University of Bialystok
Faculty of Biology and Chemistry

Edyta Jermakowicz

Phylogeography, genetic diversity and demography of boreal-montane orchid *Malaxis monophyllos* (L.) Sw.

PhD thesis

Supervisor: prof. dr hab. Emilia Brzosko
Auxiliary supervisor: dr hab. Ada Wróblewska

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This thesis is dedicated to my husband and son

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PhD thesis abstract

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Phylogeography, genetic diversity and demography of boreal-montane orchid *Malaxis monophyllos* (L.) Sw.

Understanding historical changes in species' geographical distribution is one of the most important issues in evolutionary biology, and provides valuable information for conservation strategies in the face of environmental changes. There is an increasing number of studies dealing with plants' response to these changes, but most of them apply demographic and genetic approaches separately. However, a full understanding of the species' range dynamic in time and space is only possible by considering together the geographic distribution of species' genetic diversity, and the historical and contemporary demographic processes ongoing in populations.

The genetic structure of plant species provides information on the mutations, gene flow, selection and genetic drift that operate in populations, within the historical background and in terms of the biological properties of species. One of the interesting aims of studies on genetic diversity distribution is to identify centres/hotspots of diversity, which may have different backgrounds (e.g. connected with glacial refuges, relict populations or presence of contact zones). Despite the variety of species' phylogeographic patterns, they allow for some general inferences for different plant groups. These patterns concern, e.g. refuges, directions and barriers for gene flow, and hybrid zones of different evolutionary lineages. Phylogeographic patterns have been interpreted with the greatest precision for plants with disjunctive distribution, in particular from the arctic–alpine group. However, the species with fragmented ranges also encompass boreal–montane taxa, associated with less severe conditions and much more widely distributed than arctic–alpine ones. Despite the increasing number of studies concerning phylogeographic patterns of plants, knowledge concerning boreal-montane species is still scarce, and there is still much ambiguity in this issue. The surveys indicate, e.g. considerable genetic differences between arctic-alpine and boreal-montane plants, with a mostly shallow phylogeographical structure in the latter. However, there are also similarities concerning the low genetic diversity or location of refuges, including Asian refuges (Beringia, Central Asia), and European macrorefugia (Alpine and Balkan) and microrefugia (Carpathians, Bohemian Massif). The causes of shallow phylogeographical structure are often complex and

present particular difficulties in interpretation, because many factors can interact: 1) post-glacial colonization from many glacial refuges, 2) multidirectional gene flow, 3) incomplete lineage sorting, and 4) species properties and populations' history. This last factor is often highlighted as being key, along with the historical causes, in the understanding of species' genetic patterns. Furthermore, it is stressed in many future climate projections that the changes for Europe will mainly concern temperature increase and decrease in precipitation. Thus, it might be particular severe for northern plants, in the context of their abundance and biological features, as well as their distribution, which will shrink and shift in a northern direction. Other authors have also proposed the concept of "warm-stage refuges" for boreal-alpine plants, which are associated with cold areas in the context of global climate warming. This might be especially important in case of the observed distribution of genetic diversity in this plant group.

Orchids belong to a plant group that is suitable for the study of evolutionary, ecological and conservation problems. The cumulative effects of orchids' life cycle traits (fungus' dependent germination, pollinators' dependent fruiting) make them important indicators of environmental changes, and they are species with a relatively high degree of threat. The most often negative responses of orchids to environmental changes support conclusions about their low resistance to future climate changes. What is more, in usually small and isolated orchid populations, genetic equilibrium might be disrupted throughout non-random mating, which causes genetic drift, and in consequence may decrease their genetic diversity and adaptability. However, the genetic depauperation of small populations cannot be taken as an *a priori* assumption, but should be tested for each species of interest with consideration of its history and biological properties.

Malaxis monophyllos (L.) Sw. is an excellent model species with which to investigate the presented problems. Its fragmented geographical range covers the areas corresponding with the range of boreal mixed forests in the north, and the lower subalpine zone of mountain ranges in Southern and Central Europe. As a member of the *Orchidaceae* family, it is characterised by specific life history traits, and as a species connected with declining natural habitats it is listed as endangered in many European countries. The choice of this species has also provided the analysis of intraspecific genetic and demographic diversity in the context of regional differences between habitats, in particular the role of anthropogenic ones in shaping species diversity patterns. This issue is connected with human activity that has destroyed natural habitats, but has also led to the creation of new ones, and ecological heterogeneities that may promote diversity. The newly founded populations provide evolutionary opportunities for rapid adaptive niche shifts. They could also act as kinds of bridges or sources that support gene flow between fragmented and isolated populations. However, there are very few studies related to the role of anthropogenic populations in shaping genetic and demographic structure at the species level.

Considering the above problems, **the primary aim of my PhD thesis was to determine the genetic and demographic patterns of the boreal-montane orchid *Malaxis monophyllos* (L.) Sw. within its fragmented geographical range.** I expected that the genetic pattern of *M. monophyllos* is affected by historical events connected with the shifting and shrinking of its range during the Quaternary Period, which affected gene-flow between populations and their subdivisions. Based on contemporary knowledge, I have also hypothesized that this pattern can be altered by species properties, particularly dispersal abilities and population size. Moreover, I assumed that subdivided geographical distribution may indicate the prolonged isolation of populations, and hence clear interregional demographic patterns. I also tested whether the differences between habitats, in particular between natural and anthropogenic ones, could promote differences in interregional genetic and demographic patterns.

In the first part of my research I investigated the phylogeographic structure of *M. monophyllos* within its Eurasian range. For this purpose I acquired samples from fifty-one European populations from the boreal, montane and upland parts of the geographical range, and from seventeen Asian populations. I analysed them using four polymorphic sequences of chloroplast DNA (cpDNA: *trn*L, *trn*L-*trn*F, *rps*16 and *acc*D-*psa*I). These analyses indicated 19 haplotypes and relatively high values of haplotype and nucleotide cpDNA diversity in the European range (**Chapter I**). Three centres of genetic diversity (Alpine, north-European and east-European) have been revealed for *M. monophyllos*. These diversity centres might be consistent with putative refuges, including Alpine ones. The peaks of genetic diversity and occurrences of rare haplotypes in Northern and Eastern European populations could imply the proximity of Central European glacial microrefuges, from which populations of *M. monophyllos* spread northwards and eastwards. It may also suggest colonization from eastern refuges located in Central Asia. Another explanation is the existence of melting pots of different colonization lineages in north-eastern Europe **(Chapter I)**. Besides the relatively high intraspecific diversity, I found a weak genetic structure in Europe, revealed in the widespread distribution of common cpDNA haplotypes through the *M. monophyllos* range, as well as in the lack of significant differences among populations' diversity indices ($N_{ST} = 0.49$ and $G_{ST} = 0.45$). Therefore, there was no evidence of a split between boreal and montane *M. monophyllos* populations in the genetic sense **(Chapter I)**.

At the next stage of my research I applied AFLP using two primer combinations (*Eco*RI-ACG/*Mse*I-CAT and *Eco*RI-AGC/*Mse*I-CAC) 1) to investigate the genetic diversity within and between *M. monophyllos* populations, and 2) to extend phylogenetic data. The fragmented populations of *M. monophyllos* in Europe were characterized by low genetic diversity (with the range of values after resampling procedure: $PPL_3 = 6.7 – 31.8$ %, $Hj_3 = 0.045 – 0.212$ and $DW_3 = 0.36 – 3.91$). These results suggested the genetic depauperation of populations during post-glacial colonization from refugia, and the important impact of small population sizes and concurrent inbreeding in shaping *M. monophyllos'* genetic structure (**Chapter II**). AFLPs only partly confirmed results referring to the existence of three centres of *M. monophyllos* cpDNA diversity, and identified a higher diversity level in boreal populations compared to those from mountain regions (**Chapter II**). The clearly lower genetic diversity in mountain populations, resulting from AFLP, may also reflected global erosions of genetic diversity in mountainous populations. The analyses using AFLP primers and cpDNA did not clearly indicate the regions of the Alps, Eastern Carpathians and the Bohemian Massif as potential glacial refugia (macro and microrefugia) for *M. monophyllos* in Europe. However, species distribution models (SDMs) for this period allow for such a possibility (**Chapter I**).

The shallow phylogeographic structure, confirmed by AFLPs, revealed a very low population differentiation, as well a lack of distinguishable genetic clusters and high admixture within populations (**Chapter II**). These results could be interpreted as evidence of a multidirectional gene flow between European populations of *M. monophyllos*, when the distribution range of this species was presumably more continuous and the population less isolated. This is consistent with paleovegetation evidence for the Quaternary history of European flora, which has indicated the presence of locally suitable habitats for boreal species, almost across central and eastern Europe even during the Last Glacial Maximum (LGM). Thus, the present distribution area of *M. monophyllos* could be the remnants of its Late Glacial and early Holocene much wider range that underwent fragmentation in later periods. This fragmentation might still be in progress, and thus populations become more isolated and may lead to complete range disjunction in the future (**Chapter I, II**).

Demographic analyses of 12 populations of *M. monophyllos* from Poland showed their extremely dynamic nature (in time and space), associated mainly with the intensive exchange of individuals (**Chapter III**). The ephemeral character of populations is consistent with the concept of "classical" metapopulation, which simultaneously allows for multidirectional gene flow between populations reflected in the observed genetic structure of *M. monophyllos* in Europe (**Chapter II**). Although *M. monophyllos* populations did not show genetic structuring in regions, demographic patterns based mainly on reproductive features can be identified. The analysis of these patterns indicated the boreal populations as those with high reproductive potential, and the boreal region as optimal for the persistence of this orchid in Europe in the face of future climate changes (**Chapter III**).

In the context of intraspecific diversity, populations of *M. monophyllos* from anthropogenic habitats in Polish uplands, despite their low reproductive potential and long-term instability, harbour equal or even higher genetic variation than some natural populations from other regions (**Chapter I, II, III**). Thus, I conclude that the main driving forces of their genetic diversity are the proximity and huge quantity of source populations, and the highly dynamic population growth in the first years of colonization. The anthropogenic populations existed under often extremely different from natural and unstable conditions. This may suggest that a species which established anthropogenic populations may also possess huge potential to deal with future environmental changes. Hence, due to their unique ecological character, they require a special approach and should be considered for proper management, as valuable elements in shaping *M. monophyllos'* future range.

Streszczenie rozprawy doktorskiej

Edyta Jermakowicz

Filogeografia, różnorodność genetyczna i demografia borealno – górskiego storczyka *Malaxis monophyllos* (L.) Sw.

Historia kształtowania się geograficznych zasięgów roślin jest jednym z ważniejszych zagadnień biologii ewolucyjnej. W dobie zachodzących zmian klimatycznych i siedliskowych, dostarcza również istotnych informacji przy planowaniu strategii ochrony gatunków. Większość doniesień dotyczących reakcji roślin na zachodzące zmiany, prezentuje osobno podejście demograficzne i genetyczne. Natomiast pełna wiedza na temat dynamiki zasięgów gatunków w przestrzeni i czasie, wymaga uwzględniania zarówno geograficznego rozmieszczenia zmienności genetycznej, jak również historycznych i współczesnych procesów demograficznych mających miejsce w populacjach.

Obraz genetycznej struktury populacji roślin w zasięgu geograficznym może pomóc zinterpretować szereg zachodzących procesów takich mutacje, przepływ genów, selekcję czy dryf genetyczny. W ostatnich latach ważnym celem badań nad rozmieszczeniem genetycznej różnorodności jest również identyfikacja centrów (ang. hotspots) genetycznej różnorodności, które mogą mieć różne pochodzenie (związane m.in. z lokalizacją refugiów lub/i populacjami reliktowymi czy stref wtórnego kontaktu). Mimo dużego zróżnicowania struktury genetycznej wśród roślin, zarysowują się często wyraźne wzorce filogeograficzne charakterystyczne dla poszczególnych grup. Dotyczą one m.in. obszarów refugialnych, kierunków i barier dla przepływu genów oraz stref kontaktu różnych linii ewolucyjnych. Wzorce te najlepiej poznano dla roślin o dysjunktywnym zasięgu, w szczególności z grupy gatunków arktyczno–alpejskich. Gatunki o podzielonym zasięgu obejmują również taksony borealno–górskie, związane z łagodniejszym klimatem i szerzej rozprzestrzenione niż gatunki arktyczno – alpejskie. Mimo wzrastającej liczby prac filogeograficznych, wiedza na temat gatunków borealno–górskich jest w dalszym ciągu ograniczona i wiele problemów wymaga wyjaśnienia. Dostępne dane, wskazują na znaczne różnice między gatunkami arktyczno–alpejskimi i borealno–górskimi. Dotyczą one przede wszystkim słabo zarysowującej się struktury filogeograficznej w przypadku tej drugiej grupy. Z kolei podobieństwa między tymi grupami, obejmują m.in. niską różnorodność genetyczną utrzymującą się na poziomie gatunku oraz lokalizację ich refugiów, w tym refugiów azjatyckich (Beringa, Azja Centralna), oraz europejskich makrorefugiów

(Alpy, Bałkany) i mikrorefugiów (Karpaty, Masyw Czeski). Słabo zarysowująca się struktura filogeograficzna jest szczególnie trudna w interpretacji, ze względu na wiele czynników, które mogą na nią wpływać, tj.: 1) post-glacjalna kolonizacja z wielu źródeł, 2) wielokierunkowy przepływ genów, 3) niekompletne rozdzielenie linii filogenetycznych, czy 4) właściwości gatunku oraz historia poszczególnych populacji. Ten ostatni czynnik jest często podkreślany, jako wyjątkowo istotny w kształtowaniu genetycznych wzorców u roślin. Ponadto, modele klimatyczne dla Europy pokazują, że przyszłe zmiany będą obejmowały głównie wzrost temperatur oraz zmniejszenia się ilości opadów. Taki scenariusz może być szczególnie dotkliwy dla gatunków o zasięgu północnym, zarówno w kontekście liczebności ich populacji, cech biologii, jak i zmian zasięgów, które mogą się zmniejszać i przesuwać w kierunku północnym. Niektórzy autorzy proponują również koncepcję „warm-stage refuges" dla gatunków górskich, czyli chłodnych ostoi, w kontekście globalnego ocieplania klimatu. Koncepcja ta może mieć znaczący wpływ na obecnie obserwowane wzorce różnorodności genetycznej tej grupy roślin.

Ogromne zróżnicowanie rodziny *Orchidaceae* sprzyja rozpatrywaniu problemów ewolucyjnych, ekologicznych, jak również związanych z ochroną gatunkową. Łączny efekt cech historii życiowych storczyków (kiełkowanie zależne od dostępności grzyba, owocowanie uzależnione od obecności zapylaczy), sprawia, że są one ważnymi wskaźnikami zmian zachodzących w siedliskach oraz stanowią jedną z najbardziej zagrożonych globalnie grup organizmów. Często obserwowana, negatywna reakcja storczyków na zmiany siedliskowe sprzyja ich niskiej odporności na przyszłe zmiany klimatyczne. Ponadto, w małych i izolowanych populacjach storczyków, na skutek nielosowego kojarzenia, może dochodzić do dryfu genetycznego, a w konsekwencji spadku różnorodności genetycznej i możliwości adaptacji gatunku do zmian środowiskowych. Genetyczne zubożenie małych populacji nie może być jednak przyjmowane *a priori*, a powinno być określane dla każdego gatunku oddzielnie, z uwzględnieniem jego historii oraz cech biologii.

Malaxis monophyllos (L.) Sw. (wyblin jednolistny) można uznać za gatunek modelowy do rozpatrywania prezentowanych zagadnień. Jego zasięg geograficzny charakteryzujący się fragmentacją, obejmuje obszar pokrywający się z zasięgiem lasów mieszanych strefy borealnej oraz z zasięgiem regla dolnego masywów górskich południowej i centralnej Europy. Jako przedstawiciel rodziny *Orchidaceae*, odznacza się specyficznymi cechami biologii, a jako gatunek związany z zanikającymi siedliskami jest uznany za zagrożony wyginięciem w wielu europejskich krajach. Wybór tego gatunku pozwolił również na analizowanie różnorodności genetycznej i demograficznej, w kontekście zróżnicowania siedliskowego, włączając w to rolę siedlisk antropogenicznych w kształtowaniu wzorców zmienności gatunku. Ta ostatnia kwestia dotyczy działalności człowieka, która oprócz niszczenia naturalnych siedlisk, prowadzi też do tworzenia nowych, oraz do powstawania ekologicznej heterogeniczności, która może różnorodności sprzyjać. Nowopowstałe populacje stwarzają możliwości do zachodzenia zmian adaptacyjnych, powodowanych rozszerzaniem się nisz ekologicznych. Mogą one również spełniać rolę pomostów lub źródeł, które wspierają przepływ genów między izolowanymi populacjami. Problem roli populacji na siedliskach antropogenicznych w kształtowaniu demograficznej i genetycznej struktury na poziomie gatunku jest bardzo słabo poznany.

Biorąc pod uwagę wyżej opisane problemy, **głównym celem mojej rozprawy doktorskiej było ustalenie genetycznych i demograficznych wzorców zmienności dla borealno - górskiego storczyka *Malaxis monophyllos* (L.) Sw., w jego podzielonym zasięgu geograficznym**. Spodziewałam się, że wzorce różnorodności genetycznej *M. monophyllos* będą kształtowane, przede wszystkim, przez czynniki historyczne, związane z przesuwaniem i kurczeniem się jego zasięgu podczas czwartorzędu, co wpływało na przepływ genów między populacjami i ich podział. Biorąc pod uwagę znane fakty, postawiłam również hipotezę, że wzorce te są w dużej mierze zależne od biologii gatunku, w szczególności możliwości dyspersji oraz wielkość populacji. Ponadto założyłam, że obserwowana fragmentacja zasięgu *M. monophyllos* w Europie oraz wyraźne różnice siedliskowe między regionami, wskazują na izolację populacji i w związku z tym, na wyraźne regionalne wzorce demograficzne. Chciałam również sprawdzić, czy zróżnicowanie warunków między siedliskami naturalnymi i antropogenicznymi oraz ich odmienna historia, dodatkowo sprzyjają powstawaniu różnic między populacjami, na poziomie genetycznym i demograficznym.

W pierwszej części moich badań określiłam strukturę filogeograficzną *M. monophyllos* w euroazjatyckiej części jego zasięgu. W tym celu pozyskałam próby z 51 europejskich populacji z borealnej, wyżynnej i górskiej części zasięgu oraz z 17 lokalizacji azjatyckich. Próby przeanalizowałam przy użyciu czterech, polimorficznych sekwencji chloroplastowego DNA (cpDNA: *trn*L, *trn*L-*trn*F, *rps*16, oraz *acc*D-*psa*I). Analizy te pokazały istnienie 19 haplotypów oraz względnie wysoką różnorodność haplotypową i nukleotydową cpDNA w europejskiej części zasięgu (**Chapter I**). Ujawniły również istnienie trzech centrów różnorodności genetycznej *M. monophyllos* w Europie (alpejskiego, północno-europejskiego oraz wschodnio-europejskiego). Te centra różnorodności mogą być zgodne z lokalizacjami refugiów dla tego gatunku, włączając refugium alpejskie. Wysokie wartości różnorodności genetycznej oraz obecność rzadkich haplotypów cpDNA w populacjach z północnej i wschodniej Europy może wskazywać na bliskość mikrorefugiów Europy Centralnej, skąd *M.*

monophyllos mógł się rozprzestrzenić w kierunku północnym i wschodnim, lub też kolonizację Europy z refugiów wschodnich zlokalizowanych w Azji Centralnej. Wynik ten może również wskazywać na istnienie genetycznego tygla różnych linii ewolucyjnych w Europie północno-wschodniej (**Chapter I**). Mimo relatywnie wysokiej wewnątrzgatunkowej różnorodności, wykazałam brak wyraźnej struktury filogeograficznej *M. monophyllos* w Europie, przejawiający się szerokim rozprzestrzenieniem pospolitych haplotypów cpDNA, jak również brakiem istotnych różnic między wartościami zróżnicowania międzypopulacyjnego (N_{ST}=0,49 i G_{ST}=0,45). Dlatego też, wyniki moich badań nie dowodzą odrębności borealnej i górskiej części zasięgu *M. monophyllos* w sensie genetycznym (**Chapter I**).

W kolejnym etapie pracy zastosowałam analizy AFLP, z użyciem dwóch kombinacji starterów (*Eco*RI-ACG/*Mse*I-CAT oraz *Eco*RI-AGC/*Mse*I-CAC), aby 1) zbadać genetyczną różnorodność wewnątrz oraz między populacjami *M. monophyllos* w Europie oraz 2) rozszerzyć i zweryfikować wyniki badań filogeograficznych uzyskanych metodą sekwencjonowania cpDNA. Populacje *M. monophyllos* w Europe charakteryzują się niską zmiennością genetyczną (zakresy wartości po procedurze resamplingu: PPL_3=6,7 – 31,8 %, Hj_3 = 0,045 – 0,212 i DW_3 = 0.36 – 3,91). Wyniki te wskazują na genetyczne zubożenie populacji, które miało miejsce w trakcie post-glacjalnej kolonizacji, oraz na wpływ małej wielkości populacji i inbredu na genetyczną strukturę *M. monophyllos* (**Chapter II**). AFLP tylko częściowo potwierdziło wyniki analizy sekwencji cpDNA dotyczące istnienia trzech centrów różnorodności. Wykazały też wyższy poziom różnorodności genetycznej w borealnych populacjach w porównaniu do populacji górskich (**Chapter II**). Wyraźnie niższy poziom zmienności AFLP w górskich populacjach, może wynikać z globalnie obserwowanego spadku liczebności i genetycznej różnorodności populacji roślin w obszarach górskich. Analizy AFLP i cpDNA nie wskazały jednoznacznie na Alpy, Wschodnie Karpaty czy Masyw Czeski jako refugia (makro- lub mikrorefugia) dla *M. monophyllos* w Europie. Z kolei klimatyczne modele rozmieszczenia gatunku dla okresu ostatniego zlodowacenia, taką możliwość dopuszczają (**Chapter I**).

Słabo zarysowana struktura filogeograficzna *M. monophyllos* w Europie została potwierdzona w analizach AFLP, które pokazały niskie genetyczne zróżnicowanie między populacjami, jak również brak wyróżniających się genetycznych klastrów oraz wysoką admiksję różnych pól genowych w większości populacji (**Chapter II**). Wyniki te mogą być dowodem na istnienie wielokierunkowego przepływu genów między europejskimi populacjami *M. monophyllos*, kiedy zasięg gatunku był prawdopodobnie bardziej ciągły, a populacje mniej izolowane. Jest to zgodne z danymi paleobotanicznymi dla czwartorzędowej historii europejskiej flory, które wskazują na możliwość istnienia siedlisk dogodnych dla gatunków borealnych na terenie centralnej i wschodniej Europy, nawet podczas ostatniego zlodowacenia. W związku z tym, obecny zasięg *M. monophyllos* może być jedynie pozostałością po jego późno-glacjalnym i wczesno holoceńskim dużo szerszym zasięgu, który uległ fragmentacji w późniejszych okresach. Fragmentacja ta może postępować w dalszym ciągu, prowadząc do zwiększenia izolacji między populacjami. W konsekwencji, przepływ genów miedzy populacjami będzie ograniczony, co może doprowadzić to do dysjunkcji zasięgu w przyszłości (**Chapter I, II**).

Przeprowadzone przeze mnie analizy demograficzne w 12 populacjach *M. monophyllos* z terenu Polski pokazały ich niezwykle dynamiczny charakter (w czasie i przestrzeni), związany między innymi z intensywną wymianą osobników (**Chapter III**). Efemeryczny charakter populacji jest spójny z koncepcją „klasycznej" metapopulacji, która dopuszcza wielokierunkowy przepływ genów między populacjami, mający odzwierciedlenie w genetycznej strukturze *M. monophyllos* w Europie (**Chapter I, II**). Mimo, że pod względem genetycznym populacje tego gatunku nie różnicują się regionalnie, możemy wyróżnić regionalne wzorce demograficzne, oparte głównie na cechach reprodukcyjnych (**Chapter III**). Analiza wzorców demograficznych wskazuje populacje z borealnej części zasięgu jako te, charakteryzujące się wyższym poziomem reprodukcji. Region borealny, z kolei, jako optymalny dla trwania tego gatunku w Europie, w warunkach przewidywanych zmian klimatycznych (**Chapter I, II, III**).

W kontekście wewnątrzgatunkowej różnorodności, populacje *M. monophyllos* z siedlisk antropogenicznych, mimo niższego potencjału reprodukcyjnego, i niestabilności liczebności w dłuższych okresach czasu (głównie na skutek sukcesji roślinności), utrzymują równą lub nawet wyższą różnorodność genetyczna niż niektóre populacje z siedlisk naturalnych (**Chapter I, II, III**). Na tej podstawie mogę wnioskować, że głównym mechanizmem kształtującym ich strukturę genetyczną jest bliskość i duża liczebność populacji źródłowych oraz szybki wzrost liczebności populacji w pierwszych latach kolonizacji nowych siedlisk. Populacje z siedlisk antropogenicznych ze względu na ich unikatowy, ekologiczny charakter i jako cenne elementy biorące udział w kształtowaniu się przyszłego zasięgu *M. monophyllos*, wymagają jednak specjalnego, konserwatorskiego podejścia.

Chapter I. Phylogeographical structure of the boreal-montane orchid *Malaxis monophyllos* as a result of multi-directional gene flow

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Phylogeographical structure of the boreal-montane orchid *Malaxis monophyllos* as a result of multi-directional gene flow

EDYTA JERMAKOWICZ[1]*, ADA WRÓBLEWSKA[1], EMILIA BRZOSKO[1], PAWEŁ MIRSKI[1] and TOOMAS HIRSE[2]

[1]*Department of Botany, Institute of Biology, University of Bialystok, ul. Świerkowa 20B, 15-950 Bialystok, Poland*
[2]*Nature Protection Department, State Forest Management Centre, Rõõmu tee 1, 51013 Tartu, Estonia*

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We investigated the phylogeographical structure of the boreal-montane orchid *Malaxis monophyllos* in its Eurasian geographical range. We analysed four sequences of plastid DNA (*trnL*, *trnL–trnF*, *rps16* and *accD-psaI*), resulting in 19 haplotypes and revealing a high level of intraspecific diversity ($H_D = 0.702$ and $\pi = 0.196 \times 10^{-2}$), but showing a lack of phylogeographical structure. This pattern might be caused by multiple phenomena and processes, e.g. broad-fronted recolonization with accompanying multi-directional gene flow between populations and expansion from at least two refugial areas. Despite the lack of phylogeographical structure, three centres of haplotype diversity were indicated in the European part of the range of *M. monophyllos*. According to these data, alpine and lowland glacial refugia located between the ice sheets in the European Alps and the Scandinavian glaciers seem most likely to be in Europe. Moreover, models of climatically suitable areas during the Last Glacial Maximum (LGM) confirmed the Alps as a possible refuge, and indicated an opportunity for the persistence of *M. monophyllos* populations in Beringia and parts of Siberia. Using two models [Model for Interdisciplinary Research on Climate (MIROC) and Community Climate System Model (CCSM)], we predicted a significant reduction in climatically suitable areas for *M. monophyllos* in the future (2080). Our study also demonstrated that the biological features of *M. monophyllos*, including breeding system and dispersal mode, seem to be crucial in understanding its phylogeographical pattern. Our results also highlighted the importance of anthropogenic habitats as reservoirs of genetic diversity and alternative habitats for this species in the context of declining natural populations.

ADDITIONAL KEYWORDS: anthropogenic habitats – cpDNA – Orchidaceae – plastid DNA diversity centres – species distribution models.

INTRODUCTION

In recent years, past and ongoing climate changes have been considered as the main factors affecting the survival prospects of species, particularly as a result of their influence on the availability of habitats (Davis & Shaw, 2001; Feehan, Harley & Van Minnen, 2009; Koopowitz & Hawkins, 2012). This factor forces plant species to respond to changing conditions by adaptation, shifting of their ranges to more suitable areas or extinction (McLaughlin *et al.*, 2002; Hampe & Petit, 2005; Crawford, 2008). Future simulations indicate that among the most threatened species as a result of habitat loss are northern plants encompassing boreal and arctic taxa (Thuiller *et al.*, 2005; Crawford, 2008; Scheffer *et al.*, 2012). Currently, the boreal zone covers a large part of the Northern Hemisphere, including taiga, tundra and boreal coniferous and mixed forests, which, in total, cover almost 13% of the global land mass and 32% of forests (Burton *et al.*, 2003; Schultz, 2005; Finnie *et al.*, 2007). This makes this area especially valuable in terms of global

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*Corresponding author. E-mail: edytabot@uwb.edu.pl biodiversity and its conservation. This also requires more detailed knowledge about boreal taxa.

Some cold-adapted plants have a strongly fragmented geographical distribution, e.g. arctic-alpine and boreal-montane types. Such distributions may be the result of different scenarios caused by multiple events and processes: (1) natural and anthropogenic changes in habitats; (2) long-distance dispersal (LDD) during post-glacial recolonization; and (3) *in situ* survival in multiple separated refugia (Larcombe, McKinnon & Vaillancourt, 2011; Mosblech, Bush & Van Woesik, 2011; Reitalu *et al.*, 2013; Sanz *et al.*, 2013). It is also well known that some of the cold-adapted species have already reached their Holocene maxima and are currently declining (Skrede *et al.*, 2006; Alsos *et al.*, 2009). As a consequence, range fragmentation is progressing in these species (De Lafontaine, Turgeon & Payette, 2010). Moreover, global climatic changes influencing species ranges are likely to continue in the future, and predictions about these can be made using different climatic models (Lesica & McCune, 2004; Thuiller *et al.*, 2005; Feehan *et al.*, 2009). A commonly used tool in this approach is species distribution modelling (Guisan & Thuiller, 2005). Species distribution modelling, in combination with phylogeographical surveys, has proved to be one of the most valuable approaches in unravelling the evolutionary history of species. Most future simulations strongly suggest that, by the end of the 21st century, the distribution of many European plant species will have been reduced, and will have shifted several hundred kilometres northwards (Thuiller *et al.*, 2005; Noguès-Bravo *et al.*, 2007; Feehan *et al.*, 2009; Alsos *et al.*, 2012; Pauli *et al.*, 2012). Some predictions have suggested that southern European species are the most sensitive to climatic changes, and that up to 60% may become extinct in the near future, in comparison with a 30% loss of northern plants. Moreover, the same studies indicate mountain and rear-edge populations as the most endangered, with up to 60% of mountain populations possibly facing extinction over the next few decades (Thuiller *et al.*, 2005). However, Crawford (2008) has suggested that some cold-adapted species may possess sufficient genetic variability to adapt to changing conditions or find sufficient heterogeneity in habitats further north.

Although phylogeographical studies on arctic plants are well represented in the literature (Abbott *et al.*, 2000; Alsos *et al.*, 2005; Swenson & Howard, 2005; Schönswetter, Popp & Brochmann, 2006; Skrede *et al.*, 2006; Birks, 2008; Schmitt, Muster & Schönswetter, 2010), boreal species, which are much more widely distributed on a global scale, are still under-represented (Alsos *et al.*, 2005; Brubaker *et al.*, 2005; Eidesen *et al.*, 2007; Ehrich, Alsos & Brochmann, 2008; Beatty & Provan, 2011; Wróblewska, 2012). Phylogeographical studies have rarely covered the whole range of boreal species, and therefore the knowledge about patterns of genetic diversity has often been incomplete. The picture that emerges from the wide range of studies on boreal taxa (Alsos *et al.*, 2005; 2012; Brubaker *et al.*, 2005; Eidesen *et al.*, 2007; 2013; Ehrich *et al.*, 2008; Wróblewska, 2012) indicates a rather shallow phylogeographical structure, with the main refugial areas located in Beringia and the central, southern and south-eastern parts of Siberia. Other data have confirmed this general pattern and have located sources of post-glacial recolonization, also to the north of the main southern European refugia (Gugerli *et al.*, 2001; Olivier, Hollingsworth & Gornall, 2006; Ehrich *et al.*, 2008; Ronikier *et al.*, 2008; Kramp *et al.*, 2009; Michl *et al.*, 2010; Beatty & Provan, 2011).

The observed lack of phylogeographical structure of boreal plants on the European scale could be a result of recent events, including broad-fronted colonization and frequent LDD (Lihová, Kudoh & Marhold, 2010; Beatty & Provan, 2011; Westergaard *et al.*, 2011; Jiménez-Mejías *et al.*, 2012). If we consider the current geographical distribution area of cold-adapted plants as remnants after their Holocene maximum range, we must take into account the occurrence of multi-directional gene flow in the past, when the species range was more continuous (Wróblewska, 2012). These predictions can be supported by genetic similarities between currently isolated European populations existing in different mountain massifs (Gugerli *et al.*, 2001; Kramp *et al.*, 2009). The other explanation for much of the intraspecific genetic variety being in central and northern Europe was given by Taberlet *et al.* (1998) and Hewitt (2004), and confirmed by further investigations identifying contact zones in these areas (Skrede *et al.*, 2006; Schmitt, 2007; Eidesen *et al.*, 2013). The unexpected genetic diversity peak north of the Alps might also be the result of colonization from microrefugial areas (Ashcroft, 2010; Michl *et al.*, 2010; Mosblech *et al.*, 2011).

A suitable species for the examination of phylogeographical patterns in boreal-montane plants has proved to be an orchid, *Malaxis monophyllos* (L.) Sw. The species includes two widely distributed varieties, the Eurasian *M. monophyllos* var. *monophyllos* and the North American *M. monophyllos* var. *brachypoda* (A.Gray) Morris & Eames (1929), distinguished by floral morphology and geographical range (Hultén & Fries, 1986; Anderson, 2006). This study concerns *M. monophyllos* var. *monophyllos*, named *M. monophyllos* from hereon. It is a component of boreal and mountain ecosystems (Zając, 1996) with a geographical distribution range covering boreal Eurasia and central European mountain ranges, with the surrounding uplands, and the north-eastern edge of North America (Hultén & Fries, 1986; Vakhrameeva *et al.*, 2008). Furthermore, in the last few decades, *M. monophyllos* has been reported to be disappearing from some natural habitats, placing it at a high level of threat in almost all countries (Holub & Procházka, 2000; Moser *et al.*, 2002; Kålås, Viken & Bakken, 2006; Zarzycki & Szeląg, 2006); however, numerous secondary populations have appeared in the Polish Uplands (Bernacki *et al.*, 1991) and have reached abundances and densities unseen in natural populations (Bernacki *et al.*, 1991; Zając, 1996; Bernacki, 1998; Jermakowicz & Brzosko, 2011). The habitats in which *M. monophyllos* grows differ between regions. In the boreal part of the range, it mainly occurs in calcareous peatlands, in the mountains in moist meadows and spruce forests. The upland populations, in turn, are connected with completely different anthropogenic habitats, including dry or moderate moisture pine forest, roadsides, railway banks or post-mining areas.

Malaxis monophyllos is presumed to be an obligatory outcrossing plant (Claessens & Kleynen, 2011), reproducing only by wind-dispersed seeds (Vakhrameeva *et al.*, 2008). Populations rarely exceed 50 individuals.

The goals of our research are to reconstruct the phylogeographical patterns of *M. monophyllos* in Europe and Asia and to predict the future distribution of this plant and preserved resources of plastid DNA diversity under climate change. For this purpose, we first tested whether geographical discontinuities in the European range of *M. monophyllos* are reflected in a genetic split between boreal and mountain regions. Secondly, we verified whether the response of *M. monophyllos* to future climate change is consistent with the general pattern for cold-adapted species, implying a shifting northern part of the range further north with a diametric contraction of the mountain part of the range. Finally, we make a prediction about plastid DNA diversity of *M. monophyllos* which will be preserved under climatic changes in the future (2080).

MATERIAL AND METHODS

Sampling

Three hundred and eighty-six individuals from 68 populations were sampled from 2009 to 2012, covering all of the European and part of the Asian geographical range of *M. monophyllos*. In this study, we distinguished, according to given geographical regions: (1) boreal populations, located in the north and east of Europe and in Asia; (2) mountain populations, distributed in the Carpathian Mountains, the Czech Massif and the Alps; and (3) upland populations connected with the Polish Uplands (Table 1). The number of collected samples was dependent on the population size (two to ten samples per population). In a few cases, it was not possible to achieve a large sample from a population because of the small size of populations and the high level of species threat in many countries. Each Asian location was represented by a single sample, which were obtained from herbarium specimens belonging to the collections of the Herbarium of Moscow State University in Moscow and the Herbarium of The Siberian Institute of Plant Physiology and Biochemistry in Irkutsk. Herbarium specimens were collected mainly during 1991–2009, and one of the samples originated from a specimen collected before 1980.

DNA extraction and plastid DNA sequencing

Total genomic DNA was extracted from dried leaf material following the manufacturer's instructions using the Genomic Mini Ax Plant Kit (A & A Biotechnology, Gdynia, Poland). Initially, 15 universal primers were screened using two randomly chosen samples per population throughout the geographical range (Taberlet *et al.*, 1991; Demesure, Sodzi & Petit, 1995; Shaw *et al.*, 2007). Then, four variable plastid DNA regions were sequenced in all samples ($\Sigma = 386$). The *trnL* (UAA) intron was amplified with primers *c* and *d*, the *trnL* (UAA)3′-*trnF* (GAA) intergenic spacer with primers *e* and *f*, sequences of the *rps16* intron and the *accD-psaI* region (Taberlet *et al.*, 1991; Shaw *et al.*, 2007). Amplification was carried out in the reaction mixture and with the PCR profiles described by Gielly & Taberlet (1994), using the following conditions: 96 °C for 2 min (one cycle); 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min (35 cycles); ending with a final extension of 30 min at 60 °C. Sequencing was performed using BigDye Terminator V 3.1 (Applied Biosystems) according to the manufacturer's protocol, and visualized with an ABI 3130 capillary sequencer (Applied Biosystems). Sequences were assembled, edited and manually aligned using BioEdit 5.0.6 (Hall, 1999). The alignments of the four plastid DNA regions were combined into a single dataset.

Data analysis

On the basis of sequence data (1528 bp from four sequences), we calculated the number of plastid DNA haplotypes. We estimated haplotype frequencies and identified private haplotypes (occurring only in one population) for all of the investigated populations. Intra- and inter-population genetic diversity were estimated by the haplotype diversity index H_D and π, and the mean number of pairwise nucleotide difference was calculated using DnaSP 5.0 (Pons & Petit,

Table 1. Population coding, geographical origin and plastid DNA haplotypes of 68 Eurasian *Malaxis monophyllos* populations. Plastid DNA variations: H_D and π values were given for 51 European populations. Population codes correspond to the codes on the distribution map (Fig. 1A, B). *N*, number of individuals used for the plastid DNA sequence analysis; Hap., plastid DNA haplotype code, bold font indicates private haplotype; H_D, haplotype diversity; π, nucleotide diversity; detected for *M. monophyllos* European populations; '–' indicates lack of data

Population code	Country		Region	Sampling location	Longitude (°)	Latitude (°)	*N*	Hap.	H_D	$\pi \times 10^{-2}$
B1	Poland	**Boreal**	East Baltic Lake District	Rospuda River Valley	53.90	22.94	10	H1, H2	0.20	0.079
B2	Poland		East Baltic Lake District	Rospuda River Valley	53.91	22.94	3	H2	0	0
B3	Poland		East Baltic Lake District	Czarna Hańcza River Valley	54.05	23.04	10	H3	0	0
B4	Poland		East Baltic Lake District	Kunis Lake	54.04	23.43	10	H1, H4, **H5**	0.64	0.180
B5	Poland		East Baltic Lake District	Daniłowce Lake	54.08	23.29	2	H1	0	0
B6	Poland		Masurian Lake District	Łabędzie Lake	53.74	19.52	2	H1, H6	1.00	0.196
B7	Poland		Masurian Lake District	Kirsznickie Lake	53.78	19.92	10	H4, H7, H8	0.64	0.108
B8	Poland		Masurian Lake District	Omulew River	53.47	20.60	10	H1, H7	0.35	0.047
B9	Poland		Masurian Lake District	Łaźnica Lake	53.51	20.84	10	H1, H6, **H9**, **H10**, **H11**	0.78	0.171
B10	Poland		Romincka Forest	Żetkiejmska Struga Reserve	54.35	22.61	10	H1	0	0
B11	Latvia		East Baltic Lake District	Krizu Mire	55.89	26.56	2	H1	0	0
B12	Latvia		East Baltic Lake District	Krizu Mire	55.77	26.41	2	H1	0	0
B13	Latvia		East Baltic Lake District	Krizu Mire	55.87	26.27	2	H1	0	0
B14	Belarus		East European Plain	Domzheritsy	55.26	29.70	10	H1, H4, H7, H12	0.78	0.145
B15	Belarus		East European Plain	Osetische	54.86	28.39	10	H1, H4, H7, **H13**	0.78	0.141
B16	Belarus		East European Plain	Shumilinsky District	50.29	19.40	5	H1, H2	0.60	0.236
B17	Finland		Scandinavian Peninsula	Tervola	66.99	25.06	10	H2	0	0
B18	Norway		Scandinavian Peninsula	Svendsrud	59.71	9.91	10	H1, H4, H7	0.38	0.065
B19	Norway		Scandinavian Peninsula	Herrambstjern	60.86	10.81	3	H1, H4	0.67	0.218
B20	Estonia		East Baltic Lake District	Tartu County/Tatra Valley	58.26	26.71	4	H1	0	0
B21	Estonia		East Baltic Lake District	Valga county/Sauniku microreserve	58.01	26.14	10	H1, H4, H8	0.64	0.180

Population code	Country		Region	Sampling location	Longitude (°)	Latitude (°)	N	Hap.	H_D	$\pi \times 10^{-2}$
B22	Estonia		East Baltic Lake District	Valga county/Pühajärve	58.05	26.46	10	H1	0	0
B23	Estonia		East Baltic Lake District	Valga county/Märdi	57.99	26.43	10	H4	0	0
B24	Estonia		East Baltic Lake District	Võru county/Miilimäe	57.75	27.09	4	H1, H4	0.67	0.218
B25	Russia		East European Plain	Smolensk Oblast/ Mutnoje Lake	55.50	31.69	1	H4	–	–
B26	Russia		East European Plain	Oryol Oblast/Orlovskoe Polesie	53.42	35.28	1	H4	–	–
B27	Russia		East European Plain	Tver Oblast/Alphimovo Village	58.00	34.94	1	H4	–	–
B28	Russia		East European Plain	Moscow Oblast/Viulka Village	56.88	37.57	1	H1	–	–
B29	Russia		East European Plain	Vladimir Oblast/ Nerechta Village	56.34	41.19	1	H4	–	–
R1	Russia		West Siberian Lowlands	Khanty-Mansiysk A.D.	62.06	71.28	1	H1	–	–
R2	Russia		South-central Siberia	Republic of Buryatia	53.79	107.86	1	H7	–	–
R3	Russia		South-central Siberia	Republic of Buryatia	52.05	107.20	1	H7	–	–
R4	Russia		South-central Siberia	Republic of Buryatia	50.89	105.14	1	H7	–	–
R5	Russia		South-central Siberia	Republic of Buryatia	51.30	104.65	1	H2	–	–
R6	Russia		South-central Siberia	Republic of Buryatia	51.56	102.18	1	H7	–	–
R7	Russia		South-eastern Siberia	Irkutsk Oblast	51.56	100.70	1	H7	–	–
R8	Russia		South-eastern Siberia	Irkutsk Oblast	52.48	102.96	1	H7	–	–
R9	Russia		South-eastern Siberia	Irkutsk Oblast	51.61	100.49	1	H7	–	–
R10	Russia		Eastern Siberia	Amur Oblast	53.79	127.27	1	H7	–	–
R11	Russia		Eastern Siberia	Chabarowski Region	48.83	138.08	1	H7	–	–
R12	Russia		Eastern Siberia	Kamchatka	53.20	158.55	1	H7	–	–
D1	Poland	**Uplands**	Śląsko-Krakowska Upland	Bukowno Walcownia	50.29	19.40	10	H1, H4, H8, **H14**	0.64	0.131
D2	Poland		Śląsko-Krakowska Upland	Sławków	50.30	19.34	10	H1, H2, H3, H4, **H15**, **H16**	0.89	0.307
D3	Poland		Śląsko-Krakowska Upland	Pogoria I	50.35	19.25	2	H1	0	0
D4	Poland		Śląsko-Krakowska Upland	Olkusz	50.29	19.49	10	H1, H2, H4, H7	0.67	0.155
D5	Poland		Małopolska Upland	Miechów	50.46	19.97	10	H1, H4, **H17**	0.51	0.124

Table 1. *Continued*

Population code	Country		Region	Sampling location	Longitude (°)	Latitude (°)	N	Hap.	H_D	$\pi \times 10^{-2}$
K1	Poland	**Carpathian Mountains**	West Beskids Mts.	Babia Góra Massif	48.59	19.58	10	H7	0	0
K2	Poland		Western Tatra Mts.	Chochołowska Valley	49.27	19.81	10	H1, H4	0.53	0.175
K3	Poland		Western Tatra Mts.	Chochołowska Valley	49.27	19.81	10	H1, H4	0.36	0.116
K4	Poland		Eastern Tatra Mts	Jaworzynka Valley	49.26	19.98	10	H1, H4	0.36	0.116
K5	Slovakia		Low Tatras Mts.	Demänowska Valley	49.00	19.57	10	H1	0	0
K6	Slovakia		Little Fatra Mts.	Šlahorka	49.23	19.08	8	H1. H4	0.43	0.140
S1	Czech Republic	**Czech Massif**	Bohemian Forest	Hajnice Nature Reserve	49.07	13.90	10	H1	0	0
S2	Czech Republic		Bohemian Forest	Klášterec	49.04	13.73	8	H1, H4, H12	0.46	0.093
S3	Czech Republic		Bohemian Forest	Soví Vrch-Dolní Vltavice	48.67	14.08	3	H4	0	0
S4	Czech Republic		Bohemian Forest	Veselka-Buk	49.02	13.83	5	H1	0	0
S5	Czech Republic		Bohemian Forest	Horní Hraničná	48.63	14.03	4	H1, H4, H7	0.46	0.185
A1	Austria	**Alps**	Foothills of the Alps	North Steiemark/ Dachstein	47.54	13.98	3	H1, H7	0.67	0.087
A2	Switzerland		Central Alps	Tradoellauenen	46.51	7.90	4	H2, H4, H7	0.83	0.273
A3	Switzerland		Central Alps	Graubünden/Usser Bündi	46.86	9.83	2	H6, **H18**	1	0.262
A4	Switzerland		Central Alps	Graubünden /Scud	46.78	10.28	8	H1	0	0
A5	Switzerland		Central Alps	Graubünden/Bergün	46.62	9.75	9	H1	0	0
A6	Italy		Eastern Alps	Domegge di Cadore	46.46	12.43	10	H1, H2, H4, **H19**	0.64	0.208
A7	Italy		Eastern Alps	Domegge di Cadore	46.45	12.43	10	H1, H2, H4	0.64	0.231
A8	Italy		Eastern Alps	Taibon Agordino	46.29	11.97	5	H1	0	0
A9	Italy		Eastern Alps	Alleghe	46.37	11.94	5	H1	0	0
A10	Italy		Eastern Alps	Canale d'Agordo	46.32	11.89	5	H1, H4	0.60	0.196
A11	Italy		Eastern Alps	Colle di Santa Lucia	46.47	12.03	5	H1, H4	0.40	0.131

1996; Rozes & Rozes, 1999). To visualize range-wide gradients in genetic diversity, both H_D and π, data interpolations were carried out using kriging in ArcGis 10.0 (ESRI, 2011), as described in Keller *et al.* (2010). In addition, to assess the loss of genetic diversity under future climate changes, we recalculated the number of plastid DNA haplotypes, $H_{D(2080)}$ and $\pi_{(2080)}$ at the species level for populations located in an area of suitable climate for *M. monophyllos* in 2080 [according to the Community Climate System Model (CCSM), described in Material and Methods under Species distribution modelling].

The parameters used to test the presence of phylogeographical structure, G_{ST} (population differentiation) and N_{ST} (genetic distance between haplotypes), were also calculated with DnaSP 5.0. These two parameters (G_{ST} and N_{ST}) were compared using a permutation test (1000 permutations). $N_{ST} > G_{ST}$ indicates that closely related haplotypes occur in the same populations, suggesting the presence of phylogenetic subdivision. Otherwise, when $N_{ST} < G_{ST,}$ the most strongly related haplotypes never occur in the same populations. Finally, when the haplotypes are similarly related, N_{ST} is equal to G_{ST} (Pons & Petit, 1996).

To investigate further the relationship between haplotypes, we applied Bayesian analysis with BEAST 1.7.4 (Drummond *et al.*, 2012). The best-fit substitution model determined by the Akaike information criterion (AIC), implemented in jModelTest (Posada, 2008), was the medium-complex model (HKY + Gamma). The output of BEAST was graphically viewed by FigTree 1.3.1 (Rambaut, 2009). We used two species, *Liparis cordifolia* Hook.f. (MC) and *Oberonia* sp. (Philippines) (OO), as outgroups, and these were clearly separated from other genera in the tribe Malaxideae (Cameron, 2005). The samples of these two species were obtained from specimens from the orchid collection at the Prague Botanical Garden. Statistical parsimony was employed to determine the relationship between haplotypes and to construct the haplotype network which implements the statistical parsimony algorithm described by Templeton, Crandall & Sing (1992) [TCS 1.2 (Clement, Posada & Crandall, 2000)].

In addition, to assess population history, we used Tajima's (1989) D and Fu's (1997) F_S as tests for neutrality. We also used mismatch distribution (MD) of the pairwise differences considering the group of haplotypes distinguished with the highest bootstrap support in Bayesian analyses (Fig. 1D). All of these analyses were performed in DnaSP 5.0.

SPECIES DISTRIBUTION MODELLING

We developed species distribution models (SDMs) (Guisan & Thuiller, 2005) to estimate the extent of potentially suitable habitats for *M. monophyllos* under climate change in Eurasia and North America. Species occurrence data for Europe (273 localities) were downloaded from the Global Biodiversity Information Facility data portal (http://www.gbif.org/; data downloaded in October 2013) and from the personally recorded positions of *M. monophyllos* populations (data from field research and collected information). Localities from the western and northern parts of North America were gained from the published sources of the Vascular Plant Collection Herbarium of the University of Alaska Museum (http://arctos.database.museum/SpecimenResults). Asian localities of populations were recorded after data obtained from the sources of the Herbarium of Moscow State University in Moscow and the Herbarium of The Siberian Institute of Plant Physiology and Biochemistry in Irkutsk. Pearson correlation was carried out on the 19 BIOCLIM variables in the WorldClim dataset (Hijmans *et al.*, 2005), based on extracted localities in which the species occurred. After removing variables that exhibited a strong correlation (Spearman's rank correlation > 0.7; Booth, Niccolucci & Schuster, 1994), we chose five variables: annual mean temperature (BIO1), isothermality (BIO3), mean temperature in the warmest quarter (BIO10), precipitation in the driest month (BIO14) and precipitation in the warmest quarter (BIO18). These variables were then used to generate SDMs employing the maximum entropy method in MAXENT 3.3.3k (Phillips, Anderson & Schapire, 2006; Elith *et al.*, 2011), with the default parameters for the convergence threshold (0.00001) and number of iterations (maximum 500). The performance of the model was tested using 25% of the occurrence data points to determine the area under the receiver operating characteristic curve (AUC). A cumulative presence was determined using the maximum training sensitivity plus specificity threshold (Liu *et al.*, 2005). The presented distribution model was projected onto reconstructed Last Glacial Maximum data (LGM, *c.* 21 000 years ago) to identify potential refugial areas, and for the future (2080) under two atmospheric circulation models constructed during the course of the Palaeoclimate Modelling Intercomparison Project Phase II (Braconnot *et al.*, 2007): CCSM and the Model for Interdisciplinary Research on Climate (MIROC). We used the A2 climatic scenario to represent the possible climates in 2080, according to the Intergovernmental Panel on Climate Change (IPCC, 2001).

The areas of the present and future ranges were calculated in ArcGis 10.0 (ESRI, 2011) using Cylindrical Equal Area projection. Parts of the predicted future ranges far outside the current distribution were excluded from the calculations.

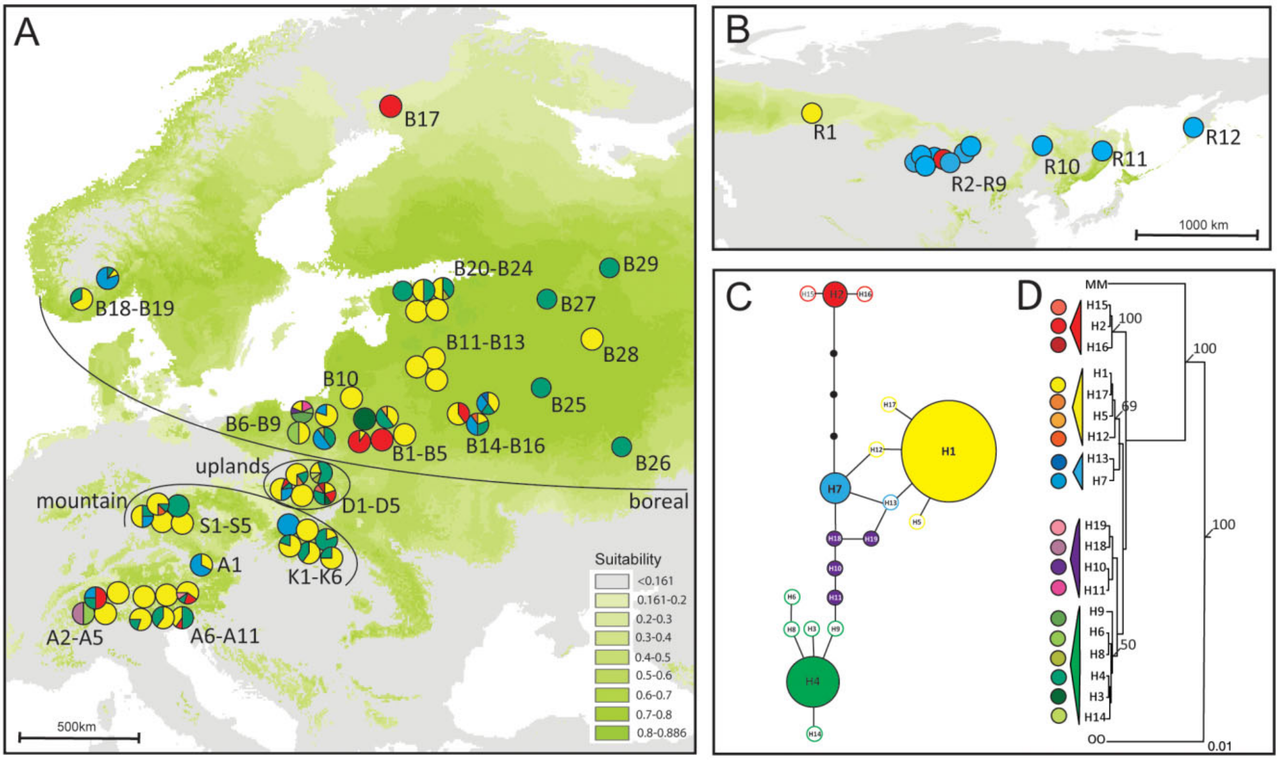

Figure 1. Distribution and frequency of plastid DNA haplotypes in the European (A) and Asian (B) populations. The green gradient corresponds to present climatic suitability. Population codes are given in Table 1. C, Statistical parsimony network of *Malaxis monophyllos* plastid DNA haplotypes. Black dots represent mutation steps. D, Consensus tree from Bayesian analysis identified by BEAST. Haplotypes grouped according to bootstrap support, whose values are equal to or greater than 50%, are given by the corresponding branches.

RESULTS

Plastid DNA variation and phylogeographical structure

Eleven nucleotide substitutions were found in the combined alignment of the four sequences, consisting of 1528 nucleotide positions. One substitution was revealed in the *trnL* sequence (630 bp) and one in the *trnL-trn*F spacer (430 bp). The *rps16* (660 bp) and *accD-psaI* (570 bp) regions were more variable, with six and three substitutions, respectively. In total, 19 plastid DNA haplotypes (H1–H19) were detected in Eurasian populations of *M. monophyllos*: 13 in boreal, seven in mountain and ten in upland populations. One, H1, was evenly distributed throughout the European part of the range, and was present in 82% of European populations with diverse frequency (10–100%) (Fig. 1A, B). Haplotype H4 also had a high frequency and occurred in 47% of populations. Haplotype H7 was present in almost 20% of European populations, but in up to > 80% (ten of 12) of Asian populations. Eleven of 19 plastid DNA haplotypes were private (almost 60% of all detected haplotypes). These haplotypes constitute 30–40% of the haplotypes presented in a given region. Two of the detected haplotypes were private for the Alps, four for boreal populations and five for upland populations (Table 1, Fig. 1A, B).

Genetic polymorphism was detected in 30 of the 51 European populations. At the species level, *M. monophyllos* possessed high values of plastid DNA diversity ($H_D = 0.702$) and nucleotide diversity ($\pi = 0.196 \times 10^{-2}$). The interpolation of haplotype diversity and nucleotide diversity values across the range of *M. monophyllos* identified three centres of genetic diversity, with an average of haplotype diversity ($H_D > 0.5$) and nucleotide diversity ($\pi > 0.2 \times 10^{-2}$). The first diversity centre was revealed for the Central Alps (Fig. 2). The second, the northern centre, included populations located in northern Poland, Scandinavia and the Polish Uplands. The third, the East European centre, included populations from eastern Poland and Belarus (Fig. 2).

A comparison of the values of parameters describing population and haplotype differentiation indicated a lack of phylogeographical structure in the *M. monophyllos* geographical range (range-wide $N_{ST} = 0.49$ and was almost equal to $G_{ST} = 0.45$). Bayesian analy-

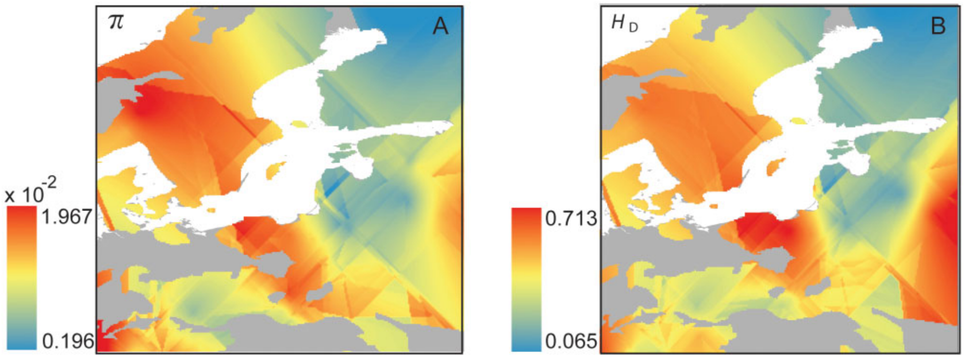

Figure 2. Geographical gradients of genetic diversity in *Malaxis monophyllos* based on nucleotide diversity (π) (A) and plastid DNA haplotype diversity (H_D) (B).

ses distinguished two haplotype groups. The first group (H2, H15 and H16) was characterized by four rare substitutions in the *rps16* sequence. Two of these haplotypes, H15 and H16, occurred exclusively in upland populations. The second group included the other haplotypes. Two subgroups in this group had bootstrap support of > 50% (Fig. 1D). Both of these two subgroups included the most frequent (H1 and H4) and private (H5, H9, H14 and H17) haplotypes. The neutrality tests and MD were estimated (Supporting Information Table S1 and Fig. S1), but showed no clear evidence for historical range expansion. Tajima's D and Fu's F_S values, estimated for all haplotypes and for three groups of haplotypes, showed no significant departures from expectation, which gave no grounds for assuming that past population expansion had occurred (Table S1). Despite this, in order to determine whether or not population structure was influenced, we used MD with the well-supported three haplogroups separately. These analyses indicated that the distribution of pairwise differences was unimodal for A and C haplogroups (Fig. S1). It revealed that the results were not clear for each group, and we did not consider that these haplogroups were under expansion.

The statistical parsimony network established H1 as the most frequent in the Eurasian part of the range of *M. monophyllos*, but it does not fulfil other objectives concerning ancestral haplotypes. In contrast, H7, which occupied the central part of the network, and has the most links with other haplotypes, may be considered as the ancestral haplotype. Although H7 was not frequent in Europe, it appeared in almost all the investigated Asian populations (Fig. 1C).

SPECIES DISTRIBUTION MODELLING

The current distribution model of *M. monophyllos* was supported by high predictive power (AUC = 0.956). The jackknife test identified annual mean temperature as the most important factor determining this model (AUC = 0.910). Other variables were also informative: isothermality (0.836), mean temperature in the warmest quarter (0.892), precipitation in the driest month (0.839) and precipitation in the warmest quarter (0.831).

Model projection to LGM showed a considerable reduction in suitable habitats for *M. monophyllos* (Fig. 3). Two of the atmospheric models used differed significantly in projection outcomes, especially in the case of Europe and western North America. CCSM yielded almost no suitable areas during the LGM in Europe, whereas MIROC showed fairly extensive areas in the lower part of the Alps and their surroundings, which extended further eastward. MIROC indicated the possibility of the existence of wide areas of suitable climate in Beringia, in contrast with CCSM, which showed a much narrower area of suitable climate in this region during the LGM. In general, in these same areas, CCSM indicated an area of suitable habitat almost half the size as that identified by MIROC.

The future distribution models (2080 for both CCSM and MIROC) showed an extensive loss of suitable climate areas for *M. monophyllos* relative to the modelled current climate envelope. Based on the A2 future scenario, we can predict, on average, a *c.* 80% reduction in suitable areas for *M. monophyllos* (71% in CCSM and 88% in MIROC). These simulations suggest that, under climate change, *M. monophyllos* will persist mainly at higher geographical latitudes and in the higher parts of mountains, but only in high massifs, such as the Alps, which, in consequence, will lead to a narrowing of the available habitats within the geographical range.

The currently observed progressive fragmentation of climatically suitable areas for *M. monophyllos* in Europe will finally (predictions for 2080) lead to the complete separation of boreal and mountain

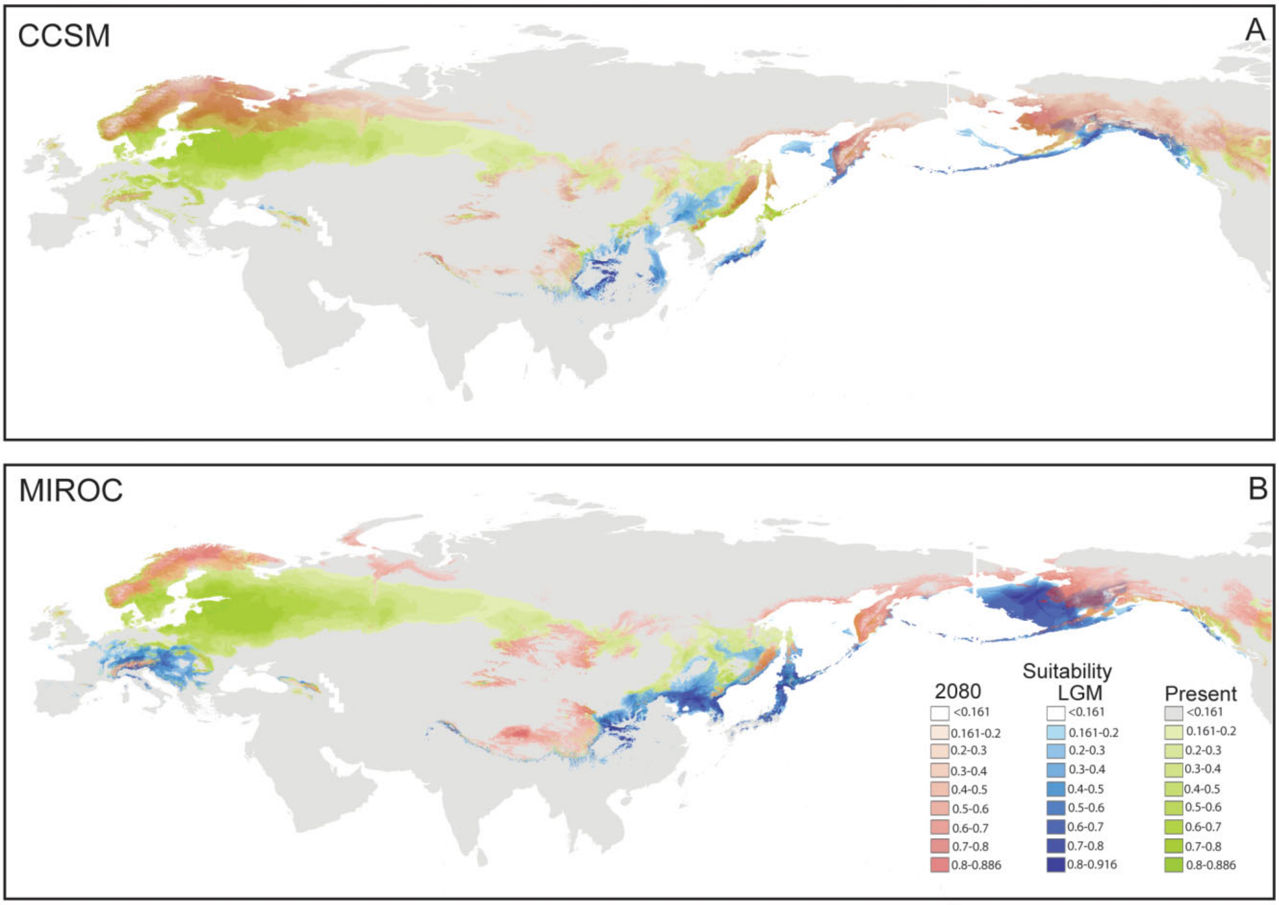

Figure 3. Modelled present, Last Glacial Maximum (LGM) (*c.* 21 000 years ago) and future (2080) distribution of climatically suitable areas for *Malaxis monophyllos* in Eurasia and North America under two atmospheric circulation models: A, Community Climate System Model (CCSM); B, Model for Interdisciplinary Research on Climate (MIROC). The suitability is shown according to the colour key. The green gradient corresponds to present, blue to LGM and red to future climatically suitable areas for *M. monophyllos*.

populations (Fig. 3). These models, in general, indicate a northward shift in the geographical distribution range of the species and the loss of suitable areas in central and eastern Europe, and in the western and central part of north Asia. The models also reveal a decrease in suitable areas in north-eastern Asia and its extension in western North America. Noticeable for Europe are the increase in suitable habitats in Scandinavia, the almost complete decline in the Carpathians and the loss of all available habitats in the Czech Massif.

In consequence, 80% of the investigated *M. monophyllos* populations will not have optimal conditions for existence and may become extinct. The recalculation of genetic diversity parameters at the species level for populations that will be able to survive future climatic changes (12 of 51 investigated populations) shows a small genetic erosion, manifested by a decrease in the value of $H_{D(2080)}$ to 0.653. The nucleotide diversity remains almost unchanged at $\pi_{(2080)} = 0.197 \times 10^{-2}$.

DISCUSSION

PHYLOGEOGRAPHICAL STRUCTURE OF *M. MONOPHYLLOS*

Our survey revealed that *M. monophyllos* exhibits high intraspecific haplotype diversity, but lacks geographical genetic structure in its Eurasian range. Thus, we cannot speculate particularly about a split between northern (boreal) and southern (montane) parts of its European range in the molecular sense. Although the observed geographical distribution of *M. monophyllos* in Europe is characterized as fragmented or even disjunct (Zając, 1996), the distance between boreal, montane and upland populations is only 300–400 km. Therefore, it is possible that there was a fairly recent and rapid fragmentation of habitats, which might not necessarily have been accompanied by genetic divergence (Young, Boyle & Brown, 1996; Schönswetter & Tribsch, 2005). Another possible explanation of the observed lack of phylogeographical structure might be incomplete lineage sorting during recent range expansion, particularly when the lineages co-occurred (Eckert & Carstens, 2008), but this phenomenon requires detailed investigation in the future using different types of markers.

Habitats in which *M. monophyllos* occurs are mostly peatlands and moist spruce forests, which, although isolated nowadays, were more widely dispersed in the glacial and early postglacial period (Tarasov *et al*., 2000; Van Andel, 2002; Williams, 2009; Allen *et al*., 2010). The observed widespread distribution of common haplotypes throughout the range of *M. monophyllos* could reflect a broad-fronted spread when the available habitats were more continuous. This type of colonization, with multidirectional gene flow, could have counteracted bottlenecks, resulting in the maintenance of a high level of variability within populations of this species.

Despite the lack of phylogeographical structure in the Eurasian range of *M. monophyllos*, three plastid DNA diversity centres were distinguished in Europe, partly consistent with putative refugial areas. This prediction was based on the assumption that the signatures of refugial areas were a high diversity coupled with private haplotypes (Comes & Kadereit, 1998; Davis & Shaw, 2001; Bhagwat & Willis, 2008; Stewart *et al*., 2010). Simultaneously, our data are based on an uneven number of samples from single locations, and therefore this kind of interpretation must be taken into account as only one of the probabilities. Nonetheless, one of the indicated centres of plastid DNA diversity was connected with the central Alps, confirmed as a source of post-glacial recolonization for alpine and arctic-alpine plants (e.g. Stehlik, Schneller & Bachmann, 2001; Tribsch & Schönswetter, 2003; Schönswetter *et al*., 2005; Bettin *et al*., 2007; Mráz *et al*., 2007; Reisch, 2008) and for boreal species (Alsos *et al*., 2005, 2009; Ronikier *et al*., 2008; Kramp *et al*., 2009; Beatty & Provan, 2011). Previous studies have indicated multiple refugial areas in the Alps, including central and peripheral areas (Schönswetter *et al*., 2005). According to paleovegetation data provided by Allen *et al*. (2010), plant communities with boreal trees (Boreal/Montane Evergreen Tree plant functional type) occupied large parts of Europe during the LGM, which were also suitable for *M. monophyllos*. Moreover, our modelling for the past (*c*. 21 000 years ago), using MIROC, narrowed available areas to the Alps and their surroundings. The explanation for such discrepancies might be partial disequilibrium between species ranges and climate, when species range is constrained by habitat accessibility. Based on data from Normand *et al*. (2011) for the current distribution, such a situation could possibly apply to more than half the plant species in Europe. These authors pointed out that such differences mostly depend on the dispersal ability of species, appropriate soil development or competition with established vegetation. We could also predict that, locally, between the ice shields in the European Alps and the Scandinavian glaciers, lowland glacial refugia for *M. monophyllos* had the opportunity to persist (Holderegger & Thiel-Egenter, 2009).

Populations that are grouped in the second and third centres of genetic diversity were located in northern (northern Poland and Scandinavia) and eastern (from eastern Poland and Belarus) Europe, respectively. The high level of plastid DNA diversity in these areas confirmed previous studies on boreal species (Ehrich *et al*., 2008; Michl *et al*., 2010; Beatty & Provan, 2011; Alsos *et al*., 2012; Eidesen *et al*., 2013) and also fitted well the idea of lowland glacial refugia, from which the populations spread further northwards and eastwards. It was also consistent with paleovegetation modelling (Allen *et al*., 2010), indicating the presence of suitable habitats for boreal species during the LGM, almost across the whole of central and eastern Europe. Moreover, Hewitt (2004) suggested the presence of a suture zone along the locations of the last remnants of the Scandinavian ice sheet, e.g. in northern Poland. Further analyses of nuclear DNA using amplified fragment length polymorphism (AFLP) would allow the verification of this hypothesis (E. Jermakowicz *et al*., unpubl. data). Different areas of Asia as sources of the colonization of Europe have been mentioned in some studies, e.g. Abbott & Brochmann (2003), Schönswetter *et al*. (2006) and Wróblewska (2012), and were confirmed by our model of climatically suitable areas for *M. monophyllos* in the past. However, because of restricted sampling outside Europe, it was not possible to clearly identify putative migration routes or corresponding refugia for Eurasian populations.

Here, we would like to pay special attention to upland populations as a region of high values of plastid DNA haplotype diversity in *M. monophyllos*. They exist on strongly disturbed, anthropogenic habitats, such as roadsides or post-mining areas. These were colonized quite recently (a few decades ago), with other rare species, after the discontinuation of mining (Nowak, 2006). However, the origins of these populations were not defined. Several explanations are possible for such high values of genetic diversity in populations in anthropogenic habitats. Populations of *M. monophyllos* were also observed in natural habitats in the uplands, and thus we can suppose that they were the source of the current anthropogenic populations. The past and ongoing gene flow from neighbouring, natural populations could be a source of variation in these new populations. Moreover, the distinct properties of anthropogenic populations in comparison with other populations (greater densities and abundance, less fruiting) (E. Jermakowicz, unpubl. data) indicate that demographic processes in this area were shaped in a different way. Two of the important features of anthropogenic populations are, indeed, the huge number of individuals and the explosive population growth (Bernacki *et al*., 1991; Bernacki, 1998; E. Jermakowicz, unpubl. data). When the growth rate of a newly founded population is high, a decrease in genetic variability may not occur (Nei, Maruyama & Chakraborty, 1975; Krüger, Hellwig & Oberprieler, 2002). The other explanation for the accumulation of genetic variation in the central European uplands could be the presence of a suture zone (or the remains of a suture zone) between colonization routes. In our opinion, upland populations could play an important role in shaping the future range of *M. monophyllos*. They could act as links (bridges) between currently isolated parts of the range. The populations existing in anthropogenic habitats could also be a reservoir of genetic diversity for this species in the context of a declining number of natural populations. Observations of orchids established in secondary habitats have been carried out in different parts of the globe (Adamowski, 1995; Nowak, 2006; Shefferson, Kull & Tali, 2008; Scheffknecht *et al*., 2010; Brzosko *et al*., 2013), but information about genetic diversity resources in these populations is sparse. In our opinion, these populations should be conserved and appropriately managed. This requires detailed knowledge of their properties, including genetic diversity.

Patterns of genetic diversity in plant species are shaped by historical events and species features (e.g. life cycles, mating system, pollination and dispersal mechanisms) and ecological variables (Loveless & Hamrick, 1984; Hewitt, 1996; Glémin, Bazin & Cherlesworth, 2006; Eckert, Samis & Lougheed, 2008; Thiel-Egenter *et al*., 2009). *Malaxis monophyllos* is presumably an obligate outcrossing, pollinator-dependent plant (Vakhrameeva *et al*., 2008; Claessens & Kleynen, 2011; E. Jermakowicz, unpubl. data). The pollinators, i.e. fungus gnats, are strongly connected with moist environments and do not move over longer distances (Claessens & Kleynen, 2011). Moreover, *M. monophyllos*, as an anemochorous plant with a high seed output, is adapted to LDD, which can support gene flow between populations and, in consequence, can counteract population subdivision (Slatkin, 1987; Glémin *et al*., 2006). In addition, this type of distribution mode can prevent the loss of genetic diversity (Thiel-Egenter *et al*., 2009; Eidesen *et al*., 2013).

In different populations various processes can dominate and shape the current pattern of diversity. A good example of populations in which history could have played a leading role may be the Carpathian populations of *M. monophyllos*. The Carpathian Mountains have been reported as a hotspot of species diversity for several European plant species, including boreal species (Kramp *et al*., 2009; Bálint *et al*., 2011). Populations of *M. monophyllos* from this region harbour less variation than populations from other parts of the range. Such low levels of genetic diversity may reflect the results of recent demographic fluctuations in isolated montane populations or of stochastic processes during post-glacial range expansion (Hewitt, 1996; Van Rossum & Prentice, 2004; Olivier *et al*., 2006) from refugial populations, located outside the Carpathian Mountains. We can suggest the same explanations for other populations of *M. monophyllos*, which showed a homogeneous structure for plastid DNA, but only the Carpathians, in general, seem to be impoverished in comparison with other regions.

FUTURE PROJECTIONS

The future projections of climatic suitability for *M. monophyllos* (both CCSM and MIROC) showed a substantial loss of climatically suitable areas across its range. The differences between these two models were quite large. CCSM indicated almost 40% more suitable areas relative to MIROC in 2080 (Fig. 3). In Europe, the decline in available habitats led to complete disjunction of the *M. monophyllos* range, separating the boreal and montane parts. Simultaneously, SDM predicted a reduction and shift in climatically suitable areas in the future, especially at higher latitudes. Habitat gain in the north has been shown for the Scandinavian Peninsula and for the north-western part of North America, which may allow for northward population expansion. This northern trend is consistent with other simulations for boreal species (Thuiller *et al*., 2005; Noguès-Bravo *et al*., 2007; Crawford, 2008; Feehan *et al*., 2009; Alsos *et al*., 2012; Pauli *et al*., 2012). However, a comparison of simulations of the future and present distribution of *M. monophyllos* indicated that $< 20\%$ of these areas overlapped (in CCSM and MIROC). This means that $> 80\%$ of present habitats may disappear in the next few decades. The 20% overlapping area was in Scandinavia, where our genetic data were sparse. Our simulations for populations that may persist to 2080 show that diversity on an intraspecific level may remain at a relatively high level, despite a huge decrease in haplotype number (seven of 19). We can associate this with the fact that the future distribution of climatically suitable areas partly overlaps with current plastid DNA diversity centres, including alpine and northern, which seems to be important for the conservation of genetic resources.

Although these future projections appear to be pessimistic, it is important to recognize that such SDMs often overpredict the loss of climatically suitable areas (Barbet-Massin, Thuiller & Jiguet, 2010) and SDMs are based only on climatic data. For a full prediction of the future distribution of *M. monophyllos*, habitat data should be included, because habitat properties (humidity, soil pH) are crucial for the persistence of *M. monophyllos*. In this case, there are different types of peatland, the structure of which is constantly changing (Gorham, 1991). Moreover, paleovegetation data for the past show that species ranges are strongly constrained by habitat accessibility (Allen *et al*., 2010). Thus, our future projections for the geographical distribution of *M. monophyllos* must be taken as a probable trend.

The maintenance of intraspecific genetic variety largely depends on the geographical distribution of a species and the distribution of genetic resources in the range of this species. Therefore, the considerable range reduction predicted for *M. monophyllos* may probably lead to a significant loss of intraspecific genetic diversity. In some geographical regions (the Carpathian Mountains and the Czech Massif), this loss might cause less harm than in others (eastern Europe, the Alps and the Polish Uplands). Our study, integrating phylogeographical analysis with range modelling, should result in the most adequate conservation strategies, when such actions become necessary.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Mismatch distributions for the three haplogroups (A, B, C) of *Malaxis monophyllos*.
Table S1. Estimates of neutrality tests for the three haplogroups of *Malaxis monophyllos*.

Figure S1.

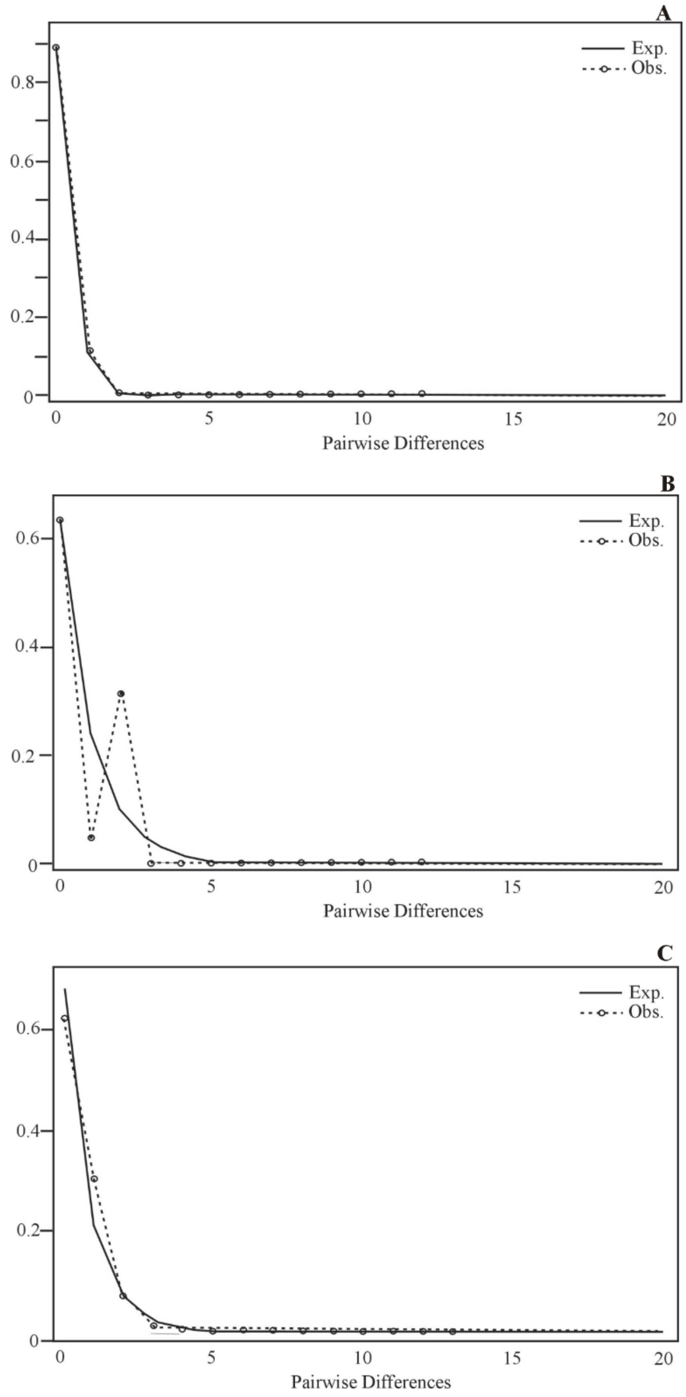
A
Exp.
Obs.
0.8
0.6
0.4
0.2
0
0
5
10
15
20
Pairwise Differences
B
Exp.
Obs.
0.6
0.4
0.2
0
0
5
10
15
20
Pairwise Differences
C
Exp.
Obs.
0.6
0.4
0.2
0
0
5
10
15
20
Pairwise Differences

Table S1.

Haplogroups[1]	Tajimas' *D*	Fu's *F*s
A (H2, H15, H16)	-1,497	-2,783
B (H1, H5, H7, H12, H13, H17)	0,070	-0,878
C (H3, H4, H6, H8, H9, H14)	-1,088	-2,785

No significance values.

[1] – haplogroups were distinguished with the highest bootstrap support in the Bayesian analyses (Fig. 1 D).

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Edyta Jermakowicz
Instytut Biologii, Wydział Biologiczno-Chemiczny
Uniwersytet w Białymstoku

Informuję, że praca pt. Genetic diversity of orchid *Malaxis monophyllos* over European range as an effect of population properties and postglacial colonization, autorstwa: Edyty Jermakowicz, Emili Brzosko, Jarosława Kotowicza oraz Ady Wróblewskiej, została przyjęta do druku w Polish Journal of Ecology i ukaże się w 1 lub 2 zeszycie 2017 roku.

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Edyta Jermakowicz contribution: research designing, field work, laboratory work, data analyses, manuscript preparation

Edyta Jermakowicz*[1], Emilia Brzosko[1], Jarosław Kotowicz[2] and Ada Wróblewska[1]

[1]Institute of Biology, University of Białystok, K. Ciołkowskiego 1J, 15-245 Białystok, Poland, *edytabot@uwb.edu.pl (*corresponding author*)
[2]Faculty of Mathematics and Informatics, University of Białystok, Ciołkowskiego 1M, 15-245 Białystok, Poland

Genetic diversity of orchid *Malaxis monophyllos* over European range as an effect of population properties and postglacial colonization

Abstract: *Malaxis monophyllos* is a rare orchid with a fragmented boreal-montane distribution in Europe where it is associated with both natural swampy and anthropogenic habitats. We employed extensive sampling of *M. monophyllos* populations from different habitat types, over its whole European geographic range, to examine its genetic diversity patterns and phylogeographic structure using amplified fragment length polymorphisms (AFLPs). Our results revealed the relatively low genetic diversity of *M. monophyllos*, with the effect of small population sizes and inbreeding as the driving forces operating within the European part of its range. The statistically highest values of genetic diversity were found in populations from the boreal region (average: PPL_3 = 21.6%, H_{j3} = 0.144, DW_3 = 1.34), while populations from mountainous regions were characterised by a reduced level (e.g. an average for Alpine populations: PPL_3 = 18.1%, H_{j3} = 0.121, DW_3 = 0.84) in comparison to boreal ones. Our results revealed also that the newly established anthropogenic populations in the Polish uplands were probably founded from numerous external sources and should be considered a significant source of the species' genetic diversity. We also confirmed the low genetic differentiation among *M. monophyllos* populations (F_{ST} = 0.074), with the lack of distinguishable genetic clusters, that supports results about the multidirectional gene flow between *M. monophyllos* populations in Europe, and directed conservation efforts on conserving all suitable for this species habitats.

Keywords: AFLPs – anthropogenic habitats – boreal-montane species– glacial refugia – *Orchidaceae* – population size

Running page headline: Genetic diversity of *M. monophyllos* in Europe

Introduction

In the northern hemisphere, climatic oscillations during the Quaternary Period produced significant changes in species distribution (Taberlet *et al*. 1998, Hewitt 2004, Parmesan 2006), which resulted in the repeated expansion and fragmentation of species' ranges and influenced their genetic diversity patterns. Cold-adapted plants (arctic and boreal) are believed to have been more severely threatened during the Quaternary Period than other plant groups (Comes and Kadereit 1998, Davis and Shaw 2001, Alsos *et al*. 2012, Eidesen *et al*. 2013). Moreover, the climate scenarios that predict future geographic distributions of northern species in Europe revealed further disadvantageous changes; by the end of the 21st century, the majority of these plants' ranges will shrink and shift northwards and to higher altitudes, into the e.g. probable warm-stage refugia in Alps (Bhagwat and Willis 2008, Gentili *et al*. 2015), leading to greater isolation of their populations or even to extinction (Thuiller *et al*. 2005, Noguès-Bravo *et al*. 2007). Consequently, a loss of genetic diversity is more expected for northern species than for temperate or Mediterranean ones (Hampe and Petit 2005, Paulus *et al*. 2013). Geographic distribution changes during the Last Glacial Maximum (LGM, ~21,000 years BP) decreased the genetic diversity of northern species compared to that of other geographic groups of species. Moreover, present-day human activity intensifies these historical changes, causing further intrapopulation genetic depauperation and increasing genetic differentiation among these populations (Jump and Peñuelas 2005, Franks *et al*. 2013). Cold-adapted plants are characterised by prolonged declines in their population sizes and geographic ranges, but also by their biological properties, like limited sexual reproduction and short-distance dispersal, which make them more susceptible to the loss of genetic diversity (Leimu *et al*. 2006, Alsos *et al*. 2012, Paulus *et al*. 2013).

Orchids are the one of the plant groups, that have undergone extensive range fragmentation and decreased population sizes in response to climate alternation and anthropogenic changes (Swarts and Dixon 2009, Hornemann *et al*. 2012), which have led to severe genetic erosion (Forrest *et al*. 2004, Philips *et al*. 2012, Brzosko *et al*. 2013, Chung *et al*. 2014). Pollen and seed-mediated gene flow are the key factors that are most frequently discussed in the context of orchid genetic structure. These factors are linked to population size and a population's spatial isolation, as well as to sexual reproduction and breeding system, which are especially variable among orchids (Forrest *et al*. 2004, Brzosko *et al*. 2009, Duminil *et al*. 2009, Jacquemyn *et al.* 2009). Although orchid population genetics are frequently studied, surveys that focused both on cold-adapted orchids and their genetic diversity patterns over a wide geographic range are still rare and necessary, given the importance of global genetic diversity preservation (Pillon *et al*. 2007, Vanden Broeck *et al.* 2014, Ilves *et al.* 2015).

An interesting species for examining genetic diversity patterns, in the context of glacial/postglacial history in Europe, life-history traits, and ongoing demographic processes, is *Malaxis monophyllos* (L.) Sw. This cold-adapted plant is characterized by a fragmented boreal-montane geographic distribution (Fig. 1A). In the north, the *M. monophyllos* distribution covers the boreal part of Eurasia and part of North America; in Central and Southern Europe it is found in the mountains, mostly in the lower mountain vegetation belt (Hultén and Fries 1986, Vekhrameeva *et al.* 2008). *M. monophyllos* grows in natural habitats, such as swamps, peat bogs, and spruce and alder forests, where the population size rarely exceeds 50 individuals, thus it is characterized by the relatively high level of threat in most regions of Europe. According to IUCN categories, *M. monophyllos* has a status from critically endangered in Czech Republic and in Norway (Holub and Procházka 2000, Kalas *et al.* 2006), through vulnerable in Lithuania and Estonia (Rašomavičius 2007, Red Data Book of Estonia 2008) to near threatened in Poland (Kaźmierczakowa *et al.* 2014). Furthermore, Jermakowicz and Brzosko (2016) reported that natural populations of *M. monophyllos* in Poland are characterised by relatively low flowering rates (36.6% and 27.7%, on average, for boreal and montane populations, respectively) and low fruit-set (22.5% and 5.3%, on average, for boreal and montane populations, respectively). Jermakowicz *et al.* (2015a) survey, based on chloroplast DNA sequences (cpDNA), revealed a weak *M. monophyllos* phylogeographic structure, influenced probably by historical multidirectional gene flow in the European distribution range. Despite the fact that the putative colonization routes and corresponding glacial refugia could not be precisely identified by cpDNA markers, high diversity has been found in the Alps and eastern and north-eastern Europe. This result highlights that these regions are the most valuable for *M. monophyllos* conservation, given future range shifting. Surprisingly, the geographic discontinuity between populations from boreal and mountainous regions in Europe was not reflected in a genetic split between them.

Another very important evolutionary aspect highlighted by Jermakowicz and co-authors (2015a) was connected with the recent history (since the eighties) and establishment of new secondary *M. monophyllos* populations in anthropogenic habitats (abandoned mining areas, railway embankments, and roadsides) in the Polish uplands. Therefore, some demographic processes are shaped there in distinct ways compared to natural populations, e.g. sexual reproduction is extremely reduced or there is a lack of fruit set (Bernacki *et al.* 1991, Jermakowicz *et al.* 2015b, Jermakowicz and Brzosko 2016). We considered that these anthropogenic populations could be a source for species survival and potentially give rise to the colonization of other areas (Nowak 2006, Esfeld *et al*. 2008), and they represent important units in terms of species evolution and conservation that require a multifaceted approach.

This survey is a continuation of an earlier investigation of *M. monophyllos* that, based on cpDNA data (Jermakowicz *et al*. 2015a), aimed to enrich knowledge about the genetic diversity patterns within the geographic range of this cold-adapted orchid in the context of its history, demography and life-history traits. Therefore, we used the AFLPs to verify putative Alpine and northern refugia in Europe for this species and to test for a lack of genetic split between populations from boreal and montane regions. We also tested the hypothesis that *M. monophyllos*, as the boreal-montane species, represented by small and fragmented populations, maintains low genetic variation, and we verified whether the anthropogenic populations with more recent histories characterised by discrete demographic processes possess distinct genetic diversity patterns compared to natural populations.

Material and methods

Study species

Malaxis monophyllos (L.) Sw. has a fragmented geographical distribution that covers boreal Eurasia, the north-western region of North America, and the Central European mountains (Hultén and Fries 1986, Vakhrameeva *et al*. 2009; Fig. 1A). It prefers humid, calcareous soils and at least moderate moss cover. In recent decades, *M. monophyllos* disappeared from many localities, due to loss of suitable habitats, but it also appeared in the Polish uplands in anthropogenic habitats (dry or moderate moisture pine forest, roadsides, railway banks, and abandoned mining areas) (Bernacki *et al*. 1991).

Malaxis monophyllos is an inconspicuous, green orchid that forms one basal pseudobulb, but vegetative propagation is not confirmed in this species. It is an outcrossing plant with minute, green flowers, which are adapted to pollination by small insects (e.g., fungus gnats from the family *Mycetophilidae*) (Vakhrameeva *et al.* 2009, Claessens and Kleynen, 2011). The life span of *M. monophyllos* is approximately 20 years (periods of unfavourable habitat conditions may prolong the life cycle via dormancy or reversion from a flowering to a non-flowering stage). In general, the subterranean phase between germination and the first appearance above ground lasts approximately 8 years, and the first flowering occurs roughly 10 years after germination. *M. monophyllos* can repeat flowering for up to four seasons. Therefore, its above-ground life span is quite short, and lasts, on average, for 5–6 years. After that period, the plant dies without experiencing a senescent phase (Vakhrameeva *et al*. 2009, Jermakowicz and Brzosko 2016).

Sampling

We sampled 315 *M. monophyllos* individuals (we collected small fragments of about 1cm^2 of leaf) from 49 populations, between 2009 and 2011, that spanned the European range of *M. monophyllos*. The samples from all populations were collected with permission of local nature conservation authority or by the persons from representative institutions. Based on the geomorphological division of Europe (Embleton, 1984), we distinguished five population groups: (1) boreal, located in northern and eastern Europe; montane populations, including locations from the (2) West Carpathians, (3) Bohemian Massif (Šumava Mountains), and (4) the Alps. Moreover, we collected samples from anthropogenic populations (5) in the Polish uplands (Śląsko-Krakowska Upland and Małopolska Upland), which we call upland or anthropogenic populations (Appendix 1, Fig. 1B). The samples were randomly chosen from these populations and the number of samples strongly depended on the population size (2–11 samples per population; Appendix 1). In a few cases, the small sizes of the populations (fewer than 10 individuals) or high conservation status of *M. monophyllos* in a given country made it impossible to collect a larger number of samples.

AFLP procedure

Total genomic DNA was extracted from dried leaf material (stored in silica gel), following the manufacturer’s instructions, using a Genomic Mini Ax Plant Kit (A & A Biotechnology, Gdynia, Poland). We followed the AFLP procedure of Vos *et al*. (1995), but we modified it according to the Applied Biosystems protocol (AFLP$^{\text{TM}}$ Plant Mapping). First, 64 primer pair combinations were tested on four selected samples. The fluorescence-labelled selective amplification products were mixed with a 500 Liz labelled size standard (Applied

Biosystems) and run on an ABI 3130 sequencer. After the preliminary analyses, we selected two primer combinations that yielded polymorphic, clear, reproducible fragments of homogeneous intensity (*Eco*RI-ACG/*Mse*I-CAT and *Eco*RI-AGC/*Mse*I-CAC). Variable fragments in the 70–500 bp size range were scored as present (1) or absent (0) using GeneMapper 4.0 (Applied Biosystems). Prior to error checking, we ensured the reliability of the genotypes. To test the repeatability of our results, 10% of the samples (35 randomly chosen individuals) from different populations were completely replicated starting from the restriction-ligation reaction of AFLP. We then assessed the genotypic error for AFLP analyses (Bonin *et al*. 2004).

AFLP analyses

To assess the level of genetic diversity in the study populations, we calculated the percentage of polymorphic fragments (*PPL*), Nei's gene diversity (*Hj*), and the rarity index (*DW*, the frequency down-weighted marker index) (Schönswetter and Tribsch 2005) as measures of divergence and long-term isolation. The genetic analyses were conducted using the R script AFLPdat (Ehrich 2006). Additionally, we used a resampling procedure to account for bias in the diversity indices because of the unequal number of individual analyses in the populations. For this purpose, we used an *ad hoc* R script to generate a large number of datasets comprising three individuals per population (based on 1000 iterations). For each population, we calculated the genetic diversity (further referred to PPL_3, DW_3, Hj_3) and finally we calculated the averages and standard errors of these indices. Afterward, we used the Kruskal-Wallis *H* test to determine differences in genetic parameters among populations and regions with data after resampling. For populations in which two samples were collected, we calculated only *DW* values prior to resampling based on the *DW* characteristic provided by Ehrich (2006), where the minimal number of individuals in populations for which *DW* might be calculated is

1. Generalized linear model (GLMs) were used to analyse the impact of population size (log transformed) (P_N) and its geographic locations (latitudes and longitudes) on genetic diversity using STATISTICA 10.0 (Stat-Soft Inc. 2011). Population structure was also examined in order to assess inbreeding in the populations via HICKORY 1.0 (Holsinger and Lewis 2003). The AFLP data were fitted to four models: a full model allowing for inbreeding; an $f = 0$ model, with no inbreeding; a *theta* = 0 model, which implies no differentiation among populations; and an *f* free model, which decouples theta estimates. The data were run with default parameters (burn-in = 50,000, number of samples = 250,000, and thinning factor = 50). To estimate the fit to the four models, deviance information criterion (DIC) with Dbar, Dhat and pD were used, following the recommendations given in the manual. A model with differences of > 6 DIC units between different models is required to indicate that one model is favoured over another model (Holsinger and Lewis 2003).

We analysed the *F* statistics among the five geographically distinguished regions (boreal, West Carpathians, Bohemian Massif, Alps, and Polish uplands), among populations within regions, and among all populations using AMOVA with ARLEQUIN 3.5.2.2 (Excoffier *et al*. 2005). We calculated 95% confidence intervals for all F_{ST} statistics by bootstrap resampling (10,000 replicates). In addition, we identified genetic relationships between the 49 populations using Principal Coordinate Analysis (PCoA) (GENALEX 6.5) based on pairwise comparisons of F_{ST} among populations (Peakall and Smouse 2012). Then we tested the differences in the average values of PCA1 and PCA2 between populations via one-way ANOVA. We assessed the presence of *IBD* (Isolation by Distance) pattern, with a Mantel-test, using genetic $[F_{ST}/(1-F_{ST})]$ and geographic [Ln(km)] distance matrices. Then, according to the method of Saitou and Nei (1987), we performed neighbour-joining analyses based on F_{ST} values with PHILIP 3.6 (Felsenstein 1993) to illustrate genetic differentiation among populations. Afterward, based on PHILIPS' results, an unrooted tree was generated in SPLITS TREE 4.13.1 (Huson and Bryant

2006), and support for the branches was estimated using 1000 bootstrap replicates. Finally, to infer population structure and assign individuals to populations, we used the model-based clustering method described by Pritchard et al. (2000), as implemented in STRUCTURE 2.3.3. The AFLP data were analysed with an admixture model with correlated allele frequencies described by Falush *et al*. (2003). Ten replicates were run for all possible values of the maximum number of clusters (*K*), up to *K*=15. Following the recommendations of Evanno *et al*. (2005), we calculated the *ad hoc* statistic ΔK based on the rate of change in the log likelihood of the data between consecutive *K* values, to assessed number of distinct genetic clusters in analysed data set. All of the runs were based on 500,000 iterations after a burn-in of 100,000 iterations.

Results

Genetic variation in M. monophyllos *populations*

There were 149 scorable polymorphic markers generated for the two primer combinations from 315 *M. monophyllos* individuals from 49 European populations. The mean genotyping error was 3.5%. Each individual possessed unique, multi-locus genotypes.

The values of the genetic parameters after the resampling procedure were correlated with the results prior to resampling [tau Kendall from 0.61 (*PPL*), 0.81 (*DW*) to 0.97 (H_j)], indicating that results for H_j were the most consistent. We found statistically significant differences in H_{j3} and DW_3, between geographic groups for about half of the compared pairs; the largest differences were observed between boreal and mountainous regions (Appendix 2). The PPL_3 ranged from 6.7–31.8% within all populations. Nei's gene diversity, H_{j3}, was also relatively low, and ranged from 0.045 to 0.212. The highest PPL_3 and H_{j3} values were found in the boreal and upland/anthropogenic population groups (21.6% and 20.5% for PPL_3 and 0.144 and 0.137 for H_{j3}, respectively) (Fig. 1C, Appendix 1). The lowest PPL_3 and H_{j3} values were noted in the Bohemian Massif (PPL_3 = 15.3 % and H_{j3} = 0.102) (Appendix 1). The frequency down-weighted marker values (DW_3) ranged between 0.36 and 3.91; they had the highest average value in the boreal region (1.34) and the lowest in the Alps (0.84). The average DW_3 in anthropogenic populations was 0.86. (Fig. 1D, Appendix 1). There were statistically significant correlations between population size (P_N) and PPL_3 and H_{j3} (Table 1). We also found a significant decrease of genetic diversity indices with latitude and longitude, towards the south-west direction of Europe (Table 1, Fig. 1C). Results from HICKORY indicated inbreeding in the European *M. monophyllos* populations (Table 2). The DIC parameter was lower in the full model than in the f=0 model (differences of 27.8 units), revealing a departure from the Hardy-Weinberg equilibrium.

Genetic structure in European, highly fragmented geographical range

The overall F_{ST} was very low (0.074) among the 49 European *M. monophyllos* populations. Mantel-test revealed a lack of statistically significant correlations between the genetic and geographic distances (r^2 = 0.0008, P = 0.29; Fig. 2). The preliminary analyses of PCoA generated high genetic divergence among populations, caused mostly by two populations (Bohemian Massif S4 and boreal B15). Analyses of scoring data for these populations revealed the existence of a lot of established, monomorphic loci, which could generate PCoA and NJ results for *M. monophyllos* in Europe, where these two populations clearly stud out of the rest of analysed populations. After excluding these two outlier populations, PCoA analyses revealed that the first two axes explained 26.6% and 15.1% of the total variation and plotted almost all populations in the central part of diagram (Fig. 3). Similarly, the neighbour-joining tree was weakly support (bootstrap values: 31 – 35%) and did not confirm that geographically close populations were genetically similar (Fig. 4). The total AMOVA, averaged over loci, confirmed the PCoA results and assigned 1.8% of the overall genetic variation among the five regions, 11.2% among the populations within regions, and 87% within populations.

The model-based clustering method implemented in STRUCTURE revealed the highest peak at $\Delta K = 3$ (Appendix 3). All *M. monophyllos* individuals represented an admixture of three genetic backgrounds and had no clear geographic structuring (Fig. 5A, B). Simultaneously, we observed differences in the dominant different gene pools in some regions. In the Bohemian Massif, for example, the first genetic cluster (green colour; Fig. 5A, B) presented with up to 80% frequency. In the central and eastern Alps and in some parts of the boreal region (north-eastern Poland), other gene pools dominated (red in the central Alps; blue in the eastern Alps and in the boreal region) (Fig. 5A, B).

Discussion

Genetic variation of M. monophyllos

As we predicted before, small and fragmented *M. monophyllos* populations were characterised by relatively low genetic diversity over the European geographical range (PPL_3 = 6.7–31.8%, H_{j3} = 0.045–0.212). This pattern is similar to other circumboreal plants, including *Chamaedaphne calyculata*, *Polygonatum verticillatum* or *Rubus chamaemorus* (Ehrich *et al*. 2008, Kramp *et al*. 2008, Wróblewska 2012). The low level of genetic diversity may, therefore, indicate the loss of alleles during recolonization from glacial refugia located mainly outside of Europe. If so, and assuming that the main source of *M. monophyllos* genetic diversity, as for other cold-adapted plant species, was located in Central Asia (Hedberg 1992, Schönswetter et al. 2006, Jermakowicz *et al.* 2015a), then the decreased genetic diversity in the south-western European *M. monophyllos* population might be evidence of this recolonization-induced loss of genetic diversity. The genetic diversity was also similar to another, closely phylogenetically related boreal orchid, *Liparis loeselii* (Cameron, 2005) (H = 0.017 – 0.146; Pillon *et al*. 2007), with overlapping habitat preferences (Vakhrameeva *et al*. 2008). *L. loeselii* is mainly an autogamous species (Pillon *et al*. 2007), while *M. monophyllos* is pollinator-dependent for setting fruits (Claessens and Kleynen 2011, Jermakowicz and Brzosko 2016). It is well known that species' genetic patterns are modulated by species properties, in particular the mode of pollination (Charlesworth and Charlesworth 1995, Pellissier *et al.* 2015). Although, in case of *M. monophyllos*, it seems that factors other than breeding systems might play a more important role in shaping of the genetic diversity pattern (Hamrick and Godt 1996, Tremblay *et al*. 2005, Frankham 1996). In *M. monophyllos*, genetic diversity depended on population sizes', pointing on this demographic factor as strongly influencing its intrapopulation genetic variation. The small sizes of the majority of *M. monophyllos* populations (rarely exceeding 50 individuals) (Jermakowicz and Brzosko 2016), with a restricted pool of reproductive individuals, can produce populations of relatives, that has often results in inbreeding.

Centres of M. monophyllos genetic diversity: refugia, relicts and/or melting pots?

The comparison of the results from application of two types of molecular markers yielded the ability to interpret the evolutionary history of *M. monophyllos* with greater precision and definiteness. Previous studies, based on cpDNA markers (Jermakowicz *et al*. 2015a), distinguished three genetic diversity centres for *M. monophyllos* in Europe. Pointing out the putative glacial refuges and/or relict populations. These are in the central Alps, the European lowlands, and areas east of Europe (Central Asian refugia). AFLP results only partly supported the cpDNA data and revealed the highest genetic diversity in a few populations from the boreal part of range of *M. monophyllos* in north-eastern Europe (i.e., Estonia and Poland). Therefore, interpretation about the proximity of northern glacial refuges or colonization from eastern refuges localized in Central Asia, as well as the location of melting pots of different colonization lineages in this region, can be confirmed (Hewitt 2004, Nordström and Hedrén 2008, Jermakowicz *et al.* 2015a). The present-day *M. monophyllos* geographic range was partly covered by an ice sheet during the LGM (~21,000 years BP) (Fig. 1A). Northern and north-eastern Europe and the Alps (including solid ice fields and montane glaciers) were widely covered by ice, while humid habitats of mountain valleys at lower altitudes in the Western Carpathians and the Bohemian Massif were free of ice (Obidowicz *et al*. 2004, Ronikier 2011, Pánek and Hradecký 2016) and had the potential to become a glacial refugia for montane plants (Kramp *et al*. 2009, Michl *et al*. 2010). However the existence of putative glacial refuges or relict *M. monophyllos* populations in these geographical regions must be interpreted with caution. The montane *M. monophyllos* populations were characterised by lower AFLP genetic diversity than boreal populations, which disagreed with published cpDNA results (Jermakowicz *et al*. 2015a). This incongruence of genetic structure could be caused by AFLPs properties, that detects also nuclear DNA polymorphism. Therefore, in case of AFLP effective population size is larger and it reach equilibrium much slower than that of cpDNA. Because of that AFLP may detect more ancestral polymorphisms, that might be shared across populations, while these of cpDNA would be fixed in some populations due to genetic drift (Petit *et al*. 2005). In case of *M. monophyllos*, we can also assume that in the preboreal and boreal periods of the Holocene (11,500-8,900 years BP), when the climatic conditions were optimal for boreal species (Latałowa 2003), it was presumably widely spread throughout Europe. Observed shifts in its range, especially to higher altitudes, e.g. to a probable warm-stage refugia in the Alps (Bhagwat and Willis 2008, Gentili *et al*. 2015), could decrease genetic diversity as a consequence of bottlenecks during movement (Williams *et al*. 2015). The observed genetic depauperation of montane *M. monophyllos* populations, in particular those from the Bohemian Massif, might be an indicator of ongoing changes in mountainous environments; that is consistent with global predictions that mountainous regions are more exposed to global climate changes than other regions of Europe (Diez *et al*. 2003, Thuiller *et al*. 2005, Nogués-Bravo *et al*. 2007).

Evidence of M. monophyllos metapopulation dynamics in Europe

Despite latitudinal and longitudinal trends in genetic variation, both the AFLP and cpDNA results revealed a weak phylogeographic structure within the European *M. monophyllos* distribution range (Jermakowicz *et al.* 2015a). The observed low genetic differentiation (F_{ST}=0.074) was probably an effect of previous processes including multidirectional gene flow and may resembles a 'classical' metapopulation model (Ellstrand 2014). Such interpretation can be supported by the demographic data which illustrated a highly dynamic spatial structure of *M. monophyllos* populations over the short time periods and a nearly complete exchange of individuals pool during the following 5–6 years (75% and 94% of individuals observed in the first year were absent after 5–6 years in two monitored populations) (Jermakowicz and Brzosko 2016).

Low genetic differentiation between populations was documented in orchids (Brzosko *et al.* 2002, Murren 2003, Jacquemyn *et al.* 2007, 2009) and was even assumed to be the rule for this plant group (Phillips et al. 2012). Although gene flow through seeds appears to be generally restricted to a few meters from maternal plants in orchids (Jersáková and Malinová 2007, Brzosko *et al.* in prep.), rare events of long-distance dispersal (LDD) should not be excluded, due to anemochory adaptations of orchids seeds (Vanden Broeck *et al.* 2014, Ilves *et al.* 2015). LDD can facilitate gene flow between populations, counteracts population subdivisions and prevents the loss of genetic diversity (Berthouly-Salazar *et al.* 2013, Eidesen *et al.* 2013). In the case of orchids, in particular these nectarless, low levels of genetic differentiation are attributed to LDD of pollen (Jacquemyn *et al.* 2007, 2009, Pillon *et al.* 2007). Nowadays however, gene flow via pollen between *M. monophyllos* populations is restricted because the geographic distances between its populations rather exceed the distances over which pollinators might migrate.

The anthropogenic M. monophyllos populations as important sources of genetic diversity

Despite orchids being endangered and sensitive to habitat changes, some of them have recently spread throughout the world in anthropogenic habitats (Hollingsworth and Dickson 1997, Bernacki *et al*. 1991, Schefferson *et al*. 2008). In some cases, they are even considered to be invasive plants (Ackerman 2007, Recart *et al*. 2013). The genetic variation of anthropogenic populations may be different than the natural ones, since the character of occupied habitats may influences a population's genetic structure (Pillon *et al*. 2007). The most recent history, with important human impacts, may enhance the genetic diversity in populations by, for example, breaking barriers between genetic lineages; it may occur via intentional species introduction or when new semi-natural and anthropogenic habitats are colonized by chance with new populations. Therefore, we expected genetic differences between the *M. monophyllos* populations from different habitat types (anthropogenic and natural). Especially, that significant differentiation was observed in the demographic properties among these population groups (Jermakowicz and Brzosko 2016). These expectations were also supported by high level of cpDNA genetic diversity and high frequency of rare haplotypes that was observed in anthropogenic populations (Jermakowicz *et al*. 2015a). AFLP markers also revealed higher level of genetic variation in anthropogenic populations in comparison to values for mountainous populations and only slightly lower then values for the most divers boreal populations. *M. monophyllos* in anthropogenic habitats in the Polish uplands were likely established by LDD from neighbouring, natural populations and further increased their sizes during the last two-three decades. Thus, among the important factors that increase/maintain genetic variation within anthropogenic populations could pointed out, an appearance from many sources, explosive population growth, and a lack of bottleneck events during colonization (Ilves *et al*. 2015, Brzosko *et al*. 2009, Krüger *et al*. 2002). In addition, when the colonizing species has possibilities for long-distance gene flow and when the source populations are numerous and located in close proximity, the negative impact of founder effect do not necessarily appear (Helsen *et al*. 2013). The lack of founder effects has been observed for other orchids: for *Epipactis palustris* colonizing a former lignin-mining area in Germany (Esfeld *et al*. 2008) and for *Orchis militaris,* which appeared in abandoned gravel and chalk quarries in Estonia (Ilves *et al*. 2015). Therefore, the anthropogenic populations, in the context of species protection, should be considered worthy of proper management because they may possess higher levels of genetic variation than some natural *M. monophyllos* populations.

M. monophyllos may be threatened in the near future, due to their lower reproductive potential (Jermakowicz and Brzosko 2016). The shallow genetic structure within the *M. monophyllos* European range means that conservation efforts should be focused on conserving all suitable habitats and the long-term conservation planning should be carefully considered due to the prognoses of future changes in species' geographic distributions.

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Compliance with ethical standards

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Table 1. Relation between population size P_N (log scale), longitude and latitude with genetic diversity indices after resampling procedure (PPL_3, H_{j3}, DW_3). Level of significance * $P < 0.05$; *ns* nonsignificant.

	Log P_N	longitude	latitude
PPL_3	$r = 0.36$ *	$r = 0.32$ *	r = 0.39 *
H_{j3}	$r = 0.36$ *	$r = 0.32$ *	r = 0.39 *
DW_3	$r = 0.06$ *ns*	$r = 0.42$ *	r = 0.31 *

Table 2. Genetic structure of *Malaxis monophyllos* populations in the European part of range analysed with HICKORY 1.0.4.

	Dbar	Dhat	pD	DIC
$F = 0$	7469.2	6333.4	1135.8	8604.9
f full	7451.8	6326.4	1125.3	8577.1
Theta = 0	10364.3	10222.6	141.6	10505.9
f free	7486.3	6331.6	1155.2	8642.0

Dbar – the posterior mean of deviance; Dhat – a point estimate of the deviance obtained by substituting in the posterior means of Theta B; pD – (Dbar-Dhat); DIC - deviance information criterion

Figure legends

Figure 1. The locations of 49 investigated European populations (A) and the geographic distribution (hatched area) of *Malaxis monophyllos* in Eurasia and North America [modified from Hultén and Fries (1986) and Vakhrameeva et al. (2008)] and (B) the distribution of *M. monophyllos* genetic diversity; the percentage of polymorphic loci (*PPL*3, C) and the rarity index (*DW*, D) values are represented by different circle sizes (legend shows sample circle sizes and the corresponding values of presented indices; the dotted lines show the limits of the Last Glacial Maximum ice sheets ~21,000 years BP).

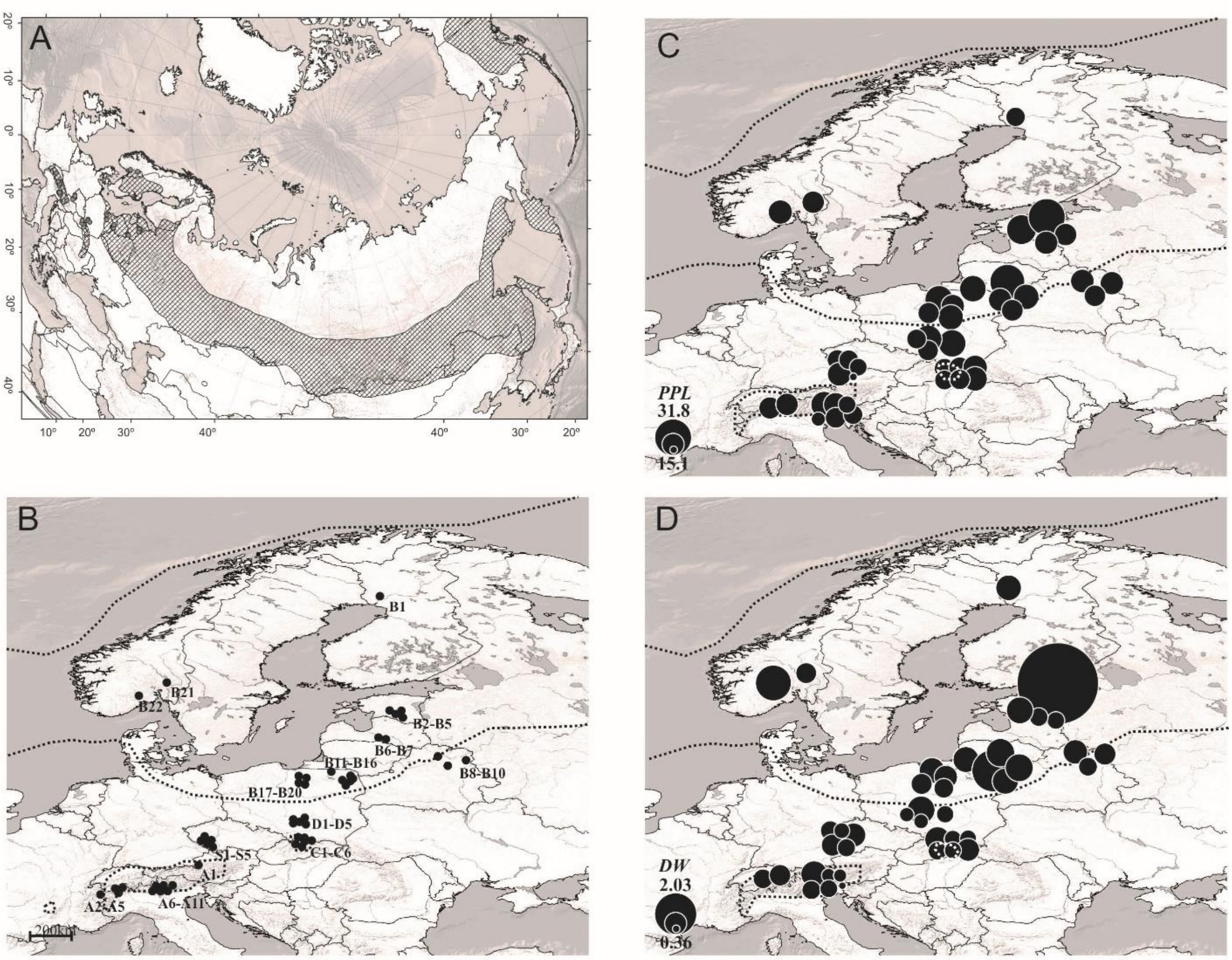


Figure 2. Isolation by distance pattern among populations of *Malaxis monophyllos* in a European range; *ns*, no significance.

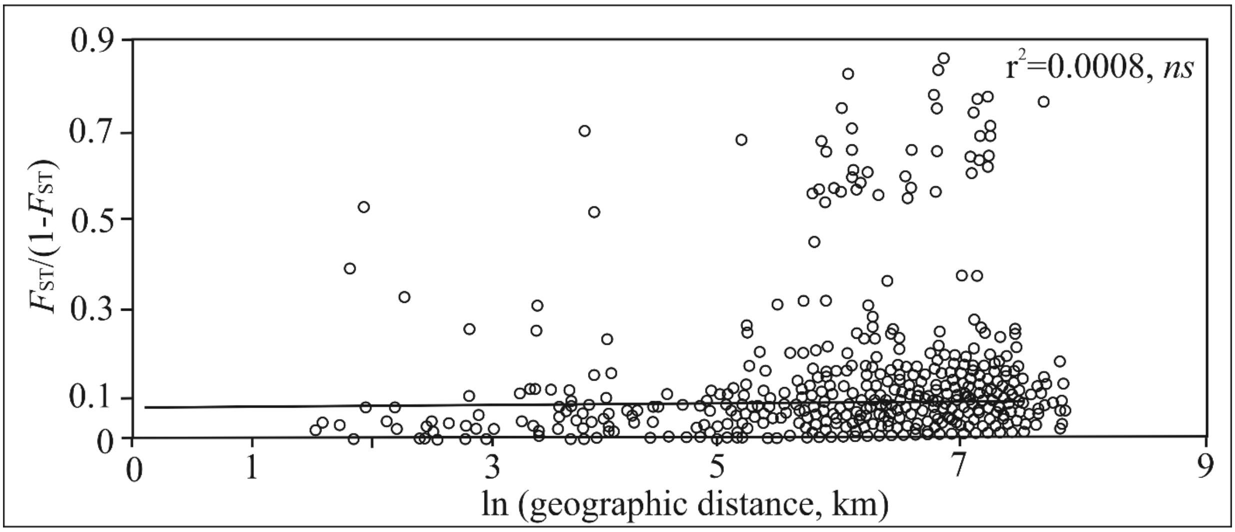


Figure 3. Principal coordinate analysis plot of 49 (A) and 47 (B) (after excluding two outlier populations: S4 and B15) *Malaxis monophyllos* populations based on AFLP data. The P values for the PC1 and PC2 axes were obtained from one-way ANOVA.

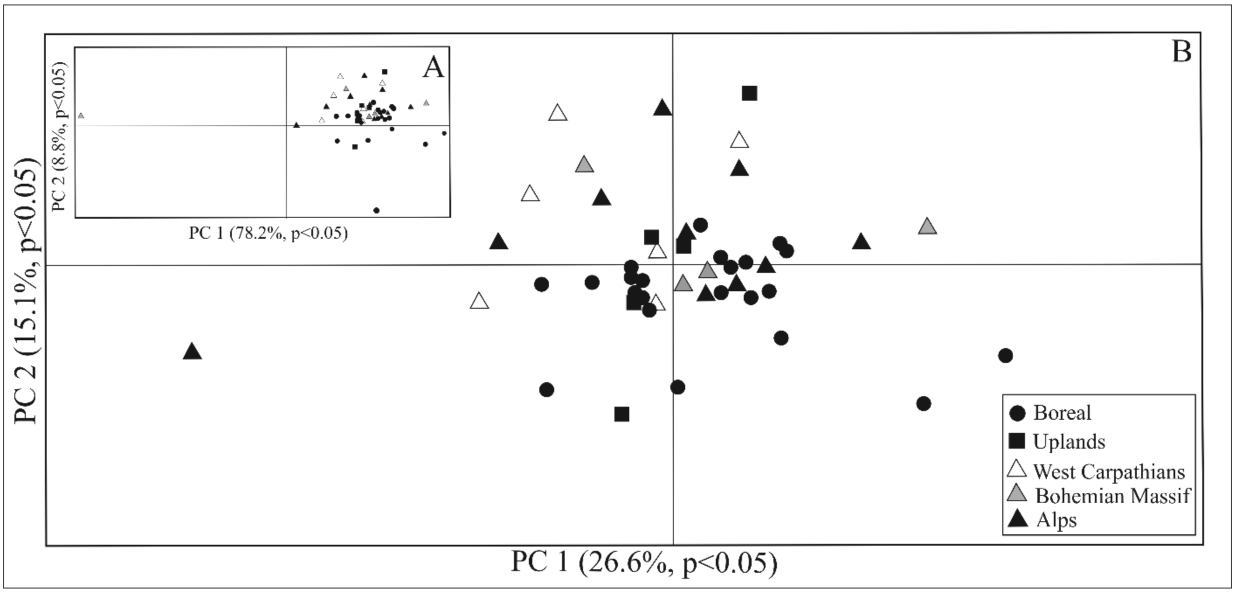


Figure 4. Neighbour-joining tree of *Malaxis monophyllos* populations, based on F_{ST} values (all bootstrap values below 35%).

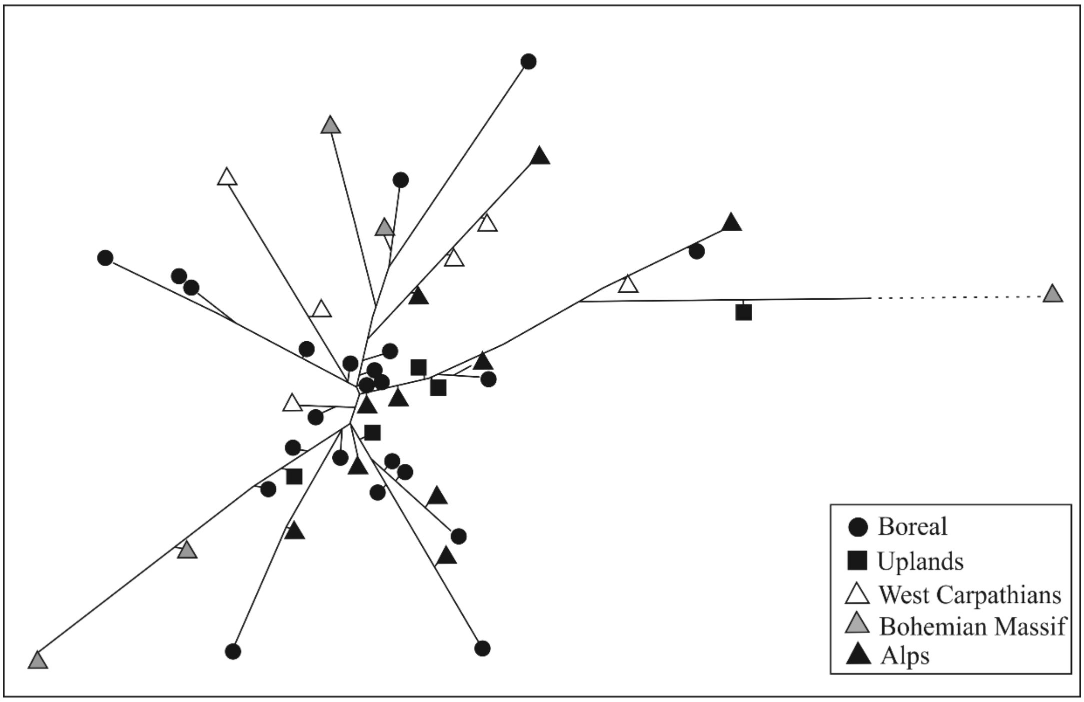
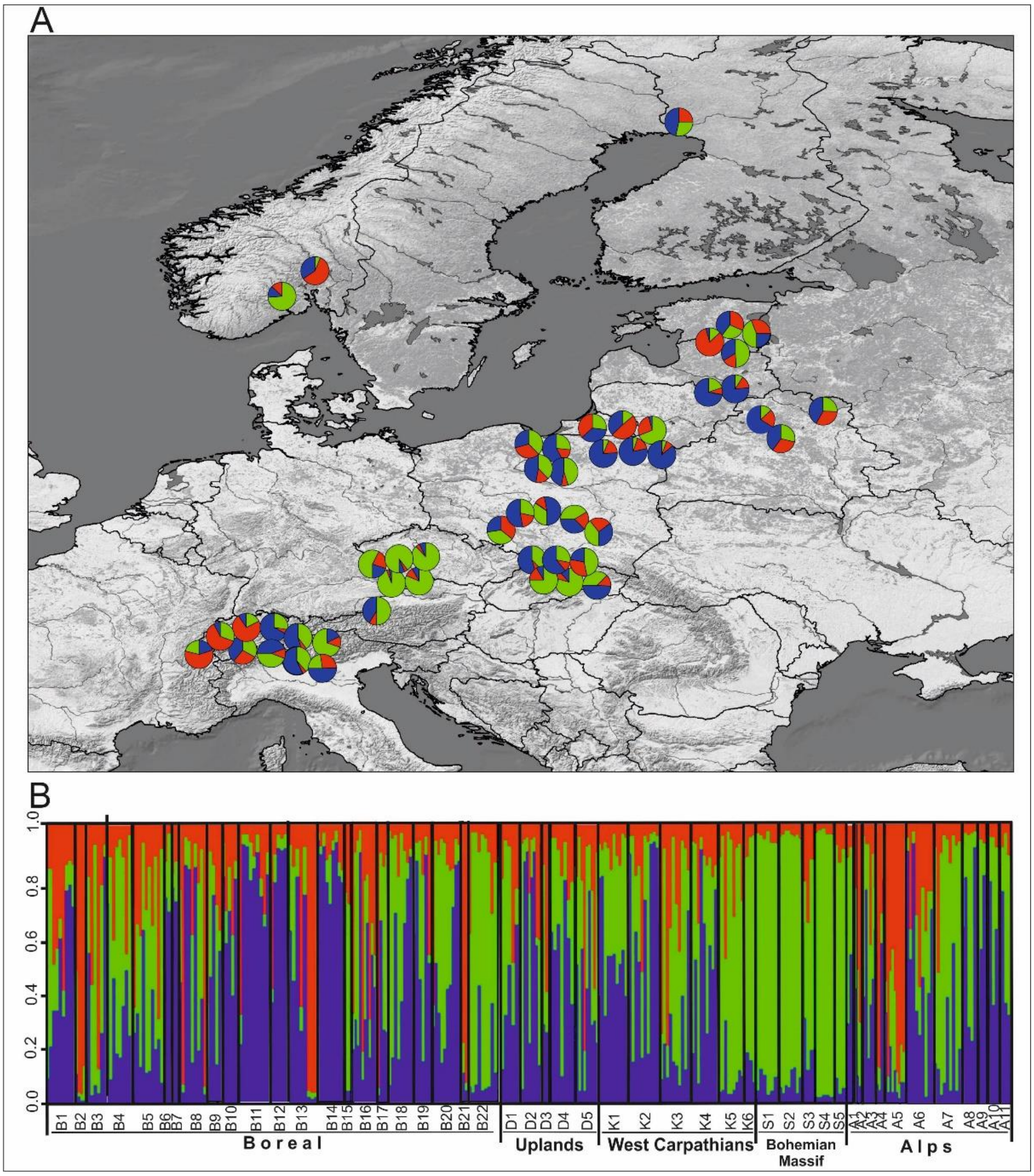


Figure 5. Geographic distribution of three AFLP genetic clusters in 49 *Malaxis monophyllos* European populations. The colours in the pie charts represent the proportional membership of individuals to definite genetic clusters (A) and the plot shows the membership probabilities of each individual in the three genetic clusters (B).



Appendices

Appendix 1. Population coding, geographical origin, population properties and characteristics of the AFLP loci for 49 European *Malaxis monophyllos* populations. P_N - number of individuals in population in the year of collection (~ approximate number of individuals), N – number of analyzed individuals, PPL - percentage of polymorphic fragments, H_j - Nei's gene diversity, DW - rarity index; PPL_3 ± SD, H_{j3} ± SD, DW_3 ± SD - characteristics calculated after resampling for three individuals per population; grey area – populations on anthropogenic habitats; - lack of data

Population code	Country/Sampling location	*Longitude* (°)	*Latitude* (°)	*Altitude m.a.s.l.*	P_N	N	PPL	H_j	DW	PPL_3 ± SD	H_{j3} ± SD	DW_3 ± SD
	Boreal											
B1	Finland/Tervola	66.99	25.06	128	~10	9	32.9	0.105	0.59	16.8 ± 2.7	0.112 ± 0.018	1.24 ± 0.44
B2	Estonia/Tatra Valley	58.26	26.71	70	~50	4	36.2	0.198	1.87	31.8 ± 1.0	0.212 ± 0.007	3.91 ± 0.56
B3	Estonia/Saunikumicro Reserve	58.01	26.14	66	~300	7	39.6	0.159	0.56	25.6 ± 2.1	0.171 ± 0.014	1.29 ± 0.31
B4	Estonia/Pühajärve	58.05	26.46	121	~100	8	34.9	0.125	0.38	20.1 ± 2.9	0.134 ± 0.019	0.89 ± 0.25
B5	Estonia/Märdi	57.99	26.43	140	~30	10	33.6	0.120	0.35	19.4 ± 2.8	0.129 ± 0.019	0.85 ± 0.18
B6	Latvia/Krizu Mire	55.77	26.41	106	20	2	-	-	0.17	-	-	-
B7	Latvia/Krizu Mire	55.87	26.27	107	4	2	-	-	0.67	-	-	-
B8	Belarus/Domzheritsy	55.26	29.70	150	46	9	35.6	0.127	0.52	20.2 ± 4.2	0.135 ±0.028	1.06 ± 0.46
B9	Belarus/Osetische	54.86	28.39	161	28	5	25.5	0.115	0.38	18.6 ± 0.9	0.124 ± 0.006	0.90 ± 0.17
B10	Belarus/Shumilinsky District	50.29	19.40	143	14	5	28.2	0.127	0.59	20.4 ± 1.8	0.136 ±0.012	1.18 ± 0.38
B11	Poland/Rospuda River Valley	53.90	22.94	120	119	9	34.2	0.118	0.55	19.9 ± 3.1	0.127 ± 0.020	1.36 ± 0.48
B12	Poland/Rospuda River Valley	53.91	22.94	120	~30	5	28.8	0.135	0.58	21.8 ± 2.1	0.145 ± 0.014	1.34 ± 0.22
B13	Poland/Czarna Hańcza River Valley	54.05	23.04	138	49	8	46.3	0.186	0.87	30.0 ± 3.5	0.200 ± 0.023	2.03 ± 0.54
B14	Poland/Kunis Lake	54.04	23.43	124	47	9	31.5	0.117	0.59	18.8 ± 2.4	0.125 ± 0.016	1.26 ± 0.25
B15	Poland/Daniłowce Lake	54.08	23.29	144	2	2	-	-	0.57	-	-	-
B16	Poland/Żetkiejmska Struga Reserve	54.35	22.61	157	53	10	40.9	0.143	0.51	22.9 ± 1.9	0.152 ± 0.012	1.24 ± 0.20
B17	Poland/Łabędzie Lake	53.74	19.52	127	4	4	28.2	0.151	0.38	24.3 ± 3.0	0.162 ± 0.020	0.95 ± 0.14
B18	Poland/Kirsznickie Lake	53.78	19.92	119	70	8	30.9	0.113	0.54	18.3 ± 3.3	0.122 ± 0.022	1.22 ± 0.39
B19	Poland/Omulew River	53.47	20.60	138	29	6	32.2	0.135	0.50	21.8 ± 3.3	0.145 ± 0.022	1.13 ± 0.22
B20	Poland/Łaźnica Lake	53.51	20.84	117	57	10	34.2	0.126	0.47	20.2 ± 1.9	0.135 ± 0.013	1.01 ± 0.29
B21	Norway/Svendsrud	59.71	9.91	270	~10	9	33.6	0.121	0.42	19.4 ± 3.1	0.129 ± 0.020	1.00 ± 0.20

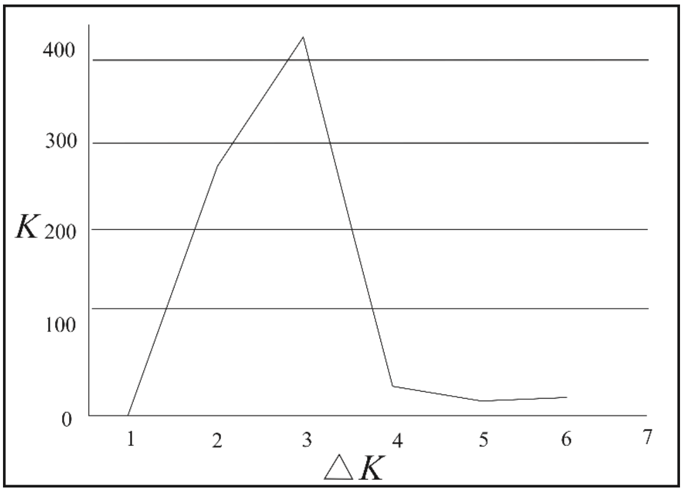
B22	Norway/Herrambstjern	60.86	10.81	242	~10	3	19.5	0.130	0.86	20.9 ± 0.4	0.139 ± 0.003	1.72 ± 0.26
Overall							**33.0**	**0.134**	**0.59**	**21.6**	**0.144**	**1.34**
	Uplands											
D1	Poland/Bukowno Walcownia	50.29	19.40	284	40	5	22.8	0.105	0.27	17.0 ± 2.8	0.113 ± 0.019	0.69 ± 0.19
D2	Poland/Sławków	50.30	19.34	289	33	9	38.2	0.131	0.58	23.8 ± 3.4	0.159 ± 0.023	1.34 ± 0.54
D3	Poland/Pogoria I	50.35	19.25	282	4	2	-	-	0.44	-	-	-
D4	Poland/Olkusz	50.29	19.49	319	~100	7	28.2	0.110	0.30	17.8 ± 2.4	0.118 ± 0.016	0.71 ± 0.21
D5	Poland/Miechów	50.46	19.97	345	152	9	38.2	0.148	0.33	23.7 ± 2.6	0.158 ± 0.018	0.84 ± 0.18
Overall							**31.8**	**0.123**	**0.38**	**20.5**	**0.137**	**0.89**
	West Carpathians											
K1	Poland/Babia Góra Massif	48.59	19.59	1021	19	10	30.9	0.109	0.54	17.5 ± 2.3	0.117 ± 0.015	1.22 ± 0.43
K2	Poland/Chochołowska Valley	49.27	19.81	1003	~100[#]	10	33.6	0.118	0.35	18.9 ± 2.4	0.126 ±0.016	0.83 ± 0.17
K3	Poland/Chochołowska Valley	49.27	19.81	1077	~100[#]	10	37.6	0.132	0.35	21.1 ± 3.0	0.141 ± 0.020	0.89 ±0.18
K4	Poland/Jaworzynka Valley	49.26	19.98	1113	~100[#]	9	34.2	0.130	0.32	21.0 ± 2.4	0.140 ± 0.016	0.82 ± 0.12
K5	Slovakia/Demänowska Valley	49.00	19.57	939	24	9	30.9	0.106	0.48	17.6 ± 1.6	0.117 ± 0.010	0.80 ± 0.11
K6	Slovakia/Šlahorka	49.23	19.08	949	11	4	19.5	0.109	0.33	16.9 ± 1.9	0.113 ± 0.013	1.10 ± 0.24
Overall							**31.1**	**0.117**	**0.39**	**18.8**	**0.128**	**0.94**
	Bohemian Massif (Šumava Mts.)											
S1	Czech/Hajnice Nature Reserve	49.07	13.90	746	46	6	24.2	0.107	0.37	17.3 ± 2.1	0.115 ± 0.014	0.95 ± 0.15
S2	Czech/Klášterec	49.04	13.73	880	19	8	27.5	0.106	0.43	17.0 ± 2.8	0.113 ± 0.019	1.06 ± 0.21
S3	Czech/SovíVrch-DolníVltavice	48.67	14.08	745	3	3	14.1	0.094	0.32	15.1 ± 0.29	0.101 ± 0.002	0.76 ± 0.07
S4	Czech/Veselka-Buk	49.02	13.83	930	10	5	9.4	0.042	0.50	6.7 ± 0.8	0.045 ± 0.006	1.16 ± 0.14
S5	Czech/HorníHraničná	48.63	14.03	820	34	4	24.2	0.129	0.35	20.5 ± 2.2	0.137 ± 0.015	0.87 ± 0.11
Overall							**19.9**	**0.095**	**0.39**	**15.3**	**0.102**	**0.96**
Alps												
A1	Austria/North Steiemark/Dachstein	47.54	13.98	880	4	2	-	-	0.21	-	-	-
A2	Switzerland/Tradoellauenen	46.51	7.90	1240	2	2	-	-	1.22	-	-	-
A3	Switzerland/UsserBündi	46.86	9.83	1430	?	2	-	-	0.37	-	-	-
A4	Switzerland/Scud	46.78	10.28	1178	?	11	33.6	0.119	0.38	19.2 ± 3.2	0.128 ± 0.021	0.95 ± 0.28
A5	Switzerland/Bergün	46.62	9.75	962	?	10	33.6	0.119	0.43	19.3 ± 2.5	0.128 ± 0.017	1.04 ± 0.20
A6	Italy/ Domegge di Cadore	46.46	12.43	826	~350	9	42.3	0.146	0.51	23.6 ± 2.9	0.157 ± 0.019	1.26 ± 0.27
A7	Italy/ Domegge di Cadore	46.45	12.43	898	~50	9	34.2	0.129	0.37	20.8 ± 2.3	0.139 ± 0.015	0.91 ± 0.12

A8	Italy/ Taibon Agordino	46.29	11.97	760	~15	5	20.8	0.094	0.34	15.2 ± 1.6	0.101 ± 0.011	0.86 ± 0.14
A9	Italy/ Alleghe	46.37	11.94	1400	~10	3	11.4	0.076	0.15	12.2 ± 0.2	0.081 ± 0.001	0.36 ± 0.01
A10	Italy/ Canaled'Agordo	46.32	11.89	1310	~15	4	21.5	0.112	0.28	17.9 ± 1.2	0.120 ±0.008	0.64 ± 0.12
A11	Italy/ Colle di Santa Lucia	46.47	12.03	1590	~30	4	18.8	0.104	0.29	16.8 ± 2.7	0.112 ± 0.018	0.68 ± 0.13
Overall							**27.0**	**0.112**	**0.41**	**18.1**	**0.121**	**0.84**

Appendix 2. The values of the Kruskal-Wallis *H* test for compared pairs of five geographical groups of *M. monophyllos* populations with the results of genetic diversity indices after the resampling procedure (PPL_3, H_{j3}, DW_3). Level of significance * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; *ns*, nonsignificant.

	Boreal	Uplands	West Carpathians	Bohemian Massif	Alps
PPL_3					
Boreal	-				
Uplands	1.76 *ns*	-			
West Carpathians	0.47 *ns*	0.53 *ns*	-		
Bohemian Massif	2.58 *ns*	0.41 *ns*	0.42 *ns*	-	
Alps	0.16 *ns*	0.01 *ns*	0.54 *ns*	3.33 *ns*	-
H_{j3}					
Boreal	-				
Uplands	1.49 *ns*	-			
West Carpathians	6.54 *	1.07*ns*	-		
Bohemian Massif	12.17 ***	4.20 *	5.97 *	-	
Alps	9.40 **	1.75 *ns*	1.48 *ns*	0.62 *ns*	-
DW_3					
Boreal	-				
Uplands	10.67 **	-			
West Carpathians	15.17 ***	1.19 *ns*	-		
Bohemian Massif	12.17 ***	0.72 *ns*	2.30 *ns*	-	
Alps	17.41 ***	2.58 *ns*	3.96 *	4.79 *	-

Appendix 3. Distribution of Δ*K* obtained for *M. monophyllos* in STRUCTURE analyses. We used 100,000 burn-in and 500,000 Markov chain Monte Carlo steps for 10 replicates for each K.



Chapter III. Demographic responses of boreal-montane orchid *Malaxis monophyllos* (L.) Sw. populations to contrasting environmental conditions

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ORIGINAL RESEARCH PAPER

Demographic responses of boreal-montane orchid *Malaxis monophyllos* (L.) Sw. populations to contrasting environmental conditions

Edyta Jermakowicz*, Emilia Brzosko

Institute of Biology, University of Białystok, K. Ciołkowskiego 1j, 15-245 Białystok, Poland

* Corresponding author. Email: edytabot@uwb.edu.pl

Abstract

In an age of changes in species' geographical ranges, compounded by climatic and anthropogenic impacts, it become important to know which processes and factors influence plant populations and their persistence in the long term.

Here we investigated dynamic and fitness components in twelve populations of *Malaxis monophyllos* (L.) Sw., situated in different geographical (regions) and ecological (type of habitat) units. Although *M. monophyllos* is a rare species, characterized by highly fragmented, boreal-montane distribution range, in last few decades it successfully colonized secondary habitats in Polish uplands. Our results indicate that *M. monophyllos* is represented mainly by small populations, which annual spatial and temporal changes might be very high, what affects the ephemeral character of these populations, regardless of the region and type of habitat. This dynamic structure, in turn, is caused by intensive exchange of individuals in populations, as well as by their short above-ground life span. Despite the large range of variation in size and reproductive traits, we can distinguish some regional patterns, which indicate boreal region as the most optimal for *M. monophyllos* growth and persistence in the long term, and with montane and upland/anthropogenic populations, due to lower reproductive parameters, as the most threatened. Although it should be considered that anthropogenic populations, despite their lower reproductive parameters and instability in the long term, present an intermediate, geographical and ecological character, therefore they may be valuable in shaping, both *M. monophyllos*' future range, as well as its potential for response on ongoing and future changes. In general, reproduction is the main factor differentiating of *M. monophyllos* populations in regions, and we can suspect that it may become the cause of the future differentiation and isolation of these populations, occurring with progressive range fragmentation.

Keywords

anthropogenic habitats; population dynamics; red listed species; reproduction

Introduction

Acquiring knowledge regarding within plant population processes and factors that influence population dynamics underpins reliable scientific knowledge based on modern conservation biology, and enables the prediction of species survival under changing environmental conditions [1]. Simultaneously, demographic changes in populations arise from the life histories of individuals which respond to selection caused by environmental changes [2–4], thus the investigation of population dynamics is also important from the evolutionary point of view.

The main globally important factors influencing plant species survival are human activity and climate changes [5–8], which are reflected at the individual and population levels [3,9–11]. The models concerning future climate changes predict huge modifications that will take place in Europe in the coming century [12,13]. The most important changes will be an increase in temperatures and the magnitude of their annual fluctuations, as well as changes in the level of precipitation [14–16]. Some habitats and some species might be more sensitive to these changes than others. Southern and mountainous species, for example, seem to be the most endangered, although northern species will also be forced to respond [17,18]. In general, plants can react to changes in their habitats by persisting in new conditions through ecological plasticity and adaptations, or they can avoid new circumstances by shifting their habitats and ranges [19–24]. As a consequence, the reactions to ongoing changes will be largely dependent on species and population properties [25,26]. The theory predicts that long life span and iterated reproduction is favored in stochastic environments [27], thus habitat-specialists with a short life span, low reproductive rates and huge demographic fluctuations are more endangered [10,28–30].

The model group of plants which, because of strong connections with other components of their habitats play a vital role in sensitivity to global and local changes and provide a warning of impending damages (acting as bioindicators), are orchids [31–33]. They very often exhibit fast responses to environmental changes, which is reflected mainly in decreases in abundances of local populations, as well in lowering their reproductive potential (e.g., [34–37]), which makes most orchid species relatively highly threatened. Moreover, high variations in demographic parameters over time and space are a property of orchids populations [38–43], and this is usually connected with differences in environmental and climatic conditions through geographical range of species, as well in differences in management [39,44–46]. Intrinsic factors, however, like genetic variability, or population age and history have also a great importance, and are highlighted as factors significantly influencing within-population processes [47]. Thus, the populations of the same species in distinct habitats may differ in structure, stages transition and in reproduction, and therefore in persistence in the short and long term.

To enrich knowledge about population dynamics in distinct geographical and ecological units, in the context of declining range, we investigated populations of the orchid *Malaxis monophyllos* (L.) Sw. This rare, boreal-montane species is represented mostly by small populations that naturally occupy calcareous fens, wet meadows, spruce and alder forests [48,49]. Despite its rarity and progressive range fragmentation in the last few decades, *M. monophyllos* has successfully colonized anthropogenic habitats in the uplands in Poland [50,51], as well as in other parts of its geographical range [48]. In general, the decrease in the abundance and reproduction of orchids as a consequence of human activity is a commonly described issue [52], although the opposite reactions to disturbance, when they colonize anthropogenic habitats, is also identified in different parts of the globe [50,53–57]. The demographic processes operating in these secondary populations are still relatively unknown, and they may be of particular value in terms of preserving the rarest species of plants [58]. Our earlier investigations revealed the significance of upland populations in shaping *M. monophyllos*' large-scale genetic structure [59]. Moreover, the extinction of *M. monophyllos* natural populations, their ephemeral character, and instability in the long term [51], with poor information regarding their life history highlight the need for detailed studies on the mechanisms and processes shaping population viability. Although the demography of orchid populations are widely discussed in many different contexts, data concerning northern species from this group are sparse [60–62] and require a deep insight, as they are particularly important and valuable in the context of the decrease of global biodiversity and its conservation.

Thus, the main aim of this study was to answer the following questions: (***i***) what is the range of the demographic variation in *M. monophyllos* populations in different parts of its geographic range in Poland and under different habitat conditions, and (***ii***) what is the potential of populations from anthropogenic habitats to persistence in the longer time scale? Thus, we hypothesize that (***i***) habitat and climatic differentiation can modify life strategies at the individual level, which is manifested in regional patterns of population structure and dynamics; (***ii***) *Malaxis monophyllos* individuals from the boreal part of the geographical range are characterized by higher levels of fitness than those from mountains, which exist under more severe environmental conditions, or than populations from uplands, which exist in anthropogenic habitats.

Material and methods

Study species

Malaxis monophyllos (L.) Sw. has a fragmented distribution, including boreal Eurasia, the northwest region of North America, and Central European mountain ranges [48,63,64] Almost everywhere it is a red-listed species [49,65,66], which despite its wide phytocenotic scale, prefers moderately humid, calcareous soils with at least moderate moss cover. In Poland it occurs both in boreal parts of the country and in the mountains [50,67,68], where it occupies different types of habitat (Tab. 1).

Malaxis monophyllos is a short-lived, inconspicuous, green orchid that reproduces exclusively by seeds and forms one basal pseudobulb. The average height of flowering stalks is approximately 20 cm ([48] and authors' observation). Flowering takes place from June to August; whereas in the south of Poland flowering starts about two weeks later (authors' observation). Fruiting occurs from July to August. *Malaxis monophyllos*

Tab. 1 Population coding, geographical origin and habitat characteristics of twelve investigated *Malaxis monophyllos* populations.

Population code	Population locality	Altitude (m a.s.l.)	*N* (min–max.)	Type of plant community
B1	Rospuda River Valley (East Baltic Lake District)	120	44–156	Alder–willow thickets (*Alnetea glutinosae*)
B2	Czarna Hańcza River Valley (East Baltic Lake District)	138	37–68	Boreal spruce bog (*Vaccinio-Piceenion*)
B3	Kunis Lake (East Baltic Lake District)	124	47–189	Alder–willow thickets on the peat layer (*Alnetea glutinosae*)
B4	Kirsznickie Lake (Masurian Lake District)	119	9–70	*Carex elata* community with the peat layer (*Magnocaricion*)
B5	Łaźnica Lake (Masurian Lake District)	117	0–83	*Picea abies* and *Betula pendula* forest in organic soil (*Vaccinio-Piceenion*)
S1	Bukowno Walcownia (Silesian-Krakow Upland)	284	40–46	*Pinus sylvestris* cultivation with succession of coniferous forests species in undergrowth (*Vaccionio-Piceetea*)
S2	Sławków (Silesian-Krakow Upland)	289	33–127	*Prunus spinosa* thickets on railway embankment (*Rhamno-Prunetea*)
S3	Olkusz (Silesian-Krakow Upland)	319	646–795#	*Pinus sylvestris* and *Betula pendula* cultivation on reclaimed heap with succession of coniferous forests species in undergrowth (*Vaccinio-Piceetea*)
S4	Miechów (Małopolska Upland)	345	152–275	*Fagus sylvatica* forest on railway embankment (*Fagion sylvaticae*)
C1	Babia Góra Massif (West Beskids Mts)	1021	19–38	*Picea abies* renewal on roadside slope (*Vaccinio-Piceenion*)
C2	Chochołowska Valley (Western Tatra Mts)	1003	22–34#	*Picea abies* forest along the montane stream (*Vaccinio-Piceenion*)
C3	Jaworzynka Valley (Eastern Tatra Mts)	1113	81–94#	Montane meadow (*Molinio-Arrhenatheretea*)

N – number of individuals in population (minimal and maximal observed number); # – number of individuals in permanent plots.

is presumably an obligatory outcrossing plant, with minute, green flowers that are adapted to pollination by small insects, i.e., fungus gnats from the Mycetophilidae family [48,69]. The life cycle of *M. monophyllos* is poorly known, and according to Vekhrameeva [48] takes approximately 20 years, including a subterranean phase between germination and the first appearance above-ground (it lasts approximately 8 years). The first flowering occurs an average of 10 years after germination, and can be repeated for up to 4 seasons. In *M. monophyllos* simultaneously to other orchids, phenomenon of dormancy (not show up of above-ground shoots) can occur. In different species of orchids dormancy can last from 1 to even 5 subsequent years, depending on environmental factors and weather [39]. Furthermore, in *M. monophyllos*, like in other species of orchids, both flowering can be disrupted by non-flowering stage, as well non-flowering one can be extended if the environmental conditions are not optimal. In case of *M. monophyllos* after flowering period, the plant dies without experiencing a senescent phase [48].

Study populations

We studied twelve populations of *M. monophyllos* situated in three regions of Poland, which differ in terms of climate conditions, for example in the level of precipitation (Tab. 1, Fig. 1). Boreal populations are located in the East Baltic Lake District (population B1, B2, B3) and in the South Baltic Lake District (Iława Lake District, B4, B5). The montane populations are situated in Beskid Żywiecki (C1), in the West (C2) and East (C3) Tatra Mountains. Populations from boreal and montane regions exist mainly in natural habitats, and only some of them in seminatural habitats. The other four populations studied are located in the Małopolska and Silesian-Krakow Uplands, all in anthropogenic habitats, but with different levels of disturbance (S1, S2, S3, S4; Tab. 1). In further analyses upland populations will be described in the ecological context as anthropogenic habitat populations, with the rest of the analyzed populations as natural ones.

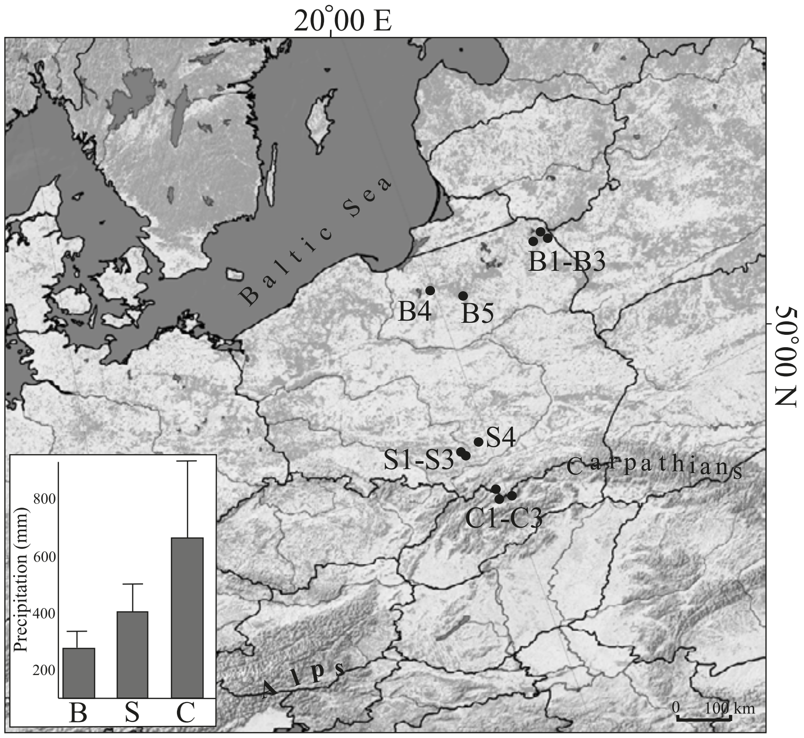

Fig. 1 Distribution of twelve study populations of *M. monophyllos* in Poland. Codes correspond with populations described in Tab. 1. Average monthly precipitation in March–June period in particular regions of Poland: B – boreal; S – upland; C – mountainous (Carpathians).

Data collection

In 2008, we began investigation of two *M. monophyllos* populations from northeast Poland (B1, B2). In subsequent years, ten more populations were included in the studies. In general, populations were investigated for 6 (2 pop.), 3 (7 pop.), or 2 (3 pop.) years. To measure population sizes (*N*) and their dynamics, in every year we counted all individuals in the population or, in the case of the biggest and most dispersed populations (S4, C2, C3), individuals on permanent plots.

In four populations from different regions and habitats (B1, B2, S2, C1), for which we were able to collect the most complete data sets (min. 3 years of observations of individual shoots), we performed analyses which allowed the determination of the basis of populations dynamics. During the first year of observation (t) we individually labeled and mapped every shoot of *M. monophyllos* to follow its history in subsequent years (t+1, t+2, …). Additionally, labeled individuals were classified according to their developmental and reproductive status into three life-stage classes: juvenile (J), nonflowering (NF), and flowering (F). This enabled us to describe the history of individuals, the rate of exchange of individuals in populations, as well as to quantify mortality and dormancy in different habitat conditions, as well to enrich knowledge regarding the life cycle of the studied species and to specify the spatio-temporal dynamics of their populations.

To estimate fitness components in all the investigated populations we assessed the reproductive potential, measured by the participation of flowering individuals (F). Additionally, as the size of individuals is one of the most important characteristics of fitness, for all the reproductive individuals we counted the numbers of flowers per inflorescence (F_{inf}), and we measured the height of shoots (H_F). Finally, as additional measures of reproductive potential, we assessed the level of fruiting (FR; ratio of fruit number to flower number). Reproductive success was assessed by the proportion of juveniles (J) in the total number of shoots in the population (as juveniles we took shoots whose leaf size did not exceed 1 cm in length).

Data analysis

We used different approaches to define the spatio-temporal dynamic of *M. monophyllos* populations in different parts of the geographical range and habitat conditions. First of all, on the basis of changes in the number of individuals in populations, we assumed a threshold of 20% change as an indicator of significant changes in population size. Changes in the number of individuals between 20% and 50% were considered moderate, and changes >50% as high. Additionally, all *M. monophyllos* occurrences mapped in the field were digitized in a vector map. Then the density of individuals in four populations was calculated with a kernel density tool in Spatial Analyst, ArcGIS 10.0 [70] using the kernel function (K_D). Each study plot had different dimensions, and therefore the kernel function parameters were set using the same assumptions with respect to the output raster extent. Mean and standard deviations were calculated for the whole output raster extent; the area varied by season, depending on the spatial distribution of individuals. The higher values of K_D indicate larger aggregations of *M. monophyllos* individuals.

Principal component analyses (PCA) were calculated using all characters describing fitness components in populations: F, F_{inf}, H_F, FR, J. PCA analyses, based on the correlation matrix, were performed in order to get insight into the overall variation on species and regional levels. The relationship between the original parameters (means per region) and the (varimax rotated) PCA scores was examined by means of rotated component loading.

Subsequently, standard analyses using the Kruskal–Wallis H test were performed to assess differences in the mean values of the most important fitness components for *M. monophyllos* populations in regions and types of habitats (analyses of the differences between populations were also made, but for clarity of results are not shown in this paper). The normality of the data was evaluated prior to the analyses, using the Shapiro–Wilk test. Spearman range correlations (*R*) were applied to measure the strength of the relationship between (***i***) individual fitness components and (***ii***) between fitness components and regions. Finally, we assessed the influence of climatic factors on populations' properties (N, F, F_{inf}, H_F, FR, J). For this purpose we used populations from which we had at least three years of observations. The data from the nearest meteorological stations were used in analyses (IMGW annual reports 2008–2013 for Suwałki, Stary Folwark, Sejny, Prabuty, Szczytno, Katowice, Jabłonka, and Polana Chochołowska). In our investigations we used precipitations in periods of year important in *M. monophyllos* ontogenesis [71]: March–June and September–December periods in the year preceding year t (t−1), and for March–June period of year t (year for which we analyzed demographic parameters). The effects were evaluated using Spearman's rank correlation coefficient in STATISTICA PL. ver. 10 [72] software packages.

The life stage transition, as well as rates of mortality and dormancy were assessed in the B1 and B2 populations, due to the largest data sets. We defined *M. monophyllos* as a dead when it did not appear above ground for two subsequent years. This assumption was made after preliminary analyses of dormancy, which revealed that only one individual among the 64 included in the analyses was dormant for two consecutive years.

Results

Population sizes and dynamics

Twelve populations of *M. monophyllos* differed considerably in size (Fig. 2a), ranging from 19 (C1 population in 2009) to almost 800 individuals (S3 population in 2010). However, our results suggest that *M. monophyllos* is mainly represented by small populations, which even at the peak of abundance rarely exceed one hundred individuals. In most cases population sizes changed significantly during the observation period (Fig. 2b). The percentage differences in the number of individuals between subsequent years exhibited moderate (B2, S4, C1, C2) or large changes, whose absolute values approached or exceeded 100% (B1, B4, B5, S2) or even 300% (B3). The highest values refer to increasing population abundance. Declines usually occurred at the 30–50% level, and only one time reached 80%. In 7 out of the 27 cases (one case is one year in one population) inter-annual differences were small (<20%; Fig. 2b).

All four populations, investigated in details, were characterized by an aggregative spatial structure, but the sizes and densities of groups of shoots in populations, described by the mean values of the kernel function (K_D), varied between populations, and fluctuated from year to year (Fig. 3). As a consequence, B2 and C1 represented a dispersed (mean $K_D = 0.012$ and $K_D = 0.082$, respectively) pattern, and populations B1 and S1 exhibited a more concentrated pattern of spatial structure (mean $K_D = 0.63$ and $K_D = 0.66$, respectively). Additionally, values of K_D can change radically from year to year, which indicates that the spatial structure of *M. monophyllos* populations, regardless of the region or type of habitat, can also be very dynamic (Fig. 3).

Fitness components

PCA. PCA reduced the total set of partly intercorrelated variables to two uncorrelated principal components including size and reproductive traits (Tab. 2). Together the two principal components explained 76.48% of total variation between regions. The first axis explained 58.24% of the variation and the second axis 18.24%. The three groups of populations represented regions were not clearly separated on the scatterplot (Fig. 4), and the particular populations represented a partly overlapping range of size and reproductive traits. The first PCA axis was most highly influenced by size parameters (H_F and F_{inf}), and less by frequency of flowering individuals (F), while the frequency of juveniles (J) and fruit set (FR) were most strongly related with the second PCA axis.

Size structure. Among the traits analyzed, study regions differ with respect to fitness components, measured by the size of individuals, particularly in the mean height of flowering shoots (H_F: H = 14.40, *df* = 2, *p* = 0.001). We noted the lowest individuals in mountainous populations where they ranged from 7.4 to 17.7 cm, with median values 14.7 cm, moderate in uplands/anthropogenic populations, ranging from 11.2 to 27.6 cm, with median values 17.6 cm, and the highest in the boreal region, from 14.7 to 27.3 cm, with a median of 21.4 cm (Fig. 5a).

Reproduction. We found no significant differences in the frequency of flowering individuals between regions (H = 0.90, *df* = 2, *p* = 0.64). In general, the highest frequency of flowering individuals was found in boreal populations (F = 65.3% and 62.2%), which ranged between 12.6–65.3%, while in uplands/anthropogenic and in mountains it appeared at the lower level, between 15.7–38.5% and 12.5–45.4%, respectively. Despite differences in the number of flowers per inflorescence between regions (means = 44.5 ±10.9; 44.7 ±12.9, 34.2 ±10.3 for boreal, upland and mountane region respectively), we found no statistically significant differences between them (F_{inf}: H = 4.81, *df* = 2, *p* = 0.09; Fig. 5b).

The second parameter which reflects reproductive potential in a given environmental conditions is the level of fruiting (FR), which strongly varied between regions (H = 15.49, *df* = 2, *p* = 0.000; Fig. 5c). The fruit set was significantly higher in the

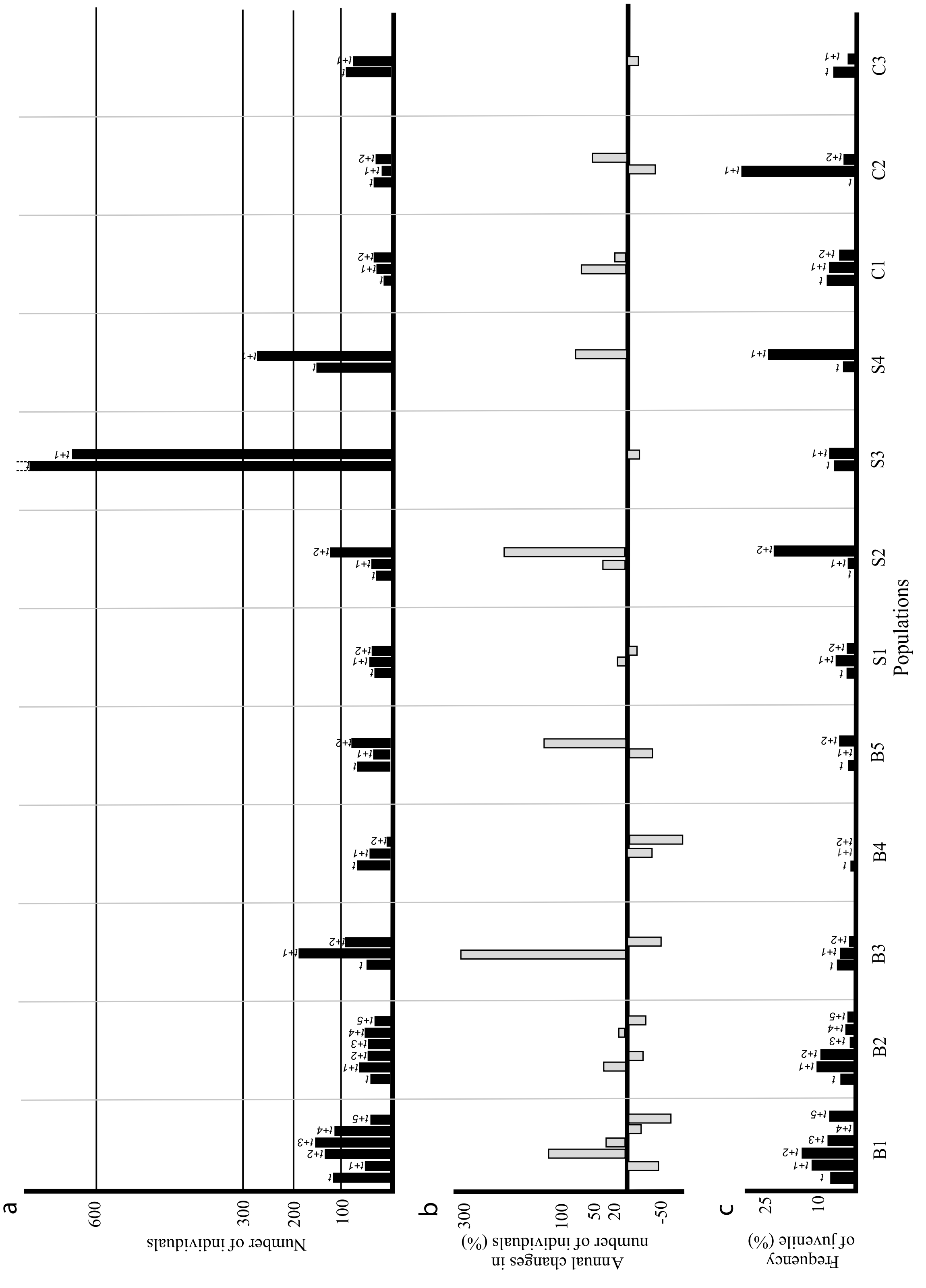

Fig. 2 Populations size changes (**a**), percentage difference in the number of individuals between two subsequent years (**b**), and frequency of juveniles (**c**) in twelve investigated populations (t – first year of observation; t+1, t+2, … – subsequent years of observation).

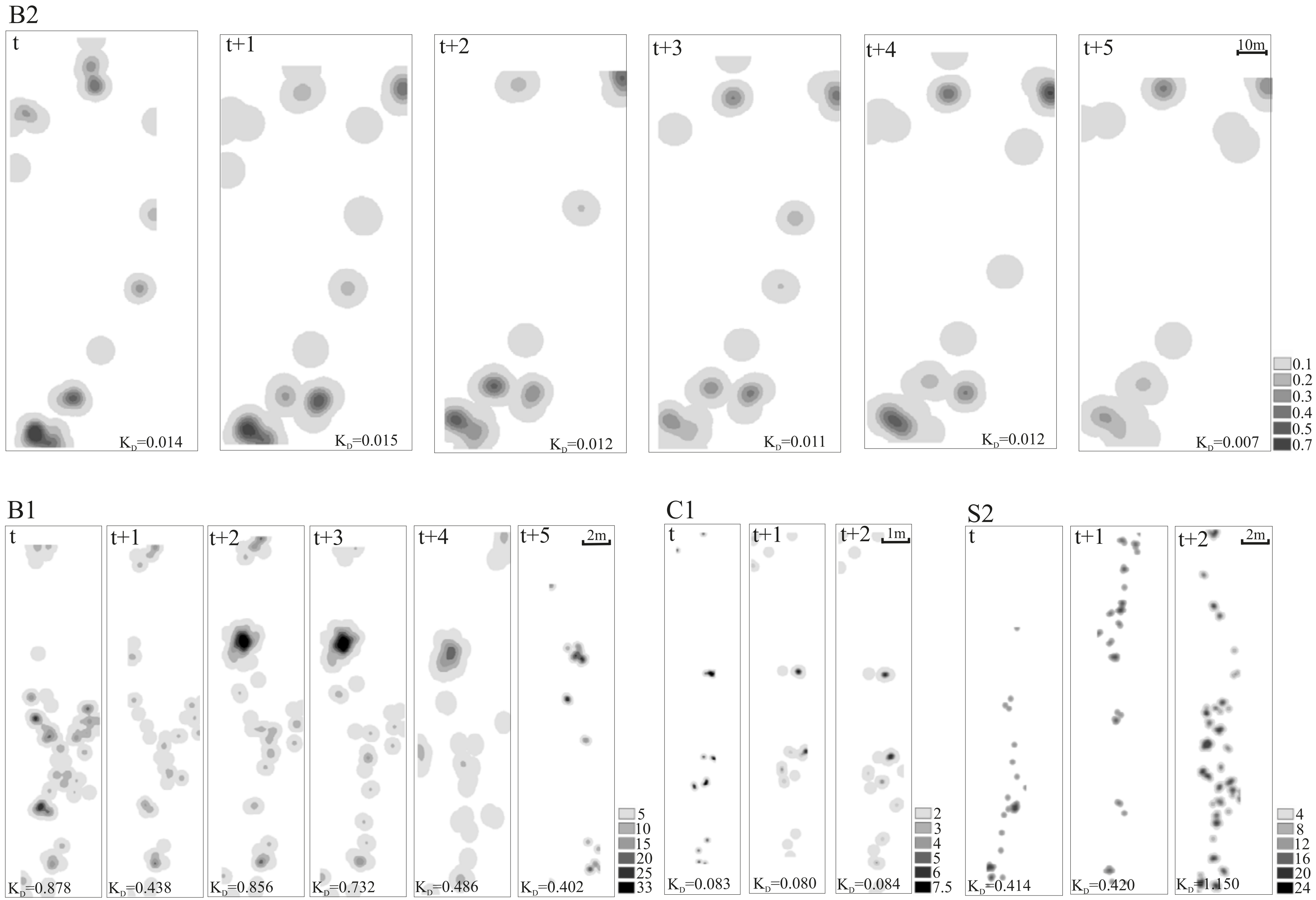

Fig. 3 Changes in spatial structure of *M. monophyllos* populations during subsequent years of observation (legend: average values of the kernel density estimator K_D; gradients show densities of individuals estimated by kernel density function).

Tab. 2 Principal component loadings of the measured size and reproductive characters (after varimax rotation). Loadings given in bold line show the highest correlation between original values and principal components scores.

	Factor loadings	
Variables	**1**	**2**
F	**−0.44**	0.34
H_F	**−0.53**	−0.29
F_{inf}	**−0.51**	−0.19
FR	−0.39	**−0.42**
J	0.34	**−0.77**
Eigenvalue	2.91	0.91
Cumulative variance (%)	58.24	18.24

F – participation of flowering individuals; H_F – height of shoots; F_{inf} – number of flowers per inflorescence; FR – level of fruiting; J – participation of juveniles.

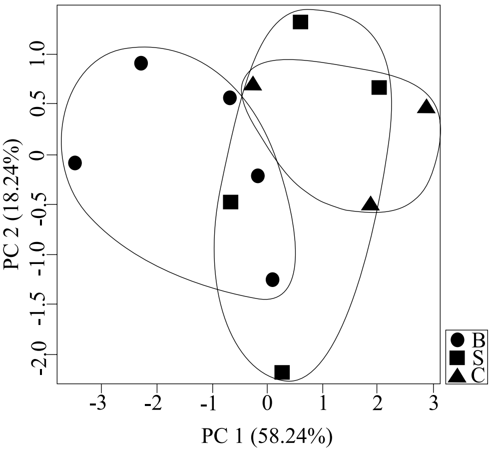

Fig. 4 Scatterplot presenting the result of principal component analysis (PCA) based on five morphological characters for twelve *M. monophyllos* populations from different regions (B – boreal; S – upland; C – mountainous).

boreal group, where the average was 22.53% (±11.51), it range from 7.5% to 46.6%, with median values 20.8%. In the remaining populations, both from uplands, as well as from mountains, fruiting rarely exceeded 10% (except in one year in population S4). Thus, it ranges from 0% to 27.9% in uplands (median = 2.6%) and from 1.5% to 11.9% (median = 7.1%) in mountains. Additionally, in boreal populations, the majority of flowering individual set fruits, while we found that fruits appeared in less than 50% of the flowering shoots in upland/anthropogenic and montane populations. The number of fruits, in turn, was correlated with the number of flowers per inflorescence in all analyzed cases, although in upland/anthropogenic populations the strength of this relationship was weaker ($R = 0.30$, $p < 0.001$) than in the boreal part and mountains ($R = 0.51$, $p < 0.001$). We also found that fruiting level depended on population size, but only in the case of boreal populations ($R = 0.45$, $p < 0.05$).

In terms of the frequency of juveniles (J), despite the fact that this is one of the traits which distinguished regions from each other in PCA analyses, there were no statistically significant differences for average values between the investigated regions ($H = 1.54$, $df = 2$, $p = 0.46$), and in the majority of populations J rarely exceeded 10% (average J for boreal populations = 5.11%, uplands/anthropogenic = 7.58%, and mountains = 8.82%; Fig. 2c).

The correlations between climatic conditions and fitness components were found only in montane populations, where the number of individuals in a given year, as well average number of flowers per inflorescence, was positively correlated with the mean monthly precipitation during October–December period in year t−1 ($R = 0.714$, $p = 0.047$ and $R = 0.857$, $p = 0.007$, respectively). Additionally, in mountains there was a negative relationship with the mean monthly precipitation in March–June period in year t−1, and frequency of juveniles in year t ($R = -0.718$, $p = 0.042$).

Dormancy, mortality, and life cycle. The lifespan of above-ground *M. monophyllos* individuals, analyzed in the two populations with the longest data sets, lasted from 1 to 6 years. Individuals in population B2 were characterized by longer life spans (3.5 years) than those from population B1 (2 years). The duration of the non-flowering stage (NF) was the same in both populations (1–4 years), while the duration of the generative stage differed between populations, and took 1–2 years in population B1 and 1–4 years in population B2.

We also found considerable differences between the B1 and B2 populations in regard to life stage transitions (Fig. 6). In population B1, for which we also found the highest annual changes in abundance, 46.9% of non-flowering individuals were in this stage during the observation period, while in population B2 more than 40% of individuals prolonged the generative stage. Populations did not differ in the frequency of individuals which transitioned to the flowering stage after the non-flowering stage, but almost twice as many individuals in population B1 returned to the non-flowering stage after flowering (Fig. 6).

The observed patterns of the life cycle in the two investigated populations are affected mainly by the rates of dormancy and mortality, which significantly differed between these populations. As a consequence of mortality, 94% of individuals in population B1 and 75% of individuals in population B2 observed during the first year (t) disappeared during the investigation period. In population B2, individuals died more frequently after flowering (60%); in population B1 after the non-flowering stage (67%). Dormancy was noted in 11% and 24% of cases in the B1 and B2 populations, respectively. In the majority of cases dormancy last only one year, and more frequently appeared after the non-flowering stage in B1 and after flowering in the B2 population.

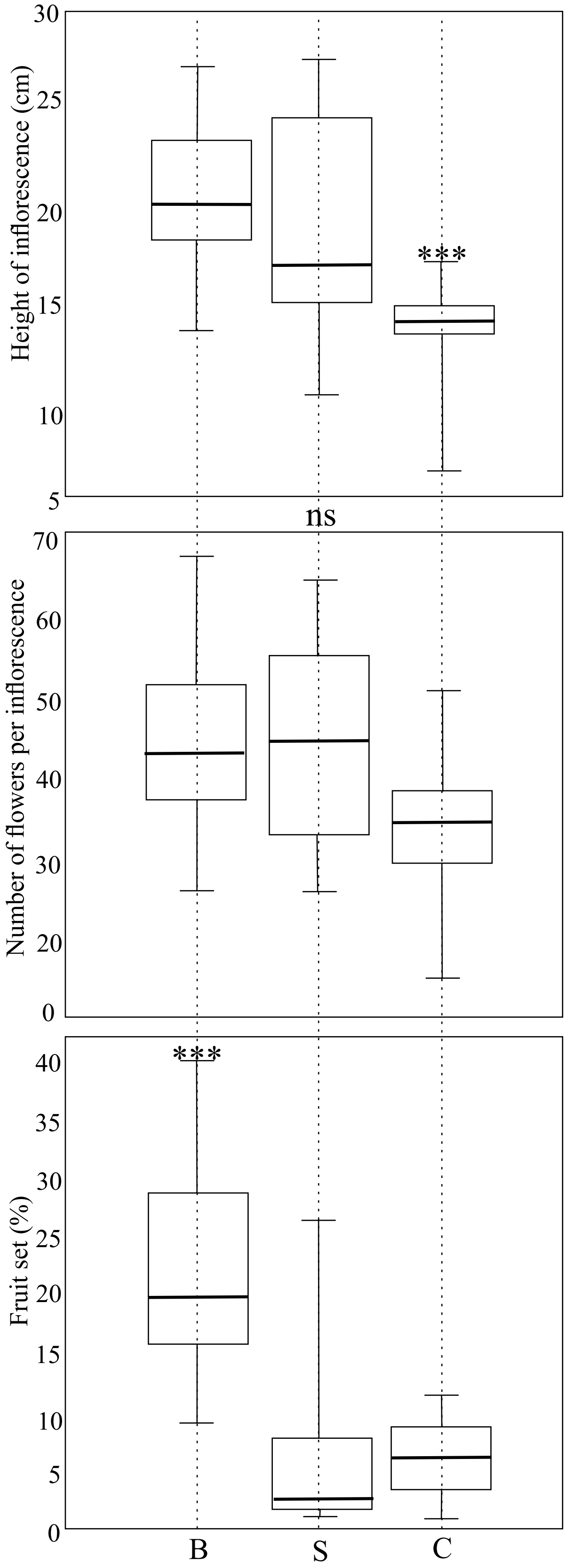

Fig. 5 Median values of the height of flowering shoot, average number of flowers per inflorescence, and the median values of the proportion between the total number of flowers and the flowers that developed into mature fruits in the populations of *M. monophyllos*, grouped according to geographical regions (B – boreal; S – upland; C – mountainous). *** $p < 0.001$; ns – not significant differences (Kruskal–Wallis H test).

Discussion

The literature data point out that *M. monophyllos* is mainly a habitat-specialist that is represented by isolated, small populations, existing in naturally fragmented habitats due to the heterogeneity of suitable environments [48,64]. Recently, natural fragmentation and isolation have intensified due to human activity, which increases the importance of ongoing detrimental processes in isolated populations.

Orchid populations are known for their highly temporal and spatial dynamic structure [39,41,42]. In *M. monophyllos* populations, in every region and type of habitat, year-to-year changes are exceptionally large, both in abundance (up to 300% differences in abundance), as well as in spatial structure (K_D = 0.414–1.150). Oostermeijer and Hartman [73], for example, observed large population dynamics for another orchid connected with swampy habitats, *Liparis loeselii*, and concluded that it depends mainly on the colonization of new, suitable habitats. Such an explanation, consistent with the metapopulation model, might be highly probable in the case of *M. monophyllos*, especially when it occurs in areas (i.e., river valleys, along mountain streams) where suitable habitats are patchy in character. This is also in accordance with Hanski [74], who stated that the metapopulation is a kind of network of colonies, which persists as the result of a balance between the extinction of individual colonies and dispersal between available habitats. This is expressed in the highly variable spatial structure of *M. monophyllos* populations, which may also reflect the distribution of sites suitable for germination [41]. The increase in abundance is mainly determined by the increase in the densities of groups of individuals, as well as by the colonization of new fragments of available habitats. The main mechanism causing changes in the abundance of *M. monophyllos* populations is an intensive exchange of individuals (94% in B1 and 75% of individuals in the B2 population observed in the first year were absent in the last year) resulting from its relatively short above-ground life span, lasting presumably from one to six years (on average 2.8 years). Some of the *M. monophyllos* populations seems to be extremely dynamic or even ephemeral in character, which may suggest the influence of local environmental disturbances, that influence process of germination and flowering [42,71].

Population sizes and their dynamics are greatly influenced by the process of reproduction. Fruiting, the common measure of the level of reproduction in orchids, significantly varied between *M. monophyllos* populations in regions and types of occupied habitats. In general, in the case of *M. monophyllos*, the fruit set is relatively low, as with many other orchids that are nonrewarding, self-incompatible and pollinators dependant for the fruit set [75,76]. Exceptionally low levels of fruiting were noted in anthropogenic populations of *Malaxis*, located in the Polish uplands, which is also reflected in studies on other species of orchids in secondary habitats. Pellegrino and Bellusci [77] noted an almost seven

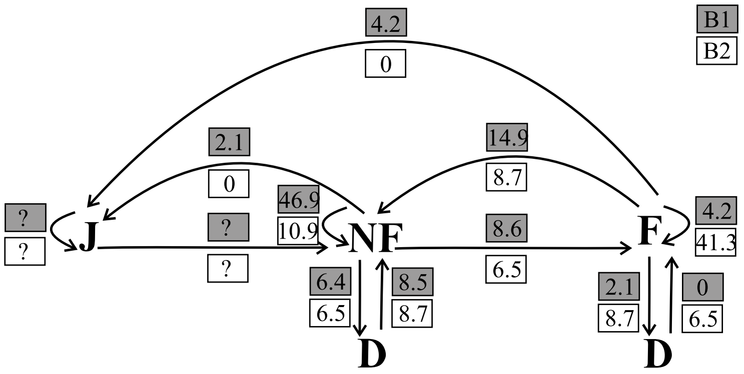

Fig. 6 Life-stage transitions in two *M. monophyllos* populations (B1, B2 – according to Tab. 1). J – juvenile stage; NF – nonflowering stage; F – flowering stage; D – dormancy; the values are given in percentages of all shoots included into analyses in a given population.

times lower fruit set in anthropogenic populations of *Serapias cordigera* in Italy than in its populations from natural habitats. In population of *Oncidium ascendens* from well-preserved rainforest from Mexico fruit production was likewise almost two times higher than in populations from synanthropic habitats [78]. Among the possible explanations for the variation in fruiting between natural and anthropogenic populations are the differences in pollinators' efficiency [79–81]. These are probably, young anthropogenic populations, that did not establish sufficient relationships with important symbionts (pollinators) which are presumably connected with wet environments [69,82]. We observed a divergence between the high level of male success (high rate of pollinia removal) and low fruit set in anthropogenic populations of *M. monophyllos*. It might suggest that a high proportion of pollinia are lost, and pollen discounting takes place [83], what may indicate that insects visiting flowers in anthropogenic populations are not pollinators. Despite low reproductive potential (fruiting) in upland populations, reproductive success (frequency of juveniles) was similar to populations from other regions. Juveniles appeared in anthropogenic habitats systematically (similar to boreal and montane populations), at least at the first stages of colonization, which enables the persistence of these populations in time, but also indicates the successional status of populations as the key to interference about population prospects. Although our data are too limited to clarify this issue, we can make some assumptions about the processes that cause instability over longer time, as reported by some authors [51]. Firstly, the emergence through a demographic explosion may suggest many neighboring sources of colonization of anthropogenic sites. Secondly, the decrease in abundance during subsequent years after the first colonization might be a symptom of disappearing external sources of seeds, an insufficiency of internal source (low fruit set). Decline of suitable sites for germination during progressive succession is also very possible. Simultaneously, the lower level of fruiting in the uplands populations may be compensated by bigger fruits [83]. Such a trade-off between the number of fruits and their size (and probably seed count) could be considered an adaptive strategy under resource limitation (pollinators limitation), and could be the confirmation of species potential for modifying its life strategy traits in disturbed habitats. In general, all these issues, in the case of anthropogenic habitats, are crucial for *M. monophyllos* populations' preservation in the long term, and require further investigation, particularly concerning the mode of pollination and breeding system.

As for many terrestrial orchids, the size of plants is an important trait that determines reproductive success [84,85]. This may indicate that the best conditions for the development of *M. monophyllos* populations in Poland are in the boreal region, which comprises the majority of its geographic range. However, individuals in some anthropogenic populations were also large, which suggests that these habitats are also suitable for *M. monophyllos*, due to unused resources or lack of competitors. In mountains (i.e., above 1000 m a.s.l.) environmental conditions are more severe (higher annual temperature amplitudes, shorter growing season) for plant growth and reproduction, which is reflected in, e.g., the smaller sizes of *M. monophyllos* individuals. Other species of plants were also smaller and have lower values of reproductive potential in montane populations, than those from lower altitudes [86,87]. The population size or floral display measured by the size of the inflorescence, are pointed to be crucial for fruiting level [76,88–92]. Although we found positive correlations between fruit set and population size only for natural populations, the relationship between fruiting and the number of flowers per inflorescence was statistically significant for all study regions.

Due to the strong fragmentation of the European part of *M. monophyllos* range, we can consider that it has two main areas of distribution: boreal and montane. Therefore, upland populations could be described as intermediate or marginal ones, especially in the ecological sense, and because of that they may have special evolutionary significance [93,94]. In such populations different traits and/or relationships with other components of the environment can depart from typical situations [41,95]. In consequence, they could be the source of increasing adaptive phenotypic plasticity because they are exposed to harsh conditions, which may influence the selection of individuals who are adapted to such environments [96,97]. Moreover, *M. monophyllos* anthropogenic populations have a special role, because they could be considered as a kind of bridge outside the present geographical distribution, and thus its impact on its future geographical range can be substantial.

In the light of recent and ongoing climatic changes, an important problem is their influence on the properties of rare species' populations and their maintenance in the long run. Despite growing interest in this problem, empirical evidence is relatively scarce. In the case of orchids, the influence of climate has most often been studied in the context of reproductive traits, which both in case of terrestrial and epiphytic orchids suggest precipitation as the main factor [98,99]. Connections of *M. monophyllos* with wet environments, together with an increase in large-scale precipitation in northeast Europe in the coming decades [15] suggest that populations in the boreal part of its range will presumably possess optimal conditions to persist. Although, we found a lack of connections between precipitation and demographic parameters of populations from the boreal region. Additionally, the Europe-wide trends of increasing temperatures might influence the reproduction and morphological traits of plants, especially of cold-adapted species [100]. Moreover, climatic changes may force them to shift their ranges further north and to higher altitudes, which in turn will be limited by the availability of habitats, and by species potential for migration. Most simulations [12,13] suggest that montane populations are especially endangered by the potential lack of reproduction and recruitment due to climatic changes. The presented study also points to montane populations of *M. monophyllos* as those with decreased individual fitness (smaller sizes, lower fruit sets), which is additionally strengthened by negative scenarios for this region, that result from SDM models for *M. monophyllos*' prospects in Europe [59].

However, because all changes on species level are a consequence of accumulated changes within populations [2], we suspect that the wide range of values of many parameters at the population and regional scale reflect *M. monophyllos*' evolutionary potential, which is quite high when we considering diversity on species level. Our results also indicate that reproduction plays a key role in the differentiation of *M. monophyllos* populations in regions, what can determine probability to survival of these populations, and may be also the cause of further progressive differentiations of populations in regions. In consequence, it could presumably lead to the formation of some biological barriers, together with progressive fragmentation and separation of the boreal and montane parts of the range [59].

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Co-authors’ statements

University of Bialystok
Faculty of Biology and Chemistry
Institute of Biology
Ciołkowskiego 1 J, 15-245 Bialystok
tel./fax: 48 (085) 738 84 14

Bialystok, 18.01.2017

Prof. dr hab. Emilia Brzosko
Plant Ecology Department
Institute of Biology, Faculty of Biology and Chemistry,
University of Bialystok
Ciołkowskiego 1J, 15-245 Bialyslok
emilka@uwb.edu.pl

Declaration

I declare that my contribution, as the coauthor of the presented papers, concerned with:

1. Jermakowicz E., Wróblewska A., **Brzosko E.,** Mirski P. and Hirse T. 2015. Phylogeographical structure of the boreal-montane orchid *Malaxis monophyllos* as a result of multi-directional gene flow. *Botanical Journal of the Linnean Society* 178: 138-154. DOI: 10.1111/boj.12268.: **participation in study conception, editing and advising the manuscript.**

2. Jermakowicz E., **Brzosko E.**, Kotowicz J. and Wróblewska A. 2017. Genetic diversity of orchid *Malaxis monophyllos* over European range as an effect of population properties and postglacial colonization. Polish Journal of Ecology X:XX: **participation in study conception, editing and advising the manuscript.**

3. Jermakowicz E., **Brzosko E.** 2016. Demographic responses of boreal-montane orchid *Malaxis monophyllos* (L.) Sw. populations to contrasting environmental conditions. *Acta Societatis Botanicorum Poloniae* 85 (1): 3488; DOI: http://dx.doi.org/10.5586/asbp.3488: **participation in study conception, advisory at all stages of manuscript preparation.**

University of Bialystok

Faculty of Biology and Chemistry

Institute of Biology

Ciołkowskiego 1 J, 15-245 Bialystok
tel./fax: 48 (085) 738 84 14

Bialystok, 18.01.2017

dr hab. Ada Wróblewska
Plant Ecology Department
Institute of Biology, Faculty of Biology and Chemistry,
University of Bialystok
Ciołkowskiego 1J, 15-245 Bialystok
adabot@uwb.edu.pl

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University of Bialystok

Faculty of Biology and Chemistry

Institute of Biology

Ciołkowskiego 1 J, 15-245 Bialystok
tel./fax: 48 (085) 738 84 14

Bialystok, 18.01.2017

Msc. Paweł Mirski
Plant Ecology Department
Institute of Biology, Faculty of Biology and Chemistry
University of Bialystok
Ciołkowskiego 1J, 15-245 Bialystok
p.mirski@uwb.edu.pl

Declaration

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Białystok, 09.09.2016 r.

MSc Toomas Hirse
Nature Protection Department
State Forest Management Centre
Rõõmu tee 1, 51013 Tartu, Estonia

Declaration

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Signed on September 28, 2016 in Tartu

Toomas Hirse
....

Białystok, 09.09.2016 r.

MSc Toomas Hirse
Nature Protection Department
State Forest Management Centre
Rõõmu tee 1, 51013 Tartu, Estonia

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