Phylogeography of *Swertia perennis* in Europe based on cpDNA markers

Jacek Urbaniak, Paweł Kwiatkowski, Paweł Pawlikowski

1Department of Botany and Plant Ecology, Wrocław University of Environmental and Life Sciences, Poland

2Department of Botany and Nature Protection, University of Silesia in Katowice, Poland

3Department of Plant Ecology and Environmental Conservation, Faculty of Biology, Biological and Chemical Research Centre, University of Warsaw

Corresponding Author:

Jacek Urbaniak

Email address: jacek.urbaniak@up.wroc.pl
Abstract

Background. *Swertia perennis* (Gentianaceae) is a perennial diploid and clonal plant species found discontinuously distributed in the peat bogs in mountains of Europe, Asia and North America as well as in European lowlands. The current geographical dispersion of *S. perennis* is probably an effect of Quaternary climatic changes that played an important role in determining the present-day distribution of *Swertia* and numerous other plant and animal species.

Methods. A presented survey of molecular studies based on combined data from chloroplast DNA markers (*trnL-trnF* and *trnH-psbA*) that were conducted to elucidate the phylogeography of *S. perennis* in European localities. Plants were collected from 28 populations that represent different localities in lowlands as well as in mountain areas of Europe (Carpathians, Sudetes, Bohemian Forest and Alps). The cDNA sequences were statistically analysed according to phylogenetical relationships in between specimens collected in separate localities.

Results. During the study, 20 haplotypes were characterized representing a high level of genetic variability, but showing a lack of phylogeographical structure. This pattern can be a result of repeated recolonization and expansion from several areas. Such genetic differentiation may also have been due to the relatively long-term isolation of *S. perennis* in Pleistocene refugia in Europe, resulting in independent separation of different cpDNA phylogenetic lineages.

Discussion. The lack of phylogeographical structure makes it impossible to indicate the centre of haplotype diversity, but refugia located between the ice sheets in the lowlands, Carpathians, Sudetes or the Alps are the most probable sites, where *S. perennis* existed in Europe. The lack of evidence for phylogeographic structure possibly indicates a high level of gene flow in the recent. The variation in nucleotide composition of cpDNA may reflect the genetic variability from the ancient period, when the landscape and the fen systems were not fragmented, especially on the lowlands, however, at present, it is difficult to speculate about relations between northern and mountain parts of its distribution range in Europe.
Introduction

The distribution of organisms as well as their genetic structure are a consequence of repeated quaternary climatic changes in ecosystems. They dramatically modified the vegetation and resulted in extinctions in the colder areas of Europe, America and the Arctic (Hewitt, 1996; Hewit, 2004; Taberlet, 1998). Climate change was also the primary reason for numerous plant or animal migrations to the southern part of Europe or warmer localities on the front of ice cover, where they survived unfavourable conditions and could start later re-migration again to the north (Ronikier, Cieślak & Korbecka, 2008; Slovak et al., 2012). Some of the commonly recognized southern refugia are located in the Mediterranean region, on the Balkan Peninsula, in isolated mountain ranges (Carpathians, Alps). However, separation from Central and Northern Europe or Northern Russia by a broad belt of lowlands could have harboring of numerous plants that inhabited similar sites or ecosystems in the Pleistocene (Schönswetter, Popp & Brochmann, 2006; Paun et al., 2008). The Alps were covered by ice during this period (Mojski 1993), which restricted the distribution of plants to isolated nonglaciated sites (nunataks), or for plants migrating to the Alps as secondary migrants, located close to the area’s peripheral refugia (Stehlik, 2000; Stehlik, 2003; Schönswetter et al., 2005; Ronikier et al., 2008). Survival in glaciated Alps on the tops of mountains, as suggested by Brockmann-Jerosch & Brockmann-Jerosch (1926), was possible in scattered localities, the nunataks on the top of the Alps, but also possible was the scenario that species immigrated into the Alps from more southern refugia or from Eastern Europe (Schönswetter, Popp & Brochmann, 2006). This scenario has been confirmed in the case of *Ranunculus pygmaeus*, which probably migrated to the Alp valleys from source populations located in Siberia via the Carpathians (Schönswetter, Popp & Brochmann, 2006). Such migrations may have been possible due to alternating warmer and glacial periods in the Quaternary that allowed gene exchange and plant migration between European lowlands and mountain ranges (Alps, Carpathians, Sudetes) (Pawłowski, 1928; Ronikier, Cieślak & Korbecka, 2008). In contrast to the Alps, the Carpathians or Sudetes were only locally or partially glaciated during the Quaternary (lower massifs in general remained ice-free) and due to the existence of numerous valleys with several ranges above 2000 m a.s.l. offered a wide spectrum of sites or ecosystems for migrants from the lowlands or as potential refugia from organisms in fragmented subranges in several countries. It seems that outside the Alps, the Sudetes, Bohemian Forest and
primarily the Carpathians played an important role as a botanical crossroad for plants migrating from Siberia, the Arctic or the Caucasus to the Alps (Schöswetter, Popp & Brochmann, 2006; Ronikier, Cieślak & Korbecka, 2008).

Such a phylogeographical hypothesis of analyzing historical-climatic processes that influence population genetic differentiation has been intensively studied, mostly in the Alps but also in the Carpathians linking together studies in the European mountain ranges (Alps, Carpathians, Sudetes, Bohemian Forest) (Schöswetter et al., 2005; Albach, Schöswetter & Tribsch, 2006; Ronikier, Cieślak & Korbecka, 2008; Ronikier et al., 2008; Alvarez et al., 2009). However, in contrast to the Alps, the phenomenon of the phylogeographical history of plants in mountain ranges of Central Europe is still poorly understood, and research are scarce (Ronikier, 2011). Similarly, research that takes into account the widespread circum-boreal plant species in the northern hemisphere inhabiting populations in the Sudetes, Bohemian Forest or Harz Mts are also scarce (Alsos et al., 2005; Kramp et al., 2009; Wróblewska, 2013; Jermakowicz et al., 2015). With the exception of studies dealing with the *Saxifraga* genus (Bauert et al., 1998; Vargas, 2001; Oliver, Hollingsworth & Gornall, 2006; Winkler et al., 2012, Winkler et al., 2013) and *Salix* sp. (Mirski et al., 2017) no studies have been concerned with plants inhabiting lowland or mountain peat bog ecosystems. Many peat bog plant species are present in a wide range of circum-boreal-alpine regions and have been able to colonize large mountain areas or migrate via Beringia to North America.

All of the populations dispersed in scattered localities are susceptible to negative effects resulting from individual population history and possible fluctuations in their size (number of individuals). Genetic drift, and founder or bottleneck effects may also influence the genetic structure of such populations which are often genetically isolated (Freeland, 2008; Hansen, Thomas & Arnholdt-Schmitt, 2009). All of the results of climatic oscillations, resulted in plant distributions and contributed to population genetic diversity that can be detected by molecular approaches (Alsos et al., 2005; Wasowicz et al., 2016). Such methods are based on nuclear ribosomal or chloroplast DNA (Taberlett et al., 1991), or DNA polymorphism (Ronikier, 2011), and allow for detailed insight into processes responsible for presently observed distributions, or for describing refugia areas for selected species. They also enable detection and better description of genetic relationships between disjunctive and close plant populations. This can
help to understand microevolutionary processes, that can take place within plant populations that have changed their previous distribution range or identify divergent lineages (Ronikier, 2011). Therefore, in the present study, we used DNA sequence nucleotide data from chloroplast markers for investigation of genetic structure among populations of the peat bog plant species – *Swertia perennis* (Polish lowlands – Carpathians – Sudetes – Bohemian Forest – Alps). The combination of samples collected from various localities together with the cpDNA markers should allow for identifying the different haplotype lineages across the 1300 km disjunctive distribution range in Europe. Based on this, we test the hypothesis on about existence the phylogeographic structure in present distribution of *S. perennis* populations and answer on question, if the present distribution can indicate the relationships between isolated populations of *S. perennis* or those surviving in isolated sites.

**Material and methods**

The Species

*Swertia perennis* Linnaeus Sp. Pl. 226, 1753, syn.: *Gentiana palustris* All. Fl. Pedem. 1: 100, 1785 is a highly variable taxon, that inhabit lowlands but can also be found at higher altitudes. The species has worldwide distribution on the Northern hemisphere, but also has discontinuous distribution from Asia to North America. It is a plant inhabiting wetlands, particularly calcareous lowland fens or high mountain in peat bogs, but is also present on wet meadows, creek shores, close sources or on wet rocks. It is a species of circumboreal distribution inhabiting the Arctic, and Northern, Atlantic and Central European and Siberian provinces (Hultén, 1968; Meusel et al., 1978; Hultén & Fries, 1986). In Europe, the species can be found in all mountain range systems of Alpine orogeny, from the Pyrenees up to the Caucasus via the Dinaric Alps and the Carpathians as well as in the Herzynian mountains (Sudetes, Bohemian Forest) and peat bogs in Central European and Eastern lowlands. *S. perennis* is a long-lived perennial rhizome herb usually producing one erect stem growing 10 to 50 centimeters tall. It a diploid (2n = 28), that flourishes in July or August (Love & Love, 1986; Pawlikowski & Wołkowycki, 2010). The species sometimes forms large populations in favourable habitats, but is also sensitive to habitat fragmentation and destruction, similar to numerous other peat bog plants.
Study area and sampling

Twenty eight populations *S. perennis* were sampled mostly in Carpathians, but also in Alps, Sudetes, Schwarzwald, Bohemian Forest and in Polish Lowlands close to Lithuanian and Belarus border (Fig. 1). This resulted in a total of 10 populations sampled in Carpathians, 8 in Sudetes, 3 in Alps, 2 in Bohemian Forest, 1 in Schwarzwald and 4 in Polish Lowland (Table 1).

The fresh plant tissues were collected in the field, placed in to plastic bags and immediately persevered by drying agent, the silica orange gel.

DNA isolation and cpDNA sequencing

The genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen; Hilden, Germany), according to the manufacturer’s protocol. Dried plant tissues were previously frozen using liquid nitrogen and disrupted from using Mixer Mill MM400 (Retsch; Haan, Germany). The quality and quantity of the DNA was determined using 1% TBE agarose gel.

In similar as Groff, Hale & Whitlock (2015), we sequenced three markers from the chloroplast genome of *S. perennis*: the *trnL-trnF* Intergenic Spacer, *trnL* Intron and the *trnH*(GUG) – *psbA* spacer. All three chloroplast DNA regions are widely used for phylogenetic studies at all taxonomic levels (Drábková et al., 2004). The *trnL-trnF* Intergenic Spacer and *trnL* Intron is often considered evolutionary conservative but employed in phylogeny and taxonomy, and some studies have found intraspecific variation in this DNA regions as biogographically informative gene that has been contradicted (Taberlet et al., 1991; Brunsfeld & Sullivan, 2005; Shaw et al., 2005; Fujii & Senni, 2006; Shaw et al., 2007; Groff, Hale & Whitlock, 2015).

Additionally, the *trnH-psbA* region of the cpDNA is often more variable than the *trnLF* region (e.g. Shaw et al., 2005). The *trnL-trnF* Intergenic Spacer together with *trnL* Intron were tested: with “c” and “f” primers (Taberlet et al., 1991) and *trnH*(GUG) – *psbA* with *trnH*(GUG) and *psbA* primers (Shaw et al., 2005). The DNA isolation from collected plants contributed to PCR and seqencing reactions. PCR reaction mix included (in the total volume of 20 µl): 1U Taq
recombinant polymerase (Thermo-Fisher Scientific), 10X Taq Buffer, 1 mM MgCl₂, 0.5 µM of each primer, 0.4 mM dNTP and 1 µl DNA template. PCR cycle was performed with a Veriti Thermal Cycler (Life Technologies, Carlsbad, CA, USA) with the following parameters: 8 min at 95°C, followed by 30 cycles of 45 s at 95°C, 45 s min at annealing temperature (49.2°C – trnL, 51.2°C – psbA) and 1min at 72°C, followed by a final extension step of 10 min at 72°C.

Prior to sequencing, PCR products were purified using GeneMATRIX PCR/ DNA Clean Up Purification Kit (Eurx, Gdańsk, Poland). Sequencing, post-reaction purification and reading were done by Genomed (Warsaw, Poland) using an ABI 377XL Automated DNA Sequencer (Applied Biosystems, Carlsbad, CA, USA). All sequences are available in GenBank (accession numbers - trnH(GUG) – psbA spacer: KY798346 - KY798347, KY817321 - KY817346; trnL-trnF Intergenic Spacer, trnL Intron: KY798346 - KY798348, KY906142 - KY906166). All molecular analysis has been done at Department of Botany and Plant Ecology Wrocław University of Environmental and Life Sciences.

cpDNA data analyses

The cpDNA sequences were aligned using DNA Baser Sequence Assembler v4 (Heracle BioSoft, 2014) and checked for nucleotide variation using BioEdit ver. 7.1.11 (Hall, 1999).

Combined both cpDNA region sequences data were concatenated and analysed together. Prior to the phylogenetic analyses, the cpDNA sequences were aligned using Muscle software (Edgar, 2004a; Edgar 2004b). We performed maximum parsimony (MP) and Bayesian inference (BI), to infer the phylogenetic relationships among selected individuals from European S. perennis populations. The trees were constructed and the topologies of the obtained trees were compared to establish and validate the phylogenetic position of the studied species. Bootstraps for MP analyses based on 1,000 replications of full heuristic searches with the tree-bisection-reconstruction (TBR) branch-swapping algorithm, and those for NJ analyses (Saito & Nei, 1987) under the JC model (Jukes & Cantor, 1969) based on 1,000 replications, were conducted using PAUP* 4.0b10 (Swofford, 2002).

The BI analyses were performed using MrBayes 3.1.2. (Ronquist & Huelsenbeck, 2003).

The substitution models used for each codon position in the BI analyses were GTR+G (1st codon...
position), GTR+I (2nd codon position), and GTR+I+G (3rd codon position), as estimated based on Aikake’s information Criterion (AIC), selected by MrModeltest 2.3 (Nylander et al., 2004) using PAUP* 4.0b10 (Swofford, 2002). The parameters of the substitution models for codon position were unlinked. The Markov chain Monte Carlo iteration was performed and stopped at 1,000,000 generations. The first 25% of generations were discarded as burn-in, whereas the remaining trees were used to calculate a 50% majority-rule tree and to determine the posterior probabilities of individual branches. The remaining trees were used to produce a majority-rule consensus tree and to calculate posterior probabilities (PP). Additionally, a statistical parsimony network was constructed by TCS v1.21:2 (Clement, Posada & Crandall, 2000).

In total, 28 sequence of *S. perennis* and five other species used as an outgroup: *Frassera speciosa*, *Lomatogonium rotatum*, *Comastoma tenellum* and *Gentianella amarella* were studied.

### Results

Molecular phylogenetic analyses cpDNA sequence data

The alignment of *trnL* intron and *trnL-trnF* spacer data cpDNA varied in length among studied individuals from European populations. We found 15 variable sites with insertions/deletions; the sequences were 827 – 838 base pairs in length, of which 33 characters were parsimony informative (Table 2). The alignment of *trnH*(GUG) – *psbA* cpDNA locus also varied in length among the studied populations as well as in sites in studied sequences. We found 19 sites with insertions/deletions (Table 3). The sequences were 410 – 428 base pairs in length, of which 38 characters were parsimony informative. Several indels at 658-665 in *trnL/F* and 70-87 in *trnH* – *psbA* were neglected because they varied inconsistently with the substitution and because indels typically mutate more frequently than substitutions (Alsos et al., 2005).

The aligned length of combined 33 sequences of *trnL/F* and *trnH* – *psbA* varied from 1485-1503 base pairs. The whole dataset contained 36 variable sites that were dispersed randomly across the entire area of analysis: from the Polish lowlands to the Alps, across the Carpathians and the Sudetes. Phylogeographic analysis showed that maximum parsimony (MP) analyses were congruent with Bayesian interference analyses (BI). We found the concatenate
sequences data more informative than single trees based on \textit{trnL/F} and \textit{trnH – psbA} and results of analysis are presented in Fig. 2. The topologies of obtained trees were congruent, and only one arbitrary selected tree from 12 most parsimonious trees is shown with bootstrap proportions (BP) from MP and BI, respectively, at the nodes.

Parsimony phylogeographic analysis of the concatenate data set of sequences resulted in more than 200 parsimonious trees (CI = 0.94, RI = 0.92). The dataset do not reveal any distinct regions within the European \textit{S. perennis} and no congruent groups were identified between populations using TCS software (Fig. 3). Within the whole \textit{S. perennis} clade (BS = 100, BP = 1.0) we found several subclades with moderately high bootstrap support, however they also do not form geographical structures of population. One of them (BS = 79, BP = 1.0) represents a common haplotype, and the same haplotype occurred in three regions: Polish lowlands, Carpathians and Alps. Another haplotype was found in five populations in the Polish lowlands and the Carpathians (Fig. 3), together with individuals from the Sudetes. Similarly, in another clade (BS = 67, BP = 0.98), individuals from 11 populations from almost all studied regions are placed, but all haplotypes significantly differed, and were limited to single populations only. This indicates genetic variation between \textit{S. perennis} populations in Europe and many of them had unique haplotypes. To be sure of that, we performed numerous additional re-sequencing reactions of the same and/or other individual \textit{S. perennis} samples to rule out the risk of confusion. This confirmed that the haplotypes are dispersed randomly across the whole of Europe.

Discussion

Genetic variability of plant populations has been influenced by complex historical processes, such as Pleistocene glaciations, migrations, bottleneck effects or gene flow and results of biological processes that can be observed using genetic analysis. However, it is difficult to point out the same rules for all plant species, particularly with respect to plant migration. A good example of this is the case of \textit{Ranunculus pygmaeus}, which probably colonized the Alps from the Taymyr region in Siberia via the Carpathians (Schönswetter, Popp & Brochmann, 2006). Another example seems to be the history of \textit{S. perennis}, in Central and Western Europe from an original Central Asian distribution. The presented results illustrate cpDNA variation between \textit{S.
perennis populations in Europe, with numerous unique and almost a lack of shared haplotypes across its geographic distribution. There is a clear lacking phylogeographic structure in Europe, without genetic evidence for distinct structural differences between geographic lineages and illustrates complicated nature of pre-glacial, as well as post-glacial plant dispersal that seems to be expressed by S. perennis individuals collected from European populations (Abbott & Brochmann, 2003). Similar to Groff, Hale & Whitlock (2015), based on the cpDNA, we did not find polymorphism within studied populations of S. perennis. To ensure that these results were not due the sampling strategy nor an artefact of performed analyses, we paid special attention to the laboratory analysis, and carried out numerous additional re-sequencing reactions of S. perennis individuals to rule out the risk of confusion. The lack of sequence polymorphism could be a result of the biology of S. perennis and plant strategy of clonal growth via root systems that form shoot-borne roots, that can fragment and be very long-lived in the ground. It is possible that a large part of the S. perennis population originated from a few individuals that formed a clone.

The sampled plant leaves, which were used for DNA extraction, may have originated from the same clone or at least from a single chloroplast lineage (Groff, Hale & Whitlock, 2015). Propagation of seeds of S. perennis is also possible; however, this was not observed by the authors.

The observed haplotypes were dispersed randomly on the phylogenetic tree across the whole of Europe and do not form closely related lineages among geographical regions (Fig. 2, 3), or unique genetic structure. The geographic relationship between cpDNA S. perennis indicates that haplotypes probably mostly originate from different localities. They also could have arisen before they spread to their current localities, from where they dispersed and mutated. Similar, genetic variability among populations of S. perennis has been detected by isozyme variation (Lienert et al., 2002) or in case observed of populations located in Hala Cebulowa and Hala Miziowa, that are situated approximately 500 m apart. Their haplotypes differed by trnH-psbA length as well as nucleotide variation in trnH-psbA and trnL-trnF. Similarly, haplotypes in individuals collected in populations from the Karkonosze range in the Sudetes mountains located about one km from each other (Kocioł Wielkiego Stawu – Kocioł Małego Stawu, Kocioł Łomniczki – Kopa) also differed by trnH – psbA and trnL – trnF length or in nucleotide variation. Other haplotypes were specific only for the sites/localities where they grow, and do not form groups of similar sequences or are located in different subtrees (Fig. 2). It seems that
closely located populations of *S. perennis* can be genetically isolated and presently form isolated population systems. This is in accordance with Lienert et al. (2002), who point out that geographic and genetic distances between *S. perennis* populations were not correlated, and gene flow between close populations was not higher than between more distant populations.

The genetic diversity of cpDNA and lack of phylogeographical structure can be an effect of the history of the range formation, and its shifting during the ice age, where a group of boreal-mountain plants emerged. During subsequent glaciations and cold temperatures, alpine plants expanded their ranges, and warm interglacials, alpine plants together with boreal and subarctic species, which came from sub-polar areas (in front of the glacier), retreated to the higher mountain localities into refugia areas located in the far north. This may have led to the distinctive geographical disjunctions seen: *S. perennis* today is present in the European mountains and in Northern Eurasia and North America (peat bogs and in boreal forests), as well as in isolated boreal ecosystems of European lowlands: Poland, the Baltic states, and Siberia (Hultén, 1968; Meusel et al., 1978; Hultén and Fries, 1986).

Similar haplotypes identified in this study may also form part of a residual lineage of haplotypes that colonized peat bog areas in both mountains and lowlands. The five most similar haplotypes (26, 28, 18, 19, 17) presented on Fig. 3, are dispersed into several disjunctive areas: in the Polish lowlands, the Carpathians and the Alps, without any geographical correlation. The present fragmented distribution of *S. perennis* seems to be a residual after more homogenous distribution, especially in the lowlands, which probably was directly linked with the main distribution range in boreal lowlands (Eurasia, Siberia). It is also possible that cpDNA mutates rapidly, and seed production or dispersal does not occur, or is scarce, with gene flow being scattered or not existing at all. European *Quercus* sp. have recolonized northern Europe from several parallel southern refugial populations since the Pleistocene, establishing a distinct pattern in cpDNA variation (Dumolin-Lapegue et al., 1997; Petit et al., 2002). At the contact zones between migrating fronts, significant mixing of haplotypes has occurred, as all populations are colonizing new territories. It is possible that the *S. perennis* migration occurred in a similar way, or in several phases. Populations located in mountains could have survived the last Wistulian glaciation, but after the end of the cold period new plants, possibly from Siberia, could have expanded to their present territory of the European lowlands. This may explain the differences in nucleotide composition in investigated cpDNA visible in populations of *S. perennis*. It is also
quite possible that the species both survived glaciations in situ and reimmigrated several times, so broadfronted repeated recolonization and glacial survival can shape genetic variation (Tausch et al., 2017). Our results may also be important for taxonomic reasons, with implications in understanding relations of the lowland and mountain populations of *S. perennis*. We did not find any cpDNA distinct lineages that could segregate both: lowland and mountain populations.

**Conclusions**

In conclusion, the lack of clear phylogeographic structure of *S. perennis* in Europe can be a result of overlapping factors, such as multidirectional gene flow in the past dispersal history, long-distance dispersal during post-glacial recolonization or surviving in several detached refugia (Cain, Milligan & Strand, 2000; Beatty & Provan, 2011; Jiménez-Mejías et al., 2012; Sanz et al., 2014). The low level of variation in haplotype structure patterns is similar to that of other northern taxon types, and may indicate the long-term process of gene flow among the population (Eidesen et al., 2007a; Eidesen et al., 2007b; Ehrich, Alsos & Brochmann, 2008; Westergaard et al., 2010; Alsos et al., 2012;). It is also possible that some of the populations occurred in Pleistocene refugia or migrated during the Holocene, and this time was enough for formation of divergent cp DNA. The lack of evidence for phylogeographic structure possibly indicates high levels of gene flow in the recent past. The variation in nucleotide composition of cpDNA in populations may also reflect the genetic variability from the ancient period, when the landscape and the fen systems were not fragmented, especially on the lowlands. Today, the gene flow is probably much smaller than previously or does not exist. Habitat fragmentation for such stenotypic specialist sp. from the peat bogs may have significantly reduced genetic variability and led to absent or only minimal gene flow between populations.

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**Figure 1** (on next page)

Sampling locations for the 28 populations of *S. perennis* examined in this study.

The numbers are adequate to number of population from Table 1. Circles with different colors correspond to lineage divergences identified by TCS and phylogenetic analyze.
Figure 2 (on next page)

Phylogenetic relationships among haplotypes and lineages detected in S. perennis.

The phylogenetic tree is based on studied trnL-trnF and trnH-psbA cpDNA sequences. Bootstrap values of MP and BI analysis are given close to branches, respectively.
Figure 3 (on next page)

Maximum Parsimony networks analysis of cpDNA haplotypes identified by TCS software.

Solid line between circles represents one mutational step between two chlorotypes based on most parsimonious algorithm. The small open circles indicate the missing chlorotypes (not sampled or extinct). Circle colors correspond to haplotype lineages, respectively, as shown in Fig. 2.
Table 1 (on next page)

Collection details of the studied plant material (S. perennis) used in the study.
| No | Locality/Code                      | Region/Country                      | Altitude (m a.s.l.) | Latitude (N)   | Longitude (E)   |
|----|-----------------------------------|-------------------------------------|---------------------|----------------|-----------------|
| 1  | Tengen, Schwarzwald               | Schwarzwald /Germany                | 700                 | 47°50'41.3"   | 08°39'08.7"     |
| 2  | Waldele, Allgauer                 | Alps/Austria                        | 1116                | 47°21'27.3"   | 10°10'07.6"     |
| 3  | Schwende, Allgauer                | Alps/Austria                        | 1028                | 47°21'50.2"   | 10°10'37.5"     |
| 4  | KlausenWald, Allgauer             | Alps/Austria                        | 1112                | 47°22'44.7"   | 10°11'22.8"     |
| 5  | Modravsky Potok, Sumava           | Bohemian Forest/Czech                | 1121                | 48°58'11.2"   | 13°29'11.0"     |
| 6  | Luzensky Potok, Sumava            | Bohemian Forest/Czech                | 1143                | 48°57'18.2"   | 13°29'16.0"     |
| 7  | Sokolnik, Karkonosze              | Sudetes/Poland                      | 1390                | 50°46'43.9"   | 15°31'36.2"     |
| 8  | Mały Śnieżny Kocioł, Karkonosze   | Sudetes/Poland                      | 1380                | 50°46'59.5"   | 15°33'18.5"     |
| 9  | Kocioł Wielkiego Stawu, Karkonosze| Sudetes/Poland                      | 1295                | 50°45'43.5"   | 15°41'22.0"     |
| 10 | Kocioł Małego Stawu, Karkonosze   | Sudetes/Poland                      | 1316                | 50°44'40.7"   | 15°41'57.6"     |
| 11 | Złote Źródło, Karkonosze          | Sudetes/Poland                      | 1414                | 50°44'31.2"   | 15°42'57.2"     |
| 12 | Kopa, Karkonosze                  | Sudetes/Poland                      | 1276                | 50°44'41.1"   | 15°43'50.9"     |
| 13 | Kocioł Łomniczki, Karkonosze      | Sudetes/Poland                      | 1234                | 50°44'23.9"   | 15°43'58.6"     |
| 14 | Slatinny Potok, Hruby Jesenik      | Sudetes/Czech Republic              | 730                 | 49°58'15.7"   | 17°12'03.1"     |
| 15 | Hala Cebulowa, Beskid Żywiecki    | Carpathians/Poland                  | 1298                | 49°32'19.3"   | 19°18'48.2"     |
|   | Location Name                      | Region            | Height (m) | Latitude         | Longitude         |
|---|-----------------------------------|-------------------|------------|------------------|-------------------|
| 16| Hała Miziowa, Beskid Żywiecki     | Carpathians/Poland| 1282       | 49°32'24.7"      | 19°18'57.0"      |
| 17| Dolne Diery, Mala Fatra           | Carpathians/Slovakia| 660       | 49°15'01.5"      | 19°04'23.8"      |
| 18| Velky Rozsutec, Mala Fatra        | Carpathians/Slovakia| 1618     | 49°13'53.5"      | 19°05'58.3"      |
| 19| Dedosova Dolina, Velka Fatra      | Carpathians/Slovakia| 717       | 48°55'20.2"      | 19°02'13.7"      |
| 20| Demanovska Dolina, Nizke Tatry    | Carpathians/Slovakia| 950       | 48°59'50.6"      | 19°34'34.8"      |
| 21| Rohacke Plesa, Zapadne Tatry      | Carpathians/Slovakia| 1646     | 49°12'31.6"      | 19°44'16.8"      |
| 22| Dolina Kościeliska, Tatry Zachodnie| Carpathians/Poland| 1010     | 49°14'24.7"      | 19°51'50.2"      |
| 23| Dolina Jaworzynka, Tatry Zachodnie| Carpathians/Poland| 1323     | 49°15'15.0"      | 19°59'51.1"      |
| 24| Ostrva, Vysoke Tatry              | Carpathians/Slovakia| 1647     | 49°09'05.2"      | 20°05'08.3"      |
| 25| Kamień, Wyżyna Lubelska            | Polish Lowland/Poland| 181       | 51°06'28.3"      | 23°34'30.1"      |
| 26| Biebrza, Krotlinia Biebrzańska     | Polish Lowland/Poland| 126       | 53°38'34.5"      | 22°35'15.9"      |
| 27| Kamienna Nowa, Krotlinia          | Polish Lowland/Poland| 131       | 53°42'35.2"      | 23°13'3.0"       |
| 28| Rowełe, Pojezierze Suwalskie      | Polish Lowland/Poland| 182       | 54°20'32.6"      | 22°54'56.6"      |
Table 2 (on next page)

Variable sites recorded in the *trnL-trnF* data in *Swertia perennis*. Nucleotide position refer to the number of nucleotide from the first position of the region.
| No | Locality/Code          | Length of \( m_{14}m_{14f} \) locus | 12 | 24 | 25 | 37 | 38 | 61 | 71 | 147 | 195 | 241 | 556 | 650 | 775 | 784 | 796 |
|----|------------------------|--------------------------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 1  | Tengen                 | 831                                  | -  | -  | A  | C  | T  | C  | T  | C  | G  | -  | -  | G  |    |    |    |
| 2  | Waldele                | 830                                  | T  | C  | -  | A  | -  | T  | C  | T  | -  | T  | G  | -  | A  |    |    |
| 3  | Schwende               | 832                                  | T  | C  | -  | A  | C  | T  | C  | -  | T  | T  | G  | -  | -  | A  |    |
| 4  | Klausen Wald           | 831                                  | -  | C  | -  | A  | C  | T  | C  | -  | T  | T  | G  | -  | -  | A  |    |
| 5  | Modravsky Potok        | 829                                  | -  | C  | -  | -  | -  | T  | C  | T  | -  | C  | G  | -  | -  | G  |    |
| 6  | Luzensky Potok         | 827                                  | -  | C  | -  | A  | -  | -  | -  | T  | C  | G  | -  | -  | G  |    |    |
| 7  | Sokolnik               | 831                                  | -  | C  | -  | A  | C  | T  | C  | -  | G  | -  | C  | G  | -  | -  | G  |    |
| 8  | Mały Śnieżny Kocioł     | 830                                  | -  | C  | -  | C  | -  | T  | C  | -  | T  | C  | G  | -  | -  | G  |    |    |
| 9  | Kocioł Wielkiego Stawu | 830                                  | -  | C  | -  | C  | -  | T  | C  | -  | T  | C  | G  | -  | -  | G  |    |    |
| 10 | Kocioł Małego Stawu    | 833                                  | -  | C  | -  | A  | C  | T  | C  | -  | T  | C  | G  | C  | A  | G  |    |    |
| 11 | Złote Źródło           | 831                                  | -  | C  | -  | A  | C  | T  | C  | -  | T  | C  | G  | -  | -  | A  |    |    |
| 12 | Kopa                   | 832                                  | -  | C  | T  | A  | C  | T  | C  | -  | T  | C  | G  | -  | -  | G  |    |    |
| 13 | Kocioł Łomniczki       | 832                                  | -  | C  | -  | A  | C  | T  | C  | A  | T  | -  | C  | G  | -  | -  | G  |    |    |
| 14 | Slatinny Potok         | 830                                  | -  | C  | -  | C  | -  | T  | C  | -  | G  | -  | C  | G  | -  | -  | G  |    |    |
| 15 | Hala Cebulowa          | 831                                  | -  | C  | -  | A  | C  | T  | C  | -  | T  | C  | A  | -  | -  | G  |    |    |
| 16 | Hala Miziowa           | 831                                  | -  | C  | -  | A  | C  | T  | C  | -  | T  | T  | G  | -  | A  |    |    |    |
| 17 | Dolne Diery            | 831                                  | -  | C  | -  | A  | C  | T  | C  | -  | T  | C  | G  | -  | -  | G  |    |    |
| 18 | Velky Rozsutec        | 831                                  | -  | C  | -  | A  | C  | T  | C  | -  | T  | C  | G  | -  | -  | G  |    |    |
| 19 | Dedosowa Dolina        | 830                                  | -  | C  | -  | A  | -  | T  | C  | -  | T  | C  | G  | -  | -  | G  |    |    |
| 20 | Demanovska Dolina      | 831                                  | -  | C  | -  | A  | C  | T  | C  | -  | T  | C  | G  | -  | -  | G  |    |    |
| 21 | Rochackie Plesa        | 830                                  | -  | C  | -  | C  | -  | T  | C  | -  | T  | C  | G  | -  | -  | G  |    |    |
| 22 | Dolina Kościeliska     | 832                                  | T  | C  | -  | C  | -  | T  | C  | -  | T  | A  | C  | G  | -  | -  | G  |    |    |
| 23 | Dolina Jaworzynka      | 830                                  | -  | T  | -  | C  | -  | T  | C  | -  | T  | C  | G  | -  | -  | G  |    |    |
| 24 | Ostrva                 | 838                                  | -  | C  | -  | A  | C  | T  | C  | -  | T  | C  | G  | -  | -  | A  |    |    |
| 25 | Kamień                 | 831                                  | -  | C  | -  | A  | C  | T  | C  | -  | T  | T  | G  | -  | -  | A  |    |    |
| 26 | Biebrza                | 831                                  | -  | C  | -  | A  | C  | T  | C  | -  | T  | C  | G  | -  | -  | G  |    |    |
| 27 | Kamienna Nowa          | 831                                  | -  | C  | -  | A  | C  | T  | C  | -  | T  | C  | G  | -  | -  | A  |    |    |
| 28 | Roele                  | 831                                  | -  | C  | -  | A  | C  | T  | C  | -  | T  | C  | G  | -  | -  | G  |    |    |
Table 3 (on next page)

Variable sites recorded in the *trnH-psbA* Data in *Swertia perennis*. Nucleotide position refer to the number of nucleotide from the first position of the region.
| No | Locality/Code            | Length of \( mth_{psbA}/GFP \) focus |
|----|-------------------------|--------------------------------------|
|    |                         | 4 15 19 55 88 97 126 127 143 145 246 277 286 399 411 |
| 1  | Tengen                  | 429 A G C C - - C - A - T T A A A C T T A |
| 2  | Waldele                 | 429 A G C C - T A - A - T G T T A A T G A |
| 3  | Schwende                | 411 A G C C - T A - A - T G T T A A T G A |
| 4  | Klausen Wald            | 429 A G C C - T A - A - T G T T A A T G A |
| 5  | Modravsky Potok         | 411 A G C C - - C - A - T T A A A C T T A |
| 6  | Luzensky Potok          | 412 C G C C - - C - A T T T A A A C T T A |
| 7  | Sokolnik                | 410 A G C C - - C - A - - T A A A C T G A |
| 8  | Mały Śnieżny Kocioł     | 413 A G C C - - C T A T T T A A A C T T A |
| 9  | Kocioł Wielkiego Stawu  | 412 A T A G - - C - A T T T A A A C T T A |
| 10 | Kocioł Małego Stawu     | 412 A G C C - - C - A T T T A A A C T T A |
| 11 | Złote Źródło            | 410 A G C C - - C - A - - T A A A C A G A |
| 12 | Kopa                    | 410 A G C C - - C - A - - T A A A C A G A |
| 13 | Kocioł Łomniczki        | 412 A G C C - - C - A T T T A A A C T T C |
| 14 | Slatinny Potok          | 410 A G C C - - C - A - - T A A A C T G A |
| 15 | Hala Cebulowa           | 412 A G C C - - C - A T T T A A A C T T A |
| 16 | Hala Miziowa            | 428 A G C C - T A - A - - G T T A A T G A |
| 17 | Dolne Diery             | 410 A G C C - - C - A - - T A A A C T G A |
| 18 | Velky Rozsutec          | 410 A G C C - - C - A - - T A A A C T G A |
| 19 | Dedosowa Dolina         | 410 A G C C - - C - A - - T A A A C T G A |
| 20 | Demanovska Dolina       | 412 A G C C A - C - A - T T A A A C T T A |
| 21 | Rochackie Plesa         | 410 A G C C - - C - A - - T A A A C T G A |
| 22 | Dolina Kościeliska      | 411 A G C C - - C - A - T T A A A C T T A |
| 23 | Dolina Jaworzynka       | 411 A G C C - - C - A - T T A A A C T T A |
| 24 | Ostrva                  | 423 A G C C - A C - T - - T A A C A T G A |
| 25 | Kamień                  | 428 A G C C - T A - A - - G T T A A T G A |
| 26 | Biebrza                 | 410 A G C C - - C - A - - T A A A C T G A |
| 27 | Kamienica Nowa          | 428 A G C C - T A - A - - G T T A A T G A |
| 28 | Rowele                  | 410 A G C C - - C - A - - T A A A C T G A |