282	fatty acid amide hydrolase isoform X2								X		X		
117418165	mitochondrial metalloendopeptidase OMA1			X						X			
117419195	amidophosphoribosyltransferase, chloroplastic					X						X	
117420845	pentatricopeptide repeat-containing protein At5g27270		X										X
117434119	Protein phosphatase 2C 37		X										X
117435101	pfkB-like carbohydrate kinase family protein		X					X					
117435688	putative methylesterase 14, chloroplastic			X					X				
117436139	villin-2		X					X					
117436296	abscisic acid receptor PYL4		X										X

117436721	voltage-gated hydrogen channel 1-like		X					X					
117437538	imidazole glycerol phosphate synthase hisHF, chloroplastic		X					X					
117438488	putative Transducin/WD40 repeat-like superfamily protein		X					X					
117441389	Mitochondrial glycoprotein		X					X					
117443212	pentatricopeptide repeat-containing protein At2g03880, mitochondrial		X										X
117444655	thyroid adenoma-associated protein homolog				X	X							

117396827	arogenate dehydrogenase 2, chloroplastic-like								X				
117398472	inositol transporter 1 isoform X2												X
117398633	phosphatidylinositol 4-kinase gamma 4-like												X
117399070	G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300												X
117399787	pentatricopeptide repeat-containing protein At3g12770		X										
117399983	phospho-2-dehydro-3-deoxyheptonate aldolase 1, chloroplastic												X
117400458	protein BPS1, chloroplastic-like												X
117400823	triacylglycerol lipase SDP1												X
117400943	aspartyl protease AED3-like												X
117402273	Dicer-like 3		X										
117402691	squamous cell carcinoma antigen recognized by T-cells 3 isoform X1												X
117402751	G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300												X
117404440	mitochondrial succinate-fumarate transporter 1												X
117404666	cytochrome P450 71AU50-like												X
117404766	187-kDa microtubule-associated protein AIR9 isoform X2												X
117405523	3-ketoacyl-CoA synthase 4												X
117405807	probable cytosolic oligopeptidase A												X
117406177	Pentatricopeptide repeat-containing protein												X
117406192	subtilisin-like protease SBT5.3 isoform X1								X				
117406421	protein NPGR2-like										X		
117407166	eukaryotic translation initiation factor 3 subunit G-like												X
117409087	protein PHOX1												X
117410859	NF-kappa-B-activating protein												X
117411073	double-stranded RNA-binding protein 3												X
117412153	4-hydroxy-3-methylbut-2-enyl diphosphate reductase												X
117412618	ribonucleoside-diphosphate reductase small chain									X			
117414857	auxin response factor 2B isoform X1												X
117414938	ATP-dependent 6-phosphofructokinase 5, chloroplastic		X										
117414940	chromatin assembly factor 1 subunit FAS2			X									
117415822	Heptahelical transmembrane protein 1												X
117416045	protein SUPPRESSOR OF GENE SILENCING 3 homolog		X										
117417052	asparagine--tRNA ligase, cytoplasmic 1-like												X
117417316	phosphatidate phosphatase PAH2	X											
117419888	Bromo adjacent homology (BAH) domain		X										
117420312	putative LRR receptor-like serine/threonine-protein kinase												X

117421191	probable RNA-dependent RNA polymerase SHL2		X										
117421413	protein WHAT'S THIS FACTOR 1 homolog, chloroplastic												X
117422597	zinc finger protein												X
117422648	choline transporter-like protein 2		X										
117423947	mitogen-activated protein kinase NPK1 isoform X2								X				
117424070	pectin methylesterase-like protein												X
117424814	histone-lysine N-methyltransferase ASHH2-like		X										
117426076	non-specific lipid-transfer protein												X
117426249	putative clathrin assembly protein At2g25430							X					
117426734	protein PSK SIMULATOR 1 isoform X1		X										
117426926	septin and tuftelin-interacting protein 1 homolog 1												X
117430818	exocyst complex component EXO84B												X
117432210	tRNA (guanine(37)-N1)-methyltransferase 1		X										
117433480	probable inactive purple acid phosphatase 28 isoform X1												X
117434014	protein ESSENTIAL FOR POTEXVIRUS ACCUMULATION 1												X
117434274	heterogeneous nuclear ribonucleoprotein H2 isoform X1		X										
117434547	toMV resistance protein Tm-1(GCR237)												X
117434660	phosphoacetylglucosamine mutase					X							
117434711	transcription factor MYB1-like								X				
117434783	F-box protein At1g67340												X
117435045	GPN-loop GTPase QQT1												X
117435503	GCN5-related N-acetyltransferase 9 isoform X2												X
117436125	acyl-lipid (8-3)-desaturase	X											
117436501	dystrophia myotonica WD repeat-containing protein-like isoform X2												X
117436516	zinc finger CCCH domain-containing protein 30-like												X
117436778	small GTP-binding protein									X			
117437995	beta-arabinofuranosyltransferase RAY1 isoform X1												X
117438016	wall-associated receptor kinase-like 20												X
117438610	Beta galactosidase small chain/ domain 5												X
117439397	mitogen-activated protein kinase kinase kinase 3												X
117439970	NDR1/HIN1-like protein 6												X
117441152	G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300			X									
117441203	class V chitinase												X
117441988	aminoacylase-1												X
117442558	twinkle homolog protein, chloroplastic/mitochondrial		X										
117443013	peroxisome biogenesis protein 6	X											
117443229	CBL-interacting protein kinase 1												X
117443680	pantothenate kinase 2 isoform X2												X
117443799	bHLH transcription factor RHL1				X								
117444692	protein DMP2												X

Table S3. Outlier SNPs found in comparisons with reference *Pinus mugo* populations.

		Grouped comparisons			Individual hybrid zone comparisons		
SNP ID	Description	Among reference PM	Hybrid PM	Reference PS	BC hybrid PM	BS hybrid PM	TZ hybrid PM
117406125	glutamine synthetase		X		X		X
117435697	adenine nucleotide transporter BT1, chloroplastic/mitochondrial				X		X
117443799	bHLH transcription factor RHL1			X	X		
117443831	mediator of RNA polymerase II transcription subunit 15A-like isoform X1		X				X
117403319	ubiquitin carboxyl-terminal hydrolase 5 isoform X1	X					
117407756	GTP cyclohydrolase 1-like	X					
117415191	homeobox-leucine zipper protein HDG11-like	X					
117419534	(R,S)-reticuline 7-O-methyltransferase	X					
117419550	auxin response factor 10						X
117422212	molybdenum cofactor sulfurase isoform X1				X		
117426821	subtilisin-like protease SBT2.5	X					
117429311	Pentatricopeptide repeat-containing protein					X	
117430520	putative pentatricopeptide repeat-containing protein At3g23330 isoform X1	X					
117431263	methionine aminopeptidase 2B	X					
117433792	protein argonaute 1	X					
117435903	uncharacterized protein LOC131026762 isoform X1	X					
117436217	serine/threonine-protein kinase VPS15						X
117437373	subtilisin-like protease SBT2.2	X					
117440211	probable protein phosphatase 2C 33	X					
117443013	peroxisome biogenesis protein 6	X					
117444655	thyroid adenoma-associated protein homolog			X			

Table S4. 29 ancestry specific markers.

SNP ID	annotation	Fixed in reference
117443799	bHLH transcription factor RHL1	*P. mugo*
117444223	protein phosphatase methylesterase 1	*P. mugo*
117420230	putative pumilio homolog 8, chloroplastic	*P. mugo*
117431689	transcription factor TCP22-like isoform X1	*P. mugo*
117436296	abscisic acid receptor PYL4	*P. sylvestris*
117444655	thyroid adenoma-associated protein homolog	*P. sylvestris*
117434739	vacuolar protein sorting-associated protein 29	*P. sylvestris*
117425397	N/A	*P. mugo*
117432804	N/A	*P. mugo*
117432944	N/A	*P. mugo*
117435266	N/A	*P. mugo*
117436437	N/A	*P. mugo*
117443055	N/A	*P. mugo*
117443202	N/A	*P. mugo*
117443269	N/A	*P. mugo*
117444389	N/A	*P. mugo*
117444678	N/A	*P. mugo*
117420110	N/A	*P. sylvestris*
117433522	N/A	*P. sylvestris*
117433680	N/A	*P. sylvestris*
117435149	N/A	*P. sylvestris*
117435341	N/A	*P. sylvestris*
117435643	N/A	*P. sylvestris*
117435704	N/A	*P. sylvestris*
117435732	N/A	*P. sylvestris*
117436126	N/A	*P. sylvestris*
117436363	N/A	*P. sylvestris*
117442819	N/A	*P. sylvestris*
117442967	N/A	*P. sylvestris*

Fig. S1. Cross entropy between ten different runs for each K in *LEA* plotted vs number of ancestral populations. The optimal number of clusters is detected by first significant drop of cross entropy at K = 2.

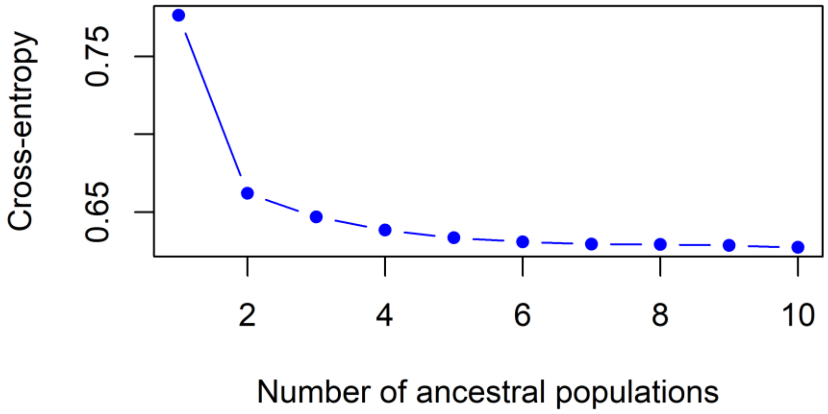


Fig. S2. Number of outliers found by three methods (P – pcadapt, O – OutFLANK and B – BayeScan) for grouped comparisons.

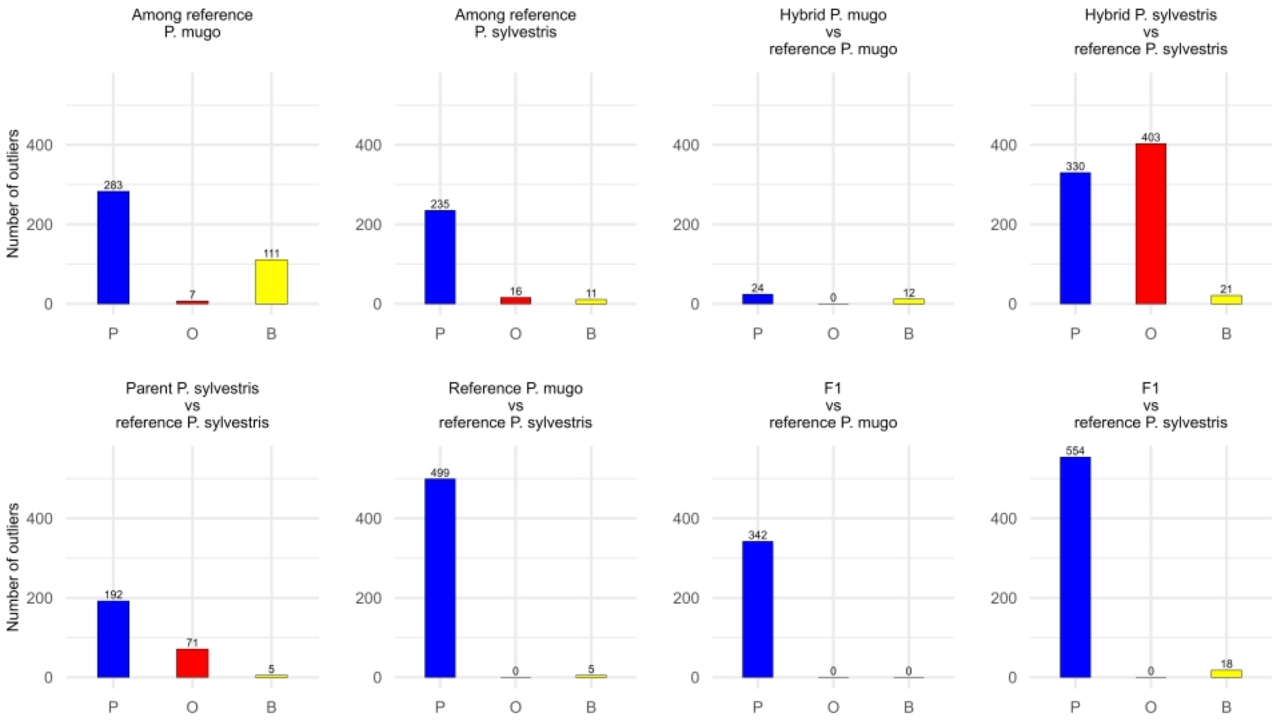


Fig. S3. Number of outliers found by three methods (P – pcadapt, O – OutFLANK and B – BayeScan) for comparisons of specific hybrid zones.

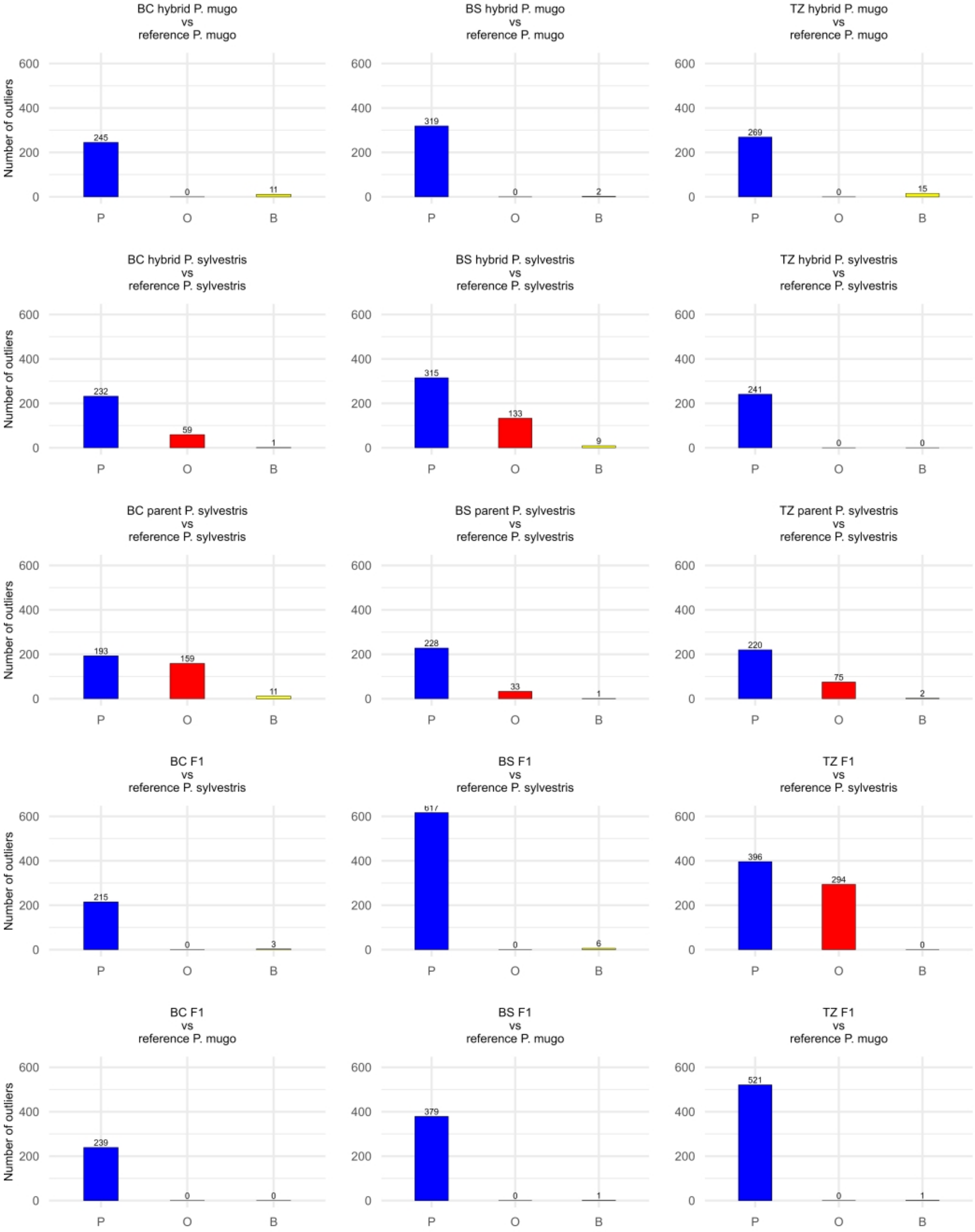


Fig. S4. Sum of all outliers found by three methods (P – pcadapt, O – OutFLANK and B – BayeScan).

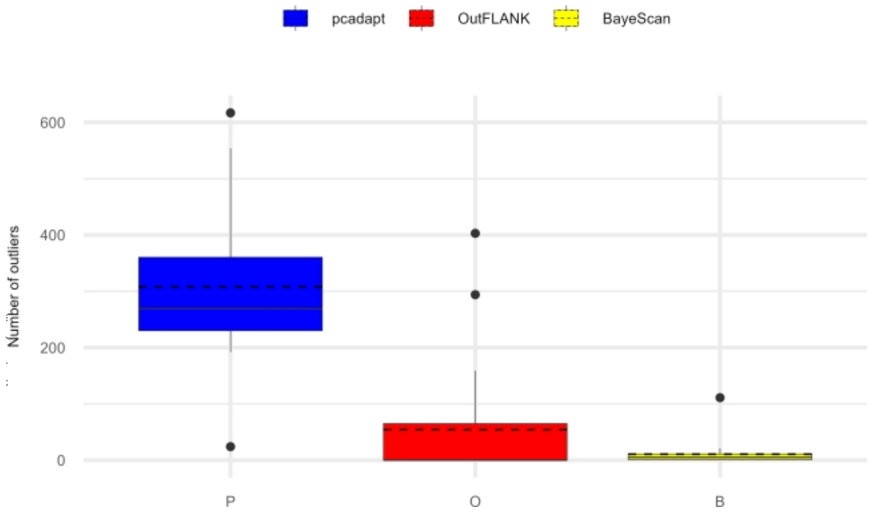


Fig. S5. Sum of all outliers found by three methods (P – pcadapt, O – OutFLANK and B – BayeScan).

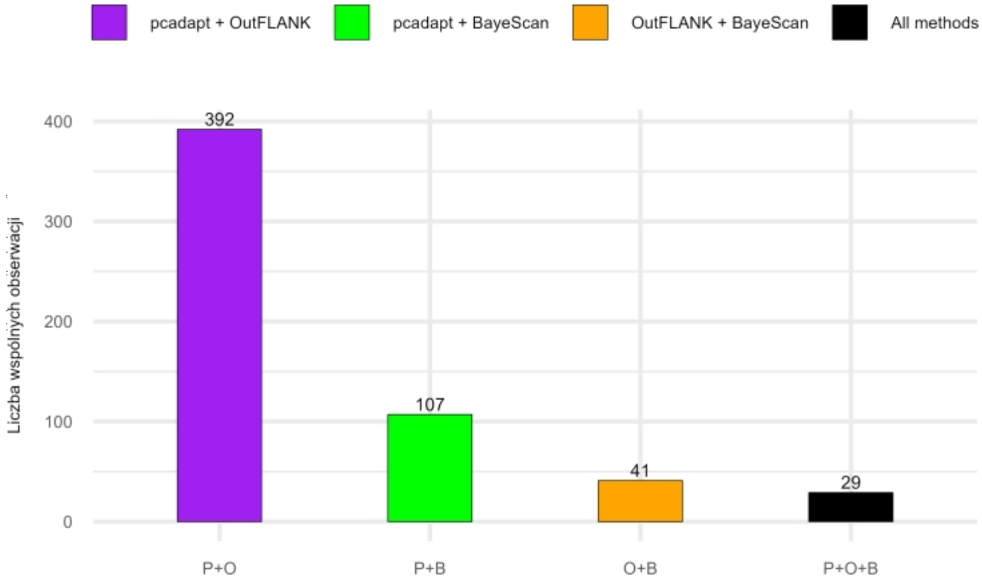


Fig. S6. Number of common outliers found by two or three methods (P – pcadapt, O – OutFLANK and B – BayeScan) for grouped comparisons.

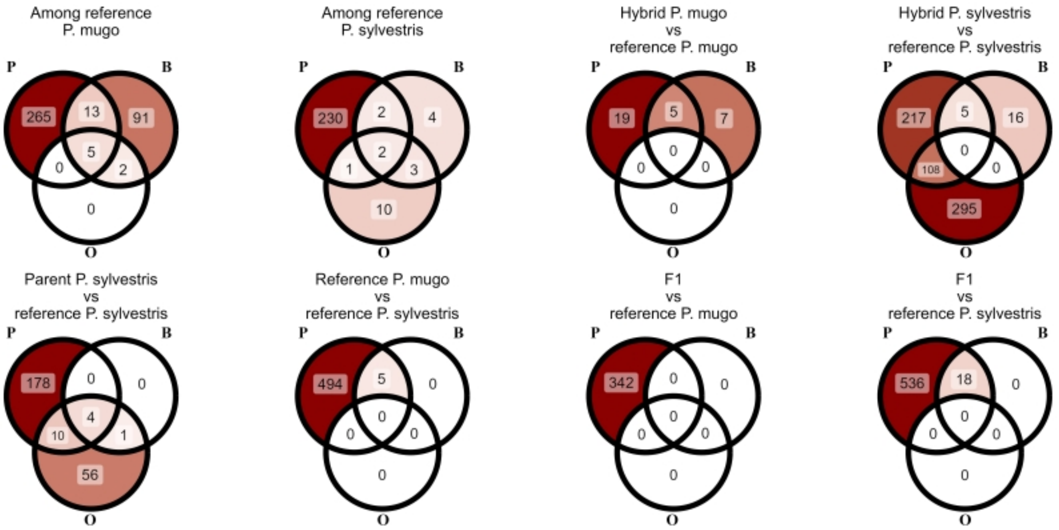


Fig. S7. Number of common outliers found by two or three methods (P – pcadapt, O – OutFLANK and B – BayeScan) for comparisons of specific hybrid zones.

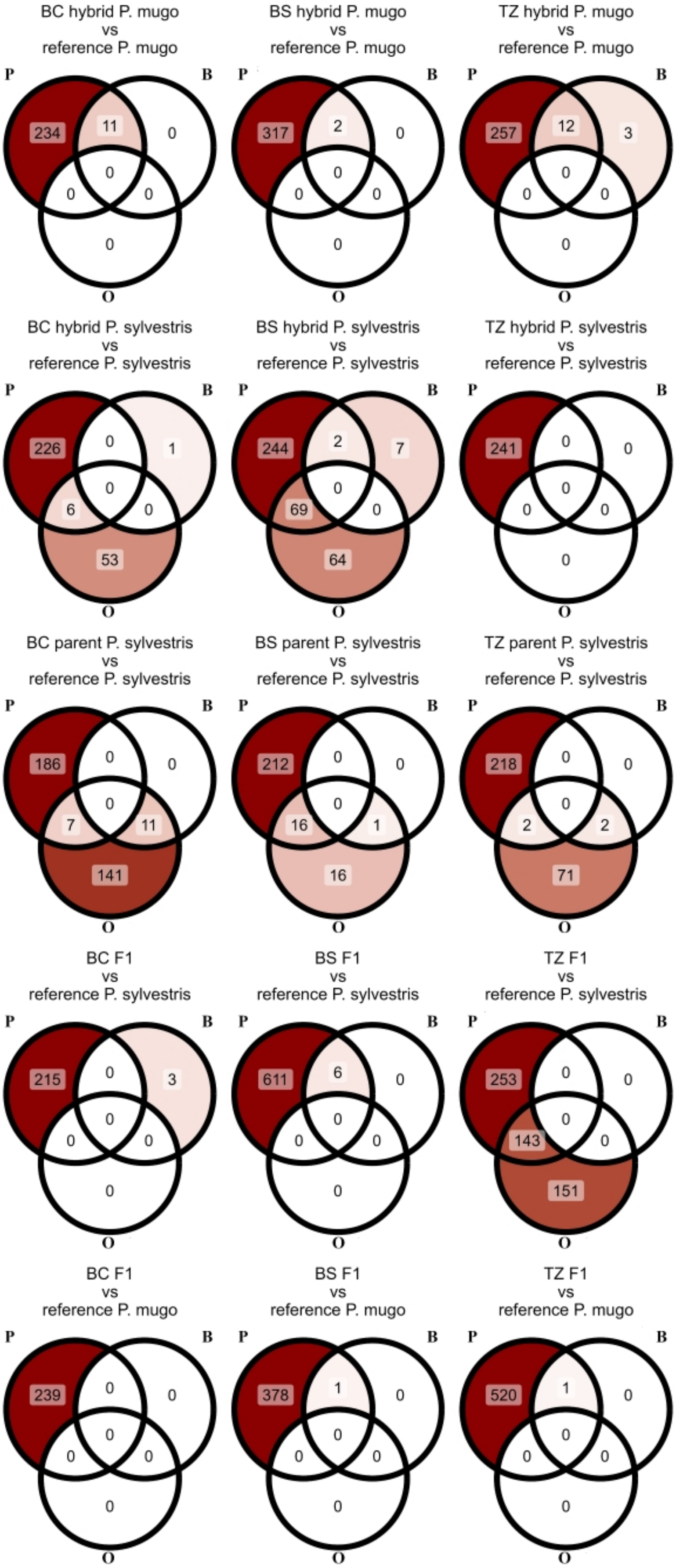


Figure S8. Gene Ontology (GO) term enrichment among outlier loci differentiating hybrid individuals from reference *Pinus sylvestris* populations. Only terms associated with at least one candidate SNP are shown. GO terms are categorized into Biological Processes, Molecular Functions, and Cellular Components. Bars represent the number of outlier SNPs annotated with each term, reflecting putative functional roles of loci under selection in hybrid genomes.

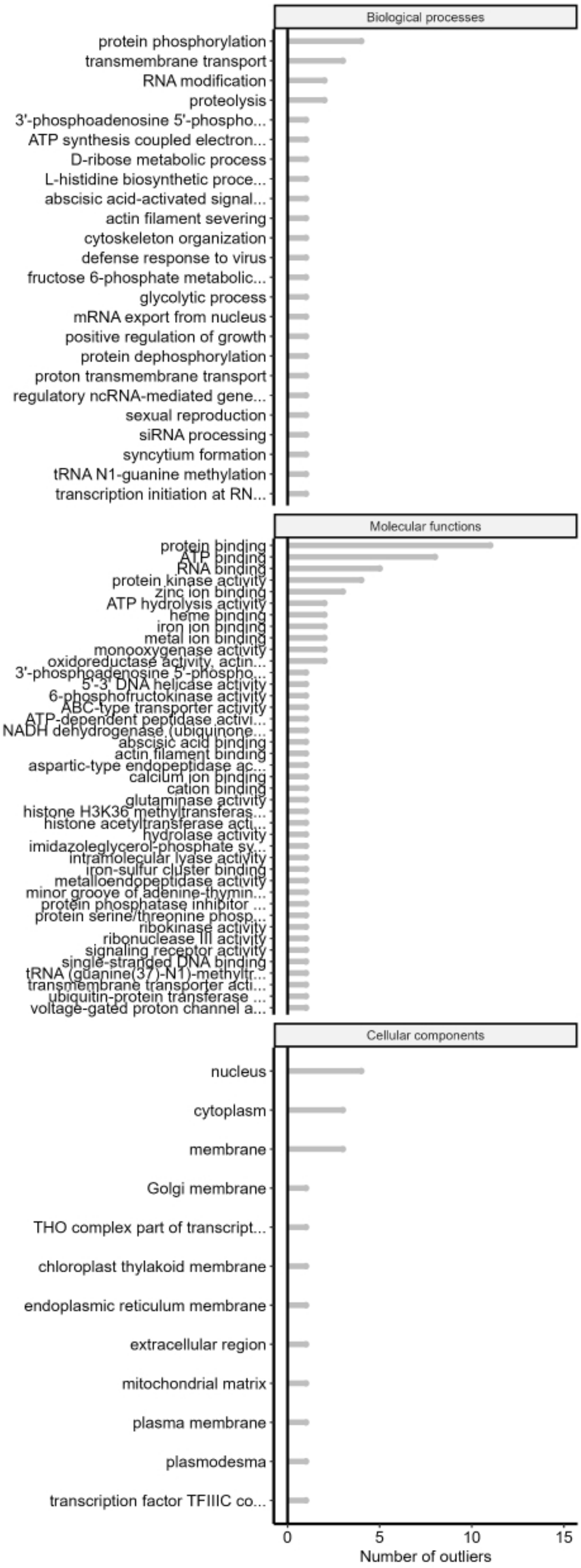


Fig. S9. Heatmap of GO terms (biological processes) found for grouped comparisons.

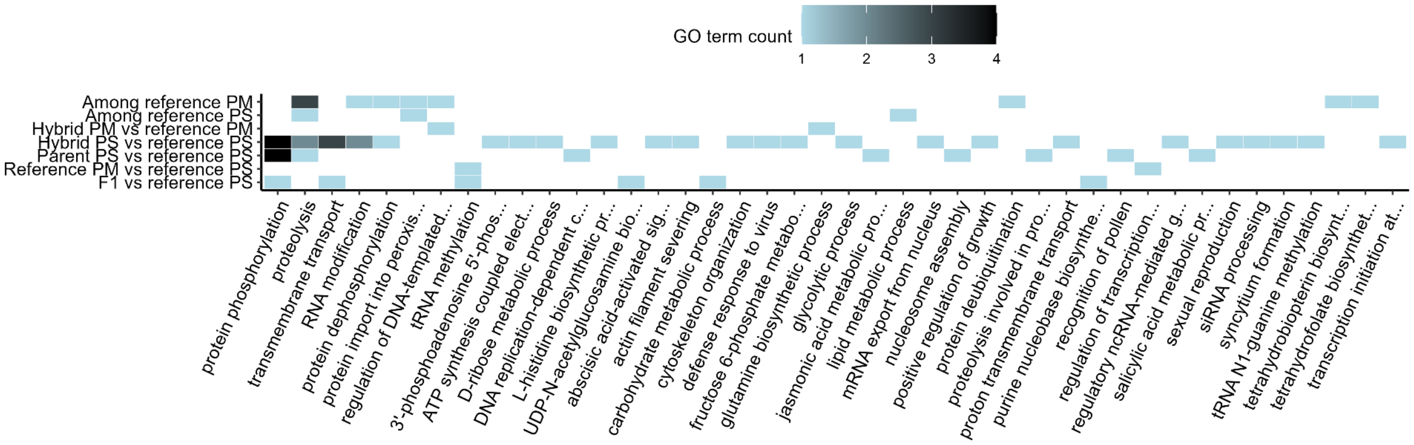


Fig. S10. Heatmap of GO terms (molecular functions) found for grouped comparisons.

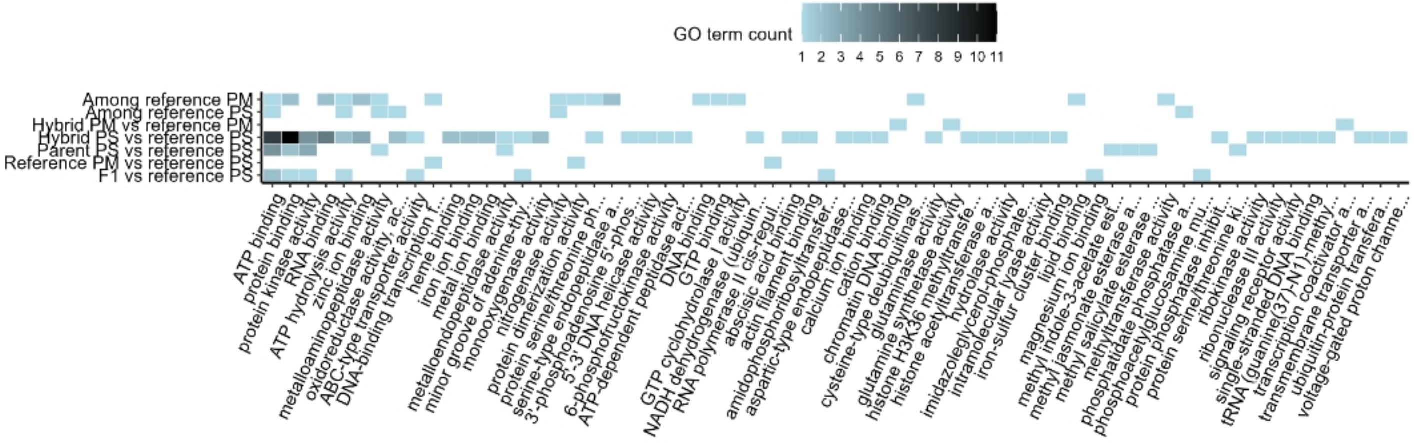


Fig. S11. Sum of common outliers for chosen comparisons of the sympatric populations.

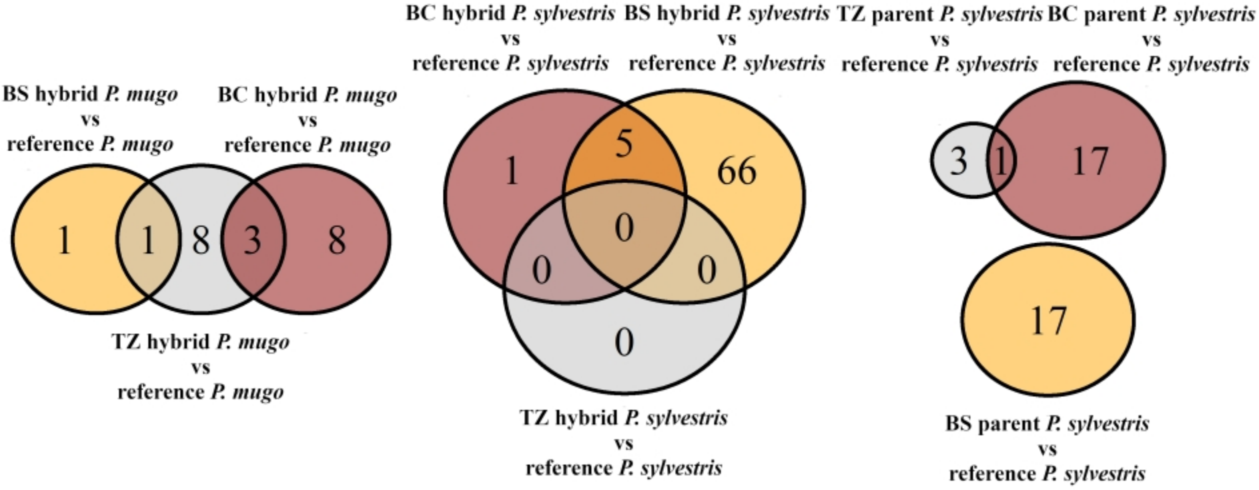


Fig. S12. Correlation between ancestry coefficients (LEA) and Hybrid Index (*gghybrid*).

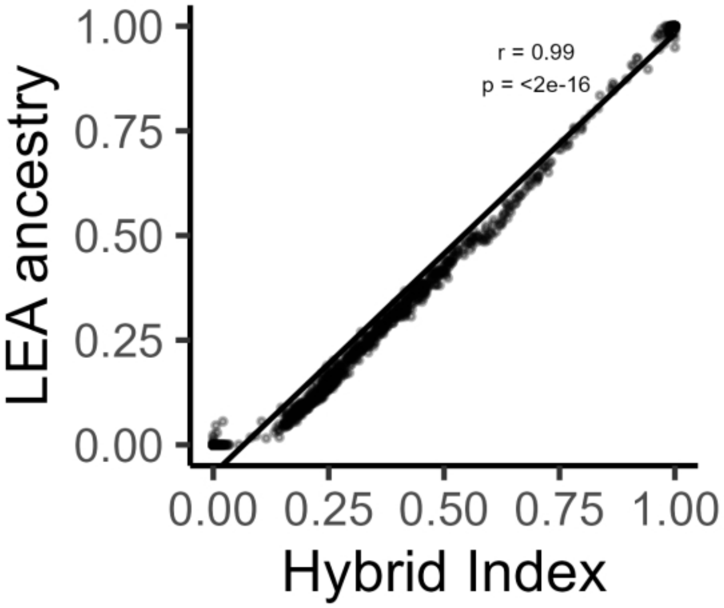
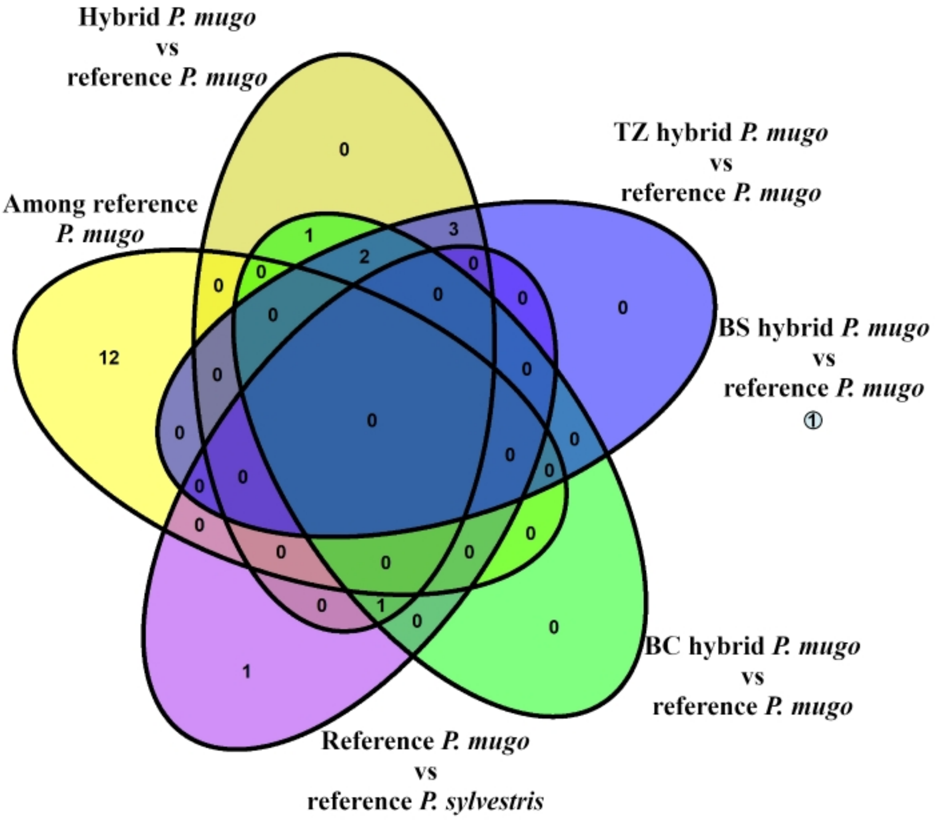


Fig. S13. Sum of common outliers for chosen comparisons with reference dwarf mountain pine populations.



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For research article:

Szczepański S, Łabiszak B, Wachowiak W. Development of a SNaPshot assay for the genotyping of organellar SNPs in four closely related pines. Dendrobiology. 2023;90:76–85.

I hereby declare that the work presented in the article, of which I am the first co-author, constitutes a part of my doctoral dissertation.

SS: Sampling, Methodology, Data acquisition and analysis, Investigation, Writing – Original Draft, Visualization; Manuscript submission; BŁ: Conceptualization, Interpretation of the results, Revising the manuscript; WW: Conceptualization, Sampling, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

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Szczepański S, Łabiszak B, Lasek M, Wachowiak W. Hybridization has localized effect on genetic variation in closely related pine species. BMC Plant Biol. 2024;24:1007.

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BŁ: Conceptualization, Data curation, Formal analysis, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. SS: Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. WW: Conceptualization, Investigation, Funding acquisition, Project administration, Resources; Supervision; Validation, Writing – original draft, Writing – review & editing.

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