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Faculty
of Science

**Downy willow (*Salix lapponum* L.) in Central Europe:
biosystematics and ecology**

PhD. thesis

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Abstract: Downy willow (*Salix lapponum*) is small dioecious shrub distributed mainly in boreal and subarctic vegetation belt of Europe. It is also present in several mountain ranges of Central and southern Europe where it forms isolated populations. This Thesis is focused on one of such isolated populations in the Krkonoše/Karkonosze Mts. (northern Czech Republic/southwestern Poland). *Salix lapponum* was studied using multi-discipline approach. At first, herbarium revision to establish the historical distribution was performed. After that, all populations were revisited in field. Sex of plants and proportion of non-reproducing individuals were surveyed in selected larger populations. Habitat preferences were established using vegetation survey and soil sample analyses. Individuals were also sampled for genetic structure analysis. Morphometric analysis was performed on herbarium specimens. The species was in the Krkonoše/Karkonosze Mts. recorded on 38 historical localities from which only 21 were confirmed in field. The habitat conditions were generally similar to those reported in the literature except of the higher content of organic nitrogen in soil samples. The sex ratio was consistently female-biased and the overall proportion of non-reproducing individuals was 35 %. Soil and vegetation analyses suggested that at least some of non-reproducing individuals grew in less favourable conditions. No correlations of sex ratio with altitude, proportion of non-flowering individuals and no differences in spatial sex segregation and microhabitat preferences were found between males and females at a fine scale within the studied populations suggesting that the biased sex ratio in *S. lapponum* probably originates during early stages of ontogenetic development. Population structure genetic analysis found low proportion of clonality, strong genetic differentiation among populations and deficit of heterozygotes in all larger populations which indicate the occurrence of inbreeding. Morphometric analysis revealed slight shift in morphology of the Krkonoše Mts. populations when compared with main range population. Moreover, occurrence of complete glabrous individuals of *S. lapponum* in the Krkonoše Mts. populations suggests that these populations are under current speciation due to their long-term isolation.

Keywords: alpine scrub, dioecy, genetic drift, habitat conditions, hybridization, Krkonoše Mts., Salicaceae, sex ratio bias, threatened species

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Abstrakt: Vrba laponská (*Salix lapponum*) je poměrně drobný dvoudomý keř, který se vyskytuje především v boreální a subarktickém pásmu Evropy. Dále se vyskytuje také v několika izolovaných populacích v horách střední a jižní Evropy. Tato disertační práce je zaměřena na jednu z těchto izolovaných makropopulací, která se nachází v Krkonoších. Ke studiu *S. lapponum* byl použit multioborový přístup. Nejprve byla provedena herbářová revize historického rozšíření. Následovala revize zjištěných lokalit v terénu. U větších nalezených populací byl stanoven poměr pohlaví a také proporce nekvetoucích jedinců. Stanovištní nároky rostlin byly stanoveny pomocí fytoocenologických snímků a analýzy půdních vzorků. Morfometrická analýza byla provedena na herbářových položkách. Pro analýzu genetické populační struktury byly sbírány listy vybraných jedinců. Na základě herbářové revize byla vrba laponská v Krkonoších zaznamenána na 38 historických lokalitách z nich 21 bylo potvrzeno terénním průzkumem. Stanovištní podmínky se, kromě vyššího obsahu organického dusíku v půdních vzorcích, významně nelišily od údajů v literatuře. Poměr pohlaví byl u většiny populací vychýlen ve prospěch samic a celková proporce sterilních rostlin činila zhruba třetinu. Analýzy půdy a vegetace v okolí sterilních rostlin naznačují, že alespoň některé sterilní rostliny se vyskytují na méně vhodných stanovištích. Absence korelace proporce pohlaví s nadmořskou výškou, proporcí sterilních jedinců v populacích a také absence rozdílů v prostorovém rozšíření a mikrostanovištních podmínkách mezi samci a samicemi naznačují, že vychýlený poměr pohlaví vzniká u *S. lapponum* již v prvotních stádiích ontogeneze. Analýza genetické populační struktury zjistila nízkou proporce klonality, silnou genetickou mezipopulační diferenciaci a deficit heterozygotů ve všech větších populacích, což může ukazovat na existenci inbreedingu. Morfometrická analýza naznačila, že rostliny z krkonošských populací se mírně odlišují od rostlin ze souvislého areálu. Tento poznatek a také výskyt zcela lysých jedinců *S. lapponum* v Krkonoších naznačují, že krkonošské populace procházejí speciací v důsledku jejich dlouhodobé izolace.

Klíčová slova: dvoudomost, genetický drift, hybridizace, Krkonoše, ohrožený druh, nevyrovnaný poměr pohlaví, Salicaceae, stanovištní podmínky, vysokohorské křoviny

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Declaration

I hereby declare that this thesis has been worked out by myself together with listed co-authors. All literary sources cited in this thesis are listed in the References section.

In Olomouc, 29 . 9. 2018

RNDr. Michal Hroneš

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Author Contributions

CHAPTER 1 – Introduction and aims of the thesis

MH wrote this text.

CHAPTER 2 – Alpine willows (*Salix*) of the Czech Sudetes – distribution and present state of populations

MH, MDa and RV conceived the study. MH revised the herbarium material for *S. lapponum* and partly for *S. bicolor*, *S. hastata* and *S. herbacea* (data not shown in thesis). BB, JŠ, RV and VN revised the herbarium material for *S. bicolor*, *S. hastata* and *S. herbacea* (data not shown in thesis). MH, BB, JŠ, RV and VN revised localities in field. MH wrote first draft of the Introduction, Material and Methods and Results and Discussion for *S. lapponum*, prepared the final version of maps and overview of the localities. BB, JŠ and VN wrote Results and Discussion chapters for *S. bicolor*, *S. hastata* and *S. herbacea* (not shown in thesis). All of the authors contributed to and approved the final manuscript.

CHAPTER 3 – Habitat conditions, stage structure and vegetation associations of geographically isolated subalpine populations of *Salix lapponum* L. (Salicaceae) in the Krkonoše Mts. (Czech Republic)

MH, SHM and MDu conceived the study. MH and SHM performed the field work and soil sample analyses. MDu and MH performed the data analyses. ZH determined the collected bryophytes. PH helped with soil sample analyses. MH wrote first draft of the manuscript. All of the authors contributed to and approved the final manuscript.

CHAPTER 4 – Female-biased sex ratio but the absence of spatial and niche segregation between sexes in alpine populations of dioecious *Salix lapponum* (Salicaceae)

MH, SHM and MDu conceived the study. MH and SHM performed the field work and soil sample analyses. MDu and MH performed the data analyses. ZH determined the collected bryophytes. PH helped with soil sample analyses. MH wrote first draft of the manuscript. All of the authors contributed to and approved the final manuscript.

CHAPTER 5 – Patterns of morphological and genetic variability in geographically isolated populations of downy willow (*Salix lapponum*, Salicaceae) in the Krkonoše Mts. (Czech Republic)

All authors conceived the study. MH collected samples in field, performed morphometrics, part of the laboratory analyses and analysed all data. MS optimized laboratory protocols and performed most of the laboratory analyses. MH wrote first draft of the manuscript. MH, MS and MDa contributed to and approved the final manuscript.

CHAPTER 6 – Typification of names related to *Salix lapponum* (Salicaceae) and its hybrids published by Ignaz F. Tausch

MH conceived the study, performed the herbarium revisions, data analysis and wrote first draft of the manuscript. BT advised on the typification process. Both of the authors contributed to and approved the final manuscript.

CHAPTER 7 – Summary and conclusions

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CHAPTER 1:

Introduction and aims of the Thesis

MICHAL HRONEŠ

Krkonoše Mts.

Although not so high, not so far-reaching and with not so diverse flora as the Alps and the Carpathians, the Krkonoše/Karkonosze Mts. are undoubtedly significant Central European mountain range. Due to their location north of the Alps, west of the Carpathians and south of the Scandinavia, they are considered as the important migration crossroad of the arctic-alpine flora (Faltysová et al. 2002; Kaplan 2012). Krkonoše/Karkonosze Mts. are located in the border of northern Czech Republic and southwestern Poland. They are part of the Sudetes Mts. that in turn are the part of the Hercynian mountains (Jeník 1998). Together with the Hrubý Jeseník Mts. and the Králický Sněžník/Śnieżnik Mts., they are the only area within the Sudetes with (sub)alpine environment, i.e., with habitats above the upper tree line (Chytrý 2012).

The upper tree line in the Krkonoše/Karkonosze Mts. is located in altitudes around 1250 m a.s.l. The climatic conditions above it are very harsh with mean temperatures ranging from 4°C to 0.2°C, mean annual precipitation ca. 1400 mm and snow cover lasting for at least six months (Faltysová et al. 2002). Nevertheless, the vegetation is very diverse with *Pinus mugo* krummholz, birch-willow scrub, tall-forb vegetation, peat bogs and various grasslands with *Avenella flexuosa*, *Calamagrostis villosa*, *Deschampsia cespitosa*, *Molinia caerulea* and *Nardus stricta* as the most notable examples (Chytrý 2012).

The glacial heritage of the last two glacial maxima (i.e., Riss and Würm) in the Krkonoše/Karkonosze Mts. can be observed in several glacial cirques (e.g., Labský důl glacial cirque, Kotelní jámy glacial cirques, Śnieżne Kotły glacial cirques). Several psychrophilous species found their refugia there (and also in tundra located in the high-elevated plateaus). Among the most interesting relicts of the colder climatic periods are *Rubus chamaemorus*, *Pinus mugo*, *Salix lapponum*, *Saxifraga nivalis*, *Pedicularis sudetica* and many other species (Kwiatkowski 2006; Kaplan 2012; Kwiatkowski & Krahulec 2016).

Dioecy in flowering plants

Phenomenon of separate sexes is widely distributed but very rare among Angiosperms. Only about 5 % of species are reported to be dioecious (Renner 2014; Käfer et al. 2017). Angiosperm phylogenies indicate that dioecy has evolved several times independently from the ancestral hermaphroditic state (Käfer et al. 2017). Orders with high representation of dioecious species include Malpighiales (e.g., Salicaceae and Phyllanthaceae), Caryophyllales (e.g., Caryophyllaceae and Polygonaceae), Asterales and Asparagales (Weiblen et al. 2000; Käfer et al. 2017).

Dioecy is sometimes considered as an evolutionary dead end, mainly because of lower species richness in dioecious compared to non-dioecious sister clades (Heilbuth 2000). However, there is a growing evidence that the opposite is true (e.g., Käfer et al. 2017; Muyle et al. 2018). New data indicate that dioecious species exhibit lower genetic drift and more adaptation compared to their non-dioecious relatives (Muyle et al. 2018). There is also some evidence that dioecy may also be reverted back to hermaphroditism or monoecy (Goldberg et al. 2017; Käfer et al. 2017).

Apart from separated generative organs, dioecy is also tied with secondary sexual dimorphism resulting in different vegetative morphology, physiology and/or phenology between sexes (Meagher 1992; Lloyd & Webb 1977; Barrett & Hough 2013). Secondary sexual dimorphism is usually explained by different intrinsic energetic needs of the sexes due to their different reproductive role (Slatkin 1984; Bierzychudek & Eckhart 1988; Dudley 2006), e.g., different allocation of resources due to different costs of reproduction, increased susceptibility to environmental stress and/or resource competition between sexes (Bierzychudek & Eckhart 1988; Delph 1999; Field et al. 2013b; Hultine et al. 2016, 2018).

Sex ratio in dioecious plant populations should be in theory 1:1 (Fisher 1930). However, there are numerous well documented examples of deviations from these theoretical expectations. For example, many species of *Salix* are reportedly female biased (e.g., Myers-Smith & Hik 2012) whereas many Apiaceae and Sapindaceae species are usually male biased (Field et al. 2013a). Several hypotheses have been pronounced to explain these observations, ranging from genetic mechanisms (Taylor 1999; Stehlik & Barrett 2005), different mortality rates in early stages, such as seeds and seedlings (Che-Castaldo et al. 2015), sex specific resource competition and

susceptibility to environmental stress (Bierzychudek & Eckhart 1988; Delph 1999), to sex specific herbivory (Dudley 2006; Che-Castaldo et al. 2015).

Family Salicaceae

In its current circumscription, Salicaceae are pantropical family distributed from northern arctic belt to southern subarctic belt (Angiosperm Phylogeny Group 2009). The family now include almost half of the former tropical Flacourtiaceae resulting in total of 55 genera with 1200 species (Chase et al. 2002; Angiosperm Phylogeny Group 2009). Members of the family are shrubs and trees, usually dioecious (except of subfamily Samydoideae), with glandular simple leaves, flowers of various structure and many stamens (Stevens 2018). Glands on leaves are usually marginal and innervated (i.e., salicoid teeth; Wilkinson 2007). Vegetative tissues content acetylsalicylic acid and its derivatives (Boeckler et al. 2011).

The origin of crown group of Salicaceae (i.e., subfamily Salicoideae; family Salicaceae in traditional sense) is estimated to be in the end of the Cretaceous or in the beginning of the Paleogene (Bell et al. 2010; Xi et al. 2012). This group can be further divided into the six tribes (Chase et al. 2002; Angiosperm Phylogeny Group 2009) from which only tribe Saliceae is of interest of this Thesis. Only two major genera are now recognized in Saliceae: *Populus* and *Salix* (Ohashi 2000; Chase et al. 2002; Chen et al. 2010). Both of these genera are fully dioecious woody plants with catkin-like inflorescences and are distributed mainly in the subarctic, boreal and temperate regions of the northern hemisphere (Chmelař & Koblížek 1990; Argus et al. 2010). They are considered as taxonomically very complex due to the extensive morphological variability, frequent hybridization, polyploidy and generally low amount of morphological characters useful for species delimitation and identification (Rechinger 1992; Skvortsov 1999). At the same time, some species of both genera are valuable in forestry, energetics, remediation and pharmacy (Dickmann 2006; Lewandowski et al. 2006; Boeckler et al. 2011) whereas other species are important parts of vegetation in floodplain or (sub)alpine areas (e.g., Wijk 1986; Skvortsov 1999; Karrenberg et al. 2002; Dudley 2006).

In *Salix*, some species (e.g., *Salix lapponum*, *S. phylicifolia*, *S. glauca*) are considered as dominants of the (sub)alpine scrub vegetation, i.e. the vegetation typical

for subarctic and boreal as well as for alpine climate where environmental and climatic conditions restrict the expansion of the forest (Mardon 1990). However, expansion of the forest is currently on the rise in these areas due to the climate change threatening the alpine flora (de Wit et al. 2014; Bebi et al. 2017). Especially the small and isolated populations of these species may be more threatened by the fast changing climatic conditions. We chose one of such species, specifically *S. lapponum*, as a topic of this Thesis (see further).

***Salix lapponum* as study object and conservation interest**

Salix lapponum is a typical member of *Salix* subgen. *Vetrix* in the way that it is moderately sized shrub with leaves with eglandular petioles, catkins with black cataphylls (i.e., bracts), two stamens and one floral nectary per flower (Skvortsov 1999). In the subgenus, it belongs to the group of 6–8, morphologically similar species treated as section *Villosae* (Fig. 1; Argus 1997; Skvortsov 1999). Typical morphological characters of this section include floral buds notably different from the vegetative ones; oblong, elliptic, (ob)ovate to (ob)lanceolate, tomentose, villous to woolly leaf blades; catkins dense with long pubescent cataphylls and (sub)sessile, densely pubescent capsules (Chmelař & Meusel 1979; Argus 1997; Skvortsov 1999). In Europe, two species of the sect. *Villosae* are recognized: *S. lapponum* and *S. helvetica* (Rechinger 1964; Skvortsov 1999). Occasionally, third species, *S. ceretana*, is distinguished from *S. lapponum* s. str. in Pyrenees (Montserrat 1950; Blanco 1993). However, taxonomic status of this species remains controversial (Baudière 1995). Another controversial taxon is *S. lapponum* var. *daphneola*, glabrous morphotype endemic to the Krkonoše Mts. (Tausch 1837; Krahulec 2006).

The main range of *S. lapponum* comprises Scandinavia, Finland, Baltic states, western Belorussia, northern part of European Russia and north western Siberia (Rechinger 1964; Jalas & Suominen 1988; Skvortsov 1999). Several extant marginal populations of main range are still present in the eastern Poland (Pogorzelec 2003; Kołos et al. 2015). Besides that, several isolated populations are scattered in mountains of Sudetes, Scotland, Massif Central, Pyrenees and northern Balkan mountain ranges Rila and Vitosha (Fig. 2; Jalas & Suominen 1988; Blanco 1993; Skvortsov 1999). Only extant Carpathian population is known from foot of the Hoverla Mt. in Ukraine

(Koblížek 2006). New locality was recently discovered also in the Black Forest Mts. in western Germany (Hügin 2005). However, it is possible that it may not represent native occurrence of *S. lapponum* (Hügin 2005; Plieninger & Lutz 2017). In the view of such distribution, it is quite remarkable that *S. lapponum* is absent from the Alps and most of the Carpathians. In these two mountain ranges, it is replaced by allopatric *S. helvetica* (Jalas & Suominen 1988; Skvortsov 1999; Koblížek 2006; Hroneš et al. 2012). Relationship of these two species remains unresolved (Hroneš 2011).



Figure 1. Morphological variability of *Salix* sect. *Villosae* in Europe. A. *S. lapponum*, B. *S. helvetica*, C. *S. lapponum* var. *daphneola*, D. *S. ceretana*.

Salix lapponum is diploid species with $2n=38$ (Engelskjoen & Knaben 1971; Büchler 1986). Due to presumable absence of prezygotic mating barriers, *S. lapponum*

freely hybridize with several other mostly diploid species throughout its range. In the Scandinavia, the most common hybrids are with *S. arbuscula*, *S. aurita*, *S. herbacea* and *S. phylicifolia* (Elven & Karlsson 2000). Several hybrids are reported also from Scotland. The more common ones are with *S. lanata*, *S. caprea*, *S. arbuscula* and *S. herbacea* (Tennant 2004). In the Sudetes and also in the Balkan mountain ranges, the most common hybrid is with Carpathian species *S. silesiaca* (Chmelař & Koblížek 1990). Recently, new hybrid combination with *S. appendiculata* was described from the Black Forest Mts. (Plieninger & Lutz 2017). On the contrary, there are no hybrids reported from the Pyrenees (Blanco 1993).

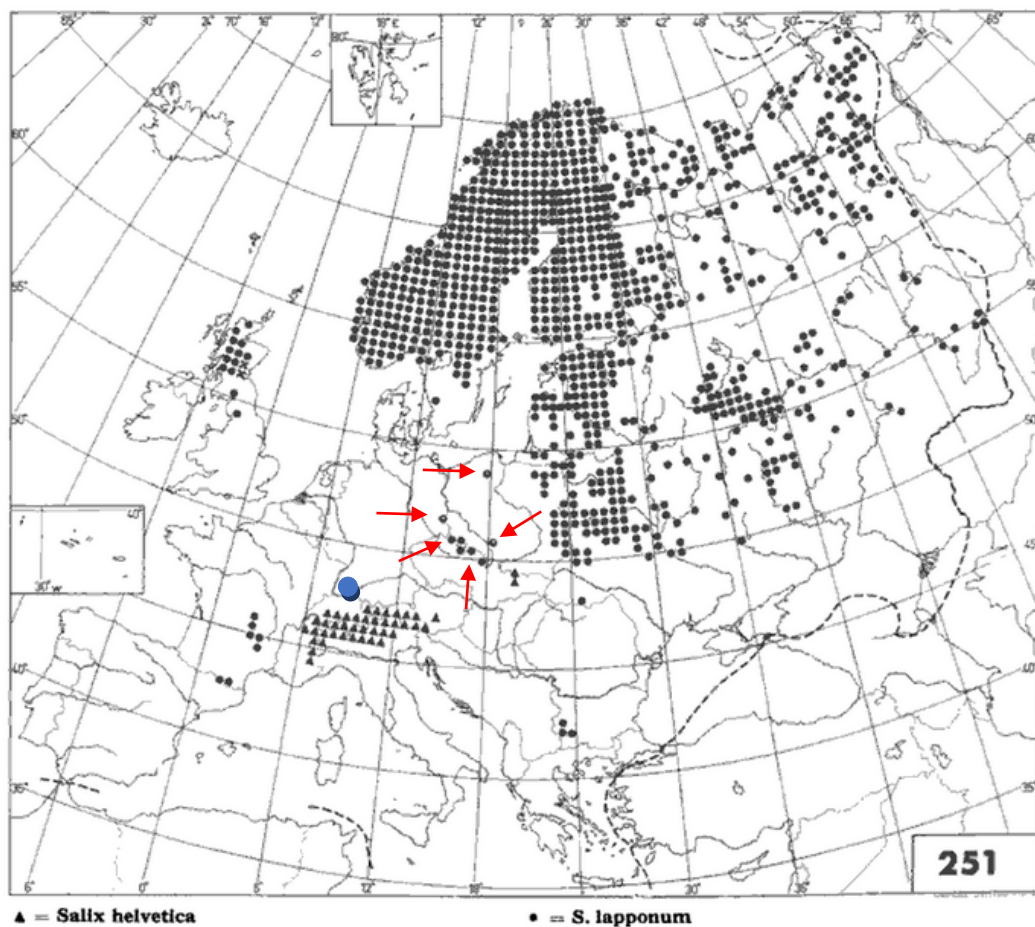


Figure 2. Distribution of *Salix lapponum* s. lat. in Europe (Jalas & Suominen 1988). Red arrows indicate erroneous records in Central Europe. Blue dot indicates the new locality reported by Plieninger & Lutz (2017).

To the typical stands of *S. lapponum* belong eutrophic and mesotrophic wetlands, wet meadows, sparse boreal forests and their openings, as well as banks of streams, glacial lakes and glacial cirques covered by tall-forb or shrubby vegetation (Chmelař & Koblížek 1990; Skvortsov 1999; Elven & Karlsson 2000). The species is

reported to be ectomycorrhizal (Milne et al. 2006), ambophilous (Peeters & Totland 1999) and anemochorous (Chmelař & Koblížek 1990). Although the species is able to reproduce vegetatively, the clonal reproduction is reportedly not significant (Stamati et al. 2007). The biotic interactions include parasitism by rust *Melampsora lapponum* (Pei 2005) and herbivory by cerambycids, leaf beetles and aphids (Wallin et al. 2017; Kmiec et al. 2018), small mammals such as mountain hares (Johannessen & Samset 1994) and voles (Shaw et al. 2010) and also by large ungulates such as red deer (Mardon 1990; Shaw et al. 2010).

Almost all populations of *S. lapponum* outside its main range are under some current threat. As a result, the species is listed in several Red Data Books and/or Red Lists, e.g., of the Czech Republic (Grulich 2012), Great Britain (Stroh et al. 2014), Lithuania (Smaliukas 2007) and Poland (Mirek et al. 2006), and it is protected by law in several countries such as Czech Republic, France and Poland. In eastern Poland where the species is on its range margin, considerable decline was observed during last 100 years (Pogorzelec et al. 2014a; Kołos et al. 2015). The change of water regime and subsequent succession was proposed as the main reason behind the decline (Pogorzelec 2008). Intensive research of these populations is in progress: from mapping the extent of current distribution (Urban & Wawer 2001; Pogorzelec 2003; Kołos & Chmielewska-Nowik 2007; Pogorzelec 2010) and establishing the habitat and ecological preferences (Pogorzelec 2003, 2008, 2009, 2010; Pogorzelec et al. 2014a) to evaluation of the experimental management interventions (Kołos et al. 2015), establishing the genetic variability of the populations (Pogorzelec & Nowosielski 2006; Pogorzelec et al. 2014b) and assessing the risks from other organisms such as insect (Kmiec et al. 2018). Similar situation is in Scotland where the intensive need for protection of mountain willow scrub led to establishment of the Scottish Montane Willow Research Group (e.g., Scottish Montane Willow Research Group 2005). Activities of this group included mapping of current distribution, establishing the rates of clonal reproduction, extent of hybridization etc. (Scottish Montane Willow Research Group 2005; Stamati et al. 2007). Overgrazing was identified as a main threat to *S. lapponum* populations in Scotland (Mardon 1990; Shaw et al. 2010).

Contrary to situation in Poland and Scotland, the Sudetes populations (which are located mainly in the area of the Czech Republic) remains vastly understudied. Research in the Krkonoše Mts. was focused mainly on uncoordinated plantings

(Lokvenc 1979a, 1979b; Vašina & Zahradníková 1998), floristics (Chejnová & Málková 1999; Kwiatkowski 2006; Hroneš et al. 2011), artificial propagation using tissue cultures (Brandová et al. 2011) and survey of fungi associated with *S. lapponum* (Chlebicki 1999). Pusz & Urbaniak (2017) studied foliar fungal diseases of *Salix* in Kocioł Łomniczki glacial cirque and in Mały and Wielki Śnieżny Kocioł glacial cirques and did not find any diseases on leaves of *S. lapponum*. To conclude, focused long-term research on the biology, ecology and taxonomy is still missing. Hrachová (2011), Hroneš (2011) and Sochor (2011) proposed several hypotheses about abovementioned in their diploma theses and put the solid foundation for the research of *S. lapponum* in the Krkonoše/Karkonosze Mts. This Thesis aims to explore these topics and hypotheses further.

Aims of the Thesis

During my research I focused on following questions:

- 1) What is exact current and historical distribution of *S. lapponum* in the Sudetes Mts.?
- 2) What are the habitat preferences of subalpine populations of *S. lapponum*?
- 3) Are there any ecological differences between sterile and fertile plants, and between plants with different sexes?
- 4) Is observed morphological variation of subalpine populations connected with genetic population structure and/or hybridization?
- 5) How geographic isolation influences the morphological and genetic patterns in *S. lapponum* populations?
- 6) Can be any taxonomic conclusions drawn from the answers to abovementioned questions?

The Thesis consists of the following parts that aim to answer the abovementioned questions:

CHAPTER 2 – Alpine willows (*Salix*) of the Czech Sudetes – distribution and present state of populations

This chapter summarise the historical and current distribution of *S. lapponum* in the Sudetes based on herbarium revision and confirmation of the state of populations in field.

CHAPTER 3 – Habitat conditions, stage structure and vegetation associations of geographically isolated subalpine populations of *Salix lapponum* L. (Salicaceae) in the Krkonoše Mts. (Czech Republic)

This chapter explores the habitat preferences and vegetation associations of *S. lapponum* populations in the Krkonoše Mts. using vegetation plots and analyses of soil samples. It also reports the proportion of the sterile and fertile individuals in large populations and discuss the causes of their occurrence.

CHAPTER 4 – Female-biased sex ratio but the absence of spatial and niche segregation between sexes in alpine populations of dioecious *Salix lapponum* (Salicaceae)

This chapter further explores the pattern of the co-occurrence of sexes. It reports the strongly biased sex ratio which, however, cannot be explained using ecological and spatial segregation. Several explanations such as role of genetic sex-determination mechanisms are proposed and discussed.

CHAPTER 5 – Patterns of morphological and genetic variability in geographically isolated populations of downy willow (*Salix lapponum*, Salicaceae) in the Krkonoše Mts. (Czech Republic)

This chapter quantify the extent of morphological and genetic variability, compare it with the situation of the main range populations and explores the possible origins of glabrous plants endemic to the Krkonoše Mts.

CHAPTER 6 – Typification of names related to *Salix lapponum* (Salicaceae) and its hybrids published by Ignaz F. Tausch

In this study, the typification of several published synonyms of *S. lapponum* or its hybrid with *S. silesiaca* is presented. Taxonomy of several names broadly used for the *S. lapponum* s. lat. populations in Central Europe is discussed.

Note on nomenclature: Nomenclature is unified throughout this Thesis using Euro+Med Checklist (Euro+Med 2006–2018) and Skvortsov (1999; for *Salix* names) unless specifically stated exceptions (i.e., Chapter 2).

CHAPTER 2:

Alpine willows (*Salix*) of the Czech Sudetes – distribution and present state of populations

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Zprávy České botanické společnosti, 2014, 49: 29–47.

(shortened and translated to English)

Abstract

Alpine willows of the Czech Republic represent a group of species with extremely fragile relic distribution. Populations of *S. lapponum* occur at rather limited number of localities and usually with low number of individuals per population in the Krkonoše Mts. and in the Hrubý Jeseník Mts. This study compares the past records of distribution of the species (based on herbarium specimens and literature records) with our recent field observations. We discuss decline trends, recent distribution and number of localities, doubtful records and future prospects of studied populations.

Keywords: alpine scrub, Hrubý Jeseník Mts., Krkonoše Mts., mountain flora, Salicaceae, threatened species

Nomenclature: Danihelka et al. (2012)

Introduction

Willows (*Salix*; Salicaceae) belong with ca. 400 recognized species to the rather species rich and broadly distributed genera of the Holarctic flora (Newsholme 2003). The genus is defined by the presence of separate sexes (dioecy), apetalous flowers arranged in catkins, simple leaves and general affinity to moist habitats. To the centers of its diversity belong temperate areas of Asia and North America. However, (sub-)arctic and alpine species that frequently have circumpolar or circumboreal range and are at the same time scattered throughout the European mountains are also common.

Willows are considered to be one of the complicated groups of the Central European flora due to their high level of phenotypic variation, reduction of generative morphological characters and frequent hybridization. Especially the combination of high level of phenotypic plasticity together with the ability to easily cross result in avoidance of their collection and even their identification by the part of the botanical public. In the Czech Republic, only few researchers focused on willows in the past 100 years. It was mainly professor J. Chmelař followed by professor J. Koblížek, who greatly contributed to the knowledge on the willow diversity and frequency of their hybridisation in the area of the former Czechoslovakia. Their effort resulted in treatment

of *Salix* for the second issue of the Flora of the Czech Republic (Chmelař & Koblížek 1990). However, despite these solid foundations, willows remained in the margin of interest of the florists. Detailed floristic contributions have been scarce, and they concerned either striking species (e.g., *Salix daphnoides*) or rare finds of new hybrids (e.g., Krkavec 1961a, b; Jansa 1977; Vašut & Pečinka 2000). Since the second issue of the Flora of the Czech Republic has been issued more than twenty years ago during which the willow research continued, we decided to publish the following work, which is focused on the distribution of rare alpine willow species *Salix lapponum* in the Sudetes Mts. and to an evaluation of the recent state of its populations.

Salix lapponum is quite distinct species with following characteristics: 1) it is species with arctic-alpine range, which is in the Czech Republic considered as glacial relic (Chmelař 1972), 2) in the Czech Republic, it is distributed only in the Krkonoše Mts. and Hrubý Jeseník Mts. (it is not present in the Králický Sněžník Mts.), 3) it has its ecological optimum in subalpine belt of these mountains (Koblížek 2002), 4) it is easily recognizable in comparison with our other autochthonous species, and 5) it is considered as endangered (Grulich 2012).

Our contribution sums-up historical knowledge (herbaria, literature) and presents results of our field revision of the *S. lapponum* distribution.

Material and Methods

Distribution of *S. lapponum* in the Czech Republic was established using the revision of specimens deposited in the following public herbaria in the Czech Republic (BRNL, BRNM, BRNU, CB, GM, HR, LIT, MJ, MP, NJM, OL, OLM, OSM, PL, PR, PRC, ROZ; acronyms sensu Hradílek et al. 1992) and Slovakia (BRA, SAV; acronyms sensu Vozárová & Sutorý 2001). Data were supplemented with the literature records and our own field observations. Localities from herbarium specimens were localized using tourstic maps (www.mapy.seznam.cz). All localities were subsequently revised in field during 2009–2011. Position of each locality, where at least one individual was still present, was recorded using GPS device. GPS coordinates in WGS-84 format were used for assembly of distribution maps in ArcGis.

Localities were sorted according to phytogeographical (sub)district (sensu Skalický 1988) and within the districts from the west to the east. Polish localities follow

after the Czech ones. Generally, the texts on labels were not modified, only some longer parts were shortened or reformulated. Labels in other than Czech language (mainly in German) were translated and are given in square brackets. In some occasions when the name of the collector was missing from the label or it was unreadable, such cases are marked by “s. coll.“ or “coll.?“, respectively. Similarly, in cases when the date of collection was missing from the label or it was unreadable, it is marked by “s. d.“ or “d.?“, respectively. Own data from field revision are noted by “not.“ and followed by the name of the finder and by year of the find. Populations which we consider extinct (i.e., they were not confirmed by our revision in field) are marked by “†“.

Results and Discussion

Salix lapponum L. – downy willow (vrba laponská)

Taxonomy

Downy willow is distributed mainly in the boreal and subarctic belt of northern and north-eastern Europe and scattered in (sub-)alpine belt of the Krkonoše Mts., Hrubý Jeseník Mts., Massif Central, Pyrenees and several mountains in the Balkan peninsula (Hultén & Fries 1986, Skvortsov 1999). *Salix lapponum* complex comprises in Central and southern Europe of two to four taxa. However, their taxonomy is still not sufficiently resolved. Generally accepted taxa are *Salix lapponum* s. str. from Krkonoše Mts., Hrubý Jeseník Mts., Massif Central Mts., Vitosha Mts. and Rila Mts., and *Salix helvetica*, from Alps and Western Carpathians. Populations of *S. lapponum* from the Pyrenees are sometimes distinguished as separate species *Salix ceretana* (Blanco 1993). The last taxon that is by some authors distinguished as a separate species is glabrous morphotype from Krkonoše Mts. which was described as *Salix daphneola* and is currently evaluated as variety of *S. lapponum* s. str., i.e., var. *daphneola*. This taxon is confined only to western Krkonoše Mts.

Historical and current distribution

The nominate variety (*S. lapponum* var. *lapponum*) was recorded on 30 historical localities in the Czech Republic, on 25 in Czech part of the Krkonoše Mts. (Fig. 3) and

on five in Hrubý Jeseník Mts. (Fig. 4, Supplementary File 1). In the Polish part of the Krkonoše Mts., it was recorded on 13 historical localities. Recently, it was re-recorded on 11 localities in the Czech part of the Krkonoše Mts. (cf. Hroneš et al. 2011), on 10 localities on the Polish part of the Krkonoše Mts. and on one in the Hrubý Jeseník Mts.

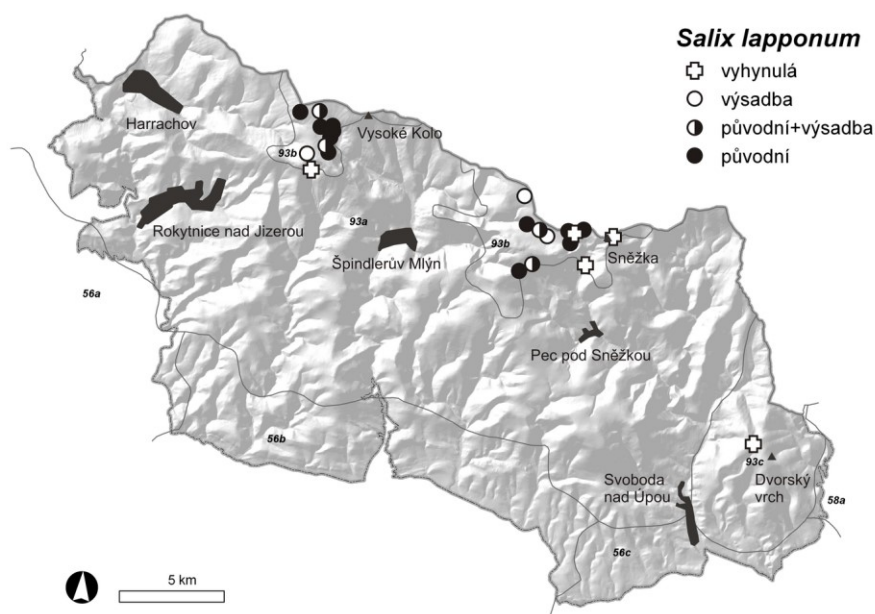


Figure 3. Distribution of *Salix lapponum* in the Krkonoše Mts. (vyhynulá = extinct, výsadba = plantation, povodní+výsadba = natural occurrence+plantation, původní = natural occurrence).

In the Czech part of the Krkonoše Mts., the *S. lapponum* is distributed mainly in three isolated areas. The first area in the eastern Krkonoše Mts. is consisted of populations in wider vicinity of Obří and Modrý důl glacial cirques, Úpské rašeliniště peat bog, Bílá louka alpine meadow, Údolí Bílého Labe valley. The second area is located in the western Krkonoše Mts. and comprises mainly of localities in Labský důl glacial cirque, Pančavská and Labská louka peat bogs. The third area lays in polish part of the Krkonoše mountains and is consisted mainly of populations located in the vicinity of the Mały and Wielky Staw lakes. Except of these main areas, *S. lapponum* grows also in the Mały Śnieżny Kocioł glacial cirque (Kwiatkowski 2006) and in the past it was also recorded in the Rýchory Mt. (cf. Šourek 1970) where it is possibly from the second half of the 20th century extinct (Štursa pers. comm.).

The delimitation of the natural occurrence of *S. lapponum* in the Czech part of the Krkonoše Mts. is further complicated by the “Reconstruction of tree stands above

the upper tree line limit” project that took place during 1952–1975 and during which *S. lapponum* was planted to several places (Lokvenc 1979b). Although the plantations are summarized by Lokvenc (1979b) and Vašina & Zahradníková (1998), in some instances it is rather complicated to determine whether the occurrence of individual and/or population is natural, introduced or the combination of both. As natural can be considered all occurrences from which the herbarium specimens were collected before 1952, i.e., before start of the introduction. In other instances, the introduced origin should be considered. However, the plantations do not represent a huge risk from point of view of the population genetics because the autochthonous material was used (from the Pančavská louka alpine meadow; Lokvenc 1979, Vašina & Zahradníková 1998,) and the plantations possess sufficient genetic variability (Sochor 2011).

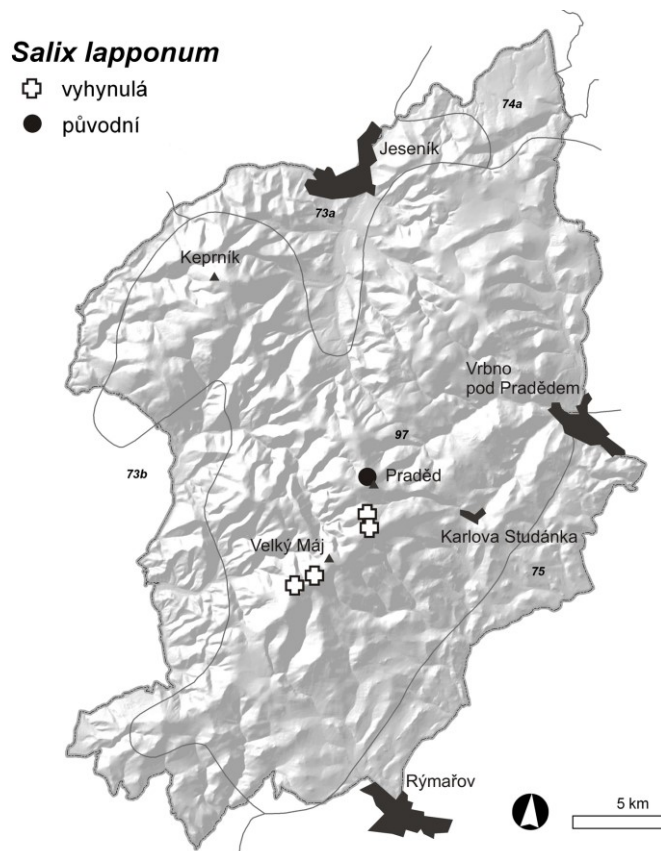


Figure 4. Distribution of *Salix lapponum* in the Hrubý Jeseník Mts. (vyhynulá = extinct, původní = natural occurrence).

In the Hrubý Jeseník Mts., *S. lapponum* can be recently found only near the Tabulové skály rocks below the summit of Praděd Mt. All herbarium specimens labeled only as Praděd are possibly identical with this site. Historical occurrence in the Petrovy kameny

rocks is somewhat controversial. It was reported from there by several sources in 19th and first half of 20th century (cf. Wimmer & Grabowski 1829, Wimmer 1844, Fiek 1881, Laus 1927) and its was recorded by several herbarium specimens from the 20th century. However, Chmelař (1972) do not report its occurrence from this locality. Therefore, it is possible that at least several of the records are based on the misslabeling the nearby Tabulové skály rocks for the Petrovy kameny rocks. *Salix lapponum* was further recorded from several other summits of the Hrubý Jeseník Mts. (i.e. Vysoká hole Mt., Jelení hřbet Mt. and Břidličná Mt.). However, from each of these localities only one specimen was recorded, and our revision failed to discover any individual on these summits. Due to the absence of any other herbarium specimens and literature records and recent unfavorable ecological conditions, any occurrence (historical or recent) of *S. lapponum* on these localities is improbable.

Salix lapponum var. *daphneola* was known only from the Pančavská louka peat bog just above the Pančava waterfall (Tausch 1837, Chmelař & Koblížek 1990). During our field revision, we have discovered other three (micro)localities in Labský důl glacial cirque and its close vicinity (Supplementary File 2, Hroneš et al. 2011). In each site, it is always present in low numbers and together with type variety.

Threats and current state of populations

Current state of the native populations of *S. lapponum* in the Krkonoše Mts. is summarized by Hroneš et al. (2011). Most of populations seem to be relatively stable with sufficient number of flowering individuals. To the identified threats belong mainly the hybridization with *Salix silesiaca* and subsequent introgression to the genome of *S. lapponum*, as was discovered in individuals from Labský důl glacial cirque and Pančavská louka peat bog populations (cf. Sochor 2011). Furthermore, the increased grazing pressure and direct destruction of the shrubs by the red deer was observed.

Population in the Hrubý Jeseník Mts. is probably comprised by only one huge female clone (Chmelař 1972). Size of the clone was in 1989 estimated to 5×10 m (Bureš 2013). Due to absence of mating partners, *S. lapponum* can therefore spread only vegetatively on very short distances. Therefore, any unexpected event (such as destruction of the shrub by red deer or introduced chamois) can be critical.

CHAPTER 3:

Habitat conditions, stage structure and vegetation associations of geographically isolated subalpine populations of *Salix lapponum* L. (Salicaceae) in the Krkonoše Mts. (Czech Republic)

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Abstract

Shrub communities are important components of the subalpine vegetation. We focused on habitat conditions and stage structure (flowering and non-reproducing individuals) of stands of the endangered *Salix lapponum* in the Krkonoše Mts., Czech Republic. Habitat conditions were determined using soil sample analyses and Ellenberg indicator values (EIVs) derived from fine-scale (1×1 m) vegetation plots. The fine-scale plots were compared with coarse-scale relevés with the occurrence of *S. lapponum* acquired from the Czech National Phytosociological Database. We found that *S. lapponum* grows on nutrient-poor, acidic soils with high relative water contents, high amounts of total nitrogen, low amounts of phosphorus, moderate amounts of magnesium and low to moderate amounts of calcium. The overall proportion of non-reproducing individuals was 35.2%, but strong variations were observed among populations (0–100%). Co-occurring species and EIVs data indicated that flowering individuals are relatively more common in humid, nutrient-rich and warmer microhabitats than non-reproducing ones. Well-developed (“typical”) stands of *S. lapponum* with a dominance of flowering individuals occur along alpine springs, streams and in glacial cirques (= association *Salicetum lapponum* Zlatník 1928), but *S. lapponum* also grows along transitional mires and peat bogs and in subalpine grasslands. Coarse-scale relevés were similar to “typical” stands of *S. lapponum* at the fine scale but were differentiated from them by high frequencies of several, mainly broad-leaved forbs and herbs typical of moist to wet and fertile soils. Threats to *S. lapponum* and the future prospects of its stands in the Krkonoše Mts. are briefly discussed.

Keywords: alpine scrub, Krkonoše Mts., flowering frequency, habitat conditions, soil conditions, vegetation composition, willow

Introduction

Alpine and subalpine shrub communities are among the most important components of the vegetation above the tree line. In harsh (sub)alpine environment, shrubs can serve as nurse species that facilitate the growth of other, mainly herbaceous, plants (Totland & Esaete 2002; Dona & Galen 2007; Zeidler et al. 2012). Alpine shrubs create specific microenvironmental conditions by modifying snow cover patterns and soil chemistry

and protecting the soil from wind erosion (Holtmeier & Broll 1992; Anthelme et al. 2003; Dona & Galen 2007).

In European mountains, subalpine shrub vegetation is composed mainly of *Alnus viridis* and various *Betula*, *Salix* and *Sorbus* species. In Central Europe, such vegetation has been traditionally classified mainly within the classes *Mulgedio-Aconitetea* Hadač et Klika in Klika et Hadač 1944 (Kočí 2010) or *Betulo carpathicae-Alnetea viridis* Rejmánek in Huml et al. 1979 (Huml et al. 1979). Arctic/alpine willows (*Salix* spp.) are among the dominant species of this vegetation. In the mountains of Sudetes, few such species of willows occur relative to the numbers in the Alps and Carpathians. In the Sudetes, the willow species are *Salix bicolor*, *S. hastata*, *S. lapponum* and *S. silesiaca* (Hroneš et al. 2014). While the first two species are very rare, occurring in only one or a few localities, *S. silesiaca* has wide distribution across the Sudetes, and *S. lapponum* is relatively abundant only in the Krkonoše/Karkonosze Mts., where it is reported to grow mainly along (sub)alpine springs and in glacial cirques (Kočí 2010; Hroneš et al. 2014).

Salix lapponum has a boreal/subarctic distribution, and its main range is in Scandinavia and western Siberia, extending into the eastern Poland, Belarus and Scotland. Geographically isolated populations persist as glacial relicts in several mountain massifs of central, southwestern and southeastern Europe (Elven & Karlsson 2000; Pogorzelec 2003; Hroneš & Trávníček 2018). As marginal or isolated populations face various threats such as fluctuations in population size and inbreeding, they might consequently shift their ecological niche (see Sexton et al. 2009 and literature cited therein). In its main range and range edges, *S. lapponum* grows in wet places with high levels of ground water and, typically, on neutral to acidic soils (Elven & Karlsson 2000; Pogorzelec 2008). It generally forms extensive stands in suitable habitats, whereas in less adequate habitats, often only a single shrub can be found (Elven & Karlsson 2000). Succession on *S. lapponum* habitats caused by decreased soil water content and increased shading by surrounding vegetation and increased herbivore pressure further threaten these isolated populations (Mardon 1990; Urban & Wawer 2001; Pogorzelec et al. 2014a; Kołos et al. 2015).

In the Czech Republic, *S. lapponum* is considered endangered (Grulich 2012). In addition, it is categorized as critically endangered in the Polish Red Book of Plants (Mirek et al. 2006) and is protected by law in the Czech Republic and Poland. Its current distribution in the Sudetes encompasses 21 localities (and several additional

plantings) in the Czech and Polish part of the Krkonoše/Karkonosze Mts. and one in the Hrubý Jeseník Mts. The number of populations is relatively stable, with only minor decline observed in the last century (Hroneš et al. 2014). As a species of considerable conservation interest in both countries, attention has been given primarily to populations in eastern Poland (e.g., Fijałkowski 1958; Urban & Wawer 2001; Kołos & Chmielewska-Nowik 2007; Pogorzelec 2010; Pogorzelec et al. 2014a, b; Kołos et al. 2015) rather than to populations in the Sudetes (Macko 1952; Chlebicki 1999; Hroneš et al. 2014; Pusz & Urbaniak 2017). Moreover, no detailed assessments of the habitat conditions of *S. lapponum* stands in the Sudetes are available. During our ongoing research on this species in the Krkonoše Mts., it became clear that populations vary in the proportion of non-reproducing individuals. The present study aims to (1) describe the habitat conditions of stands of *S. lapponum* populations in the Krkonoše Mts. using soil sample analysis and bioindication based on vegetation survey, (2) quantify the proportion of flowering individuals in each population and explore its relationship with (micro)habitat conditions, and (3) characterize vegetation patterns and co-occurring species.

Material and Methods

Field work, fine-scale vegetation sampling and soil analyses

The field work was carried out during the years 2009–2013 in the Czech part of the Krkonoše Mts. (Czech Republic). All known indigenous populations of *S. lapponum* with more than one individual (see Hroneš et al. 2014) were surveyed, totalling nine populations (sites) as follows (ordered from east to west): Úpská jáma glacial cirque (UJ), Úpské rašeliniště peat bog (UR), alpine meadow near Výrovka chalet (VY), spring in Modrý důl glacial valley (MD), Hančova louka alpine meadow (HL), Pančavská louka peat bog (PL), eastern part of Navorská jáma glacial cirque (SZ), northern part of Navorská jáma glacial cirque (NJ), and spring of Pančava rivulet (HPL; Supplementary File 3). The exact location and approximate altitude of each individual were determined by a GPS device (Garmin GPX-60 Cx) and by its position in the orthophotomap provided by the Krkonoše NP administration (available at <http://gis.krnep.cz/map/>). In situ identification of individuals was possible because preliminary analyses using microsatellite markers (SSR-loci; Hroneš et al., in prep.) showed that individuals form

discrete shrubs and that the occurrence of clonality (e.g., root suckering or layering, stem fragmenting) is low. It was generally possible to distinguish individual plants visually based on combination of several traits (growth direction of branches, patterns of shallowly buried stems, and colour and hairiness of the leaves).

During the vegetation season in 2009, each population was repeatedly visited to determine the optimal timing for the examination of flowering frequency. Then, the all individuals were repeatedly surveyed for flowering in June and July between 2010 and 2013. Individuals were classified into two categories according to their flowering status: flowering (i.e., the individual had flowered in at least one of the surveyed years) and non-reproducing (i.e., the individual did not flower during any surveyed year). The numbers of flowering and non-reproducing individuals were counted within each population studied. We also searched the stands of each population for the occurrence of any seedlings.

A fine-scale vegetation survey was performed at four selected readily accessible (i.e., without climbing equipment) population sites that contained at least 6 individuals and optically with relatively low clonality (NJ, PL, SZ, UJ; Supplementary File 3; fine-scale data set) during July 2010 (NJ, PL) and July 2013 (SZ, UJ). A vegetation plot of 1×1 m was established around each individual *Salix* plant with its caudex positioned in the middle of the plot. Plots were established in this manner at all sites except the NJ site; here, 39 plots were established around randomly selected plants due to the large size of the population and the difficult terrain. This resulted in total of 207 sampled vegetation plots from these four populations. The cover of each species of vascular plant and bryophyte was recorded using a 7-grade Braun-Blanquet abundance-dominance scale (Braun-Blanquet 1964). The cover of each layer was estimated visually as a percentage. Slope inclination was estimated with a clinometer, and slope orientation was measured with a compass in GPS device.

Soil samples (207 in total) were collected from each plot of the vegetation survey populations (NJ, PL, SZ, UJ; Supplementary File 3) over two days at the end of August 2013. Soil was sampled with a soil sampler at 2–10 cm depth below the surface at several points around the centre of each plot. The subsamples from each plot were placed together in a plastic bag and immediately weighed. After transportation to the laboratory, the soil samples were oven dried at 60°C for 48 hours to constant weight. Soil relative water content (SRWC) was calculated by dividing the dry weight of the

sample by the wet weight. Samples were then passed through a 2-mm sieve. The pH of each sample was determined in water solution using a pH 70+DHS portable pH meter (XS Instruments). The concentration of total nitrogen (N) in each sample was determined after mineralization with sulphuric acid and Se-K catalyst using a DK 20 heating digester (Velp Scientifica) and subsequent distillation with water vapour into boric acid on a Vapodest 30 s distillation apparatus (Gerhardt). The final concentration of N was established by titration with 0.01 M HCl using a TitroLine 6000 titrator (SI Analytics). For analyses of phosphorus (P), calcium (Ca) and magnesium (Mg), Mehlich II extract was prepared (Mehlich 1978). The concentration of phosphorus was estimated colourimetrically using HACH PhosVer 3 reagent powder pillows on a DR 2800 spectrophotometer (HACH). The concentrations of calcium and magnesium were estimated using an Avanta Σ atomic absorption spectrophotometer (GBC).

Data extraction

All available phytosociological relevés with the presence of *S. lapponum* from the Krkonoše/Karkonosze Mts. stored in the Czech National Phytosociological Database (CNFD; Chytrý & Rafajová 2003) were acquired, totalling in 22 relevés. Those relevés with recording date information were recorded between 1924 and 1998. Most relevés had unknown recording dates; however, most of these were published by Matuszkiewicz & Matuszkiewicz (1975) and therefore had to have been recorded before 1975. Relevé area ranged from 2 to 200 m² with mean area (\pm SD) of 101 \pm 61 m²; two relevés had unknown areas. Most relevés (64%) also lacked exact location information, which was listed only as the Krkonoše Mts. Another source of relevés with the occurrence of *S. lapponum* from Karkonosze Mts. (Macko 1952) was not extracted because only synthetic relevés were presented by the author.

Data analyses

Fine-scale vegetation plots were stored in Turboveg 2.0 (Hennekens & Schaminée 2001) and then submitted to CNFD, where they are available upon request. Mean Ellenberg indicator values (EIVs; Ellenberg et al. 1992) for nutrients, light, temperature, moisture and soil reaction were calculated in software Juice 7.0 (Tichý 2002) for both

vascular plants and bryophytes (except for nutrients) without species weighting for each plot for both datasets (i.e., fine and coarse datasets). Basic descriptive statistics of soil conditions and EIVs for each plot (where available) and the proportion of non-reproducing individuals at each locality were calculated in NCSS 9 (Hintze 2013).

Microhabitat differentiation between flowering and non-reproducing individuals was analysed by partial detrended correspondence analysis (pDCCA; Šmilauer & Lepš 2014) of a matrix of floristic composition and by partial redundancy analysis (pRDA; Šmilauer & Lepš 2014) of a matrix of soil characteristics of fine-scale plots from two populations with sufficient numbers of both non-reproducing and flowering individuals (SZ, UJ). Presence of either non-reproducing or flowering *Salix* within the plot was considered as a categorical explanatory variable. Because floristic composition and soil parameters within a plot might be influenced by *Salix* cover (Totland & Esæte 2002), *Salix* cover was included in the analyses as a covariate. Locality was included as covariate defining block. The effect of the explanatory variable was tested by Monte Carlo permutation test with 999 permutations limited to within blocks. A second set of analyses examined the relationships between interpopulation differences in the proportion of non-reproducing plants and both floristic composition (pDCCA) and soil characteristics (pRDA) of fine-scale plots over four populations that were studied in detail, including two populations with low or no presence of non-reproducing individuals. The proportion of non-reproducing individuals in populations was considered as an explanatory variable, and *Salix* cover was included as a covariate. An effect of the explanatory variable was tested by Monte Carlo permutation test with 999 permutations. Spearman correlation coefficient was used to explore the relationship between EIVs and the first canonical axis in the pDCCA, while a t-value biplot was used to identify soil variables that significantly responded to the explanatory variable in the pRDA. Bonferroni correction of P-values was applied in the case of multiple comparisons testing.

Floristic composition of the fine-scale plots was classified using modified divisive TWINSpan classification (Roleček et al. 2009) with pseudospecies cut levels of 0, 5, 10, and 30. We also compared the floristic composition of coarse-scale relevés with that of our plots sampled at the fine-scale. We identified diagnostic species differentiating classified groups or studied data sets based on fidelity analysis using the phi coefficient (Chytrý et al. 2002) in Juice 7.0. Species with a statistically significant

phi coefficient higher than 0.20 (using Fisher's exact test at $P \leq 0.05$) were considered to be diagnostic (= an indicator) of a particular group/data set.

Results

Habitat conditions of S. lapponum stands in the Krkonoše Mts.

Both the fine- and coarse-scale data sets indicated similar geomorphological and altitudinal characteristics of *S. lapponum* stands in the Krkonoše Mts. (Table 1). *Salix lapponum* occurred in the subalpine zone at elevations ranging from ca. 1200 to 1420 m a. s. l. The microrelief surrounding tagged *Salix* individuals was mostly slightly (1–5 degrees) to strongly sloping (up to 40 degrees) with a prevailing north-eastern to south-eastern aspect (Table 1a). Coarse-scale relevés were recorded at slightly more diverse relief with sloping up to 45 degrees and aspects ranging from north-western through north to south-eastern (Table 1b).

Table 1. Abiotic characteristics (elevation, aspect, slope), cover of shrub, herb and moss layers, and EIVs for selected ecological factors calculated separately for (a) fine-scale plots sampled within four *S. lapponum* populations and (b) coarse-scale relevés with the occurrence of *S. lapponum* from various localities in the Krkonoše/Karkonosze Mts. Cover variables of coarse data set should be interpreted with caution because only 11 relevés had reliable data on cover. Due to rather similar values for elevation, aspect and slope of fine-scale plots within each population, mean value per site (fine-scale set) entered analyses.

Variable	(a) Fine-scale plots				(b) Coarse-scale relevés			
	Mean	± SE	Min	Max	Mean	± SE	Min	Max
Abiotic and structure characteristics								
Elevation (m a. s. l.)	1299	39	1256	1367	1302	58	1200	1420
Aspect (degrees) ¹	99	11	68	135	65	12	23	360
Slope (degrees)	17	9	1	40	21	3	1	45
Cover of shrub layer (%)	51	4	5	90	82	7	20	100
Cover of herb layer (%)	49	2	5	90	65	4	30	80
Cover of moss layer (%)	44	2	0	100	39	6	20	80
Ellenberg indicator values								
Light	6.5	0.2	5	7.8	6.5	0.5	5	7.3
Temperature	3.5	0.1	0	5	3.3	0.3	2.8	3.8
Continentality	4.7	0.1	3	6.3	4.6	0.2	3.9	5.4
Moisture	7.3	0.2	4.8	10	7.2	0.5	5.6	8.6
Soil reaction	2.8	0.1	1.3	4.8	4.3	0.4	2.4	5.6
Nutrients	2.8	0.1	1	5	4.3	0.2	2.3	6.4

¹ Circular statistics were used

The soil was typically moist or damp, with SRWC generally exceeding 60%, although several plants were found on drier soils (SRWC ~17–25%). The chemical analyses of soil sampled from the fine-scale plots (Table 2) showed that *S. lapponum* occurred on soils with low pH, with the majority of pH values being between 4.2 and 4.7. The amount of total nitrogen varied greatly, ranging from 0.001 to 47.7 g.kg⁻¹, with a high mean value (22.6 g.kg⁻¹). Soil samples with an N content below 1.5% (39% of the samples) were typical of moist sites, while soils with N contents > 1.5% were typical of damp sites with the high levels of organic matter (Spearman correlation SRWC-N: $r_s = 0.72$, $P < 0.001$). The soil samples generally contained low amounts of P, generally between 5 and 30 mg.kg⁻¹, moderate amounts of Mg (generally between 90 and 230 mg.kg⁻¹) and low to moderate but highly variable amounts of Ca (generally between 300 and 1200 mg.kg⁻¹; Table 2). High values of both Ca and Mg were found in damp soils with high amounts of N (Ca-N: $r_s = 0.54$, $P < 0.001$, Mg-N: $r_s = 0.69$, $P < 0.001$).

According to the EIVs of the fine-scale plots, *S. lapponum* grows in stands with plants indicative of well-lit places but also occurring in partial shade, and more or less infertile, acidic soils that are fresh to constantly moist or damp but not wet (Table 1a). The coarse-scale relevés had mean indicator values almost identical to those of the fine-scale plots for all factors except two (soil reaction, nutrients). However, the coarse-scale relevés indicated lower-acid (acidic to moderately acidic) and more fertile (less infertile to moderately fertile) soils relative to the soils of the fine-scale plots (Table 1b).

Frequency of non-reproducing individuals in populations and their relationships with (micro)habitat

In total, 961 individuals from 9 populations were examined for their reproductive status. The overall proportion of non-reproducing individuals was 35.2%, but the proportion varied greatly among the populations, ranging from a completely non-reproducing to completely flowering population (Fig. 5). No seedlings were observed in the studied population stands over the research period.

Table 2. Basic soil properties of stands of four largest *S. lapponum* populations in the Krkonoše Mts.

	PL (n=51)				SZ (n=81)				UJ (n=36)				NJ (n=39)				Total (n=207)			
	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	± SD	Min	Max
SRWC (%)	67.3	18.6	17.6	93.2	70.4	16.2	35.8	91.8	80.6	10.2	43.1	93.3	79.2	10.9	55.3	91.6	3.1	15.9	17.6	93.3
pH	4.2	0.2	3.9	4.6	4.4	0.3	3.9	5.1	4.9	0.5	4.1	6.0	4.6	0.4	3.8	5.9	4.5	0.4	3.8	6.0
N (g.kg ⁻¹)	16.7	11.0	3.2	43.3	19.5	11.0	3.8	44.2	31.3	10.5	0.001	46.3	29.0	12.7	7.9	47.7	22.6	12.6	0.001	47.7
P (mg.kg ⁻¹)	16	25	0	169	22	20	1	99	19	20	2	70	18	17	1	69	19	21	0	169
Ca (mg.kg ⁻¹)	610	458	66	1845	575	446	53	2064	1258	696	123	2490	853	618	131	2477	755	587	53	2490
Mg (mg.kg ⁻¹)	116	66	10	279	149	81	43	378	298	152	51	618	182	70	81	372	173	111	10	618

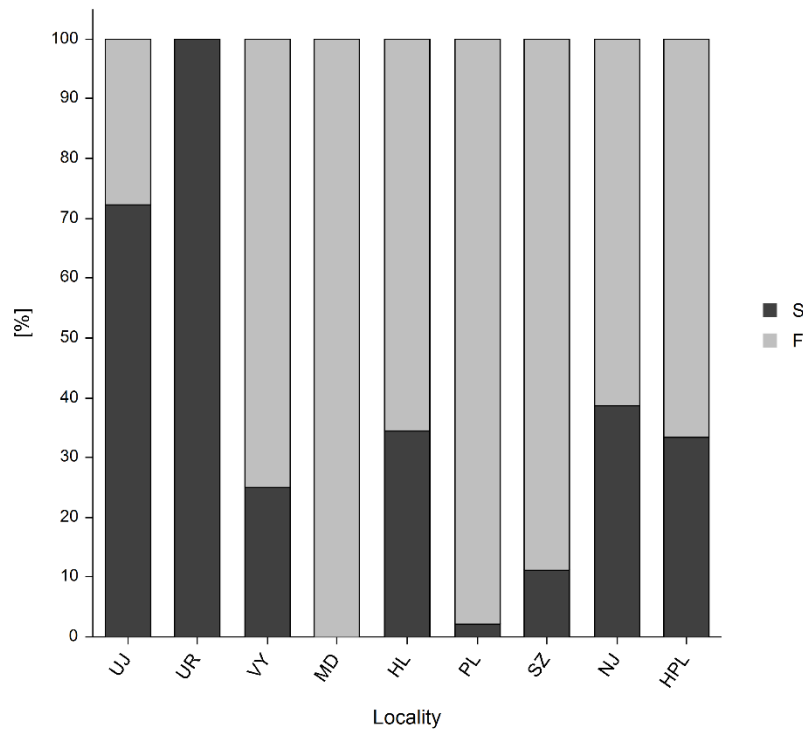


Figure 5. Proportion of non-reproducing (i.e., non-flowering during surveyed period; S) and flowering (F) individuals in each locality.

The composition of co-occurring plants differed significantly between plots with non-reproducing individuals and those with flowering individuals (pDCCA, pseudoF = 1.7, $P = 0.022$), although the adjusted explained variation was only 0.63%. Flowering individuals co-occurred to a greater extent with species (Fig. 6a) indicative of relatively more humid, well-lit and less continental microhabitats in comparison with non-reproducing ones, as evident from the correlation of EIVs with the first canonical axis (EIV for moisture $r_s = -0.32$, $P < 0.001$; light: $r_s = -0.55$, $P < 0.001$; continentality: $r_s = 0.29$, $P = 0.003$; other factors: all P non-significant). In contrast, no difference in soil conditions was found between plots with non-reproducing individuals and those with flowering individuals (partial RDA, pseudoF = 0.4, $P = 0.834$).

At the inter-population level, populations with different proportions of flowering individuals significantly differed in species composition (pDCCA, pseudoF = 5.3, $P = 0.002$; adjusted explained variation = 2.0%) and soil conditions (pRDA, pseudoF = 27.4, $P = 0.002$; adjusted explained variation = 11.4%). *Salix lapponum* populations with higher proportions of flowering individuals were associated with the occurrence of species indicating more humid, warmer and nutrient-rich microhabitats (Fig. 6b; correlation EIVs with the first canonical axis: moisture $r_s = -0.20$, $P < 0.001$; nutrients:

$r_s = -0.30$, $P < 0.001$; temperature: $r_s = -0.15$, $P = 0.029$; other factors: all P non-significant). Concerning the analysis of soil variables, populations with higher proportions of flowering individuals generally occurred on soils with lower concentrations of Ca and Mg and correspondingly lower soil pH (t-value $> |2.0|$ in t-value biplot).

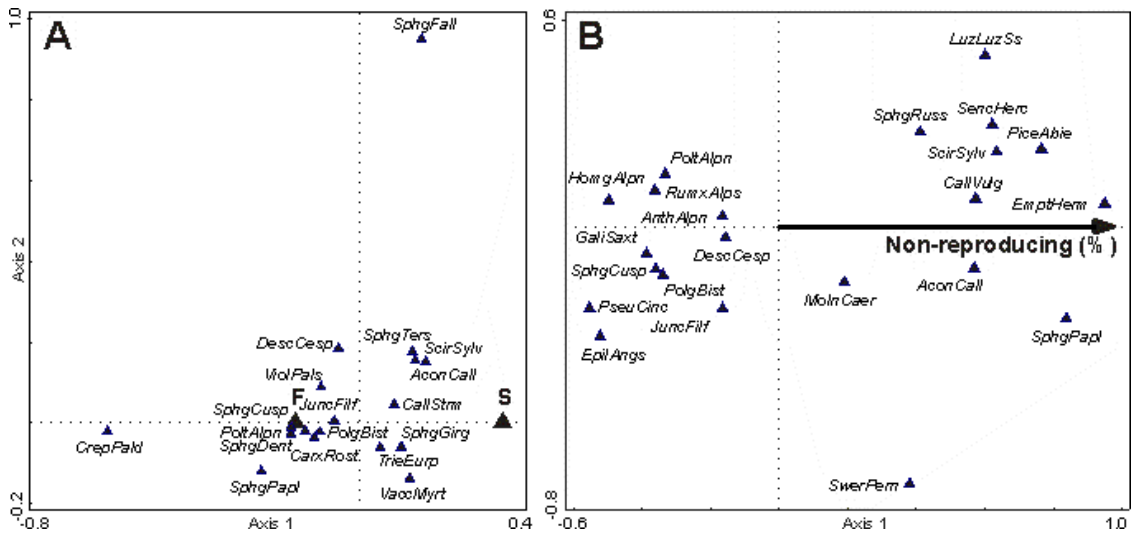


Figure 6. Ordination results for partial Detrended Canonical Correspondence Analyses (pDCCA): (A) The difference in species composition of co-occurring plants between plots with non-reproducing (S) and flowering (F) individuals of *S. lapponum*. (B) The difference in species composition between populations with different proportion of non-reproducing individuals of *S. lapponum* (Non-reproducing (%)). Only species with higher fit ($> 1.0\%$ in A and $> 4\%$ in B, respectively) on the horizontal (canonical) axis are showed.

Abbreviations: AconCall = *Aconitum plicatum*, AnthAlpn = *Anthoxanthum alpinum*, CallStrm = *Calliargon stramineum*, CallVulg = *Calluna vulgaris*, CarxRost = *Carex rostrata*, CrepPald = *Crepis paludosa*, DescCesp = *Deschampsia cespitosa*, EmpHerm = *Empetrum hermaphroditum*, EpiAngs = *Epilobium angustifolium*, GaliSaxt = *Galium saxatile*, HolAlp = *Homogyne alpina*, JuncFill = *Juncus filliformis*, MolnCaer = *Molinia caerulea*, PiceAbie = *Picea abies*, PolgBist = *Bistorta officinalis*, PoltAlpn = *Polytrichastrum alpinum*, PseuCinc = *Pseudobryum cinclidioides*, RumxAlps = *Rumex alpestris*, ScirSylv = *Scirpus sylvaticus*, SencHerc = *Senecio hercynicus*, SphgCusp = *Sphagnum cuspidatum*, SphgDent = *Sphagnum denticulatum*, SphgGirg = *Sphagnum girgensohnii*, SphgPapI = *Sphagnum papillosum*, SphgRuss = *Sphagnum russovii*, SphgTers = *Sphagnum teres*, SwerPern = *Swertia perennis*, TriEurp = *Trientalis europaea*, VaccMyrt = *Vaccinium myrtillus*, VioPals = *Viola palustris*

Co-occurring plants and vegetation structure

In total, 64 bryophyte taxa and 70 taxa of vascular plants co-occurred with *S. lapponum* in the 207 plots sampled at a fine scale at four localities. Most species co-occurred at low frequencies with *S. lapponum*. Only two vascular plants were present in relevés at frequencies higher than 50% (*Calamagrostis villosa*: 58%, *Trientalis europaea*: 54%), and four species had frequencies between 40 and 50% (*Molinia caerulea*, *Viola palustris*, *Deschampsia cespitosa*, *Veratrum album* subsp. *lobelianum*). Bryophytes co-occurred less frequently with *S. lapponum* than did vascular plants. The most frequent bryophytes, *Sphagnum cuspidatum* and *S. recurvum* agg., were each found in only 29% of plots (Table 3). The covers of shrub, herb and moss layers were rather similar among the plots (Table 1) and typically reached 50%, with the cover of the moss layer being the most variable.

Divisive classification divided the fine-scale dataset into three groups of plots (Table 3). The most common group (C; 57% of the fine-scale plots) represents stands with the highest frequency of flowering *Salix* individuals (88.9%). The stands are differentiated by diagnostic taxa of broad-leaved forbs (*Rumex arifolius*, *Veratrum album* subsp. *lobelianum*), grasses (*Calamagrostis villosa*, *Deschampsia cespitosa*), *Bistorta officinalis*, *Viola palustris*, *Sphagnum cuspidatum* etc. and represent relatively wet, less acidic, warmer and nutrient-rich microhabitats along streams and in glacial cirques. The second most frequent group (B; 22%) represent stands with rather common occurrence of flowering *Salix* (84.8%) that are differentiated by the presence of the diagnostic taxa *Molinia caerulea*, *Carex rostrata*, *Juncus filiformis*, *Eriophorum vaginatum*, and *Sphagnum* mosses (*S. recurvum*, *S. denticulatum*). These stands represent the wettest but less nutrient rich microhabitats typical of margins of transitional mires as well as subalpine spring vegetation (presence of *Bartsia alpina*, *Swertia perennis*, *Carex echinata*). The last group (A; 21%) has a higher frequency of non-reproducing (37.2%) *Salix* plants. Stands are differentiated by the presence of some *Sphagnum* species (*S. russowii*, *S. papillosum*), *Racomitrium sudeticum* and ericoid plants (*Vaccinium myrtillus*, *V. vitis-idaea*, *V. uliginosum*, *Calluna vulgaris*, *Empetrum nigrum*) indicating relatively drier, cool and nutrient poor conditions typical of either poor acidic mires with regularly decreasing water level or subalpine heathlands (Table 3).

Analysis of the 22 relevés acquired from CNFD revealed 40 bryophyte taxa and 96 taxa of vascular plants co-occurring with *S. lapponum*. Seven species co-occurred with *S. lapponum* at frequencies greater than 50% (e.g., *Bistorta officinalis*: 91%; *Deschampsia cespitosa*: 86%; *Calamagrostis villosa*: 77%), and six other species had frequencies between 40 and 50% (Table 3). Compared with vascular plants, bryophytes co-occurred less frequently with *S. lapponum*; the most frequent co-occurring bryophytes, *Rhizomnium punctatum* and *Philonotis seriata*, were found in 45 and 32% of relevés, respectively (Table 3). Data on layer cover were available for 11 relevés. Cover of *S. lapponum* reached on average 82%, while cover of both the herb and moss layers typically reached ca 40–50% (Table 1).

Comparison of plot data recorded at the fine- and coarse-scale revealed small but significant differences in species composition (Mann-Whitney U for similarity of relevé groups, $P < 0.001$, percentage difference: 7.4%; Table 3). The floristic composition of coarse-scale relevés was differentiated from fine-scale plots by several, mainly robust, broad-leaved forbs typical of moist and fertile soils (e.g., *Cicerbita alpina*, *Chaerophyllum hirsutum*, *Aconitum plicatum*, *Adenostyles alliariae*) and herbs typical of wet soils of springs and peats (e.g., *Viola biflora*, *Crepis paludosa*). In addition, most of the typical bryophytes in coarse-scale relevés (e.g., *Philonotis seriata*, *Dicranella palustris* and *Scapania uliginosa*) indicate the acidic, well-lit soils of mountain springs. In contrast, most of the abovementioned species were either rare or not recorded at fine-scale plots. The fine-scale plots were differentiated from coarse-scale relevés by the occurrence of some grasses (i.e., *Molinia caerulea*, *Anthoxanthum alpinum*), small herbs (*Viola palustris*, *Trientalis europaea* and *Solidago virgaurea* subsp. *minuta*), and slightly higher frequencies of various *Sphagnum* species (Table 3).

Table 3. Frequencies of co-occurring plant species with *S. lapponum* in fine-scale plots (207 plots) and coarse-scale plots acquired from ČNFD (22 relevés) in the Krkonoše Mts. Fine-scale plots were classified by divisive classification (TWINSPAN) into three groups (A, B, C). For each fine-scale group of plots, mean(SD) of EIVs for selected factors and cover of each layer were calculated. Species positively discriminating respective group (= indicator species with high fidelity; phi coefficient > 0.2 and Fisher exact test $P \leq 0.05$) are in frames. Other species present at least in 10% of relevés of one of the groups are displayed. Species are sorted according to layer and decreasing frequencies within respective layer/plot group.

	Layer	A	B	C	Total fine-scale data set	Coarse-scale data set
No of plots or relevés		43	46	118	207	22
Light		6.4(0.6)	6.7(0.5)	6.5(0.5)		
Temperature		3.1(0.6)	3.4(0.4)	3.5(0.4)		
Continentality		4.5(0.4)	4.6(0.5)	4.6(0.6)		
Moisture		6.4(0.6)	7.6(0.6)	7.2(0.8)		
Soil reaction		2.2(0.5)	2.8(0.6)	2.8(0.6)		
Nutrients		2.2(0.4)	2.5(0.4)	2.9(0.5)		
Cover E2 (%)		54(19)	45(22)	51(21)		
Cover E1 (%)		54(23)	51(18)	47(20)		
Cover E0 (%)		53(32)	45(30)	45(34)		
Indicator species						
<i>Vaccinium myrtillus</i>	E1	63	9	14	23	5
<i>Calluna vulgaris</i>	E1	49	13	3	14	9
<i>Avenella flexuosa</i>	E1	40	.	19	19	9
<i>Vaccinium vitis-idaea</i>	E1	30	.	3	8	.
<i>Empetrum hermaphroditum</i>	E1	12	.	.	2	5
<i>Vaccinium uliginosum</i>	E1	12	.	.	2	5
<i>Sphagnum russowii</i>	E0	47	4	4	13	.
<i>Sphagnum papillosum</i>	E0	16	.	.	3	.
<i>Racomitrium sudeticum</i>	E0	9	.	.	2	.
<i>Molinia caerulea</i> s.lat.	E1	67	80	21	44	9
<i>Juncus filiformis</i>	E1	9	63	37	37	32
<i>Carex rostrata</i>	E1	7	43	32	29	27
<i>Eriophorum vaginatum</i>	E1	5	26	3	9	23
<i>Dactylorhiza *psychrophila</i>	E1	.	26	3	7	.
<i>Swertia perennis</i>	E1	5	17	3	7	41
<i>Carex echinata</i>	E1	.	7	.	1	9
<i>Bartsia alpina</i>	E1	.	7	.	1	14
<i>Sphagnum recurvum</i> agg.	E0	19	61	20	29	5
<i>Sphagnum denticulatum</i>	E0	2	13	2	4	.
<i>Calypogeia sphagnicola</i>	E0	.	7	.	1	.
<i>Calamagrostis villosa</i>	E1	47	33	72	58	77
<i>Deschampsia cespitosa</i>	E1	28	17	66	47	86
<i>Bistorta officinalis</i>	E1	2	2	64	37	91
<i>Veratrum *lobelianum</i>	E1	21	20	61	43	68

Table 3. Continued.

	Layer	A	B	C	Total fine-scale data set	Coarse-scale data set
<i>Viola palustris</i>	E1	5	33	58	41	14
<i>Rumex alpestris</i>	E1	.	2	27	16	45
<i>Homogyne alpina</i>	E1	9	2	23	15	5
<i>Galium saxatile</i>	E1	.	2	12	7	.
<i>Carex canescens</i>	E1	.	2	9	6	14
<i>Scirpus sylvaticus</i>	E1	.	.	7	4	.
<i>Senecio hercynicus</i>	E1	.	.	6	3	50
<i>Sphagnum cuspidatum</i>	E0	7	7	46	29	18
<i>Salix silesiaca</i>	E2	.	.	1	1	36
<i>Crepis paludosa</i>	E1	.	13	6	6	68
<i>Viola biflora</i>	E1	68
<i>Cicerbita alpina</i>	E1	.	.	3	2	50
<i>Aconitum plicatum</i>	E1	.	4	4	3	41
<i>Chaerophyllum hirsutum</i>	E1	.	2	3	2	41
<i>Adenostyles alliariae</i>	E1	36
<i>Alchemilla glabra</i>	E1	36
<i>Myosotis palustris</i> agg.	E1	.	2	.	1	32
<i>Hypericum maculatum</i>	E1	.	.	3	2	27
<i>Athyrium distentifolium</i>	E1	.	4	.	1	23
<i>Rhizomnium punctatum</i>	E0	2	.	.	1	45
<i>Philonotis seriata</i>	E0	.	2	3	2	32
<i>Dicranella palustris</i>	E0	.	.	2	1	27
<i>Pellia</i> sp.	E0	23
<i>Scapania uliginosa</i>	E0	23
Other species						
<i>Betula pubescens</i> subsp. <i>carpatica</i>	E3	.	4	2	2	.
<i>Salix lapponum</i>	E2	100	100	100	100	100
<i>Sorbus aucuparia</i>	E2	5	.	.	1	18
<i>Rubus idaeus</i>	E2	.	.	2	1	14
<i>Trientalis europaea</i>	E1	51	57	53	54	23
<i>Anthoxanthum alpinum</i>	E1	23	35	47	39	14
<i>Potentilla erecta</i>	E1	26	41	40	37	27
<i>Solidago virgaurea</i> subsp. <i>minuta</i>	E1	28	30	36	33	5
<i>Nardus stricta</i>	E1	30	26	7	16	9
<i>Ranunculus platanifolius</i>	E1	.	.	1	1	18
<i>Epilobium nutans</i>	E1	18
<i>Epilobium palustre</i>	E1	18
<i>Valeriana sambucifolia</i>	E1	18
<i>Geranium sylvaticum</i>	E1	14

Table 3. Continued.

	Layer	A	B	C	Total fine-scale data set	Coarse-scale data set
<i>Pedicularis sudetica</i>	E1	14
<i>Polytrichum commune</i>	E0	30	15	13	17	18
<i>Calliergon stramineum</i>	E0	5	24	18	16	18
<i>Sphagnum girgensohnii</i>	E0	9	7	18	14	23
<i>Sphagnum squarrosum</i>	E0	18
<i>Sphagnum capillifolium</i>	E0	14

Discussion

Although considerable attention has been given to the ecology of *S. lapponum* within its main range or at its range margins (e.g., Fijałkowski 1958; Urban & Wawer 2001; Totland & Esaete 2002; Pogorzelec 2003, 2008, 2009; Pogorzelec et al. 2014a, b; Scottish Montane Willow Research Group 2005; Kołos et al. 2015), no extensive studies have been performed on isolated (sub)alpine populations of this species from central and southern Europe. Therefore, our study presents the first comprehensive analysis of the habitat conditions and population structure of these geographically isolated stands. Isolated populations may differ from those in the main range due to lower genetic diversity and absence of among-population gene flow. Consequently, their responses to various abiotic and biotic interactions may also differ. Such responses are probably species dependent and may thus vary among taxa (Sexton et al. 2009). Concerning *S. lapponum*, we found that habitat conditions of its isolated populations are rather similar to those in the main range. Particular similarities and differences are discussed below.

Habitat conditions of S. lapponum stands

All of the four studied populations of *S. lapponum* in the Krkonoše Mts. grow in places with high relative SRWC (Table 1), consistently with most published studies. Typically, stands of *S. lapponum* in the Krkonoše Mts. occur in local depressions around subalpine springs, at the periphery of mires, along streams or along the ledges or upper slopes of glacial cirques. Such sites tend to exhibit shallow, concave relief with poor drainage or

occur on shallow to steep slopes with shallow soil layers, often with great snow accumulation (Kočí 2010). Long snow lie is considered an important factor for stands of *S. lapponum* in Scotland (McVean & Ratcliffe 1962) and Scandinavia (Dahl 1956) because it shelters the plants from frost, especially during the spring, and supplies water during snow melt.

Generally, *Salix lapponum* is reported to occur on acidic soils on acidic bedrock (Fijałkowski 1958; Chmelař & Meusel 1979; Pogorzelec 2003). However, according to Elven & Karlsson (2000) *S. lapponum* is indifferent to soil reaction. Stands of lowland populations from (north)eastern Poland vary in soil pH from almost neutral (6.7) in the Puszcza Knyszyńska (Kołos et al. 2015) through slightly acidic (4.2–6.9) in the Polesie National Park (Pogorzelec 2003; Pogorzelec 2008) to strongly acidic (3.5–4.0) in the Sobibor Landscape Park (Urban & Wawer 2001). Substrates under *S. lapponum* stands in the Krkonoše Mts. develop on acid bedrock, which is formed mainly by biotite granite of Variscan orogeny (Chaloupský 1989) and belong mostly to the various subtypes of podzols (often with shallow peat layer), histosols or rarely gleys (www.geology.cz). Accordingly, we found the soil pH of *S. lapponum* sites to range from strongly to slightly acidic (Table 1), consistent with previous work (see also Hadač & Váňa 1967).

Stands of *S. lapponum* in Poland and Scotland are reported to occur on oligo- or mesotrophic soils that typically have low concentrations of nutrients (Pogorzelec 2008, Mardon 1990). In the Krkonoše Mts., *S. lapponum* sites show variable but generally higher concentrations of soil N due to the poorly decomposable biomass. It follows that nutrients are bounded as organic compounds in peat or raw humus (Hájek & Hájková 2011). Indeed, the concentrations of Ca, Mg, and P in the soils of subalpine populations in the Krkonoše Mts. are generally low. However, we found high variation in Ca (and in Mg to some extent) in soil samples among and even within populations. This suggests that at least some individuals grow on microsites enriched in minerals. It cannot be excluded that at least some of these microsites contain slowly depleting particles of dolomitic lime (Semelová et al. 2008) as residuals of past aerial liming that was applied in the Krkonoše Mts. during 1983–1990 to slow acidification (Matějka et al. 2010) or allochthonous material (e.g., dolomitic lime, cinder, basaltic rock) used in the past for track stabilization (Müllerová et al. 2011). Alternatively, these microsites might contain streaks of mineral-rich rock in otherwise acidic bedrocks (Pokorná 1978).

The mean EIVs show similar patterns as the soil data. The species co-occurring with *S. lapponum* in the Krkonoše Mts. are mainly acidophytes and indicators of moderate to high sunlight conditions and constantly moist to damp, less infertile to moderately fertile soils. This pattern agrees with indicator values for *S. lapponum* derived for Polish and British floras by Zarzycki & Korzeniak (2002) and Hill et al. (1999), respectively. However, *S. lapponum* is considered by Hill et al. (1999) as an indicator of weakly acid to weakly basic conditions, which is absent from very acid soils. This characteristic is consistent with the frequent occurrence of *S. lapponum* on highly to moderately calcareous schist and limestone in the higher mountains of the Scottish Highlands despite its rare occurrence on rather acid substrates (Rodwell 1991). This strong contrast to the occurrence of *S. lapponum* on acid granite in the Krkonoše Mts. might be due to the pressure from herbivores that restricted the Scotland populations to outcrops of base-rich substrates (Mardon 1990).

Environmental factors may influence proportion of non-reproducing individuals

The occurrence of non-reproducing plants in populations may be caused by two different phenomena. Non-reproducing individuals may be juvenile plants that continue to grow to the reproductive stage, or they may be adult individuals that do not flower due unsuitable environmental conditions (Alliende & Harper 1989; Takkis et al. 2013). We have shown that the frequency of non-reproducing individuals of *S. lapponum* is greater than 30% in five of the nine populations examined in the Krkonoše Mts. In addition, we did not observe the establishment of any *Salix* seedlings during our five years of field work. Very low seedling establishment was similarly reported for *S. lapponum* populations in Scotland (Scottish Montane Willow Research Group 2005; Stamati et al. 2007) and Poland (Pogorzelec et al. 2014a).

The estimated period from seedling establishment to the first flowering in willows is short, e.g., six to ten years in *Salix cinerea* (Alliende & Harper 1989) and two to three years in *S. bicolor* (Chmelař & Koblížek 1990). However, no such data are available for *S. lapponum*. In addition, very little is known regarding the longevity and population dynamics of *S. lapponum*. However, one record indicates that *S. lapponum* individuals can live for a considerable period of time: One female individual below the summit of the Praděd Mt. (Hrubý Jeseník Mts.) was reported by early *Salix* researchers

and is still present today (Grabowski 1843; Chmelař 1972; Hroneš et al. 2014). Even if the clonality rate is quite low in *S. lapponum* (Stamati et al. 2007; Pogorzelec et al. 2014b), this individual has been able to persist at its locality for more than 160 years. This observation is in strong contrast with the statement of Chmelař & Koblížek (1990) that the expected longevity of *S. lapponum* individuals is approximately 30 years. Similar or even greater longevity has been observed also in other alpine shrubs, e. g. *Juniperus sabina* (<770 years; Wesche et al. 2005), *Rhododendron ferrugineum* (<280 years; Pornon et al. 2000) and *Salix herbacea* (<450 years; de Witte et al. 2012).

Stamati et al. (2007) explained the apparent lack of seedlings in populations of *S. lapponum* as either the overlooking of very rare seedling establishment or occasional bursts of seed and seedling production under optimal conditions. The optimal conditions for seedling recruitment in *S. lapponum* are considered bare soil or mechanically disturbed microsites without dense vegetation cover and without strong competition from other plants (Mardon 1990; Shaw et al. 2010). Similar conditions for seedling recruitment have been described for many riparian *Salix* species in which new populations are established via the simultaneous recruitment of a large number of seedlings in newly disturbed sites created by flooding (Scott et al. 1996); such species show an even-aged, high-density population structure from an early stage of development (Alliende & Harper 1989). Our analyses and observations showed that most sites with present occurrence of *S. lapponum* in the Krkonoše Mts. are densely covered by herb and/or moss layers and that open microsites are uncommon. The establishment of seedlings in populations of *S. lapponum* in the Krkonoše Mts. may thus be a rare event, which might be partly compensated by the longevity of individuals. Other possible explanations of seedlings absence are herbivore pressure towards seedlings (Shaw et al. 2010) and/or inbreeding influencing the survival of seedlings (Naito et al. 2005). Populations of *S. lapponum* in the Krkonoše Mts. thus appear to be in the mature stage, with little recent recruitment. However, we recorded the spontaneous establishment of some likely hybridogenous *Salix* plants along touristic trails with disturbed surfaces near one of the studied *Salix* sites. This observation suggests that recruitment of *S. lapponum* might be limited by the availability of disturbed sites in natural conditions.

The short time from seedling establishment to the first flowering together with the probability of high longevity of individuals indicates that the non-reproducing plants

may not be juveniles but are more likely older plants that do not flower because they are growing outside of the ecological optimum. We are aware of interpretational limits of our analyses stemming from the fact that only a limited number of populations were studied in detail, and we do not know the exact ages of the plants. However, the multivariate analyses at two spatial scales (within and between populations) and the classification of species composition produced rather consistent findings: Flowering plants of *S. lapponum* occur in more humid, more nutrient-rich, warmer and less continental microhabitats than do non-reproducing ones. Concerning the soil variables, the soils of population stands with higher proportion of non-reproducing individuals typically had higher concentrations of Ca and Mg and correspondingly higher pH than did the soils of stands with lower proportions of such individuals. In agreement with our results, several sources report optimal performance of *S. lapponum* on sites with high levels of soil moisture (Dahl 1956; McVean & Ratcliffe 1962) and soils with lower pH (Fijałkowski 1958). Pogorzelec (2008) found that the water of stands with uncommon occurrences of *S. lapponum* significantly differs from that of stands with abundant occurrences of *S. lapponum* in having higher P and Mg concentrations but lower Ca concentrations.

Vegetation associations of S. lapponum

Salix lapponum is reported to grow in environments including peat bogs, mires, lake shores, stream banks, swampy boreal forests and glacial cirques (Elven & Karlsson 2000; Pogorzelec 2008). It forms extensive scrub and is typically accompanied by other alpine/boreal willow species, e.g., *S. lanata*, *S. arbuscula* and *S. glauca* (Mardon 1990; Elven & Karlsson 2000). Situation in the Krkonoše Mts. is partly different, because *S. lapponum* forms either mostly monospecific stands within the shrub layer where other shrub species (e.g., *Salix silesiaca*) rarely occur or scattered stands of isolated individuals. Consequently, scrub vegetation with dominant *S. lapponum* was described as an endemic association *Salicetum lapponum* Zlatník 1928 in this region (Kočí 2010) but was recently reported to also occur in Massif Central, France (de Foucault 2012).

Association *Salicetum lapponum* has been traditionally classified within the alliance *Adenostylion alliariae* Br.-Bl. 1926, tall-forb and fern-rich chionophilous communities at high altitudes (Kočí 2010) because of the frequent occurrence of its

diagnostic species. However, a recent critical account of European vegetation (Mucina et al. 2016; see also Kliment et al. 2010) corrected the assignment of subalpine communities of deciduous shrubs (incl. subalpine stands of *S. lapponum*), and excluded them from the class *Mulgedio-Aconitetea* and classified them into the class *Betulo carpaticae-Alnetea viridis* which represents subalpine and subarctic herb-rich alder and willow scrub and krummholz of the Alps, the Carpathians, Sudetes, the Balkans, the Caucasus, Northern Europe and Greenland. The classification of fine-scale plots (Table 3) showed that such “typical” stands of *S. lapponum* with frequent co-occurrence of upper montane and (sub)alpine broad-leaved species of wet and nutrient richer soils (e.g., *Rumex arifolius*, *Veratrum album* subsp. *lobelianum*, *Cicerbita alpina*, *Senecio nemorensis*) represented approximately half of the observed plots. Individuals of *S. lapponum* were also found in less optimal microhabitat conditions on the periphery of acidic, nutrient poor mires with fluctuating water level belonging to various vegetation types within the class *Scheuchzerio palustris-Caricetea nigrae*, the subalpine spring vegetation on nutrient-poor siliceous bedrock (alliance *Swertio perennis-Dichodontion palustris*), the subalpine grasslands dominated by *Molinia caerulea* of the alliance *Calamagrostion villosae* or the subalpine heathlands of the alliance *Genisto pilosae-Vaccinion* (see also Macko 1952; Hadač & Váňa 1967).

Similar vegetation types to “typical” stands with a dominance of *S. lapponum* can also be found in alpine areas of Scandinavia (Dierßen 1996) and partially even in Scotland (Rodwell 1991) and the Pyrenees (Danton & Baffray 1995). Stands of *S. lapponum* on the periphery of transitional mires and peat-bogs and mires are partially similar to *S. lapponum* stands in eastern Poland where *S. lapponum* grows mainly in (sub)boreal mesotrophic wetlands and transitional mires (i.e. classes *Phragmito-Magno-Caricetea* Klika in Klika et Novák 1941, *Scheuchzerio palustris-Caricetea nigrae* Tüxen 1937) and also temporarily persist in their successional stages such as order *Molinietalia caeruleae* Koch 1926 (Pogorzelec 2009; Kołos et al. 2015).

Comparing of the data between vegetation plots at coarse- and fine-scales, we found that some tall-forb species typical for this vegetation type (e.g., *Aconitum plicatum*, *Adenostyles alliariae*, *Cicerbita alpina*) generally avoid growing below the willow canopy. Totland & Esaete (2002) showed that the canopy of *S. lapponum* did not affect abiotic conditions (i.e., pH, SRWC, temperature) drastically. However, the canopy is generally dense and therefore does not provide sufficient space for larger

plants. Another explanation for the compositional differences between the two groups of vegetation plots might be less light income inside the canopy (Totland & Eisaete 2002), promoting the occurrence of forest species above the tree line due to the so-called nurse effect (Dona & Galen 2007; Zeidler et al. 2012). However, this appears to be unlikely in the present case because the EIVs indicated similar light conditions in both datasets. In addition, some type of bias may theoretically contribute to the species composition differences between coarse- and fine-scale plots when joint analysis (classification, ordination) of vegetation plots differing in size recorded within relatively homogeneous vegetation is done (Dengler 2003; Otýpková & Chytrý 2006). Therefore, we have not analysed the fine- and coarse-scaled plots together. Our analyses also showed that coarse-scale plots were likely preferentially recorded in the most suitable places with well-developed *S. lapponum* vegetation (i.e., in the way that typical phytosociologists work; Hédl 2007), whereas we sampled all of the individuals in each population, including those growing outside of their optimal conditions and outside the well-developed stands.

Threats to S. lapponum and future prospects of its populations in the Krkonoše Mts.

The studied populations are located in the most valuable parts of the Krkonoše Mts., i.e., in glacial cirques and mountain plateaus, where they form part of the subalpine landscape, which is otherwise very rare in the Sudetes. Direct destruction of shrubs by large herbivores (mainly red deer) is considered a main threat to *S. lapponum* in the Sudetes Mts. (Hroneš et al. 2014). Similar large herbivore pressure to alpine willows, especially at higher elevations, has been observed in Scotland (Mardon 1990; Shaw et al. 2010). High browsing by herbivores results in decreased numbers of shoots and decreased radial growth of stems (Scottish Montane Willow Research Group 2005; Shaw et al. 2010; Speed et al. 2013). Shaw et al. (2010) showed that reductions in shoot number lead to lower numbers of inflorescences, fewer pollinator visits and consequently lower seed production. We have observed another negative impact of deer on *S. lapponum* in the Krkonoše Mts. Twigs of large willow individuals were broken by animals and then damaged by frost in the winter. In eastern Poland, *S. lapponum* is not threatened by herbivores but by decrease of soil water content and by shading caused by succession (Urban & Wawer 2001; Kołos & Chmielewska-Nowik 2007; Pogorzelec et

al. 2014a; Kołos et al. 2015). Climate change-enhanced succession may be a serious future threat to subalpine populations in the Krkonoše Mts. but might also stimulate the growth of some populations as observed for *Alnus viridis* in the Alps (Anthelme et al. 2003). Future active protection measures for *S. lapponum* in the Krkonoše Mts. should be focused mainly on the reduction of the red deer numbers and on the creation of places suitable for seedling germination (i.e. artificial creation of gaps in the vegetation).

CHAPTER 4:

Female-biased sex ratio but the absence of spatial and niche segregation between sexes in alpine populations of dioecious *Salix lapponum* (Salicaceae)

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Abstract

Dioecious plants often exhibit deviations from expected 1:1 sex ratios. Genus *Salix* is a notable example of the female-biased sex ratio. Quite surprisingly, there are very few studies retesting observed bias patterns from the different parts of the species range. We have determined whether isolated subalpine populations of *Salix lapponum* exhibit a biased secondary sex ratio, measured the size of the plants, and tested the spatial and ecological correlates of the bias at fine and broad scales. Males were generally taller than females, suggesting that a different allocation of resources may occur in both sexes. Despite this, we found consistent secondary female bias with females on average twice as common as males in most populations studied. No correlations of sex ratio with altitude as a proxy of environmental harshness and proportion of non-flowering individuals were found. Additionally, no differences in spatial sex segregation and microhabitat preferences were found between males and females at a fine scale within the studied populations. Our results suggest that the biased sex ratio in *S. lapponum* is not environment-dependent and probably originates during early stages of ontogenetic development (seeds).

Key words: dioecy, niche differentiation, secondary sexual dimorphism, sex ratio bias, spatial sex segregation, willow

Introduction

Dioecy, a state when each individual bears only one sex, is a relatively rare phenomenon among flowering plants. Theoretical models based on negative frequency-dependent selection predict a 1:1 primary sex ratio in populations of dioecious organisms (Fisher 1930); however, there is solid evidence that deviations from this expectation are common in Angiosperms, usually measured as the difference in the frequency of reproducing plants of each sex (e.g., Delph 1999; Myers-Smith & Hik 2012; Sinclair et al. 2012; Field et al. 2013a, b). Field et al. (2013a) found that approximately half of the 243 analysed dioecious species diverged from the 1:1 ratio, with the male-biased sex ratio being twice as common as female-biased in mature populations.

The mechanisms driving the bias are still not well known and are attributed to various genetic and ecological factors (Delph 1999, Field et al. 2013a, b; Pucholt et al. 2017). Primary sex ratio observations based on direct analysis of gametes or seeds that require sex-specific markers are scarce (e.g., Stehlik & Barrett 2005; Charlesworth 2016). The observation of the primary sex ratio is mainly possible in species with well-developed sex chromosomes. Bias in such species can be caused by Y-chromosome degeneration due to the accumulation of deleterious mutations (Stehlik & Barrett 2005), sex chromosome meiotic drive (Taylor 1999) and pollen competition (i.e., certation; Taylor 1999; Stehlik et al. 2008). However, in the majority of dioecious species without sex chromosomes, only the observation of reproducing plants (i.e., secondary sex ratio) is possible.

The secondary sex ratio is affected by secondary sexual dimorphism (i.e., sex characters that are not directly related to gamete production; Meagher 1992). Secondary sexual dimorphism may be morphological, physiological (e.g., photosynthesis efficiency, growth rates, use of resources; Lloyd & Webb 1977; Barrett & Hough 2013) and phenological (e.g., timing of flowering; Delph 1999) and its evolution is usually explained by the dimorphic niche scenario, which is based on the assumption that females and males differ in their intrinsic energetic needs due to their different reproductive roles (Slatkin 1984; Bierzychudek & Eckhart 1988; Dudley 2006). Therefore, a biased secondary sex ratio may arise as a result of the different allocation of resources due to different costs of reproduction, differences in mortality, increased susceptibility to environmental stress and/or resource competition between sexes (Bierzychudek & Eckhart 1988; Delph 1999; Field et al. 2013b; Hultine et al. 2016, 2018). This may be apparent in the different growth rates of each sex or different sizes in first flowering (Delph 1999; Obeso 2002) and/or can lead to the segregation of sexes at various spatial scales (SSS; Dudley 2006; Myers-Smith & Hik 2012; Che-Castaldo et al. 2015). Generally, females are considered as the sex showing greater trade-offs with other life history traits compared to males because of their higher resource investment in reproduction (Delph & Meagher 1995), making the secondary sex ratio male-biased (Field et al. 2013b). However, in clonal herbs and shrubs with abiotic pollen dispersal that need to produce massive quanta of pollen to be able to successfully reproduce, the higher reproductive cost may be on the side of males (Harris & Pannell 2008), resulting in female-biased secondary sex ratios (Sinclair et al. 2012; Field et al. 2013a).

The secondary sex ratios in the dioecious genus *Salix* (Salicaceae) are reported to be female-biased in several mainly arctic-alpine species, e.g., 2:1 in *S. alaxensis*, *S. glauca* (both Myers-Smith & Hik 2012), *S. arctica* (Dawson & Bliss 1989), *S. cinerea* (Alliende & Harper 1989), *S. sachalinensis* (Ueno et al. 2007) and *S. sitchensis* (Che-Castaldo et al. 2015); 3:1 in *S. barrattiana*, *S. pulchra*, *S. richardsonii* (all Myers-Smith & Hik 2012) and *S. repens* (de Jong & van der Meijden 2004); and 3:2 in *S. herbacea* and *S. polaris* (Crawford & Balfour 1983). Given that in *Salix* the sex chromosomes are not present, and females are probably the heterogametic sex (Pucholt et al. 2015), several known prezygotic mechanisms responsible for a female sex bias, such as Y-chromosome degeneration (Stehlik & Barrett 2005) and certation (Taylor 1999), should be excluded. Moreover, reports on significant ecological correlates of secondary sexual dimorphism in *Salix* are quite anecdotal but also counterintuitive to explain the observed female-biased sex ratio. Ecological causation also predicts spatial segregation of sexes along underlying habitat gradients, but there are only two studies reporting a significant SSS in *Salix*. In several populations of *S. arctica* and *S. glauca*, the sexes display significant spatial segregation, with females more often occupying mesic, nutrient-rich habitats, whereas males more often occupy more stressful environments, i.e., xeric, nutrient-poor places (Dawson & Bliss 1989; Dudley 2006). Such ecological differentiation is related to physiological differences between the sexes. Dudley (2006) found that females possess higher phosphorus concentrations in seeds than males have in pollen in *S. glauca*, and Dawson & Bliss (1989) found higher turgor pressures and higher rates of stomatal conductance in the leaves of males relative to females in *S. arctica* growing in xeric conditions. Such physiological differentiation between sexes may lead, in theory, to higher resource uptake capacity and consequently to the competitive superiority of males over females, eventually leading to a male-biased sex ratio (Lloyd & Webb 1977). Paradoxically, the abovementioned authors (Dawson & Bliss 1989; Dudley 2006) observed a female-biased sex ratio in studied populations. Further authors have only speculated about compensatory processes to explain this paradox, such as sex bias in germination or seedling mortality (Che-Castaldo et al. 2015 but see de Jong & van der Meijden 2004) or the differences in dispersal between pollen and seeds (de Jong et al. 2002), and biotic factors, such as herbivory (Dudley 2006; Che-Castaldo et al. 2015), which might counteract the effect of a dimorphic niche scenario and shift the sex ratio to female-biased.

It is obvious that the factors determining the sex ratio in *Salix* are potentially wide-ranging and may strengthen or weaken one another (Hultine et al. 2018), and their contribution to the bias may vary depending on species, location and/or age of its populations. Moreover, very few studies have concentrated on a larger geographical scale or on the retest of observed patterns of sex ratios in the different parts of the species range. For example, Field et al. (2013b) have found only 13 species with more than 3 populations sampled on a larger scale, and among them, only one *Salix* species. The Arctic/alpine species *Salix lapponum* represents a good model for such a study due to the nature of its range. Observations of its sex ratio were already published based on its main range in Sweden by Hughes et al. (2010), who observed a female-biased sex ratio (0.42; calculated as M/F) but no evidence of SSS. To add more data on the sex ratio and its possible precursors in *S. lapponum*, we have chosen alpine populations of this species in the Krkonoše Mts. (Czech Republic). In this paper, we aim to explore whether their secondary sex ratio is biased, and if so, whether there are any ecological factors (i.e., sex-specific spatial segregation, microhabitat preferences) and differences in morphological sexual dimorphism, which could explain the observed sex ratios. Given that in absence of sex-specific genetic markers the primary sex ratio is almost impossible to obtain in wild populations, we are focusing on factors tied to the dimorphic niche scenario in this study.

Materials and methods

Study species

The downy willow (*Salix lapponum* L.) is a dioecious shrub predominantly distributed in the boreal belt of Europe and western Siberia, with isolated populations in the (sub)alpine zone of the central, south eastern and south western European mountains (Skvortsov 1999). Pollination of *S. lapponum* is reported to be ambophilous, i.e., 50:50 insect:wind pollination (Peeters & Totland 1999). The seeds are dispersed by wind. Vegetative reproduction occurs rarely and reportedly does not play a significant role in population structure (Stamati et al. 2007). Typical stands are in conditions that have oligo- to mesotrophic soils with acidic to neutral pH and a high level of underground water, i.e., peat bogs and mires (Skvortsov 1999; Pogorzelec 2008). In the Krkonoše

Mts., it is present mainly in glacial cirques and in the vicinity of springs, generally on mild slopes with long-lasting snow cover and a high water content exceeding 60 % in altitudes of 1200–1500 m a. s. l. (Hroneš et al. 2018).

Secondary sex ratios, plant size, vegetation and soil analyses

The fieldwork was carried out during 2009–2013 in the Czech part of the Krkonoše Mts. (the Czech Republic). All known populations where more than one individual has been found (i.e., nine; Hroneš et al. 2018; for locality information see Supplementary File 4) were surveyed in 2009. First, the exact location and approximate altitude of each individual was determined by using a GPS device (Garmin GPX-60 Cx) and its position in the orthophotomap provided by the Krkonoše NP administration (available at <http://gis.krnep.cz/map/>). Then, the individuals were repeatedly surveyed for their sex each June/July between 2009 and 2013. They were categorized into three groups according to their sex: female, male and nonreproductive, i.e., plants that did not flower during our study. Individual plants could usually be distinguished visually, but when plants were located close to one another, we examined the growing direction of branches and the colour of the leaves. In only the NJ population, the clonality optically seemed to be much higher, and the employed method of identifying the individuals failed. Moreover, the population existed in troublesome terrain that limited the possibility of obtaining the exact location of each individual. Therefore, after an attempt to count all individuals for their sex, one transect approximately 115 m long and 25 m wide was established, and all individuals within the transect were subsequently positioned and analysed.

In three large populations (NJ, PL, SZ), the vegetation survey, soil samples (see below) and biometric measurements were carried in 2013. Biometric attributes (i.e., length, width, and height of the individual) were established using a tape measure. The length of the individual was defined as the widest part of the individual, and the width was determined as the orthogonal projection of the length. The area of each individual was calculated as length \times width.

A vegetation plot of 1 \times 1 m was carried out around each *S. lapponum* plant with its caudex in the middle of the plot. The cover of each species of vascular plants and

bryophytes was recorded using the 7-grade Braun-Blanquet abundance-dominance scale (Braun-Blanquet 1964).

Soil samples were taken in each plot over two days at the end of August 2013. The soil was sampled with a soil sampler at a depth of 2–10 cm below the surface at several points around the centre of each plot, and subsamples were mixed together, stored in plastic bags and immediately weighed. After transportation to the laboratory, soil samples were oven dried at 60°C for 48 hours to a constant weight. Soil relative water content was calculated by dividing the dry weight by the crude weight of the sample. The pH of each sample was determined in a water solution using a pH 70+DHS portable pH meter (XS instruments, Italy). The concentration of organic nitrogen (N) in each sample was determined after mineralization with sulphuric acid and Se-K catalysator on DK 20 (Velp Scientifica, Italy) and subsequent distillation with water vapour into boric acid on Vapodest 30s (Gerhardt, Germany). The final concentration of N was established by titration with 0.01 M HCl using a TitroLine 6000 titrator (SI Analytics, Germany). For the analysis of phosphorus (P), calcium (Ca) and magnesium (Mg), the Mehlich II extract was prepared (Mehlich 1978). The concentration of P was estimated colorimetrically using HACH PhosVer 3 reagent powder pillows on a DR 2800 spectrophotometer (HACH, USA). The concentrations of Ca and Mg were estimated using atomic absorption spectrophotometer Avanta Σ (GBC, Australia).

Data analysis

The secondary sex ratio was calculated as the proportion of males over the total number of reproducing individuals (males/[females+males]; Field et al. 2013a). A χ^2 test was used to examine if the sex ratio was significantly different from 0.5 separately for each population with a sufficient sample size. Heterogeneity χ^2 -test (Sokal & Rohlf 1995) was used to assess if there was a significant among population heterogeneity in sex ratios. Only five populations with sufficient sample sizes ($n > 30$; HL, NJ, PL, SZ, UJ) were included in the analysis. Spearman correlation coefficients (r_s) were used to investigate if the variation in sex ratio among populations was related to the proportion of nonreproductive individuals, population size and altitude (as a proxy of environmental harshness) in these five populations. Bonferroni correction of P-values was used to counteract the problem of multiple comparisons.

Three large populations with a sufficient number of male and female individuals (NJ, PL, and SZ) were further analysed in detail for biometric, spatial and microhabitat differentiation between males and females. A linear mixed model with fixed effects design was used for testing the effects of sex and locality on height and area of individual plants. Locality was modelled with a different variance in each group. Univariate analyses were performed in NCSS 9 (NCSS, USA).

To assess the spatial relationship between males and females, we examined bivariate spatial interactions using Ripley's $K_{12}(t)$ and its standardized $L_{12}(r)$ modification (Lotwick & Silverman 1982) using the software PASSaGE 2.0 (Rosenberg & Anderson 2011) separately for each population. K and L were calculated for distances ranging up to 25 % of the maximum distance among points, and rescaling for the counts-based overlap with the study boundary was applied (Rosenberg & Anderson 2011) in order to limit the influence of the margin effects (Haase 1995). Randomisation tests were used to construct 95 % confidence limits in conjunction with the values obtained from the analysis of the original coordinates. For testing, „Test of the associations conditional on current locations” was applied (Rosenberg & Anderson 2011).

To test for microhabitat differentiation between males and females, we used redundancy analyses (RDA; Legendre & Legendre 2012) of two data matrices (matrix of species composition and matrix of soil variables). Analyses were done separately for each population. We used a specialized type of variation partitioning, in which the variation explained by a set of explanatory variables is separated from the variation explained by the spatial positions of individuals. To identify spatial explanatory variables, we used principal coordinates of neighbouring matrices (PCNM; Borcard & Legendre 2002), resulting in the PCNM variables (Borcard et al. 2004). First, we computed a pairwise geographic (Euclidean) distance matrix from the geographic coordinates of the plot locations. Then, we determined the cut-off threshold distance to cover the nearest-neighbour of each plot, with all the distances larger than the cut-off distance replaced by the specific value (Legendre & Legendre 2012). Subsequently, we performed principal coordinate analysis (PCoA) to compute the principal coordinates of this modified distance matrix. Because of the large number of PCNM variables created by PCoA, we used forward selection in order to obtain a manageable number of variables. Only those PCNM variables with an adjusted P lower than 0.05 were

selected. P values were adjusted by the “False discovery rate” method (Benjamini & Hochberg 1995). Lastly, effects of sex, spatial (PCNM) variables and cover of *Salix* on the overall pattern in species composition and soil conditions were analysed using RDA. Ordinal cover values in the species occurring in the herb and moss layers were transformed using the Hellinger transformation prior to the analyses. Values of soil variables were log-transformed ($x+1$) when needed and Z-transformed before analysis. Relative cover (%) of *S. lapponum* was used as another explanatory variable to separate the potential effect of shrub cover on species composition and soil conditions from the effect of sex. We used a specific partitioning algorithm proposed by Peres-Neto et al. (2006) that provides unbiased estimates of explained variation (adjusted R^2), using RDA to quantify pure and shared effects of sex, cover of *Salix* and spatial (PCNM) variables. The significance of the pure and marginal effects of the explanatory variables was tested using a Monte Carlo permutation test with 999 permutations. Analyses and Venn diagrams were performed using the CANOCO 5 package (Šmilauer & Lepš 2014) and the library vegan (Oksanen et al. 2014) in the R language (R Core Team 2015).

Results

Female bias was observed in seven out of eight populations. However, due to the small population size of four populations, statistical tests were done for only five populations, three of which showed a significant female bias (Table 4). The four largest populations did not show any among-population heterogeneity in sex ratios ($\chi^2 = 5.86$, $P = 0.119$). When summing over all populations, females were two times more frequent than males (sex ratio = 0.323; Table 4).

Despite the observed large variation in proportions of nonreproductive individuals among populations ranging from zero to unity ($\chi^2 = 70.6$, $P < 0.001$), nonreproductive individuals were in the minority in seven out of nine populations, including the four largest populations (Table 4). If we considered nonreproductive plants as males, the sex ratio in most populations would become either even or male-biased (Table 4).

Table 4. Comparison of number of non-reproducing, male and female individuals for nine populations of *S. lapponum* from the Krkonoše Mts. χ^2 test was used to determine whether sex ratios differ significantly from 0.5 (i.e. 1:1), such cases are given in bold. Only large-sized populations were statistically analysed. Overall sex ratio over all populations and potential sex ratio where all sterile individuals were considered as males were also calculated.

Population	ID	n	Females (F)	Males (M)	Sterile (S)	Proportion of steriles (S/n)	Secondary sex ratio [M/(M+F)]	Chi ²	P	Potential sex ratio [(M+S)/n]	Chi ²	P
Upske raseliniste	UR	2	0	0	2	1.000	-	-	-	1.000	-	-
Modry dul	MD	3	3	0	0	0.000	0.000	-	-	0.000	-	-
Vyrovka	VY	4	2	1	1	0.250	0.333	-	-	0.500	-	-
Horni Pancava	HPL	6	3	1	2	0.333	0.250	-	-	0.500	-	-
Upska jama	UJ	36	5	5	26	0.722	0.500	0.00	1.000	0.861	18.78	<0.001
Pancavska louka	PL	51	32	18	1	0.020	0.360	3.92	0.048	0.373	3.31	0.069
Schustlerova zahradka	SZ	81	41	31	9	0.111	0.431	1.39	0.239	0.494	0.01	0.912
Hancuv pomnik	HL	90	40	19	31	0.344	0.322	7.46	0.006	0.556	1.11	0.292
Navorska jama	NJ	688	296	126	266	0.387	0.299	68.48	<0.001	0.570	13.40	<0.001
Total	-	961	422	201	338	0.352	0.323	78.39	<0.001	0.561	14.24	<0.001

Height was significantly affected by sex ($F_{1, 126.7} = 8.5, P = 0.004$) and locality ($F_{2, 89.1} = 11.4, P < 0.001$), but not their interaction ($F_{2, 89.1} = 1.8, P = 0.188$). Males were generally taller (mean \pm SE; 96 \pm 4 cm) than females (82 \pm 3 cm), and plants at NJ were taller (104 \pm 5 cm) than plants at SZ (88 \pm 3 cm) and PL (75 \pm 4 cm). The area of individual shrubs was significantly affected by locality ($F_{2, 87.2} = 3.9, P = 0.024$), while neither sex ($F_{1, 118.4} = 0.1, P = 0.993$) or interaction sex \times locality ($F_{2, 87.2} = 2.6, P = 0.078$) had significant effects.

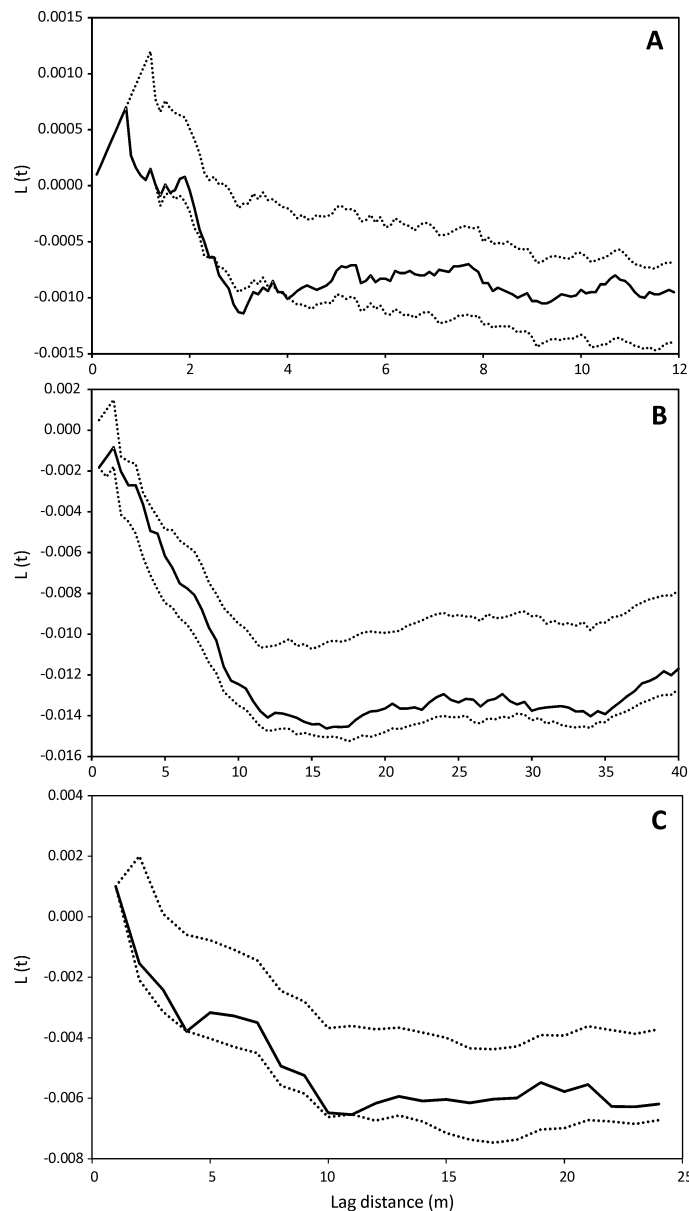


Figure 7. Second order bivariate Ripley's $L_{12}(t)$ analyses for males vs. females inside three large populations (A: SZ population, B: PL population, C: NJ population). Solid line is $L_{12}(t)$, while dashed lines represent the 95 % confidence envelopes under the random labelling null model. Points above the confidence envelope show positive spatial interactions (attraction), points below the confidence envelope show negative spatial interaction (repulsion), while points located inside the envelope show no significant spatial interaction.

Males and females in PL and NJ populations showed no significant spatial segregation. In the SZ population, negative interactions between sexes occurred from 2 to 4 m, whereas no significant spatial interactions between sexes were found for other distances (Fig. 7).

Table 5. Pure and marginal effects of sex, cover of *Salix* and space (PCNM variables) and of all variables combined, on the species composition and soil conditions at the three sites (SZ, PL and NJ). Degrees of freedom, test statistics, probabilities of type I error and adjusted coefficient of determination (R^2) values are given for each variable. Adjusted R^2 can be negative for any fraction and such values are interpreted as zeroes (Legendre & Legendre 2012). The significances were tested using a Monte Carlo permutation test with 999 permutations.

Variables	Pure effects				Marginal effects			
	df	Adjusted R^2 (%)	F	P	df	Adjusted R^2 (%)	F	P
(a) SZ population								
<i>Species composition</i>								
All variables	-	-	-	-	7	17.0	3.079	0.001
Sex	1	0.5	1.419	0.092	1	1.6	2.174	0.008
Cover of <i>Salix</i>	1	0.1	1.717	0.026	1	2.5	2.821	0.001
PCNM variables	5	12.9	3.139	0.001	5	15.6	3.616	0.001
<i>Soil conditions</i>								
All variables	-	-	-	-	5	17.9	4.109	0.001
Sex	1	0.0	0.608	0.640	1	0.1	1.084	0.319
Cover of <i>Salix</i>	1	1.3	2.068	0.093	1	2.0	2.455	0.062
PCNM variables	3	15.6	5.367	0.001	3	17.2	5.919	0.001
(b) PL population								
<i>Species composition</i>								
All variables	-	-	-	-	4	6.8	2.002	0.001
Sex	1	0.0	0.599	0.928	1	0.1	0.702	0.830
Cover of <i>Salix</i>	1	1.8	2.5	0.003	1	2.1	2.647	0.001
<i>Soil conditions</i>								
All variables	-	-	-	-	4	0.5	1.128	0.316
Sex	1	0.3	1.147	0.301	1	0.7	1.256	0.234
Cover of <i>Salix</i>	1	0.0	0.902	0.425	2	0.2	1.105	0.339
(c) NJ population								
<i>Species composition</i>								
All variables	-	-	-	-	4	9.7	2.215	0.001
Sex	1	-0.3	0.966	0.458	1	-0.3	0.981	0.402
Cover of <i>Salix</i>	1	4.5	3.051	0.002	1	6.2	3.813	0.002
PCNM variables	2	4.0	1.92	0.007	2	5.4	2.213	0.003
<i>Soil conditions</i>								
All variables	-	-	-	-	4	0.4	1.0	0.412
Sex	1	-2.3	0.167	0.974	1	-2.3	0.157	0.976
Cover of <i>Salix</i>	1	-1.2	0.569	0.717	2	0.6	1.229	0.298
PCNM variables	2	2.1	1.380	0.188	2	3.9	1.773	0.103

The analysis of pure and shared effects of space, cover of *Salix* and sex on species composition and soil conditions within three populations showed that all variables had an overall poor explanatory power (6.8–17.0 % and 0.4–17.9 % of total variation in species composition and soil conditions, respectively). When included in the model, spatial variables accounted for a substantial part of the explained variation. The pure effect of sex was negligible and non-significant for both species composition and soil conditions, while *Salix* cover had a small but significant pure effect on the species composition. Shared effects between sex and *Salix* cover were sometimes pronounced for both species composition and soil conditions (Table 5, Fig. 8).

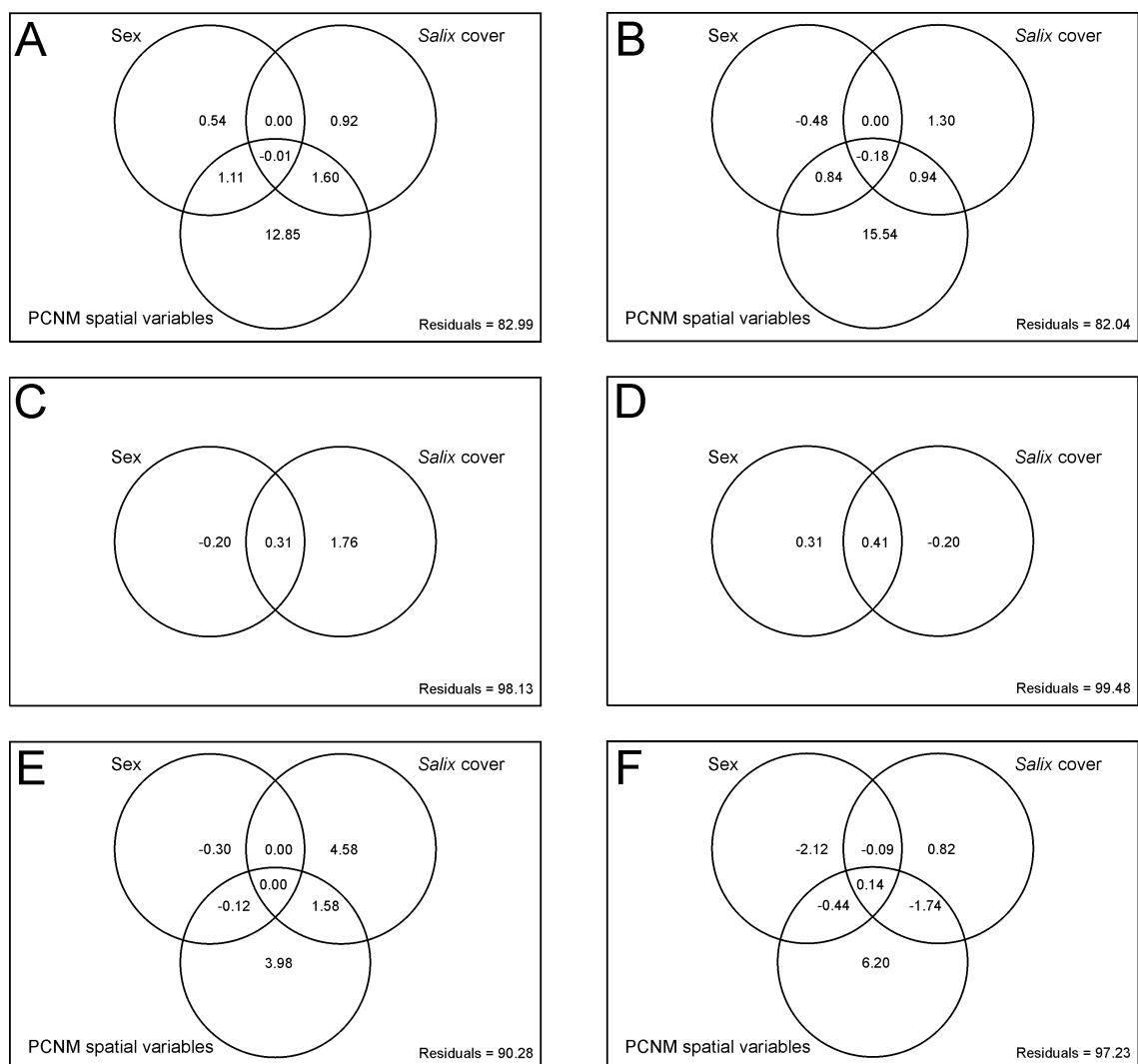


Figure 8. Variation partitioning showing the effects of sex, cover of *Salix* and space (PCNM spatial variables) on the species composition (A, C, E) and soil variables (B, D, F) at the localities SZ (A, B), SL (C, D) and PL (E, F). Adjusted R² values (%) are given for each pure and shared fraction of explained variation (Peres-Neto et al. 2006). Adjusted R² can be negative for any fraction and such values are interpreted as zeroes (Legendre & Legendre 2012). According to Legendre (2008), the negative values of explained variation are interpreted as zeroes when they correspond to cases where the explanatory variables explain less variation than random normal variables.

Altitude as a proxy of harshness of environment did not correlate significantly with any population parameters (sex ratio: $r_s = -0.10$, $P = 0.850$; proportion of nonreproductive individuals: $r_s = 0.41$, $P = 0.492$; population size: $r_s = -0.62$, $P = 0.269$).

Discussion

We found strong female-biased secondary sex ratios (approx. 0.3) in seven out of the nine populations of *S. lapponum* studied. Hughes et al. (2010) reported a similarly female-biased sex ratio in *S. lapponum* from Sweden. Our results also fit well with the generally observed pattern of secondary sex ratios in the genus *Salix* (e.g., Crawford & Balfour 1983; Myers-Smith & Hik 2012; Che-Castaldo et al. 2015). The mechanisms driving the observed bias may be basically twofold: ecological consequences of sexual dimorphism (Bierzychudek & Eckhart 1988) and early acting mechanisms introducing the bias in the plant's early life stages (Taylor 1999).

The separation of male and female sexual functions into discrete individuals initiates the evolution of secondary sexual dimorphism, including morphological, physiological and phenological characters (Delph 1999; Obeso 2002). The differentiation in secondary characters is rooted in the intrinsic differences in reproductive allocation between sexes (Agren 1988). The higher reproductive costs in individuals of one sex usually result in their lower growth rates, smaller absolute sizes, and consequently, increased mortality (Lloyd & Webb 1977; Delph 1999). When resources are limited, niche differentiation may also occur between the sexes and can result in their spatial segregation at various spatial scales (Cox 1981). It can be apparent on a fine spatial scale when the sexes segregate in response to heterogeneously distributed resources within a population (Bierzychudek & Eckhart 1988) or on broad scales when different performances and survivorship of the sexes across environmental gradients might result in different sex ratios between populations (Grant & Mitton 1979; Cox 1981). Because higher reproductive expenditure is pronounced usually in more stressful environments (e.g., higher altitudes and latitudes, low light levels, drought) when resources are limited (Bierzychudek & Eckhart 1988; Field et al. 2013b), strong SSS should be observed in stressful environmental conditions, with the sex with higher

reproductive expenditure less common in more stressful (micro)sites (Bierzychudek & Eckhart 1988; Dawson & Bliss 1989).

In dioecious woody plants, females are expected to have a higher reproductive cost due to the energy allocation to the costlier fruits (Delph 1999; Obeso 2002). On the other hand, in species with abiotic pollen dispersal, the sex with higher reproductive expenditure may be males due to their production of large amounts of nitrogen-rich pollen (Harris & Pannel 2008). In *Salix*, which is woody and usually at least partly anemophilous, these two expectations are clearly in conflict. Concerning *S. lapponum*, we have not found any significant among-population variation in the sex ratio despite altitudinal differences among populations and no spatial and niche segregation of the sexes within populations related to environmental variation, despite the populations having female-biased sex ratios. Our results are consistent with other studies that found no niche and spatial segregation in several *Salix* species (Alliende & Harper 1989; Crawford & Balfour 1983; Ueno et al. 2007; Che-Castaldo et al. 2015) but are in contrast with patterns observed in *S. arctica* (Dawson & Bliss 1989) and *S. glauca* (Dudley 2006), where sexes were spatially segregated, with males usually occurring on drier sites. Concerning *S. lapponum*, Hughes et al. (2010) found an almost significant SSS in one of five studied transects of *S. lapponum* in Sweden. In that case, females have been found to grow in lower (and therefore wetter) mean elevations than males.

Though we did not directly study the growth rates in *S. lapponum*, we found that males are significantly taller. Taller males were also observed in *S. alaxensis* (Myers-Smith & Hik 2012) and are probably characteristic of shrubs in general (Obeso 2002). This may indicate that males allocate more resources to growth than females, i.e., the growth of females might be partly hampered by increased reproductive allocation (Delph & Meagher 1995). However, it is counterintuitive to explain the observed female-biased sex ratio in *S. lapponum* with this fact. However, in some other *Salix* species with female-biased sex ratios, females also possess lower biomass production and/or lower nutrient contents in their leaves due to their higher allocation of resources to reproduction compared to males (Allen & Antos 1993; Turcotte & Houle 2001; Ueno et al. 2006; Che-Castaldo et al. 2015).

How can these contradictory findings be explained? The sex-specific allocation of resources might be pronounced only in more contrasting environmental conditions (Delph 1999). Dudley (2006) found in the alpine species *S. glauca* that the growth rates

of individuals of each sex were dependent on the availability of water: females had higher growth rates than males in mesic sites, while in xeric sites males significantly outperformed females. A similar pattern was observed by Dawson & Bliss (1989) in *S. arctica*, where females had a higher leaf area than males in wet conditions, whereas it was the opposite in xeric conditions. These data suggest that females may maximize resource uptake in high-resource habitats, while males may maximize resource use efficiency that allows them to survive in less favourable habitats (Hultine et al. 2016, 2018). However, in *S. lapponum*, the observed patterns of SSS, environmental niches and morphology (height and area) of the sexes were similar over three localities that differed in available resources as mirrored by the significant locality effect (see also Hroneš et al. 2018). This might mean that the habitat conditions, though variable both within and between populations, are not stressful in any of the populations studied (Hultine et al. 2018) and that the increased height of males may be an adaptive trait for better pollen dispersal in anemophilous species (Pickup & Barrett 2012). This indeed may be the case for *S. lapponum*, where male catkins are mostly crowded at the apex of twigs (personal observation). Males may also experience delayed maturation or irregular flowering due to their reproductive costs, which are associated with high levels of pollen production and may result in their identification as nonreproductive individuals (Harris & Pannel 2008). In the most extreme cases, when all nonreproductive plants of *S. lapponum* will be considered males, the sex ratio would become even or slightly male-biased in some populations (Table 4). However, abovementioned explanations of the observed secondary sex ratios and morphological differences but the absence of the spatial and niche segregation between the sexes in *S. lapponum* are rather speculative. To prove their relevance, additional experimental tests using common garden experiments with detailed examinations of sexually dimorphic physiological traits are needed. However, in the absence of sex-specific genetic markers, the primary sex ratio is almost impossible to test in wild populations.

Alternatively, the female-biased sex ratio found in *S. lapponum*, despite observed niche similarity of the sexes, might be explained by early-acting factors, i.e., genetic mechanisms affecting the primary seed sex ratio (Taylor 1999; Stehlik & Barrett 2005). Genetic mechanisms known to induce a female bias have been thoroughly investigated in *Rumex* and *Silene* (e.g., Taylor 1999; Stehlik & Barrett 2005). However, these are linked to genetic systems with males as a heterogametic sex, whereas in *Salix*,

the heterogametic sex was found to be represented by females in some species (Pucholt et al. 2005). Despite this, both Alström-Rapaport et al. (1997) and de Jong & van der Meijden (2004) found a biased seed sex ratio in experimental populations of *S. viminalis* and *S. repens*, suggesting that the bias is of a genetic nature and not caused by seed or seedling mortality. Recently, Pucholt et al. (2017) proposed a genetic mechanism responsible for the biased sex ratio in *S. viminalis*. They have found that the bias is a result of the absence of a specific combination of alleles, which very likely excludes a portion of the males from the population during seed development (Pucholt et al. 2017). Given the relatively close phylogenetic relationship of *S. lapponum* and *S. viminalis*, it is possible that a similar mechanism may also be behind the biased sex ratio in this species.

CHAPTER 5:

Patterns of morphological and genetic variability in geographically isolated populations of downy willow (*Salix lapponum*, Salicaceae) in the Krkonoše Mts. (Czech Republic)

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Abstract

Populations of alpine species represent interesting model to study the effect of isolation on their genetic and morphological variability. We focused on the geographically isolated populations of *Salix lapponum* in the Krkonoše Mts. to examine if some changes in their morphological and genetic diversity can be observed in comparison with main range populations in Scandinavia. We also tested whether these populations are endangered by hybridization with *S. silesiaca*. Furthermore, we focused on the origin of endemic glabrous variety *S. lapponum* var. *daphneola*. Specifically, we tested whether the origin of var. *daphneola* can be explained by hybridization. To answer our questions, we measured seven morphological characters and analysed six microsatellite loci. We found that morphological variability *S. lapponum* in the Krkonoše Mts. is slightly shifted when comparing with main range populations. We also found that all larger populations exhibit the significant deficit of heterozygotes that may indicate occurrence of inbreeding. On the other hand, the extent of hybridization was quite low, and we were able to discover only three possible introgressants. Regarding var. *daphneola*, we propose that its origin cannot be explained by hybridization but rather by recessive state of alleles or mutation in gene controlling indumentum development. Populations *S. lapponum* in the Krkonoše Mts. can be under recent speciation due to their long-term isolation.

Key words: alpine plants, genetic drift, hybridization, *Salix daphneola*

Introduction

Isolated alpine populations, especially those with limited number of individuals, may face many environmental, stochastic and/or genetic threats (Lynch et al. 1995; Keller & Waller 2002). Due to the small number of reproductively active individuals any variation in environmental factors (e.g., climatic factors) critical for reproduction and survival may lead to extinction of such population (Lande 1993). Similarly, any random variation in birth and mortality rates and sex ratios among individuals in such population can be critical (Lande 1993). Moreover, reduced genetic diversity resulting from bottleneck effect, long-lasting isolation and/or inbreeding can be also expected (Lynch et al. 1995; Keller & Waller 2002). Reduction of fitness by inbreeding (i.e.,

inbreeding depression) may arise from mating among relatives which increase the proportion of homozygotes (Keller & Waller 2002). Inbreeding is more frequent in populations with deficit of suitable partners and in absence of the gene-flow from other populations (Austerlitz et al. 2012). As the selection is expected to be less effective in small populations, the deleterious mutations may tend to accumulate (Lynch et al. 1995). Also, the contribution of genetic drift by which alleles are randomly fixed or excluded from gene-pool of population may gradually erode the fitness and increase the extinction probability of an isolated population (Lande 1995). Isolated populations are also more prone to the hybridization and subsequent erosion of parental genomes due to introgression (Todesco et al. 2016). In cases when the parental species (or genetically distant populations) produce considerable amounts of hybrids which have decreased fitness relative to their parents in the first or later generations, outbreeding depression may occur (Fenster & Galloway 2000; Todesco et al. 2016). In high instances of outbreeding depression even low rates of hybridization can threaten small and isolated populations (Edmands 2007; Todesco et al. 2016). However, hybridization can also act oppositely and increase the fitness of the inbred population (i.e., genetic rescue) by increasing the allele diversity (Ingvarsson 2001). All these above noted processes may constitute the threat to persistence of isolated populations but at the same time the isolated populations may exhibit the perfect model for studying these processes.

One of the examples of isolated plant populations is *Salix lapponum* L. in the Krkonoše/Karkonosze Mts. (hereafter referred to as the Krkonoše Mts.; Hroneš et al. 2018). The species is distributed mainly in the northern Europe with disjunct populations in the Sudetes Mts., Massif Central, Pyrenees and several mountain ranges in the Balkan Peninsula (Skvortsov 1999). The (sub)alpine vegetation of the Krkonoše Mts. has been isolated by forest at the beginning of the Holocene ca. 11,500 years ago (Tremel et al. 2008; Chytrý 2012). Therefore, *S. lapponum* is considered to be the glacial relic in the Krkonoše Mts. (Kwiatkowski & Krahulec 2016). The nearest extant populations of *S. lapponum* survived in the Hrubý Jeseník Mts. in the Czech Republic and in the Poleski National Park in the eastern Poland, more than 100 km and 520 km apart, respectively (Hroneš et al. 2014; Kołos et al. 2015).

In the Krkonoše Mts., the hybridization is quite frequent with *Salix silesiaca* Willd. Rare hybrids with *Salix aurita* L. and *Salix caprea* L. have been also reported, mainly from the Polish part of the mountains (Kwiatkowski 2006). *Salix lapponum* and

S. silesiaca are both diploid species (Skvortsov 1999) hence no pre-zygotic mating barriers possibly exist and post-zygotic barriers such as hybrid sterility are not known either. Apart from hybridization, considerable morphological variation has been observed in populations of *S. lapponum* (Tausch 1837, Hroneš & Trávníček 2018). The most striking variation is observed in leaf and capsule indumentum, ranging from normally indumented individuals (i.e., with tomentose leaves and capsules) to completely glabrous ones. This variability led Tausch (1837) to believe that glabrous individuals represent different species and he described them as *Salix daphneola* Tausch. However, its taxonomic status remains controversial and the glabrous plants are now considered as variety of *S. lapponum*, i.e. *S. lapponum* var. *daphneola* (Tausch) Wimmer (hereafter *S. *daphneola*; Danihelka et al. 2012, Hroneš & Trávníček 2018). Given that such an extent of morphological variation is not reported from elsewhere of the range of *S. lapponum*, *S. *daphneola* is considered to be endemic to the Krkonoše Mts. (Krahulec 2006).

Two hypotheses may be postulated to explain the origin of *S. *daphneola* (and to some extent overall increased morphological variability of *S. lapponum* in the Krkonoše Mts.): 1) the glabrous morphotypes may originate as a product of introgressive hybridization of *S. lapponum* and other species (*S. silesiaca* which is the only sympatrically occurring willow species thus it is the most likely candidate) or 2) they may represent the recent speciation due to the long-lasting isolation of the populations in the Krkonoše Mts. In this study using the combined morphometric and microsatellite loci analysis, we aimed to assess extent of the intra- and interpopulation morphological and genetic variability of *S. lapponum* in the Krkonoše Mts., to determine whether the isolated populations exhibit signs of genetic drift and inbreeding as would be expected, to assess the extent of introgressive hybridization with *S. silesiaca* and explore the origin of glabrous morphotypes.

Material and methods

Plant material

Plant material for microsatellite loci (SSR) analysis was collected during the years 2008–2013. Altogether, 268 individuals were collected. From that count, 202

individuals of *S. lapponum* from 19 populations originated from the Krkonoše Mts., one individual from the Hrubý Jeseník Mts. and 11 individuals from various parts of the Scandinavia (Table 6). Additionally, 12 individuals of *S. *daphneola* from four populations in the Krkonoše Mts., 11 individuals of *S. ×nepetifolia* from the Krkonoše Mts. and 31 individuals of *S. silesiaca* from various parts of its range were also included to the analyses (Tab. 6). Several leaves from each individual were put into tea-bag and stored in silica-gel. Herbarium specimens were taken from each population and stored in herbarium of the Department of Botany, Palacký University in Olomouc (OL).

Morphometric analysis

Morphometric analysis was performed on herbarium specimens from the Czech Republic deposited in BRNU, HR, LIT, MJ, MP, PRC and ROZ herbaria and own samples deposited in OL (herbaria acronyms according to Thiers 2018). Altogether, 119 specimens of *S. lapponum*, 9 specimens of *S. *daphneola*, 8 specimens of *S. ×nepetifolia* and 10 specimens of *S. silesiaca* were evaluated (Supplementary File 5). Morphological characters were measured using digital calliper and stereo microscope (Intraco Micro, Tachlovice). In total, 7 vegetative (length, width and indumentum of adaxial and abaxial part of lamina, length of petiole, number of main veins and presence of stipules) and 5 generative characters (length of catkin, length and indumentum of capsule, length of capsule stalk and indumentum of cataphylls) were examined. Indumentum was examined using five grade scale, with 1 corresponding to completely glabrous leaves to 5 corresponding to 75 %–100 % of lamina covered by hairs. Due to the ephemeral nature of male catkins, only female plants with well-developed leaves and catkins were included into the analysis. Data were analysed using a set of R functions contained in MorphoTools version 1.01 (Koutecký 2015). Basic descriptive statistics (average, minimum, maximum) were calculated for each morphological character and studied taxon. Tukey-Kramer multiple comparison tests at $p \leq 0.01$ were performed to determine which characters show significant differences among taxa. Correlations of morphological characters were tested using Pearson's correlation coefficient and Principal component analysis (PCA; Sneath & Sokal 1973) was employed to test the morphological homogeneity within the putative taxa. Two PCAs were run: first with the whole data set and second with only *S. lapponum* to examine

extent of morphological variability between its isolated and main range populations (i.e., Krkonoše Mts. Hrubý Jeseník Mts. and Scandinavia).

Table 6. List of localities of *S. lapponum* sampled for SSR loci analysis. N - number of individuals collected.

Abbreviation	Locality	Coordinates	N
HL	Krkonoše Mts., Hančova louka alpine meadow	50°45'32.2"N, 15°32'42.0"E	3
HLL	Krkonoše Mts., Labská louka alpine meadow, below spring of the Labe river		1
HPL	Krkonoše Mts., spring of Pančava rivulet	50°46'7.9"N, 15°32'19.3"E	6
HRS	Karkonosze Mts., upper edge of the ridge between Kocioł Wielkiego and Małego Stawu glacial cirques	50°45'5.7"N, 15°41'41.7"E	9
JES	Hrubý Jeseník Mts., Praděd Mt., Tabulové skály rocks	50°5'8.1"N, 17°13'47.6"E	1
MS	Karkonosze Mts., upper edge of Kocioł Małego Stawu glacial cirque	50°44'51.0"N, 15°41'41.3"E	1
NJ	Krkonoše Mts., northern part of Navorská jáma glacial cirque	50°46'3.1"N, 15°32'48.9"E	39
NLB	Krkonoše Mts., alpine meadow between Luční bouda chalet and state border	50°44'17.8"N, 15°41'56.7"E	1
OB	Karkonosze Mts., <i>Pinus mugo</i> scrub west of Dom Śląski chalet	50°44'26.7"N, 15°43'23.7"E	1
PL	Krkonoše Mts., Pančavská louka peat bog	50°45'39.6"N, 15°32'36.6"E	61
PLB	Krkonoše Mts., alpine meadow near Luční bouda chalet	50°44'6.2"N, 15°41'45.3"E	11
PO	Karkonosze Mts., Polana peat bog	50°45'59.9"N, 15°42'13.7"E	4
SCA	Scandinavia, various parts	-	11
SLO	Karkonosze Mts., north slope of Stříbrný hřbet Mt., under Słonecznik rocks	50°45'49.2"N, 15°41'9.8"E	4
SZ	Krkonoše Mts., eastern part of Navorská jáma glacial cirque	50°45'54.3"N, 15°32'47.5"E	44
SZL	Krkonoše Mts., Úpská jáma glacial cirque, Sněhový žlab gully	50°43'55.5"N, 15°42'54.5"E	1
UJ	Krkonoše Mts., northern part of Úpská jáma glacial cirque	50°44'18.4"N, 15°43'22.0"E	3
UR	Krkonoše Mts., Úpské rašeliniště peat bog	50°44'11.4"N, 15°42'50.7"E	1
VY	Krkonoše Mts., alpine meadow near Výrovka chalet	50°43'3.6"N, 15°40'59.4"E	4
WS	Karkonosze Mts., upper edge of Kocioł Wielkiego Stawu glacial cirque	50°45'23.1"N, 15°41'22.5"E	5
WSV	Karkonosze Mts., upper edge of Kocioł Wielkiego Stawu glacial cirque near viewpoint	50°45'19.4"N, 15°41'28.7"E	3

DNA extraction and SSR analysis

Genomic DNA was extracted using 50 mg of silica-dried leaf tissue according to the cetyl trimethyl ammonium bromide protocol described by Doyle & Doyle (1987). The DNA concentration was measured with a spectrophotometer Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA), and the quality of the extracted DNA was verified on a 1.5 % agarose gel stained with UltraPower™ Nucleic Acid Stain (BioTeke Corp., Beijing, China).

Six SSR loci, i.e. SB24, SB80, SB100, SB201, SB210 and SB233 (Barker et al. 2003), were examined under published PCR conditions with minor individual modifications. The basic thermal cycling on a PTC-200 (MJ Research Inc., Reno, NV, USA) consisted of an initial denaturation step (2 min at 95 °C), followed by a specific number of cycles of 0.5 min at 95 °C, 0.5 min at the selected annealing temperature (Supplementary File 6), 0.5 min (or 0.75min) at 72 °C and ending with a final extension step (5 min at 72 °C). Amplification was performed in 10 µl reaction volumes using 5 ng of genomic DNA. Reaction components were mixed according to the manufacturer's protocol (Promega, Madison, WI, USA) and contained 1× GoTaq (Promega) reaction buffer, 0.2 mM dNTPs, 1 µM of primers and 0.25 U of GoTaq polymerase. Using a T-REX (Thermo Scientific Owl Separation Systems, Rochester, NY, USA) sequencing gel electrophoresis device, the PCR products were separated by denaturing polyacrylamide gel electrophoresis using a 6 %, 0.4 mm-thick polyacrylamide gel (0.5× TBE buffer) and consequently visualised by silver staining. SSR alleles were scored based on fragment lengths (in base pairs; bp), which were determined using the 30–330 bp AFLP® DNA ladder (Invitrogen, Carlsbad, CA, USA).

SSR data analysis

The grouping of identical multilocus genotypes, the probability of identity (P_{ID} , i.e., the probability that two identical genotypes have been derived independently by sex), the average number of alleles per locus (A), the observed and expected heterozygosity (Nei 1973), coefficients of inbreeding F_{IS} and ρ_{IS} (based on the infinite alleles model and the stepwise mutational model, respectively) as well as locus statistics and analysis of molecular variance (AMOVA) were calculated using GENEALLEX 6.503 (Peakall &

Smouse 2006, 2012). AMOVA was used to partition variation within and among populations and was based on F_{ST} (infinite alleles model) with 999 permutations. The proportion of different genotypes (PD) was calculated as the number of distinct genotypes (nG) divided by number of individuals (N; Ellstrand & Roose 1987). Mean number of alleles per locus after rarefaction (A_r) with standardised sample size of five individuals was computed in ADZE 1.0 (Szpiech et al. 2008). Fixation indices F_{ST} and ρ_{ST} , the score test for heterozygote deficit/excess and the exact G test for population differentiation were performed in GENEPOP 4.7.0 (Rousset 2008) with the following parameters: 10 000 dememorization iterations; 200 batches (runs); and 10 000 iterations per batch. Population structure was analysed using a Bayesian clustering approach implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000). The analysis was set as follows: admixture model, no prior population information, K in the range 1–30 with ten replicates for each K, 80,000 burnin iterations followed by 800,000 MCMC iterations. STRUCTURE output files were summarized and graphically interpreted using CLUMPAK software (Kopelman et al. 2015) where the calculation of similarity coefficients (SC) between replicate runs and posterior probabilities of K using ΔK (Evanno et al. 2005) were performed.

Results

Morphological variation

Salix lapponum s. lat. (incl. *S. *daphneola*) and *S. silesiaca* significantly differed in almost all morphological characters examined. *Salix lapponum* and *S. *daphneola* significantly differed in indumentum of lamina, capsules and cataphylls. The hybrid *S. ×nepetifolia* was generally closer to *S. lapponum* from which it differed by presence of stipules, higher average number of veins, size of leaves and length of petiole (Fig. 9, Table 7). No pairs of highly correlated characters ($r > 0.90$) were found. Therefore, the entire dataset was used in the multivariate analyses. Three main groups corresponding to *S. lapponum*, *S. silesiaca* and *S. *daphneola* were revealed in PCA. *Salix lapponum* and *S. silesiaca* were separated along the first component axis and *S. lapponum* and *S. *daphneola* were separated by second component axis in the principal component analysis (the first, second and third axis explained 47 %, 19 % and 11 % of variation, respectively; Fig. 10a, b). Individuals of *S. ×nepetifolia* were distributed along the first

axis closer to *S. lapponum*. In second PCA data set, individuals of *S. lapponum* from the Krkonoše Mts. and the Hrubý Jeseník Mts. were slightly separated from those of Scandinavia by the second axis (the first, second and third axis explained 29.8 %, 19.2 % and 12.5 % of variation, respectively; Fig. 10 c, d).

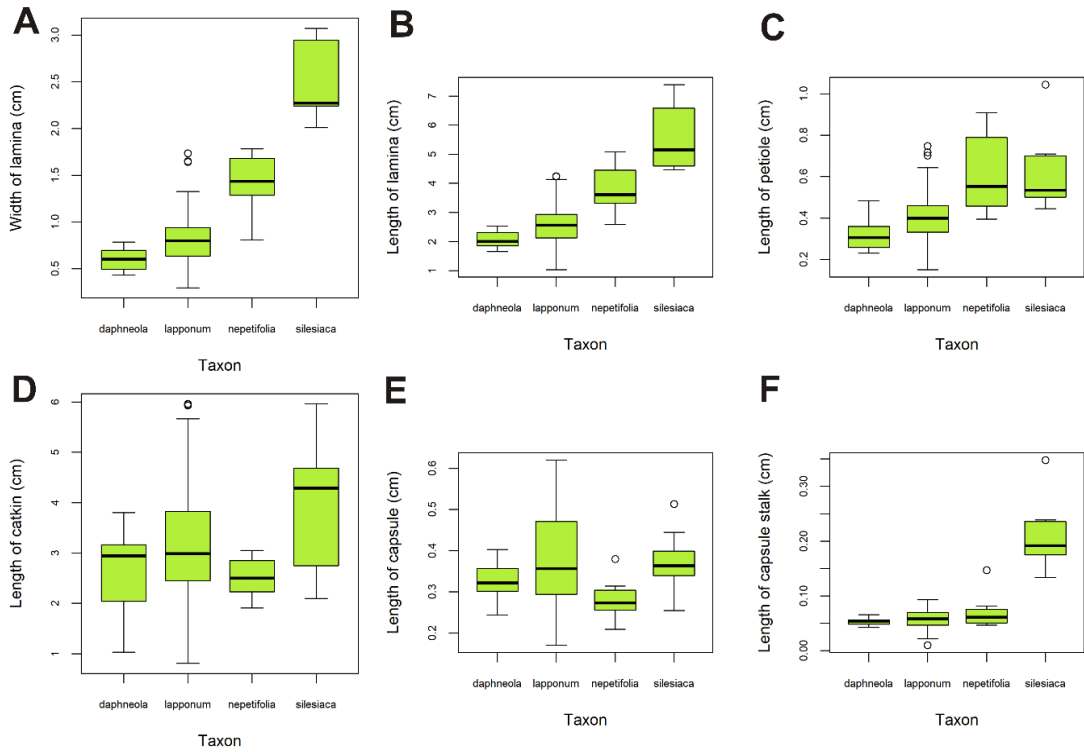


Figure 9. Variability of selected morphological characters. Rectangles define the 25th and 75th percentiles, horizontal lines show the median, whiskers are from the 10 to 90 percentiles, circles show extreme values.

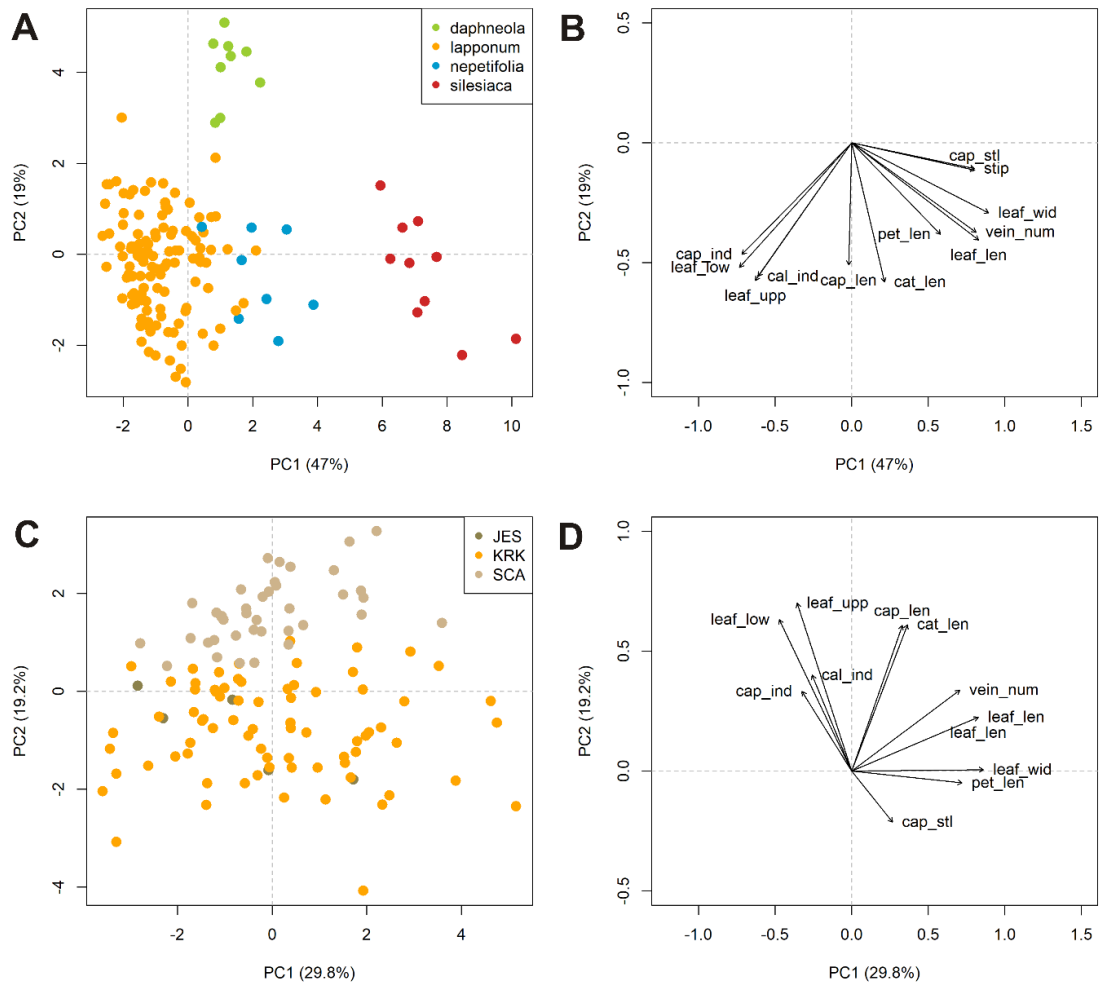


Figure 10. Principal component analysis of 12 morphological characters examined in *S. lapponum*: A) whole dataset, individuals, B) whole dataset, variability of analysed characters, C) *S. lapponum* only, individuals, D) *S. lapponum* only, variability of analysed characters. cap_ind – capsule indumentum, cal_ind – cataphyll indumentum, cap_len – capsule length, cap_stl – length of capsule stalk, cat_len – catkin length, leaf_len – lamina length, leaf_low – indumentum of lower leaf side, leaf_upp – indumentum of upper leaf side, leaf_wid – lamina width, stip – presence of stipules, pet_len – petiole length, vein_num – veins number.

Table 7. Extent of morphological variability measured on herbarium specimens (min – minimum, max – maximum, \pm SD – standard deviation). Second row in *S. lapponum* showing results of statistics for populations (JES – Jeseniky Mts., KRK – Krkonoše Mts., SCA – Scandinavia). Upper case letters marks mean significantly different from other means as calculated by Tukey-Kramer multiple comparison tests at $p \leq 0.01$ (d – *daphneola*, l – *lapponum*, n – *nepetifolia*, s – *silesiaca*).

Character	<i>S. daphneola</i>			<i>S. lapponum</i>				<i>S. \timesnepetifolia</i>		<i>S. silesiaca</i>	
	(min)mean(max)	\pm SD		(min)mean(max)			\pm SD	(min)mean(max)	\pm SD	(min)mean(max)	\pm SD
				JES	KRK		SCA				
Indumentum of lower leaf side	(1)1.3(2) ^{l,n}	0.43		(2)4.4(5) ^{d,s}		0.89		(2)3.75(5) ^{d,s}	0.89	(1.0)1.4(2) ^{l,n}	0.5
			(4)4.8(5)	0.45	(2)4.1(5)	0.98	(4)4.9(5)	0.22			
Indumentum of upper leaf side	(1)1.2(2) ^{l,n}	0.35		(2)3.3(5) ^{d,s}		0.86		(2)2.6(3) ^d	0.52	(1)1.45(2) ^l	0.5
			(3)3.8(5)	0.84	(2)2.9(5)	0.72	(2)4(5)	0.59			
Lamina length (cm)	(1.6)2.1(2.5) ^{n,s}	0.29		(1.0)2.6(4.2) ^{n,s}		0.66		(2.6)3.8(5.1) ^{d,l,s}	0.8	(4.5)5.5(7.4) ^{d,l,n}	1.02
			(2.3)2.9(3.8)	0.55	(1)2.5(4.2)	0.7	(1.6)2.7(4.2)	0.57			
Lamina width (cm)	(0.4)0.6(0.8) ^{n,s}	0.13		(0.3)0.8(1.7) ^{n,s}		0.25		(0.8)1.4(1.78) ^{d,l,s}	0.32	(2.0)2.5(3.1) ^{d,l,n}	0.39
			(0.6)0.8(0.9)	0.16	(0.3)0.8(1.7)	0.28	(0.5)0.8(1.3)	0.17			
Petiole length (cm)	(0.2)0.3(0.5) ^{n,s}	0.1		(0.15)0.4(0.7) ^{n,s}		0.12		(0.4)0.6(0.9) ^{d,l}	0.2	(0.4)0.6(1.0) ^{d,l}	0.18
			(0.2)0.3(0.5)	0.1	(0.2)0.4(0.7)	0.12	(0.2)0.4(0.7)	0.1			
Veins number	(6)6.5(7) ^{n,s}	0.5		(4)7(9) ^{n,s}		0.86		(7)8.4(10) ^{d,l,s}	1.15	(8.5)10.9(13.5) ^{d,l,n}	1.41
			(6)6.8(7.5)	0.67	(4)6.8(9)	0.93	(6)7.3(9)	0.65			
Stipules presence	0	0		0		0		1	1	1	1
Capsule indumentum	(1)1(1) ^{l,n}	0		(3)4.8(5) ^{d,s}		0.53 ^{d,s}		(5)5(5)	0	(1)1.1(2) ^{l,n}	1
			(5)5(5)	0	(5)5(5)	0	(4)4.9(5)	0.31			
Capsule length (cm)	(0.2)0.3(0.4)	0.04		(0.1)0.4(0.6)		0.12		(0.2)0.3(0.4)	0.05	(0.3)0.4(0.5)	0.07
			0.2(0.3)	0.04	(0.2)0.3(0.6)	0.1	(0.3)0.5(0.6)	0.09			
Capsule stalk length (cm)	(0.04)0.05(0.07) ^s	0.01		(0.02)0.06(0.09) ^s		0.02		(0.05)0.07(0.15) ^s	0.03	(0.1)0.2(0.4) ^{d,l,n}	0.06
			(0.04)0.06(0.07)	0.01	(0.01)0.06(0.09)	0.02	(0.03)0.05(0.09)	0.01			
Catkin length (cm)	(1.0)2.6(3.8)	0.9		(1.1)3.2(6.0)		1.07		(1.9)2.5(3.1)	0.4	(2.1)4.0(6.0)	1.2
			(1.5)2.0(2.2)	0.29	(0.8)2.9(4.9)	0.8	(1.5)3.9(6.0)	1.18			
Cataphyll indumentum	(1)2(3) ^{l,n,s}	1		(3)4.8(5) ^{d,s}		0.46		(2)4.1(5) ^{d,s}	1	(2)2.9(4) ^{d,l,n}	0.9
			(3)4.2(5)	1.1	(3)4.8(5)	0.47	(4)5	0.18			

SSR analysis

The results of locus statistics are summarised in Table 8. A maximum of two alleles per locus per individual was detected as expected in diploid species. Proportion of distinguishable genotypes (PD) was high among all analysed populations and taxa except *S. *daphneola*. The majority of individuals (82.5 %) had unique multilocus genotypes (MLG). The probability of identity P_{ID} , i.e. probability of false identification of two nonclonal individuals as one genotype, was 4.1×10^{-7} . However, 20 sets of putative clones (identical MLG as determined by the six microsatellite loci) were revealed. Majority of clones in *S. lapponum* were found within the same population in the distances up to 10 m (7 clones in PL, 4 clones in SZ, 2 clones in HRS and 1 clone in WS). Two clones were shared between the populations: between HPL and PL and between NJ and SZ, ca. 720 and 315 m apart respectively. Two clones were found in *S. silesiaca*. In *S. *daphneola*, three putative clones were revealed. The observed heterozygosity (H_{obs}) was generally high (0.33–0.72) and usually similar to or higher than the expected heterozygosity (H_{exp}). *Salix lapponum* populations NJ, PL, SCA, SZ and WS as well as *S. *daphneola*, *S. ×nepetifolia* and *S. silesiaca* samples showed significant deficit of heterozygotes with $F_{IS}/\rho_{IS} > 0$ (Table 9).

Table 8. Results of loci statistics. NA – number of obtained alleles, H_{obs} – observed heterozygosity, H_{exp} – expected heterozygosity, F_{IS} – coefficient of inbreeding, P value for Hardy-Weinberg equilibrium test for all populations on genet level.

Locus	NA	H_{obs}	H_{exp}	F_{IS}	FST	P
SB24	30	0.638	0.876	-0.079	0.369	0.007
SB80	13	0.390	0.528	-0.167	0.265	0.000
SB100	8	0.575	0.609	-0.285	0.304	0.001
SB201	24	0.590	0.795	0.007	0.382	0.000
SB210	17	0.426	0.747	0.036	0.312	0.000
SB233	18	0.638	0.829	-0.045	0.347	0.000

Table 9. Within-population characteristics based on SSR data. N – number of individuals sampled in population, nG – number of genotypes, PD – proportion of distinct genotypes, H_{obs} – observed heterozygosity, H_{exp} – expected heterozygosity, A – mean number of alleles per locus, A_r – mean number of alleles after rarefaction, F_{IS} – coefficient of inbreeding, ρ_{IS} – inbreeding coefficient counted from allele size, HE – p value of heterozygote excess, HD – p value of heterozygote deficit. Population abbreviations as in Table 6.

Population	N	nG	PD	H_{obs}	H_{exp}	A	A_r	F_{IS}	ρ_{IS}	HD	HE
HLL	1	1	-	0.667	0.333	1.667	1.667	-	-	-	-
HPL	6	5	0.83	0.694	0.551	2.667	2.412	-0.174	-0.433	0.925	0.090
PL	61	52	0.85	0.691	0.728	8.167	3.131	0.058	0.038	<0.001	1.000
NJ	39	39	1.00	0.468	0.564	6.000	2.514	0.179	-0.004	<0.001	1.000
SZ	44	38	0.86	0.444	0.494	3.667	2.227	0.110	0.224	0.001	0.999
HL	3	3	1.00	0.722	0.546	2.667	2.344	-0.091	-0.916	0.577	0.459
VY	4	4	1.00	0.514	0.539	3.167	2.695	0.182	-0.046	0.088	0.923
PLB	11	11	1.00	0.644	0.616	4.500	2.769	0.005	-0.009	0.266	0.734
UR	1	1	-	0.333	0.167	1.333	1.333	-	-	-	-
UJ	3	3	1.00	0.500	0.509	2.833	2.389	0.217	0.185	0.132	0.882
SZL	1	1	-	0.500	0.250	1.500	1.500	-	-	-	-
NLB	1	1	-	0.500	0.250	1.500	1.500	-	-	-	-
OB	1	1	-	0.500	0.250	1.500	1.500	-	-	-	-
MS	1	1	-	0.333	0.167	1.333	1.333	-	-	-	-
HRS	9	7	0.78	0.537	0.464	3.833	2.301	-0.099	-0.367	0.758	0.253
WS	5	4	0.80	0.350	0.463	2.833	2.180	0.4	0.84	0.003	0.997
WSV	3	3	1.00	0.417	0.424	2.333	2.044	0.243	0.037	0.120	0.976
PO	4	4	1.00	0.625	0.594	3.167	2.765	0.091	0.564	0.286	0.728
SLO	4	4	1.00	0.458	0.458	3.000	2.399	0.143	0.65	0.107	0.914
dap	12	7	0.58	0.556	0.557	3.667	2.389	0.046	0.042	0.020	0.981
JES	1	1	-	0.333	0.167	1.333	1.333	-	-	-	-
SCA	11	11	1.00	0.437	0.700	6.500	3.231	0.388	0.294	<0.001	1.000
nep	11	11	1.00	0.527	0.724	7.333	3.365	0.314	0.009	<0.001	1.000
sil	31	29	0.94	0.501	0.815	13.000	3.808	0.393	0.288	<0.001	1.000
MEAN	11	10	0.92	0.511	0.472	3.729	2.297	0.142	0.082		

The overall genetic differentiation among all analysed individuals was very strong with a mean overall F_{ST} value of 0.25. Inferring from pairwise F_{ST} and ρ_{ST} indices most populations had moderate to strong genetic differentiation (Supplementary File 7). However, some populations pairs in the Krkonoše Mts. (e.g., HPL vs. HRS, PL vs. PLB, HRS vs. VY) showed low F_{ST} value indicating the occurrence of gene flow. AMOVA revealed that most of the variation (83 %) occurred within populations while

only 10 % and 7 % occurred among populations and regions, respectively. Bayesian clustering implemented in the STRUCTURE software using ΔK estimate (Evanno et al. 2005) identified the $K=2$ as the most suitable number of clusters, the second most suitable number of clusters was $K=5$ (Fig. 11).

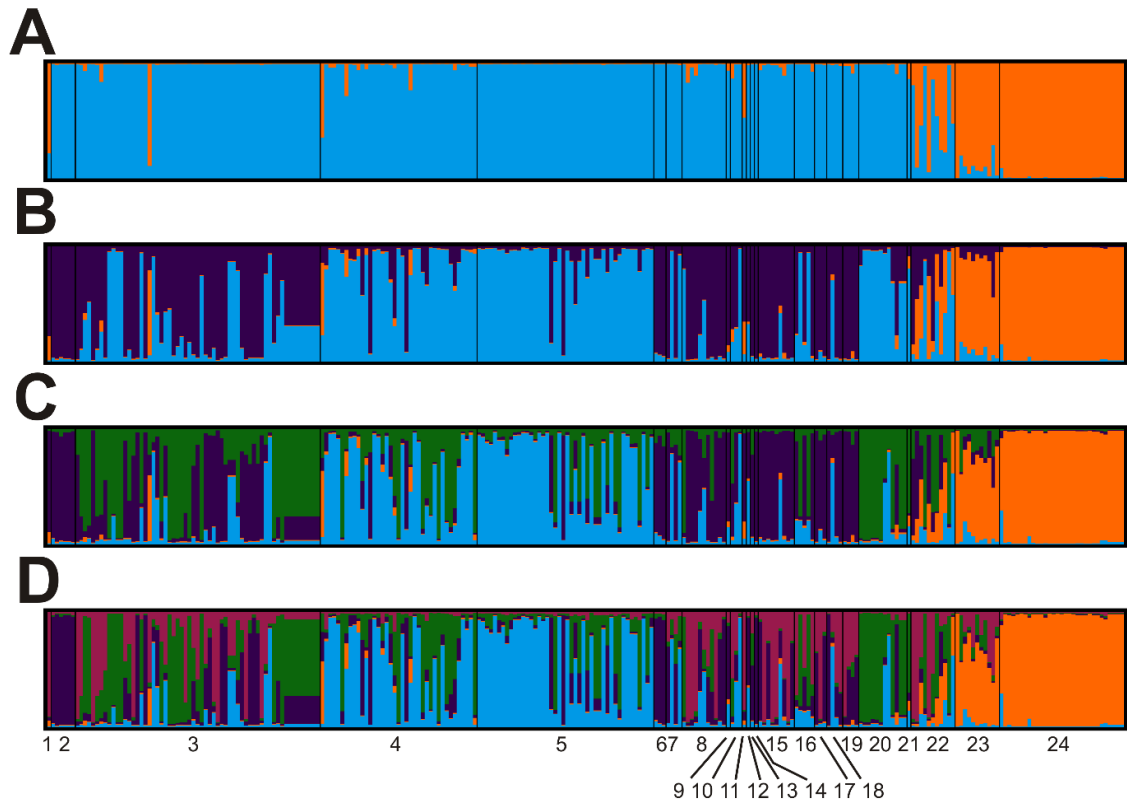


Figure 11. Levels of population admixture using Bayesian structuring of *S. lapponum* and allied taxa in STRUCTURE: A) $K=2$, B) $K=3$, C) $K=4$, D) $K=5$. Each bar represents one individual, vertical black lines separate the populations (1 – HLL, 2 – HPL, 3 – PL, 4 – NJ, 5 – SZ, 6 – HL, 7 – VY, 8 – PLB, 9 – UR, 10 – UJ, 11 – SZL, 12 – NLB, 13 – OB, 14 – MS, 15 – HRS, 16 – WS, 17 – WSV, 18 – PO, 19 – SLO, 20 – *S. *daphneola*, 21 – JES, 22 – SCA, 23 – *S. ×nepetifolia*, 24 – *S. silesiaca*; population abbreviations as in Table 6).

Discussion

Genetic variation and population structure

Isolated populations are expected to undergo several phenomena that affect their genetic diversity such as bottleneck, genetic drift and inbreeding depression (Lande 1995; Lynch et al. 1995; Keller & Waller 2002). Number of reproducing individuals in the Czech part of the Krkonoše Mts. is ca. 600 (Hroneš et al. 2018) a number low enough to

enable manifestation of inbreeding under particular conditions (Ellstrand & Elam 1993). Our data showed relatively high values of observed heterozygosity (0.511) and mean number of alleles per locus (except the one-individual populations). The obtained observed heterozygosity data were similar to other species of *Salix* (e.g., Stamati et al. 2007; Trybusch et al. 2012; Sochor et al. 2013). All larger populations exhibit the significant deficit of heterozygotes which may point to inbreeding due to drop of effective size of population and lack of the gene flow (Frankham 2015). This is in line with the low number of observed reproducing individuals in the Czech part of the Krkonoše Mts. (Hroneš et al. 2018) and the high overall F_{ST} that suggests the strong reproductive isolation among populations in the Krkonoše Mts. Even higher deficit of heterozygotes was observed in *S. lapponum* in Scotland (Stamati et al. 2017).

We detected only 13 clone pairs (or triplets in two cases) which were separated only by very small distances up to 10 m. This is in agreement with results of Stamati et al. (2007) and Pogorzelec et al. (2014) who also observed very low rates of clonality in *S. lapponum*. In another two cases, multilocus genotypes (MLGs) were shared between the populations. These two cases can be explained either by low number of microsatellite loci used that were not able to discriminate the MLGs or by long-distance dispersal of plant fragment. At least in the case of the HPL and PL population which shared the MLG at ca. 720 m distance, the clone occurrence can be explained by the dispersal of twig fragment by Pančava rivulet. Similar long-distance dispersal by water was observed in other members of Salicaceae (Krasny et al. 1988; Karrenberg et al. 2003). Observation of low clonality rates therefore further confirms the assumption that *S. lapponum* is propagating mainly sexually despite the apparent lack of observed seedlings (see Stamati et al. 2007 and Hroneš et al. 2018).

The AMOVA revealed low genetic variation among the populations. According to Nybom (2004), the long-lived and outcrossing species are able to preserve most of their genetic variation within their populations. Pairwise F_{ST} suggest the strong isolation between the most populations. Similarly, STRUCTURE analysis revealed several geographically close populations (HPL vs. PL., SZ and NJ vs. PL) that were separated to different cluster. This is in strong contrast with the general belief that the *Salix* seeds can be dispersed by wind to the long distances which results in the intensive gene flow and low genetic differentiation (e.g., Hörandl et al. 2002). On the contrary, very limited gene flow resulting in strong interpopulation structure were observed in other *Salix*

species (e.g., Sochor et al. 2013; Pogorzelec et al. 2014b). This may be explained by the fact that the establishment of seedlings in *S. lapponum* is a very rare event requiring coincidence of disturbance, wind and suitable habitat conditions (Stamati et al. 2007; Pogorzelec et al. 2014b; Hroneš et al. 2018).

Morphological variation in S. lapponum

Marginal or isolated populations may exhibit the shift in their morphology due to long-term isolation, bottleneck event and/or genetic drift (Lande 1995; Lynch et al. 1995; Keller & Waller 2002). *Salix lapponum* from the Krkonoše Mts. showed such shift in its morphology compared to analysed populations from the Scandinavia (i.e., main range). Plants from the Krkonoše Mts. had generally less hairy leaves with wider lamina when compared to plants from Scandinavia. They were at the same time also more variable in these traits. An extreme case of this variation is a glabrous morphotype (i.e., *S. *daphneola*). Variable leaf shape and size was observed also in marginal populations of *S. lapponum* in eastern Poland (Fijałkowski 1957). Increased variability in leaf morphology in the Krkonoše Mts. than in main range can be explained by different environmental conditions, role of hybridization and/or genetic drift. Environmental conditions, especially soil moisture, concentration of nutrients and pH were observed to modify leaf morphology (Castro-Díez et al. 1997; Todeschini et al. 2011). However, the soil conditions of stands in the Krkonoše Mts. are same or very similar to conditions elsewhere in the range of *S. lapponum* (Hroneš et al. 2018). Hybridization and subsequent introgression can bring the new alleles to the population gene pool (Ellstrand & Schierenbeck 2000). Observed increased variation would require large scale introgression which we failed to observe (see further). Although we are aware of the several limitations of our study (only one sympatrically growing willow species included, morphometric data collected on herbarium specimens), our observations suggest that long term isolation and genetic drift is responsible for increased morphological variation of *S. lapponum* in the Krkonoše Mts.

Hybridization with S. silesiaca

Interspecific hybridization is considered to be one of the main reasons of *Salix* taxonomic complexity (e.g., Rechinger 1992, Skvortsov 1999). *Salix* hybrids are expected to be intermediate between parents by some authors (e.g., Skvortsov 1999) while others view them as a mosaic of parental morphological traits (e.g., Elven & Karlsson 2000). Although our study was focused mainly on morphological and genetic variation in *S. lapponum*, several individuals of *a priori* identified hybrids and *S. silesiaca* were also included. From the morphological point of view, the hybrids were closer to *S. lapponum* by their short stalked and hairy capsules while in other traits such as lamina length and width they were intermediate between parents. The hybrids had also shorter catkins and capsules than their parents (although not significantly). This may indicate the lower fitness of the hybrids (Gramlich & Hörandl 2016).

Frequent hybridization may lead to the extinction of the isolated population (Todesco et al. 2016). Backcrossing of hybrid to one or both of its parents can result in hybrid swarms such as in *S. eriocephala-sericea* hybrid zone (Hardig et al. 2000). We investigated whether the increased morphological variability in *S. lapponum* may be result of such introgression. In STRUCTURE diagrams, *S. silesiaca* was clearly distinguished from *S. lapponum* and plants *a priori* identified as hybrids showed affinity to both *S. silesiaca* cluster and at least one of the *S. lapponum* clusters. Only two other plants, which were *a priori* identified as *S. lapponum* (PL40 and NJ1), may be later generation hybrids or introgressants. However, the observed putative introgression seems to be very low and at this stage it seems to not threaten the populations of *S. lapponum* in the Krkonoše Mts.

Origin of glabrous morphotype

The occurrence of glabrous plants of *S. lapponum* has puzzled botanists since their discovery by Tausch (1837). They were treated as separate species, subspecies or variety of *S. lapponum* or not recognized as separate at all (Tausch 1837; Wimmer 1866; Dostál 1989; Hroneš & Trávníček 2018). Our analysis of morphological characters showed that it differs from typical *S. lapponum* not only by absence of indumentum of leaves and capsules but also by smaller leaves. On the contrary,

glabrous plants cannot be distinguished from typical *S. lapponum* using microsatellite markers showing the genetic affinity to the respective population in which they were sampled.

Parallels to *S. lapponum* versus *S. *daphneola* can be found in members of sect. *Villosae* (to which *S. lapponum* also belongs) in the North America: *S. alaxensis* forms morphotype with glabrous to sparsely villous, glaucous leaves which is treated as var. *longistylis* and glabrescent morphotypes of *S. candida* are treated as f. *denudata* (Argus 2010). Argus (2010) also speculates that glabrous morphotypes of *S. candida* may be of the hybrid origin. Similar expectation may be postulated also for origin of *S. *daphneola* in the Krkonoše Mts. In that scenario, *S. silesiaca* would be the only suitable sympatrically occurring willow species due to its subglabrous leaves and capsules. However, our morphological and SSR data showed that *S. *daphneola* is clearly distinguished from *S. lapponum* × *silesiaca* hybrids and it is at the same time genetically undistinguishable from the typical *S. lapponum*. Therefore, hybrid origin with other now extinct willow species can be also excluded and the glabrous morphotype of *S. lapponum* is most probably not of a hybrid origin. The second option is that the glabrous plants may be recessive homozygotes in one or few genes controlling the indumentum (Kärkkäinen & Ågren J 2002; Widén 2018). Such explanation favours the facts that glabrous plants had $F_{IS}/\rho_{IS} > 0$ which suggest the deficit of heterozygotes (Allendorf & Luikart 2007). Moreover, they showed quite high proportion of clonality despite the positive F_{IS} that is quite paradoxical (but see Reichel et al. 2016). Only seven out of twelve sampled *S. *daphneola* plants possessed own distinguishable genotype. Another option can be the mutation in gene that controls the development of indumentum. This mutation can be further spread in populations by drift (Yeaman & Otto 2011). Therefore, we suggest that the glabrous morphotype may originate polytopically and is at least partially maintained by drift and/or vegetative propagation.

Despite the absence of clear genetic differentiation of glabrous morphotype from typical *S. lapponum*, the plants display striking morphological differences. Therefore, we believe that they deserve recognition on some lower taxonomic rank such as variety or form. Given that glabrous morphotype has not been found elsewhere in the range of *S. lapponum* and shows the strong geographical affinity only to the western Krkonoše Mts., the status of variety would be more appropriate for its conservation.

Taxonomical treatment of the *Salix lapponum* s. lat. in the High Sudetes

Salix lapponum Linnaeus

Typus: Habitat in Alpibus Lapponiae ubique, Helvetiae, sine auctorum, sine dato, LINN 1158.57, upper female specimen (Jonsell & Jarvis 1994)

Salix lapponum var. *lapponum*

= *Salix argyroides* Tausch

= *Salix arenaria* var. *atropurpurea* Tausch

= *Salix arenaria* var. *marrubiifolia* Tausch ≡ *Salix marrubiifolia* (Tausch) Andersson;
Salix helvetica subsp. *marrubiifolia* (Tausch) B. Flod.; *Salix lapponum* subsp.
marrubiifolia (Tausch) Dostál

= *Salix arenaria* var. *nivea* Tausch (1837, p. 339)

Salix lapponum var. *daphneola* (Tausch) Wimmer

Typus: A. d. Sümpfen des Riesengebirges, Tausch, sine dato, Dendrotheca Bohemica
PRC 400850 (Hroneš & Trávníček 2018)

≡ *Salix daphneola* Tausch; *Salix lapponum* subsp. *daphneola* (Tausch) Dostál

CHAPTER 6:

Typification of names related to *Salix lapponum* (Salicaceae) and its hybrids published by Ignaz F. Tausch

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Abstract

Typifications and nomenclatural notes on seven names (two at the rank of species and five varietal ones) of willows published by I. F. Tausch in 1837 are provided. The name *Salix arenaria* var. *latifolia* is deemed illegitimate. Four names (*Salix arenaria* var. *atropurpurea*, *S. arenaria* var. *nivea*, *S. arenaria* var. *marrubiifolia* and *S. argyroides*) are considered synonymous to *S. lapponum* var. *lapponum* and one (*S. arenaria* var. *denudata*) to its hybrid with *Salix silesiaca*. In contrast, *Salix daphneola* applies to a unique glabrous variety of *S. lapponum* (var. *daphneola*) endemic to the Krkonoše Mountains, Czech Republic.

Introduction

Downy willow (*Salix lapponum* L.) is a small deciduous shrub with appressed silvery hairy leaves and capsules. It is distributed mostly in northern Europe and western Siberia, but also scattered in the mountains of central and southern Europe. In the Czech Republic, the species is very rare, occurring only in the Krkonoše Mountains and Hrubý Jeseník Mountains in the northern part of the country (Hroneš et al. 2014). Several interspecific hybrids with *S. lapponum*, mostly from Scandinavia and Scotland, have been formally described (Elven and Karlsson 2000, Tennant 2004). In the Krkonoše Mountains, *S. lapponum* mostly hybridizes with *S. silesiaca* Willd. This hybrid was described by the Czech botanists J. and C. Presl as *S. ×nepetifolia* J. Presl & C. Presl (Danihelka et al. 2012). Hybrids with *S. caprea* L. and *S. aurita* L. have also been occasionally reported (Kwiatkowski 2006).

The taxonomy of downy willow has not been sufficiently resolved yet and several related taxa have occasionally been recognized by various authors, including *S. ceretana* (P. Monts.) J. Chmelař, *S. marrubiifolia* (Tausch) Andersson and *S. daphneola* Tausch (Floderus 1943, Rechinger 1964, Blanco 1993, Danihelka et al. 2012). The last two names, together with several others, were published by I. F. Tausch in his contribution to the taxonomy of mountain willows (Tausch 1837) from the Krkonoše Mountains. During our ongoing biosystematic research on *S. lapponum*, we revealed that the species exhibit more morphological variation in this mountain range than in other areas (Hroneš et al. unpubl.). This variation was noticed also by Tausch, who intended to describe a lot of morphologically divergent individuals as new taxa and

distributed them in his exsiccata series. Unfortunately, only some of the relevant names were validly published by Tausch (1837). Most of these described taxa differ only slightly from the typical *S. lapponum* and can be synonymized with it. Still, the typification of many names of willows established by Tausch is necessary because Tausch usually cited no specimens in his publications. Here, we provide these typifications with notes on the taxonomic identity of the taxa concerned.

Historical background

Ignaz Friedrich Tausch, born in 1793, was a Czech botanist of German origin. During his life, he was associated with Count Joseph E. M. von Canal's garden and was employed as a professor of economic and technical botany at Charles-Ferdinand Univ. of Prague (now Charles Univ. in Prague; Maiwald 1904). He is widely recognized as an extremely skilled and precise botanist with a particular eye for detail (Kirschner et al. 2007, Krahulec 2012). Tausch described many new taxa and created new plant names for the flora of central Europe, as well as for exotic plants cultivated in Canal's garden, e.g. from Australia (Tausch 1836). However, his work was mainly focused on the exploration of the flora of the Sudetes, especially the Krkonoše Mountains (Krahulec 2012). Among other taxa, he discovered and described several species endemic to this small mountain range, such as *Sorbus sudetica* (Tausch) Bluff, Nees & Schauer, *Galium sudeticum* Tausch, and many *Hieracium* species.

His exceptional collection effort resulted in several thematic series of exsiccata, with the most important being 'Plantae Selectae Florae Bohemicae', 'Dendrotheca Bohemica' and 'Herbarium Florae Bohemicae' (Kirschner et al. 2007). The most complete extant sets of these exsiccata series are deposited in the Herbarium of the Charles Univ. in Prague (PRC) and in the Herbarium of the National Museum in Prague (PR). Numerous duplicates were also distributed to various herbaria across Europe (Stafleu and Cowan 1986, Kirschner et al. 2007). The specimens of 'Herbarium Florae Bohemicae' were sent without labels and accompanied only by hand-written list of localities. Specimens forming this series bear paper 'tags' with exsiccata series numbers that were made by Tausch himself (Fig. 12). After Tausch's death, all localities of 'Herbarium Florae Bohemicae' were summarized by Ott (1851a, b). Specimens of the

other exsiccata were distributed with printed labels, probably also made by Tausch himself (Tausch 1833).

After his expeditions to the Krkonoše Mountains, which likely took place during the 1820s, Tausch described several species and intraspecific taxa of willows (Tausch 1837). He subordinated several of these taxa to *Salix arenaria* L., which was in the first half of the 19th century widely confused with *S. lapponum* (Villars 1789, Schkuhr 1803, Willdenow 1805, Wimmer 1866). This confusion mostly arose from the ambiguous protologues of *S. lapponum* and *S. arenaria* provided by Linné in ‘Species Plantarum’ (Linné 1753). The name *S. arenaria* has now been typified and fixed to a taxon closely related to *S. repens* agg. (Jarvis 2007). Therefore, most of the taxa published by Tausch and attributed to *S. arenaria* should belong to *S. lapponum*.

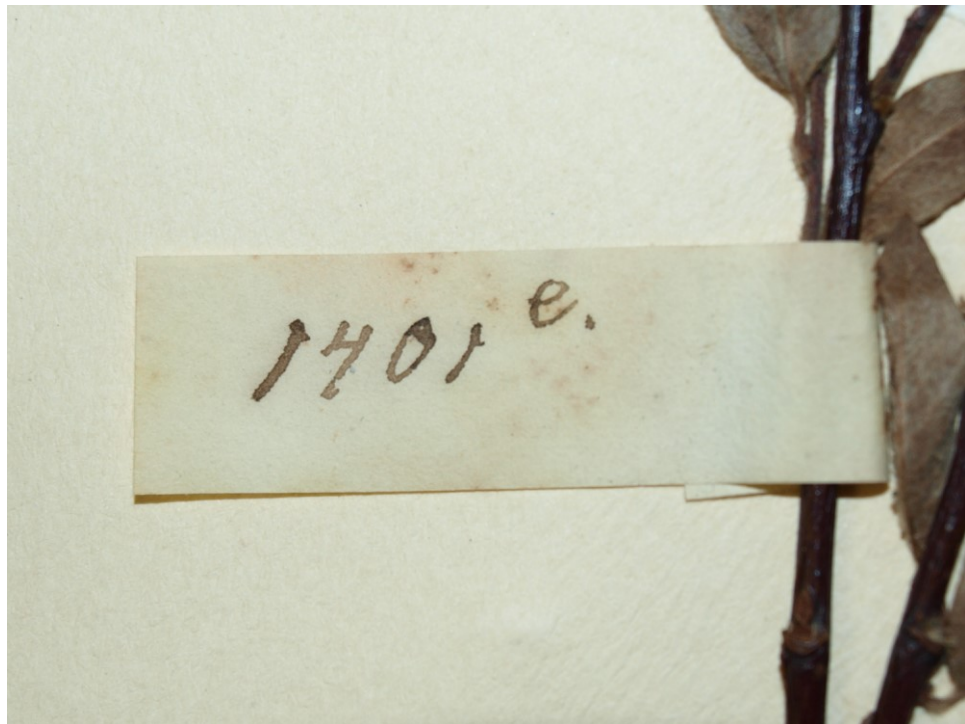


Figure 12. Example of Tausch’s handwriting.

Material and methods

The typification process followed the instructions of the International Code of Nomenclature for Algae, Fungi and Plants (the Melbourne Code; McNeill et al. 2012). Herbarium material associated with the names published by Tausch (1837) was

searched for in PRC and PR. However, recognizing original material among other specimens was somewhat difficult, as Tausch himself never associated his collections with a label in the modern sense. Thus, as a reference collection we have chosen Tausch's series 'Herbarium Florae Bohemicae', in which specimens bear handwritten small paper 'tags' with exsiccata series numbers that were made by the original author and as such can be considered as part of the original material (Fig. 12). Some exceptions were made in cases where the protologue points directly to other exsiccata series. Considering that Tausch never distributed his exsiccata with 'proper' labels and never gave any information about the date of their collection and therefore no one can unambiguously determine whether or not a particular specimen was collected before or after the date of publication, we have chosen to refer to all selected type specimens as 'lectotype or neotype'. The accepted names for the taxa were adopted from the most recent version of the Checklist of vascular plants of the Czech Republic (Danihelka et al. 2012).

Nomenclature notes and typifications

Salix arenaria β [var.] *latifolia* Tausch (1837, p. 339)
nom. inval. under ICN Art. 26.2.

Notes

In the protologue for this variety, Tausch included in its synonymy '*S. arenaria* L. spec. 1447', pointing to the page of description of *S. arenaria* L. in 'Species Plantarum' ed. 2 (Linné 1763). Therefore, he included the type of *S. arenaria* and the name should be deemed as invalidly published under Art. 26.2 (McNeill et al. 2012).

Salix arenaria δ [var.] *atropurpurea* Tausch (1837, p. 339)

Lectotype or neotype (designated here): [Czech Republic] Am Sümpfen des Riesengebirges ['on peat bogs of the Krkonoše Mountains'], Tausch, sine dato, 'Herbarium Florae Bohemicae' no. 1382d, PRC 400860 (Fig. 13).

Accepted name: *Salix lapponum* L. var. *lapponum*.



Figure 13. Type of *Salix arenaria* var. *atropurpurea* Tausch.

Notes

Tausch described this variety as “ramis atropurpureis”; however, reddish twigs should be attributed to individual variability and do not have any taxonomical importance. We traced two relevant specimens deposited at PRC from the ‘Herbarium Florae Bohemicae’. The first specimen (PRC 400860) consists of two fragments. One (on the left) with fully hairy leaves and the second (on the right) with somewhat denudate, larger leaves. The label for this specimen is written by charcoal and bears the following text: “Am Sümpfen des Riesengebirges”. The second specimen (PRC 415452) bears three fragments and a label written by ink that reads “V. kleinen Teiche im Riesengrunde”. The locality information on the second specimen is clearly wrong. First of all, glacial lakes (Teiche) are located on the opposite side of mountain massif than Obří důl glacial cirque (Riesengrund). Second, the locality for the collection ‘Herbarium Florae Bohemicae’ no. 1382d is given as “Am Sümpfen des Riesengebirges” in Ott

(1851a). For these reasons, we chose the right-hand fragment on the specimen PRC 400860 as the type.

Salix arenaria ϵ [var.] *nivea* Tausch (1837, p. 339)

Lectotype or neotype (designated here): [Czech Republic] Aus dem Elbegrunde des Riesengebirges [‘from Labský důl glacial cirque in Krkonoše Mountains’], Tausch, sine dato, ‘Herbarium Florae Bohemicae’ no. 1378, PRC 415451 (Fig. 14).

Accepted name: *Salix lapponum* L. var. *lapponum*.

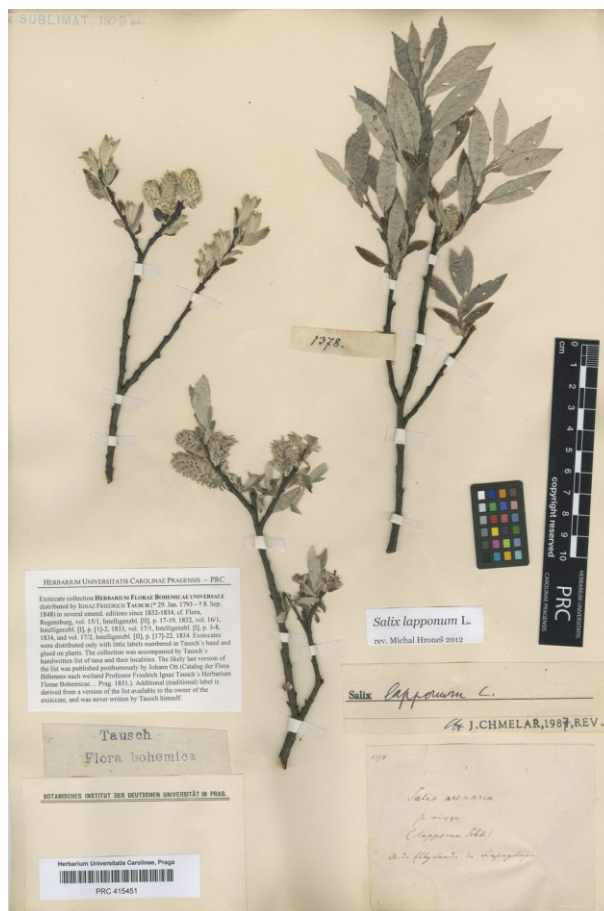


Figure 14. Type of *Salix arenaria* var. *nivea* Tausch.

Notes

In the protologue, Tausch described this variety as “foliis supra canis, subtus niveo-tomentosis” and accompanied the description with the note “(α & β)”. The part in parentheses is pointing to the varieties α (i.e. var. *arenaria*) and β (i.e. var. *latifolia*), and therefore suggesting the shape of leaves. We found only one relevant specimen at

PRC, accompanied by a small, glued paper tag with a hand-written number. It bears three fragments, all with female catkins. As the type, we chose the specimen to the right, which fully agrees with Tausch's description. This type fits well into the variability of the *Salix lapponum* var. *lapponum*.

Salix arenaria η [var.] *denudata* Tausch (1837, p. 339)

Lectotype or neotype (designated here): [Poland] Von kleinen Teiche im Riesengebirge [‘from small glacial lakes in the Krkonoše Mountains’], Tausch, sine dato, ‘Herbarium Florae Bohemicae’ no. 1382, PRC 400869 (Fig. 15).

Accepted name: *Salix* × *nepetifolia* J. Presl & C. Presl (*Salix lapponum* × *S. silesiaca*).



Figure 15. Type of *Salix arenaria* var. *denudata* Tausch.

Notes

This variety was described as “foliis utrinque virescentibus plusminusve denudatis, imo demum glaberrimis (α & β)”. The specimen we chose as the type bears one fragment

with denudate large leaves with uneven secondary venation. The overall appearance of the specimen points to introgression with *Salix silesiaca*. Therefore, we consider this name to be a synonym of *Salix* ×*nepetifolia*.

Salix arenaria θ [var.] *marrubiiifolia* Tausch (1837, p. 339)

Based on the same type: *Salix marrubiiifolia* (Tausch) Andersson (1868, p. 276). – *Salix helvetica* subsp. *marrubiiifolia* (Tausch) B. Flod. (1943, p. 77). – *Salix lapponum* subsp. *marrubiiifolia* (Tausch) Dostál (1984, p. 3).

Lectotype or neotype (designated here): [Poland] V. kleinen Teiche im Riesengebirge [‘from small glacial lakes in the Krkonoše Mountains’], Tausch, sine dato, ‘Herbarium Florae Bohemicae’ no. 1382c, PRC 400846 (Fig. 16).

Accepted name: *Salix lapponum* L. var. *lapponum*.

Notes

This broad-leaved variety of *S. lapponum* was described by Tausch as “foliis subrotundatis subrepando-undulatis rugosissimis”. The description and epithet points to a very unusual appearance of the leaves, which, based on their shape and undulate margins, resemble the leaves of common horehound (*Marrubium vulgare* L.). In fact, the specimen seems to be somewhat damaged by environmental conditions, most probably by frost.

The name was misinterpreted by Floderus (1943) and Rechinger (1964), who used it in a different meaning for various populations from the Sudetes and Carpathians. Floderus used the name *S. helvetica* subsp. *marrubiiifolia* for populations from the Tatra Mountains. However, he most probably never examined Tausch’s original material, which is very different from western Carpathian plants of *S. helvetica*. Rechinger, clearly influenced by the work of Floderus, in his treatment of *Salix* for the first edition of ‘Flora Europaea’ applied the name *S. marrubiiifolia* (as ‘*marrubifolia*’) to plants from the Sudetes Mountains and the Carpathian Mountains. In his circumscription, two species now generally accepted as different were included – *Salix lapponum* from the Sudetes and Eastern Carpathians and *S. helvetica* from the Western Carpathians, respectively. Yet again, the name was not used in the original sense of Tausch.

We found a single herbarium specimen at PRC from the ‘Herbarium Florae Bohemicae’. It bears four fragments, three with fully developed leaves only and one

without leaves and with an undeveloped male catkin. This composition suggests that the sheet contains a mixture of at least two individual gatherings that were obtained at different times. Therefore, we chose as the type the left-hand fragment, with a paper tag bearing the number of ‘Herbarium Florae Bohemicae’.



Figure 16. Type of *Salix lapponum* var. *marrubifolia* Tausch.

Salix daphneola Tausch (1837, p. 343)

Based on the same type: *Salix lapponum* var. *daphneola* (Tausch) Wimmer (1866, p. 41). – *Salix lapponum* subsp. *daphneola* (Tausch) Dostál (1989, p. 124, comb. inval. ICN Art. 41.5).

Lectotype or neotype (designated here): [Czech Republic] A. d. Sümpfen des Riesengebirges [‘on peat bogs of the Krkonoše Mountains.’], Tausch, sine dato, ‘Dendrotheca Bohemica’, PRC 400850 (Fig. 17).

Accepted name: *Salix lapponum* var. *daphneola* (Tausch) Wimmer.

Salix argyroides Tausch (1837, p. 340)

Lectotype (designated here): [icon] tab. 317 g in Schkuhr, Bot. Handbuch (1803).

Accepted name: *Salix lapponum* L. var. *lapponum*.

Notes

The name *Salix argyroides* represents another case in which Tausch referred to a previously published work (as “Schkr. man. t. 317 g” = Schkuhr 1803) and indicated a specific collection in his protologue, i.e. ‘Dendrotheca Bohemica’. The referenced plate in Schkuhr (1803) portrays a typical plant of *S. lapponum* var. *lapponum*. According to Tausch, it differs from *S. arenaria* (i.e. *S. lapponum*) by its pedicellate capsule, a trait which has no taxonomic value and falls into the variability of *S. lapponum*. Therefore, we chose the plate 317 g in Schkuhr (1803) as the lectotype (Fig. 18).



Figure 18. Lectotype of *Salix argyroides* Tausch.

CHAPTER 7:

Summary and conclusions

MICHAL HRONEŠ

The effective conservation of any species requires prior knowledge of its life-history, demography, genetics and biotic interactions (Oostermeijer et al. 2003). Although willows are usually omitted by general botanical audience, they present a set of interesting ecological as well as evolutionary problems. The isolated populations of *S. lapponum* in the Krkonoše Mts. are quite similar to other isolated and main range populations but at the same time they differ in many respects from these.

The habitat conditions of stands of *S. lapponum* in the Krkonoše Mts. are generally similar to other populations except of higher content of nitrogen and lower pH of soil. Contrary to extensive scrub consisted of several willow species that is typical for *S. lapponum* vegetation in its main range, the stands of *S. lapponum* in the Krkonoše Mts. are either mostly monospecific within the shrub layer or scattered stands of isolated individuals are present. Monospecific *S. lapponum* scrub is further supplemented by mountain and alpine tall-forb species forming an association *Salicetum lapponum* Zlatník 1928 which were considered endemic to the Sudetes Mts. Recent reports of the same vegetation unit from Massif Central (de Foucault 2012) need further confirmation.

Analysis of genetic population structure revealed relatively low clonality but also indicated the absence of gene flow and lack of heterozygotes in all larger populations. This may constitute a threat for future preservation of the Krkonoše populations. The long-term isolation of *S. lapponum* populations in the Krkonoše Mts. also probably resulted in overall morphological shift towards the more glabrous leaf blades and catkins in comparison with main range populations. This indicates that populations are undergoing recent speciation due to absence of gene flow from main range and the role of genetic drift. On the contrary, the role of hybridization can be considered as relatively insignificant at present state of knowledge.

The conservation effort in the Krkonoše Mts. should be focused mainly on the support of the establishment of the new seedlings and also on the protection of the old individuals from large herbivores. The inspiration can be drawn from similar efforts in Scotland and Poland. The future research should be focused mainly on resolving the relationships of the Krkonoše populations with other, particularly Hercynian and Balkan populations, on the fate of the planted populations and also on the biotic interactions (e.g., foliar diseases, mycorrhiza, herbivores).

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SUPPLEMENTARY FILES

Supplementary File 1 – Distribution of *Salix lapponum* var. *lapponum* compiled from herbarium specimens, literature and own field observations.

93a. Krkonoše lesní: U Špindlerovy boudy (1928 Dostál, PRC). – Modrý důl (1954 Šourek, PR; 1967 Faltys, MP; 1973 Hodoval, BRA; not. Hroneš & Hrachová, 2009). – Obří důl, louka Na Dolech (1953 Horák & Šourek, MP; 1954 Soják, PR; 1954 Šourek, PR) [pravděpodobně †]. – **93b. Krkonoše subalpínské:** Navorská louka, při cestě z Labské louky k Vosecké boudě (2005 Grulich, BRNU; 2010 Hroneš, OL). – Prameniště Velké Mumlavy (2010 Hroneš, OL). – Kotelní jámy (1959 Čábera, CB). – Malá Kotelní jáma (1907 Čelakovský, PR). – Labská louka (1883 Velenovský, PR; 1890 Kabát, BRNU, PR; 1913 Schustler, PR; 1927 Sillinger, PR; 1928 Krajina, PRC; 1933 Horák, MP; 1947 Křístek, GM; s. d. Tausch, PRC). – Prameniště 150 m JZ Labské boudy (1995 Hanousek, HR). – Labská bouda (1907 Domin, PRC; 1959 Čábera, PR). – Pančavská louka (1880 Freyn, PRC; 1882 Baenitz, PRC; 1883 s. coll., PRC; 1883 Velenovský, PRC; 1895 Lukeš, BRNU; 1895 Tocl, PR; 1896 Limpricht, BRA; 1896 Tocl, PRC; 1905 Čelakovský, PR; 1907 Domin, PRC; 1920 Behr, BRNU; 1921 s. coll., PR; 1922 Puchmajerová, PRC; 1925 Thenius, BRNU; 1930 s. coll., PR; 1931 s. coll., PR; 1933 Horák, MP, PRC; 1933 Kluge, MP; 1934 Dostál & Novák, PRC; 1950 Míkyška, PR; 1950 Kurka, CB; 1952 Hrabětová, BRNU; 1960 Chmelař, BRNL; 1967 Hentschel, BRNL; 1968 Šťastný, LIT; 1971 Chmelař, BRNL; 1975 Belicová, HR; s. d. Domin, PRC). – Pančavská louka, Z okraj (1967 Businský, ROZ). – Pančavská louka, u pramenů Pančavy (1927 Puchmajerová, PRC; 2010 Hroneš, OL). – Pančavská louka, u Pančavského vodopádu (1880 Freyn, PRC; 1880 Hora, PRC; 1890 Cypers, PR; 1907 s. coll., PRC; 1927 Puchmajerová, PRC; 1936 s. coll., PRC; 1949 Šourek, PR; 1952 Chmelař, BRNL; 1959 Chmelař, BRNL; 1962 Zlatník, BRNL; 1967 Krahulec, BRNL; not. Hroneš & Hrachová, 2010). – Navorská jáma (1980 Šrůtek, ROZ). – Labský důl (1927 Puchmajerová, PRC; 1971 Chmelař, BRNL; s. d. Tausch, PR, PRC; s. d. Litenský, PR). – Labský důl, svah pod Hančovým pomníkem (1962 Zlatník, BRNL; 2010 Hroneš, OL). – Labský důl, hrana nad karem se Schustlerovou zahrádkou (1975 Jeslík, ROZ). – Stráž u můstku přes Labský vodopád (1955 Hostička, MP). – Čertova louka, rašeliniště vlevo od cesty (1975 Belicová, HR). – Stříbrné návrší (1901 Cypers, PR). – Výrovka, při cestě k Luční boudě (1980 Procházka, MP; 1989 Kubát, LIT; not. Hroneš & Hrachová, 2009). – Údolí Bílého Labe (1881 Purkyně, PL, PRC; 1922

Schustler & Zlatník, PR; 1934 Dostál & Novák, PR, PRC; not. Hroneš & Hrachová, 2009). – Bílá louka (1810 s. coll., PRC; 1887 Krátký, ROZ; 1887 s. coll., PR; 1922 Krajina, PRC; 1936 s. coll., PRC; 1954 Šourek, PR; 1957 Dvořák, BRNL; 2010 Hroneš, OL). – Úpské rašeliniště (1956 Skalický, PR; 2010 Hroneš, OL). – Pláň mezi Obří a Luční boudou (1959 Deyl, OLM) [pravděpodobně †]. – Studniční hora (s. d. Traxler, HR). – Jižní úbočí Studniční hory, v pásu kosodřeviny (1946 Horák, MP, PRC). – Obří důl (s. d. Tausch, BRNU). – Obří důl, Sněhový žlab (1971 Chmelař, BRNL; not. Hroneš, 2010). – Obří důl, Úpská jáma (1938 Kavka, BRA). – Úpská jáma, Krakonošova zahrádka (1926 s. coll., PRC). – Úpská jáma, mezi vodopády Úpy a Úpičky (1949 Hendrych, PR; 2010 Hroneš, OL). – **93c. Rýchory:** Rýchory, na náhorní planině blíže cestičky při vstupu do bukového pralesa (1959 Šourek, PR) [†]. – **97. Hrubý Jeseník:** Tabulové skály na Pradědu (1877 Oborny, PRC; 1893 Schierl, BRNM; 1904 Oborny, BRNM; 1929 Laus, OSM, PRC; 1930 Laus, BRNU, PR, PRC; 1933 Kresl, PRC; 1934 s. coll., PRC; 1935 Laus, OLM; 1935 Otruba, OLM; 1936 Otruba, OLM, PR; 1946 Pospíšil, BRNM; 1947 Šmarda, BRNM; 1950 Zavřel, BRA; 1954 Zavřel, BRA; 1956 Šula, OLM; 1960 Chmelař, BRNL, CB, OL; 1976 Bureš, OLM; s. d. Spatzier, PR; not. Hroneš, 2011). – Praděd (1883 Freyn, BRNM; 1893 Anders, PR; 1894 Rothe, BRNU; 1909 Laus, BRNM, GM, OLM; 1909 s. coll., BRNU; 1922 Bayer, BRNL; 1929 Laus, OLM; 1933 Rypáček, PRC; 1934 Preis, PRC; 1937 Laus, NJM; 1938 s. coll., PRC; 1946 Medlinová, PRC; 1950 Vězda, BRNL; 1959 Hrabětová, BRNU; 1960 Chmelař, BRNL; 1962 Vaněček, CB; 1964 Deyl, OLM; 1966 Procházka et al., MP; 1972 Kubát, LIT; s. d. Formánek, BRNM; s. d. Grabowski, PRC; s. d. Spatzier, LIT; not. Hroneš, 2011). – Praděd při cestě na Švýcárnu (1980 Kubát, LIT). – Petrovy kameny (1946 Krajina, PRC; 1960 Dostál, PR) [†]. – Vysoká hole (1960 Dostál & Bednář, PR) [pravděpodobně †]. – Jelení hřbet (1965 Dostál, PR) [pravděpodobně †]. – Břidličná hora [„Schieferstein“] (1931 s. coll., PR) [pravděpodobně †]. – **Karkonosze:** Polední kámen [„Mittagstein, Słonecznik“] (1890 coll?, HR, MP; 1962 Deyl, OLM). – Na žluté turistické stezce cca 350 SV od Poledního kamene (not. Hroneš, 2012). – Hrana Rybníků [„Teichrand“] (1876 Cypers, PRC; 1876 s. coll., PR; 1878 Sterneck, PRC; not. Hroneš, 2012). – Velký Rybník [„Grosser Teich, Wielki Staw“] (1880 s. coll., PRC; 1893 Proschwitzer, PRC; 1898 Vilhelm, PRC; 1909 Missbach, BRNM, BRNU, PRC; 1919 Bayer, BRNL; 1925 s. coll., PRC; 1932 Bayer, BRNL; 1932 Kavka, MP; 1962 Deyl, OLM; 1964 Sýkora, OLM; not. Hroneš, 2012). –

Polana (not. Hroneš, 2012). – Bouda prince Jindřicha [„Prinz Heinrich Baude, Schronisko im. Księcia Henryka“] (1907 Domin, PRC; 1909 Houfek, CB; 1913 Kavina, PR; 1923 s. coll., MP; 1936 coll?, PRC; not. Hroneš, 2012). – Předělový hřeben mezi Malým a Velkým Rybníkem (1975 Jeslík, ROZ; not. Hroneš, 2012). – Malý Rybník [„Kleiner Teich, Mały Staw“] (1843 Poech, PRC; 1843 Röttig, MJ; 1869 coll?, OLM; 1870 coll?, BRNM; 1879 Freyn, BRNM; 1881 Traxler, HR, PR; 1884 Fiek, PRC; 1886 Puchmajerová, PRC; 1891 Čelakovský, PR; 1894 Baenitz, PRC; 1909 Missbach, PR; 1913 Schustler, PR; 1922 Traxler, PRC; 1926 Puchmajerová, PRC; 1932 Karel, MP, SAV; 1932 Kavka, BRA; 1932 Salzmann, PR; 1932 Traxler & Karel, HR; 1946 Šourek, PR; 1959 Kuczyńska, BRNU; 1975 Jeslík, ROZ; s. d. Kablíková, BRNU, LIT, PR; s. d. Tausch, BRNU, MJ, PR, PRC; s. d. s coll., PRC; not. Hroneš, 2012). – U cesty Česko-polského přátelství, asi 400 m Z od Obřího sedla (not. Hroneš, 2012). – Cestou od Sněžky na sever, pravděpodobně † (1912 Adámek, GM).

Broadly located herbarium records:

Sudety (1843 Poech, PR; s. d. Tausch, MJ). – Krkonoše (1836 Wagner, PR; 1859 Ettl, PR; 1896 Toel, PR; 1902 Smolák, ROZ; 1932 Bayer, BRNL; 1933 s. coll., ROZ; s. d. Domin, PRC; s. d. Fiek, BRNM, PRC; s. d. Kablíková, PR; s. d. Kynčl, PRC; s. d. Tausch, PRC; s. d. Vinikláš, PRC). – Jeseníky (s. d. Grabowski, BRNM).

Additional literature records:

Karkonosze: Malá Sněžná jáma [„Mały Śnieżny Kocioł“] (Kwiatkowski 2006). – Pielgrzymy [„Dreisteine“] (Schube 1903). – Jáma Lomničky [„Melzergrund, Kocioł Łomniczki“] (Kwiatkowski 2006).

Supplementary File 2 – Distribution of *Salix lapponum* var. *daphneola* compiled from herbarium specimens, literature and own field observations.

93b. Krkonoše subalpínské: Pančavská louka, u vodopádu Pančavy (1919 Schustler, PR; 1925 Thenius, BRNU; 1949 Šourek, MP, PR; 1959 Chmelař, BRNL). – Pančavská louka (1889 Fiek, PRC; 1895 Hirte, PRC; 1920 Behr, BRNU; 1922 Puchmajerová, PRC; 1934 Dostál & Novák, PRC; 1950 Kurka, CB; not. Hroneš & Hrachová, 2010). – Severní okraj Navorské jámy (not. Hroneš, 2010). – Jižní okraj Navorské jámy, u pravostranného přítoku Labe (not. Hrachová, 2010). – Labský důl, svah pod Hančovým pomníkem (not. Hroneš, 2010).

Broadly located herbarium records:

Krkonoše [„In den Sümpfen des Riesengebirges“] (s. d. Tausch, OLM, PR, PRC).

Supplementary File 3 – Overview of all sampled populations of *S. lapponum* in the Krkonoše Mts. with exact number of individuals used for respective analysis. S – non-reproducing, F – flowering, N – not sampled.

Population	Acronym	GPS coordinates (WGS-84)	No. of individuals	S	F	Analysis	
						Fine-scale plots	Soil samples
Úpská jáma glacial cirque	UJ	50°44'18.4"N, 15°43'22.0"E	36	26	10	36	36
Úpské rašeliniště peat bog	UR	50°44'11.4"N, 15°42'50.7"E	2	2	0	N	N
alpine meadow near Výrovka chalet	VY	50°43'3.6"N, 15°40'59.4"E	4	1	3	N	N
spring in Modrý důl glacial valley	MD	50°43'16.8"N, 15°41'29.6"E	3	0	3	N	N
Hančova louka alpine meadow	HL	50°45'32.2"N, 15°32'42.0"E	90	31	59	N	N
Pančavská louka peat bog	PL	50°45'39.6"N, 15°32'36.6"E	51	1	50	51	51
eastern part of Navorská jáma glacial cirque	SZ	50°45'54.3"N, 15°32'47.5"E	81	9	72	81	81
northern part of Navorská jáma glacial cirque	NJ	50°46'3.1"N, 15°32'48.9"E	688	266	422	39	39
spring of Pančava rivulet	HPL	50°46'7.9"N, 15°32'19.3"E	6	2	4	N	N

Supplementary File 4 – Overview of all surveyed populations of *S. lapponum* for their sex in the Krkonoše Mts. with exact number of individuals. S – non-reproducing, F – female, M – male.

Population	Acronym	GPS coordinates (WGS-84)	No. of individuals	S	F	M
Úpská jáma glacial cirque	UJ	50°44'18.4"N, 15°43'22.0"E	36	26	5	5
Úpské rašeliniště peat bog	UR	50°44'11.4"N, 15°42'50.7"E	2	2	0	0
alpine meadow near Výrovka chalet	VY	50°43'3.6"N, 15°40'59.4"E	4	1	2	1
spring in Modrý důl glacial valley	MD	50°43'16.8"N, 15°41'29.6"E	3	0	3	0
Hančova louka alpine meadow	HL	50°45'32.2"N, 15°32'42.0"E	90	31	40	19
Pančavská louka peat bog	PL	50°45'39.6"N, 15°32'36.6"E	51	1	32	18
eastern part of Navorská jáma glacial cirque	SZ	50°45'54.3"N, 15°32'47.5"E	81	9	41	31
northern part of Navorská jáma glacial cirque	NJ	50°46'3.1"N, 15°32'48.9"E	688	266	296	126
spring of Pančava rivulet	HPL	50°46'7.9"N, 15°32'19.3"E	6	2	3	1

Supplementary File 5 – List of specimens used for morphometric analysis. Data are presented in following format: locality, date of collection (s.d. = without exact date), collector (coll.? = collectors name not deciphered, s.coll. = without any specific collector), herbarium acronym according Thiers (2018), number of specimens analysed in parentheses.

S. lapponum

Krkonoše Mts., s.d., s.coll., HR (2). – Krkonoše Mts., s.d., I.F. Tausch, PRC (16). – Krkonoše Mts., 8.1811, s.coll., PRC (2). – Krkonoše Mts., 15.7.1933, coll.?, MP (1). – Krkonoše Mts., Bílá louka alpine meadow, 7.1887, Krátký, ROZ (1). – Krkonoše Mts., alpine meadow near Luční bouda chalet, 7.1922, V. Krajina, PRC (1). – Krkonoše Mts., Úpská jáma glacial cirque, Krakonošova zahrádka gully, 7.7.1926, s.coll., PRC (1). – Krkonoše Mts., south foot of Studniční hora Mt., 23.7.1946, V. Horák, MP (1). – Krkonoše Mts., Navorská jáma glacial cirque, 14.8.1980, M. Šrůtek, ROZ (1). – Krkonoše Mts., Navorská jáma glacial cirque, 7. 1927, M. Puchmajerová, PRC (1). – Krkonoše Mts., Pančavská louka alpine meadow, 22.6.1934, J. Dostál & F.A. Novák, PRC (2). – Krkonoše Mts., Pančavská louka alpine meadow, 2.6.1925, Thenius, BRNM (6). – Krkonoše Mts., Pančavská louka alpine meadow, 12.7.1933, V. Horák, MP (1). – Krkonoše Mts., Pančavská louka alpine meadow, west margin of peat bog, 10.7.1967, R. Businský, ROZ (1). – Krkonoše Mts., Pančavská louka alpine meadow, near Pančava river springs, 8.1927, M. Puchmajerová, PRC (1). – Krkonoše Mts., Pančava waterfall, 27.7.1907, s.coll., PRC (1). – Krkonoše Mts., Labská louka alpine meadow, s.d., I.F. Tausch, PRC (1). – Krkonoše Mts., Labská louka alpine meadow, 11.6.1890, J.E. Kabát, BRNM (1). – Krkonoše Mts., Labská louka alpine meadow, 21.7.1933, V. Horák, MP (1). – Krkonoše Mts., near Labská bouda chalet. 6.7.1907, K. Domin, PRC (1). – Karkonosze Mts., near Mały Staw glacial lake, s.d., I.F. Tausch, BRNM, PRC (14). – Karkonosze Mts., near Mały Staw glacial lake, s.d., J. Kablíková, BRNM, LIT (2). – Karkonosze Mts., near Mały Staw glacial lake, 7.1922, R. Traxler, PRC (1). – Karkonosze Mts., near Mały Staw glacial lake, 29.7.1843, B. Röttig, MJ (2). – Karkonosze Mts., near Mały Staw glacial lake, 10. and 12.6.1932, R. Traxler & J. Karel, HR (2). – Karkonosze Mts., near Mały Staw glacial lake, 6.1932, J. Karel, MP (1). – Karkonosze Mts., near Wielky Staw glacial lake, s.d., I.F. Tausch, PRC (1). – Karkonosze Mts., banks of Wielky Staw glacial lake, 6.1932, V. Kavka, MP (1). – Karkonosze Mts., Polední kámen rocks (east of Malý Šišák Mt.), 5.7.1890, coll.?, HR (1). – Karkonosze Mts., upper edge of Kocioł Małego Stawu glacial cirque, 21.6.1975, Jeslík, ROZ (3). – Karkonosze Mts., upper edge of Kocioł Wielkiego Stawu glacial cirque, 7.1909, R. Missbach, BRNM (2). – Karkonosze Mts., ridge between Kocioł Małego and Wielkiego Stawu glacial cirques, 21.6.1975, Jeslík, ROZ (1). – Hrubý Jeseník Mts., Praděd Mt., 1966, Procházka et al., MP (2). – Hrubý Jeseník Mts., Praděd Mt., s.d., Spatzier, LIT (1). – Hrubý Jeseník Mts., Praděd Mt., 23.6.1972, K. Kubát, LIT (1). – Hrubý Jeseník Mts., Praděd Mt., near road to Švýcárna chalet, 19.6.1980, K.

Kubát, LIT (1). – Norway, Ringeby NP, 1.7.2008, M. Hroneš, OL (2). – Norway, Dovrefjell NP, 1.7.2008, M. Hroneš, OL (6). – Norway, Svartisen NP, 2.7.2008, M. Hroneš, OL (6). – Norway, Ragø NP, 4.7.2008, M. Hroneš, OL (6). – Norway, Ånderdalen NP, 7.7.2008, M. Hroneš, OL (7). – Finland, Käsivarsi NP, 9.7.2008, M. Hroneš, OL (6). – Finland, Lemmenjoki NP, 13.7.2008, M. Hroneš, OL (6).

*S. *daphneola*

Krkonoše Mts., s.d., I.F. Tausch, PRC (4). – Krkonoše Mts., Pančavská louka alpine meadow, near Pančava waterfall, 13.7.1949, J. Šourek, MP (2). – Krkonoše Mts., Pančavská louka alpine meadow, 22.6.1934, J. Dostál & F.A. Novák, PRC (2). – Krkonoše Mts., Pančavská louka alpine meadow, 1922, M. Puchmajerová, PRC (1).

S. ×nepetifolia

Krkonoše Mts., s.d., I.F. Tausch, PRC (6). – Karkonosze Mts., Polana peat bog, 24.6.2012, M. Hroneš, OL (1). – Rila Mts., Sedemte Ezera glacial lakes, 28.6.2014, M. Hroneš, OL (1).

S. silesiaca

Krkonoše Mts., 30.6.1935, J. Pačes, ROZ (1). – Krkonoše Mts., s.d., J. Kablíková, ROZ (1). – Krkonoše Mts., Obří důl valley, by the bridge across the Úpa river, in lower part of the valley, 24.6.2012, M. Hroneš, OL. – Krkonoše Mts., Obří důl valley, near abandoned forge Rudník, 24.6.2012, M. Hroneš, OL. – Krkonoše Mts., Obří důl valley, peat bog right bank of Úpa river, 24.6.2012, M. Hroneš, OL. – Velká Fatra Mts., north slope of Kriváň Mt., 20.7.1967, J. Sachl, ROZ (1). – Nízke Tatry Mts., Ludárův důl glacial cirque, 10.7.1976, J. Sachl, ROZ (1). – Západné Tatry Mts., Kamenistá dolina glacial cirque, 11.8.1978, P. Pyšek, ROZ (1). – Slovakia, near Spišské Vlachy village, 5. 1857, Veselský, ROZ (1). – Fagaras Mts., valley of Podragul brook below cabana Podragul, 24.7.1974, Huml, Lepš, Prach & Rejmánek, ROZ (1).

Supplementary File 6 – List of SSR loci (Barker et al. 2003) showing the adjusted annealing temperature (T_a) and the number of cycles for PCR (N_c).

locus	T_a [°C]	N_c
SB24	55.5	31
SB80	64.3	29
SB100	50.0	36
SB201	50.0	27
SB210	59.7	32
SB233	50.0	31

Supplementary File 7 – Pairwise F_{ST} (below diagonal) and ρ_{ST} (above diagonal) inferred from six microsatellite loci.

	HLL	HPL	PL	NJ	SZ	HP	VY	PLB	UR	UJ	SZL	NLB	OB	MS	HRS	WS	WSV	PO	SLO	dap	JES	SCA	nep	sil
HLL	-	0.408	0.175	0.453	0.720	0.471	0.595	0.050	0.764	0.449	0.676	0.659	0.690	0.812	0.423	0.608	0.508	-0.703	0.323	0.344	0.824	0.262	0.122	0.411
HPL	0.219	-	0.087	0.068	0.399	0.008	-0.070	0.022	-0.015	0.027	-0.057	-0.030	-0.089	0.073	0.004	0.097	-0.030	0.164	-0.071	0.116	-0.082	0.027	0.060	0.367
PL	0.136	0.132	-	0.085	0.240	-0.023	0.091	0.000	-0.161	0.039	-0.029	-0.181	-0.291	0.167	0.052	0.114	0.114	0.116	0.089	0.044	-0.100	0.006	0.032	0.128
NJ	0.294	0.177	0.093	-	0.124	-0.064	0.059	0.111	-0.317	-0.083	0.133	-0.115	-0.202	0.288	0.017	-0.044	0.163	0.325	0.093	0.002	-0.263	0.052	0.181	0.295
SZ	0.388	0.244	0.110	0.042	-	0.231	0.431	0.381	-0.178	0.147	0.578	0.212	0.239	0.674	0.300	0.079	0.547	0.606	0.435	0.079	0.138	0.355	0.475	0.418
HP	0.264	0.130	0.079	0.093	0.162	-	0.042	-0.027	0.047	-0.079	0.232	0.112	0.012	0.369	-0.047	-0.046	0.127	0.120	-0.041	-0.047	0.017	-0.073	0.046	0.233
VY	0.206	0.120	0.027	0.044	0.053	0.062	-	0.024	0.024	0.027	-0.070	0.016	-0.087	0.140	0.000	0.119	-0.073	0.189	-0.141	0.123	-0.149	0.017	0.066	0.380
PLB	0.196	0.133	0.042	0.130	0.130	0.147	0.049	-	-0.131	0.036	-0.206	-0.181	-0.315	-0.011	0.035	0.137	0.018	0.001	0.016	0.084	-0.150	-0.021	-0.025	0.218
UR	0.727	0.261	0.117	0.109	0.137	0.173	-0.061	0.098	-	-0.533	0.633	0.189	0.301	0.793	-0.103	-0.901	0.155	-0.061	-0.447	-0.328	0.319	-0.255	-0.006	0.104
UJ	0.154	0.200	0.056	0.082	0.075	0.127	-0.023	0.033	-0.072	-	0.074	-0.142	-0.432	0.259	-0.039	-0.203	0.149	0.197	-0.033	-0.067	-0.672	-0.029	0.139	0.302
SZL	0.650	0.244	0.024	0.157	0.160	0.222	0.037	0.042	0.688	0.073	-	0.399	0.528	0.388	0.061	0.253	-0.376	-0.530	-0.533	0.129	0.758	-0.170	-0.249	0.296
NLB	0.588	0.184	0.108	0.148	0.210	0.091	0.025	0.231	0.706	0.174	0.647	-	0.235	0.588	-0.041	-0.196	0.007	-0.167	-0.571	-0.151	0.424	-0.194	-0.152	-0.013
OB	0.632	0.324	0.069	0.209	0.280	0.167	0.056	0.164	0.706	0.042	0.684	0.647	-	0.772	-0.191	-0.331	-0.107	-0.379	-0.695	-0.254	0.490	-0.485	-0.243	0.018
MS	0.727	0.179	0.116	0.149	0.228	0.060	-0.066	0.132	0.500	0.076	0.688	0.688	0.737	-	0.198	0.436	-0.261	-0.247	-0.330	0.292	0.960	0.105	-0.030	0.450
HRS	0.404	0.269	0.099	0.210	0.171	0.234	0.158	0.041	0.239	0.118	0.143	0.391	0.342	0.266	-	0.037	0.051	0.230	-0.022	0.045	-0.127	0.008	0.089	0.299
WS	0.270	0.252	0.070	0.115	0.105	0.130	-0.035	0.092	0.087	0.046	0.029	0.156	0.110	0.146	0.193	-	0.277	0.357	0.082	-0.026	-0.820	0.097	0.241	0.339
WSV	0.318	0.228	0.073	0.160	0.150	0.154	0.009	0.057	-0.009	0.035	0.071	0.274	0.287	0.074	0.096	0.086	-	0.111	-0.189	0.200	0.035	0.055	0.013	0.376
PO	0.043	0.098	0.070	0.134	0.162	0.022	-0.010	0.075	0.073	0.060	0.085	-0.016	0.122	-0.033	0.188	0.097	0.117	-	0.151	0.249	-0.167	0.122	0.020	0.349
SLO	0.342	0.269	0.125	0.281	0.282	0.231	0.133	0.155	0.356	0.215	0.129	0.232	0.323	0.330	0.231	0.110	0.129	0.153	-	0.119	-0.475	0.051	0.045	0.346
dap	0.306	0.193	0.102	0.070	0.151	0.099	0.121	0.176	0.223	0.171	0.262	0.217	0.314	0.244	0.286	0.184	0.193	0.184	0.308	-	-0.228	0.050	0.162	0.211
JES	0.714	0.274	0.171	0.101	0.222	0.189	0.001	0.267	0.733	0.082	0.737	0.643	0.722	0.714	0.456	0.168	0.311	0.087	0.372	0.200	-	-0.350	-0.020	0.268
SCA	0.084	0.157	0.016	0.096	0.105	0.055	-0.025	0.053	-0.032	0.015	-0.097	0.018	-0.043	0.022	0.103	0.029	0.025	0.063	0.114	0.100	0.025	-	0.018	0.237
nep	-0.012	0.095	0.048	0.062	0.123	-0.013	-0.005	0.088	0.005	0.034	-0.004	-0.146	0.038	-0.025	0.164	0.064	0.063	-0.015	0.146	0.091	-0.007	0.035	-	0.217
sil	-0.024	0.171	0.142	0.219	0.262	0.094	0.136	0.185	0.140	0.131	0.110	-0.023	0.062	0.149	0.252	0.175	0.185	0.096	0.182	0.176	0.082	0.126	0.064	-

Olomouc, 2018

Palacký University in Olomouc
Faculty of Science
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Faculty
of Science

**Downy willow (*Salix lapponum* L.) in Central
Europe: biosystematics and ecology**

Summary of the PhD. Thesis

Author: **RNDr. Michal Hroneš**
Supervisor: **Doc. RNDr. Bohumil Trávníček, Ph.D.**

Olomouc, 2018

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Experimental Botany, Šlechtitelů 27, Olomouc.

The Ph.D. thesis is available in the Library of the Biological Departments of
Faculty of Science at Palacký University, Šlechtitelů 27, Olomouc.

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1 Introduction

Salix lapponum (Salicaceae) is a small dioecious shrub with oblong, elliptic, (ob)ovate to (ob)lanceolate, villous leaf blades and dense catkins with long pubescent cataphylls and (sub)sessile, densely pubescent capsules (Chmelař & Meusel 1979; Skvortsov 1999). Its main range comprises Scandinavia, Finland, Baltic states, western Belorussia, northern part of European Russia and north western Siberia (Jalas & Suominen 1988; Skvortsov 1999). It is also present in several isolated populations that are scattered in mountains of Sudetes, Scotland, Massif Central, Pyrenees and northern Balkan mountain ranges Rila and Vitosha (Jalas & Suominen 1988; Skvortsov 1999).

It is diploid species with $2n=38$ (Büchler 1986). Due to presumable absence of prezygotic mating barriers, *S. lapponum* freely hybridize with several other mostly diploid species throughout its range including *S. silesiaca* in the Sudetes (Chmelař & Koblížek 1990).

To the typical stands of *S. lapponum* belong eutrophic and mesotrophic wetlands, wet meadows, sparse boreal forests and their openings, as well as banks of streams, glacial lakes and glacial cirques covered by tall-forb or shrubby vegetation (Chmelař & Koblížek 1990; Skvortsov 1999; Elven & Karlsson 2000).

Salix lapponum is considered as endangered in many countries. The change of water regime and subsequent succession as well as overgrazing are usually proposed as the main threats (Pogorzelec 2008; Shaw et al. 2010). Complex, multi-approach research of *S. lapponum* populations was carried mainly in Poland and Scotland. On the contrary, the Sudetes populations remains vastly understudied.

2 Aims of the thesis

During my research I focused on following questions:

- 1) What is exact current and historical distribution of *S. lapponum* in the Sudetes Mts.?
- 2) What are the habitat preferences of subalpine populations of *S. lapponum*?
- 3) Are there any ecological differences between sterile and fertile plants, and between plants with different sexes?
- 4) Is observed morphological variation of subalpine populations connected with genetic population structure and/or hybridization?
- 5) How geographic isolation influences the morphological and genetic patterns in *S. lapponum* populations?
- 6) Can be any taxonomic conclusions drawn from the answers to abovementioned questions?

3 Material and methods

Herbarium revision and distribution assessment

Distribution of *S. lapponum* in the Czech Republic was established using the revision of specimens deposited in the following public herbaria in the Czech Republic (BRNL, BRNM, BRNU, CB, GM, HR, LIT, MJ, MP, NJM, OL, OLM, OSM, PL, PR, PRC, ROZ) and Slovakia (BRA, SAV; acronyms sensu Thiers 2018). Data were supplemented with the literature records and our own field observations. Localities from herbarium specimens were localized using touristic maps (www.mapy.seznam.cz). And GPS coordinates derived from these maps were used for assembly of distribution maps in ArcGis.

Herbarium material for typification purposes was searched for in PRC and PR. The typification process followed the instructions of the International Code of Nomenclature for Algae, Fungi and Plants (the Melbourne Code; McNeill et al. 2012).

Field work

All localities of *S. lapponum* in the Czech part of the Krkonoše Mts. (Czech Republic) resulting from herbaria revision were subsequently revised in field during 2009–2011. Position of each locality, where at least one individual was still present, was recorded using GPS device Garmin GPX-60 Cx.

All known indigenous populations with more than one individual were surveyed, totalling nine populations (sites) as follows (ordered from east to west): Úpská jáma glacial cirque (UJ), Úpské rašeliniště peat bog (UR), alpine meadow near Výrovka chalet (VY), spring in Modrý důl glacial valley (MD), Hančova louka alpine meadow (HL), Pančavská louka peat bog (PL),

eastern part of Navorská jáma glacial cirque (SZ), northern part of Navorská jáma glacial cirque (NJ), and spring of Pančava rivulet (HPL). The exact location and approximate elevation of each individual were marked into orthophotomap provided by the Krkonoše NP administration (available at <http://gis.krnep.cz/map/>).

During the vegetation season in 2009, each population was repeatedly visited to determine the optimal timing for the examination of flowering frequency. Then, all individuals were repeatedly surveyed for flowering in June and July between 2010 and 2013. Individuals were classified into three categories according to their flowering status: females, males and non-reproducing (i.e., the individual did not flower during any surveyed year). The numbers of flowering and non-reproducing individuals were counted within each population studied.

A fine-scale vegetation survey was performed at four selected population sites (NJ, PL, SZ, UJ) during July 2010 (NJ, PL) and July 2013 (SZ, UJ). A vegetation plot of 1×1 m was established around each individual *Salix* plant in the population. Plots were established in this manner at all sites except the NJ site; here, 39 plots were established around randomly selected plants due to the large size of the population and the difficult terrain. This resulted in total of 207 sampled vegetation plots from these four populations. The cover of each species of vascular plant and bryophyte was recorded using a 7-grade Braun-Blanquet abundance-dominance scale (Braun-Blanquet 1964). The cover of each layer was estimated visually as a percentage. Slope inclination was estimated with a clinometer, and slope orientation was measured with a compass in GPS device.

Plant material for microsatellite loci (SSR) analysis was collected during the years 2008–2013. Altogether, 268 individuals of *S. lapponum*, *S. lapponum* var. *daphneola*, *S. ×nepetifolia* and *S. silesiaca* were collected in the Krkonoše/Karkonosze Mts., Hrubý Jeseník Mts., various parts of Scandinavia and the Carpathians. Several leaves from each individual were put into tea-bag and stored in silica-gel. Herbarium specimens were taken from each population and stored in herbarium of the Department of Botany, Palacký University in Olomouc (OL).

Soil sample analyses

Soil samples (207 in total) were collected from each plot of the vegetation survey populations (NJ, PL, SZ, UJ) over two days at the end of August 2013. Soil was sampled with a soil sampler at 2–10 cm depth below the surface at several points around the centre of each plot. The subsamples from each plot were placed together in a plastic bag and immediately weighed. After transportation to the laboratory, the soil samples were oven dried at 60°C for 48 hours to constant weight and soil relative water content (SRWC) was calculated. Samples were sieved, and their pH was determined using a pH 70+DHS portable pH meter (XS Instruments). The concentration of total nitrogen (N) in each sample was determined after mineralization with sulphuric acid and Se-K catalyst using a DK 20 heating digester (Velp Scientifica) and subsequent distillation with water vapour into boric acid on a Vapodest 30 s distillation apparatus (Gerhardt). The final concentration of N was established by 0.01 M HCl titration using a TitroLine 6000 titrator (SI Analytics). Analyses of phosphorus (P), calcium (Ca) and magnesium (Mg) were performed using Mehlich II extract (Mehlich 1978). The concentration

of P was estimated colourimetrically using HACH PhosVer 3 reagent powder pillows on a DR 2800 spectrophotometer (HACH). The concentrations of Ca and Mg were estimated using an Avanta Σ atomic absorption spectrophotometer (GBC).

Vegetation and soil data analyses

Two vegetation data sets were analysed: fine-scale data resulting from our own sampling and coarse-scale relevés acquired from the Czech National Phytosociological Database (CNFD; Chytrý & Rafajová 2003). Fine-scale vegetation plots were stored in Turboveg 2.0 (Hennekens & Schaminée 2001). Mean Ellenberg indicator values (EIVs; Ellenberg et al. 1992) for nutrients, light, temperature, moisture and soil reaction were calculated in software Juice 7.0 (Tichý 2002) for both vascular plants and bryophytes (except for nutrients) without species weighting for each plot for both datasets (i.e., fine and coarse datasets). Basic descriptive statistics of soil conditions and EIVs for each plot (where available) were calculated in NCSS 9 (Hintze 2013).

Microhabitat differentiation between flowering (i.e., females and males) and non-reproducing individuals was analysed by partial detrended correspondence analysis (pDCCA) and partial redundancy analysis (pRDA; Šmilauer & Lepš 2014) in two populations with sufficient numbers of both non-reproducing and flowering individuals (SZ, UJ). A second set of analyses examined the relationships between interpopulation differences in the proportion of non-reproducing plants and both floristic composition (pDCCA) and soil characteristics (pRDA) of fine-scale plots over four populations that were studied in detail, including two populations with low or no presence of non-reproducing individuals. Spearman correlation coefficient was used to

explore the relationship between EIVs and the first canonical axis in the pDCCA, while a t-value biplot was used to identify soil variables that significantly responded to the explanatory variable in the pRDA. Bonferroni correction of P-values was applied in the case of multiple comparisons testing. Floristic composition of the fine-scale plots was classified using modified divisive TWINSpan classification (Roleček et al. 2009). The floristic composition of coarse-scale relevés with that of our plots sampled at the fine-scale were also compared in Juice 7.0.

Secondary sex ratio analyses

The secondary sex ratio was calculated as the proportion of males over the total number of reproducing individuals (males/[females+males]; Field et al. 2013). NCSS 9 software was used for calculation of χ^2 test, heterogeneity χ^2 -test (Sokal & Rohlf 1995) and Spearman correlation coefficients. Only five populations with sufficient sample sizes ($n > 30$; HL, NJ, PL, SZ, UJ) were analysed. Bonferroni correction of P-values was used to counteract the problem of multiple comparisons.

Three large populations with a sufficient number of male and female individuals (NJ, PL, and SZ) were further analysed in detail for biometric, spatial and microhabitat differentiation between males and females. Univariate analyses were performed in NCSS 9. Spatial relationship between males and females were examined using the software PASSaGE 2.0 (Rosenberg & Anderson 2011). Microhabitat differentiation between males and females were tested using redundancy analyses (RDA; Legendre & Legendre 2012) in the CANOCO 5 package (Šmilauer & Lepš 2014) and the library vegan (Oksanen et al. 2014) in the R language (R Core Team 2015).

Morphometric analyses

Morphometric analysis was performed on herbarium specimens from the Czech Republic deposited in BRNU, HR, LIT, MJ, MP, PRC and ROZ herbaria and own samples deposited in OL (Thiers 2018). Altogether, 148 specimens of *S. lapponum*, *S. *daphneola*, *S. ×nepetifolia* and *S. silesiaca* were evaluated. Morphological characters were measured using digital calliper and stereo microscope. In total, 7 vegetative and 5 generative characters were examined. Due to the ephemeral nature of male catkins, only female plants with well-developed leaves and catkins were included into the analysis. Data were analysed using a set of R functions contained in MorphoTools version 1.01 (Koutecký 2015). Basic descriptive statistics were calculated for each morphological character and studied taxon. Tukey-Kramer multiple comparison tests at $p \leq 0.01$ were performed to determine which characters show significant differences among taxa. Correlations of morphological characters were tested using Pearson's correlation coefficient and Principal component analysis (PCA; Sneath & Sokal 1973) was employed to test the morphological homogeneity within the putative taxa. Two PCAs were run: first with the whole data set and second with only *S. lapponum* to examine extent of morphological variability between its isolated and main range populations (i.e., Krkonoše Mts., Hrubý Jeseník Mts. and Scandinavia).

Microsatellite loci analyses

Genomic DNA was extracted using 50 mg of silica-dried leaf tissue using CTAB (cetyl trimethyl ammonium bromide) protocol described by Doyle & Doyle (1987). Six SSR loci from Barker et al. (2003) were examined under published PCR conditions with minor individual modifications. The PCR

products were separated by denaturing polyacrylamide gel electrophoresis using a 6 % polyacrylamide gel (0.5× TBE buffer) and consequently visualised by silver staining. SSR alleles were scored based on fragment lengths (in base pairs; bp), which were determined using the 30–330 bp AFLP® DNA ladder (Invitrogen, Carlsbad, CA, USA).

Molecular data analyses

The grouping of identical multilocus genotypes, the probability of identity, the average number of alleles per locus, the observed and expected heterozygosity (Nei 1973), coefficients of inbreeding F_{IS} and ρ_{IS} (based on the infinite alleles model and the stepwise mutational model, respectively) as well as locus statistics and analysis of molecular variance (AMOVA) were calculated using GENEALX 6.503 (Peakall & Smouse 2006, 2012). AMOVA was used to partition variation within and among populations and was based on F_{ST} (infinite alleles model) with 999 permutations. Mean number of alleles per locus after rarefaction with standardised sample size of five individuals was computed in ADZE 1.0 (Szpiech et al. 2008). Fixation indices F_{ST} and ρ_{ST} , the score test for heterozygote deficit/excess and the exact G test for population differentiation were performed in GENEPOP 4.7.0 (Rousset 2008). Population structure was analysed using a Bayesian clustering approach implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000). STRUCTURE output files were summarized and graphically interpreted using CLUMPAK software (Kopelman et al. 2015).

4 Survey of results

Salix lapponum was found on 43 historical localities: on 25 localities in Czech part of the Krkonoše Mts., on 13 localities in the Polish part of the Karkonosze Mts. and on five localities in Hrubý Jeseník Mts. It was re-recorded on 11 localities in the Czech part of the Krkonoše Mts., on 10 localities on the Polish part of the Karkonosze Mts. and on one in the Hrubý Jeseník Mts. The current distribution in the Krkonoše/Karkonosze Mts. is concentrated mainly to the area around Kocioł Małego and Wielkiego Stawu glacial cirques in the eastern part of the mountain range and also to the area around Pančavská louka alpine meadow and Navorská jáma glacial cirque in the western part of the range.

We found that *S. lapponum* grows on nutrient-poor, acidic soils with high relative water content, high amounts of total nitrogen, low amounts of phosphorus, moderate amounts of magnesium and low to moderate amounts of calcium. Well-developed stands of *S. lapponum* with a dominance of flowering individuals occur along alpine springs, streams and in glacial cirques (= association *Salicetum lapponum* Zlatník 1928), but *S. lapponum* also grows along transitional mires and peat bogs and in subalpine grasslands. Coarse-scale relevés were similar to “typical” stands of *S. lapponum* at the fine scale but were differentiated from them by high frequencies of several broad-leaved herbs typical of moist to wet and fertile soils.

The overall proportion of non-reproducing individuals was 35.2%, but strong variations were observed among populations (0–100%). Co-occurring species and EIVs data indicated that flowering individuals are relatively more common in humid, nutrient-rich and warmer microhabitats than non-reproducing ones. Reproducing individuals exhibited strong female

bias with females in average almost twice as common as males. Males were generally taller than females, suggesting that a different allocation of resources may occur in both sexes. Despite this, no correlations of sex ratio with altitude and proportion of non-reproducing individuals were found. Additionally, no differences in spatial sex segregation and microhabitat preferences were found between males and females within the studied populations. Our results suggest that the biased sex ratio in *S. lapponum* is not environment-dependent and probably originates during early stages of ontogenetic development (seeds).

Regarding morphological and genetic variability, we found that populations of *S. lapponum* in the Krkonoše Mts. are morphologically slightly different in comparison with main range populations. We also found that all larger populations exhibit significant deficit of heterozygotes that may indicate occurrence of inbreeding. On the other hand, the extent of hybridization was quite low, and we were able to discover only three possible introgressants. Regarding var. *daphneola*, we propose that its origin cannot be explained by hybridization but rather by recessive state of alleles or mutation in gene controlling indumentum development. Populations of *S. lapponum* in the Krkonoše Mts. can be under recent speciation due to their long-term isolation.

As a consequence, typifications and nomenclatural notes on seven names of willows published by I. F. Tausch were provided. The name *Salix arenaria* var. *latifolia* is deemed illegitimate. Four names (*Salix arenaria* var. *atropurpurea*, *S. arenaria* var. *nivea*, *S. arenaria* var. *marrubiiifolia* and *S. argyroides*) are considered synonymous to *S. lapponum* var. *lapponum* and one (*S. arenaria* var. *denudata*) to its hybrid with *Salix silesiaca*. In contrast, *Salix daphneola* applies to a unique glabrous variety of *S. lapponum* (var. *daphneola*) endemic to the Krkonoše Mts.

5 Conclusions

The effective conservation of any species requires prior knowledge of its life-history, demography, genetics and biotic interactions. During my research, I applied multi-discipline approach on endangered *Salix lapponum* in the Krkonoše/Karkonosze Mts.

Comparison of historical and current distribution of the species revealed almost 50 % populations decline, mainly in the Czech part of mountains. At least the larger populations occur in habitats that are very similar to those in the main range. However, at least part of the non-reproducing individuals in these populations was found to grow in suboptimal conditions (i.e., on drier soils with higher concentrations of Ca and Mg and correspondingly higher pH). On the contrary, there were no differences in habitat preferences between sexes despite the strongly female biased sex ratio. This suggest that the biased sex ratio in *S. lapponum* is not environment-dependent and probably originates during early stages of ontogenetic development. Quite surprisingly, low amount of introgression from *S. silesiaca* was detected. Therefore, the *S. lapponum* populations are not under eminent threat by hybridization. Population structure genetic analysis found low proportion of clonality, strong genetic differentiation among populations and deficit of heterozygotes in all larger populations that may indicate the occurrence of inbreeding. Morphometric analysis revealed slight shift in morphology of the Krkonoše Mts. populations when compared with main range populations. These observations suggest that populations of *S. lapponum* in the Krkonoše/Karkonosze Mts. are going through recent speciation due to their long-term isolation and subsequent role of the genetic drift. Prime

example of this speciation is the presence of glabrous plants that are not known from anywhere else of the range of *S. lapponum* and are not of the hybrid origin. Although they are strikingly different from typical *S. lapponum* by means of morphology, they do not differ genetically. Therefore, the current rank of variety (i.e., *S. lapponum* var. *daphneola*) is appropriate for these plants.

To conclude, the populations of *S. lapponum* in the Krkonoše/Karkonosze Mts. deserve to be protected not only because they represent the glacial relicts but also because they may be unique in comparison with other *S. lapponum* populations.

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8 Souhrn (Summary, in Czech)

Vrba laponská (*Salix lapponum*, čeled' Salicaceae) je poměrně drobný dvoudomý keř s hustě chlupatými, eliptickými až obkopynatými listovými čepelemi a hustě chlupatými, přisedlými semeníky. Areál výskytu druhu je situován do boreálních až subarktických oblastí Evropy a západní Sibiře. Druh se dále vyskytuje v několika izolovaných areálech v horách střední a jižní Evropy (Krkonoše, Hrubý Jeseník, Skotská vysočina, Massif Central, Pyreneje, Rila, Vitoša). Tato disertační práce je zaměřena na jednu z těchto izolovaných makropopulací, která se nachází v Krkonoších. Ke studiu *S. lapponum* byl použit multioborový přístup s využitím herbářové revize historického rozšíření, revize zjištěných lokalit v terénu, stanovení poměru pohlaví a také proporce nekvetoucích jedinců, analýz vegetace, půdních vzorků, genetické populační struktury s využitím mikrosatelitových markerů a také morfometriky. Výsledky ukázaly na vliv dlouhodobé izolace krkonošských populací, která se projevuje v mírné morfologické odlišnosti rostlin v porovnání s populacemi v hlavním areálu a také ve zvýšené frekvenci homozygotních jedinců. Stanovištní podmínky krkonošských populací se příliš neliší od podmínek jiných populací publikovaných v literatuře. Přesto však byl v některých populacích zaznamenán poměrně častý výskyt sterilních jedinců. To může znamenat, že ne všechny rostliny rostou na nejvhodnějších mikrostanovištích. Žádné rozdíly naopak nebyly detekovány v prostorovém uspořádání ani mikrostanovištních preferencích jednotlivých pohlaví, což je poněkud v rozporu se zjištěným vychýleným poměrem pohlaví ve prospěch samic. Lze tedy soudit, že vychýlený poměr pohlaví není způsobený prostředím, ale spíše vzniká již v raných stádiích vývoje jedince.