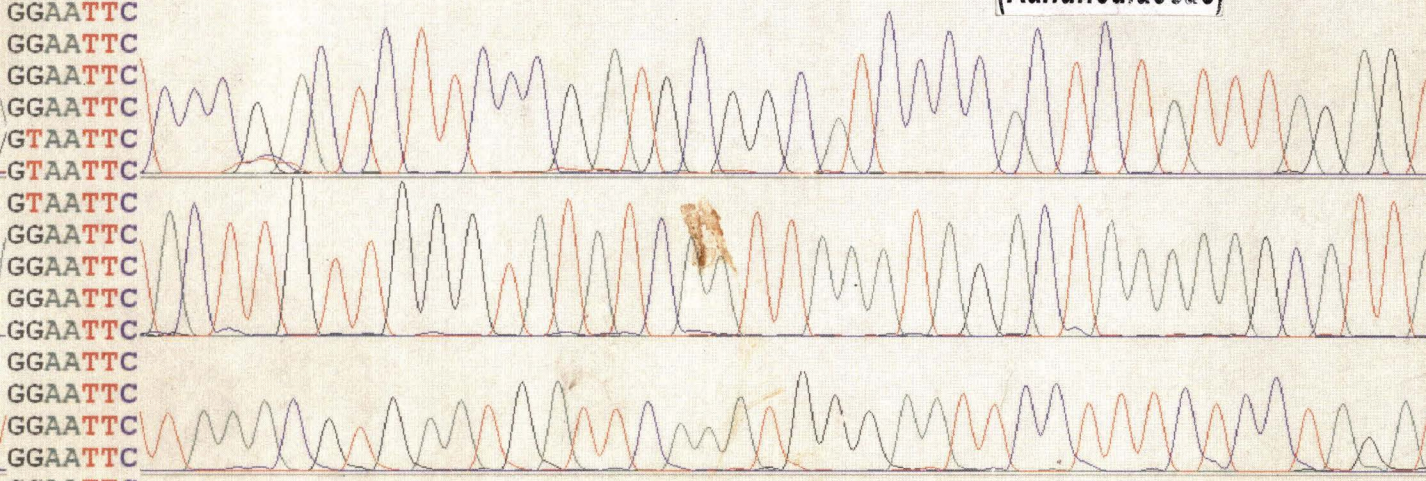
Michał Ronikier

Genetic diversity and phylogeography of *Pulsatilla vernalis* (L.) Mill.

(*Ranunculaceae*)



W. SZAFER INSTITUTE OF BOTANY – POLISH ACADEMY OF SCIENCES

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Genetic diversity and phylogeography of *Pulsatilla vernalis* (L.) Mill. (*Ranunculaceae*)

Ph. D. thesis completed at the
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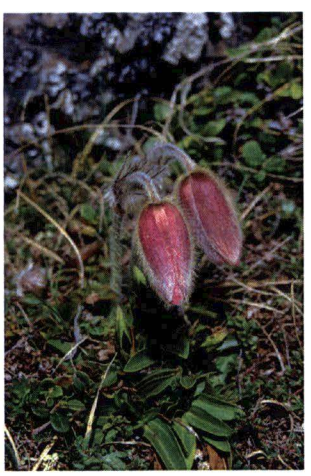
INTRODUCTION AND AIMS OF THE STUDY



1. INTRODUCTION

1.1. PHYLOGEOGRAPHY – IMPORTANCE OF DNA ANALYSES FOR THE HISTORICAL BIOGEOGRAPHY

Biogeography is an integrative and multifaceted discipline, which tends to: (i) describe the contemporary – and, when possible, also past – geographical distributions of living organisms, as well as interpret patterns of these distributions, (ii) seek for historical factors which are responsible for shaping these patterns, and (iii) analyze contemporary factors which are involved in the formation and spatial dynamics of populations (Cain 1974, Kornaś & Medwecka-Kornaś 2002, Avise 2004). Ultimately, the biogeographical studies should "achieve comprehensive understandings of biological and physical processes (at both ecological and evolutionary time-frames) that have shaped the spatial arrangements of the Earth's species and biotas" (Cox & Moore 1993, after Avise 2004).

Historical biogeography gathers and interprets information, which can be used for inference of past migrations and their causal factors. The most explicit sort of data are contemporary species' distributions themselves (often analysed together with infraspecific taxonomy), based on which – and along with climatological, geological etc. knowledge – past events can be deduced. In order to test hypotheses founded on present distribution patterns, specific, historically relevant sources of information have to be employed. In the time scale of last geological periods data from palynological analyses (eg. Huntley & Birks 1983, Ralska-Jasiewiczowa 2004) and macrofossils (eg. Birks & Birks 2000) bring the most direct information on former presence of taxa in space. In addition to that, methods of indirect inference of historical information can be applied (often in concert). Contemporary phenotypic variability, manifesting microevolutionary processes undergoing in isolated populations, was studied to look for historical relationships between populations (eg. discussed by Ehrendorfer 1968). Further on, information registered in the plant genome has been extensively explored. First, karyological analyses were found to be useful for reconstruction of distribution ranges' histories (Favarger 1961). Studies of ploidy levels and their geographical distribution in species' populations proved to be an efficient tool for insight into processes of range evolution and infraspecific differentiation (eg. Skalińska 1963, Küpfer 1974). A further step was the direct employment of the molecular polymorphism of the very carrier of the genetic information – the DNA. The beginning of such attempts dates back to the end of 1970s, when first works employing the mitochondrial DNA (mtDNA) variation to study natural animal populations started, and resulted in first large-scale geographical analysis

of genetic lineages (Avise et al. 1979). A term of "phylogeography" was introduced (Avise et al. 1987) to describe the quickly developing field dealing with analysis of geographic distribution of genetic variation, especially within and among closely related taxa, and inference of phylogenetic relationships between species' populations (and parts of distribution ranges) based on gene lineages. Application of molecular markers showed to be one of milestones in biogeographical studies. These tools already proved to be efficient in finding answers to several hitherto unresolved biogeographical problems. Mining the genetic variability data potentially allows to find traces of historical events, even in case where no morphological differentiation occurs or no direct palaeobotanical data are available. Phylogeography, studying the influence of historical factors on geographic distribution of gene lineages, is accompanied by ecogeography, emphasizing the role of contemporary natural selection processes on geographic distribution of genetic variation (Avise 1998, 2000); outputs of studies in these two overlapping fields should be used together for the most efficient insights into biogeographical problems (Wiens & Donoghue 2004).

Phylogeographical studies mainly concerned lineages of animal mtDNA (Avise 1998), while application of phylogeographical approach in plants was retarded. Nevertheless, mainly thanks to the introduction of studies of variability of (basically) uniparentally inherited and haplotypic chloroplast DNA (cpDNA), works dealing with plants became more popular in the 1990s (although, still, lower mutation rate of cpDNA makes it less straightforward for use than the animal mtDNA). Together with accumulation of empirical data, also development of new conceptual approaches to their analysis, aiming at reinforcing conclusions, have been introduced, eg. the nested clade analysis (NCA), which helps to discern between recent population processes and historical processes, such as isolation by distance, long-distance dispersal events etc., by interrelated analysis of geographical distribution, spatial extension of genotypes and their phylogenetic relationships (Templeton 1998).

Beginning of plant phylogeography and its largest application was in studies of tree species, and this was most probably due to presence of most complete palaeobotanical data for this group (and therefore possibility of verification of sound palaeobotanical hypotheses), as well as to economic and ecological importance of forests and potential usefulness of information on their provenience and distribution of genetic diversity across large areas (Newton et al. 1999). Among the first was an European-wide study of cpDNA in *Fagus sylvatica* (Demesure et al. 1996) demonstrating the survival of the species and higher genetic richness in populations from southern refugia and marked bottleneck effect in postglacial recolonization of temperate Europe from one refugium. Further on, complex programs were carried out to

obtain phylogeographical data for all important species building European forests, with especially meticulous cpDNA studies of European *Quercus* spp. (eg. Petit et al. 2002 and references therein). At present, cpDNA variability data are available for most important European trees allowing extensive comparative analyses (eg. Konnert & Bergmann 1995, King & Ferris 1998, Petit et al. 2002, Grivet & Petit 2003, Palmé et al. 2003, Heuertz et al. 2004) in a clear contrast to herbaceous plant species.

Phylogeographic approach was also extensively and efficiently applied during recent years to studying the Quaternary history of the arctic and alpine flora. In fact, unlike many other areas, arctic and alpine habitats are particularly suitable for testing clearly formulated hypotheses in many scientific fields and serve as most complex "natural experiments" (Körner 2001). Especially, questions addressed for a century by biogeographers and concerning the survival of plants during Quaternary glacial periods, when most of their usual habitats in the Arctic as well as in the mountains were heavily glaciated, were reconsidered, using information from genetic variability of populations (Gugerli & Holderegger 2001, Ronikier 2001a). A relatively complex molecular dataset for the Arctic biota was recently summarized by Abbott & Brochmann (2003) and Brochmann et al. (2003), indicating that postglacial formation of the North Atlantic biota was due to complete re-immigration (involving several trans-Atlantic long dispersal events) rather than *in situ* "nunatak" survival, considered as indispensable by several traditional biogeographers to explain present shape of distributional disjunctions. A number of papers also appeared presenting results of studies on glacial survival in the European Alps, and confirmed both *in situ* survival of some alpine plants (Stehlik et al. 2001), as well as re-immigration from several peripheral refugia (Schönswetter et al. 2004c). Recently, the question of potential influence of Quaternary glaciations not only on structuring the infraspecific genetic variability, but also on rapid speciation processes, was adressed by studying complexes of closely related taxa (eg. Comes & Kadereit 2003).

Phylogeographical analyses can be efficient mainly in studying relatively recent (in geological time scale) historical events, where "footprints" in distribution of genetic lineages are well detectable and not too much obscured by sequences of distributional shifts. Nevertheless, sometimes complex phylogenetic data, at higher taxonomic levels, analyzed in concert with present geographical distributions of interrelated taxa, can bring very useful information for old key events in the evolution of floras, as demonstrated eg. in analysis of phylogenetic patterns in the Eastern Asian/North American disjunction (Xiang et al. 1998) or a study of phylogenetic relationships in Balsaminaceae (Yuan et al. 2004).

Several case studies of phylogeographic analyses are available now for animals and plants, and some comparative analyses were made to search for common patterns in distribution of genetic lineages (eg. for Europe – Taberlet et al. 1998, Hewitt 2000). Only a small degree of congruence was found among phylogeographical patterns across Europe (Taberlet et al. 1998), although some general similarities in postglacial migration routes were also formulated, unraveling main refugial areas, most important barriers in range expansions (mainly the Alpine arch for lineages from Italian refugium), as well as common suture zones for lineages from different refugia. No common geographical patterns were found in a preliminary study of plants nowadays united in a common plant association (arctic-alpine snow-bed community) suggesting, that plant associations are not persistent through time and are newly formed in given ecological and climatical context (Taberlet et al. 2001).

First comparative phylogeographical studies showed no simple synthesis possible, but very interesting prospects. Possibility of future comparative studies is claimed to be essential to make substantial advance in understanding reciprocal relationships between genetic patterns and ecological properties of organisms, as well as to understand better the processes of landscape evolution (Bermingham & Moritz 1998). Moreover, phylogeographical studies have an essential value for identification of areas for efficient conservation of genetic diversity (Moritz & Faith 1998, Petit et al. 2003, Wiens & Donoghue 2004).

The availability of data, especially in case of plants (in the European perspective) is to date very uneven with respect of geography as well as ecological groups and floristic elements. Much effort in studying plant taxa representing different groups, with an appropriate, dense sampling across study areas, is to be done to really allow an extensive analysis of (infraspecific) genetic diversity patterns across Europe, their causes and potential consequences. The present study aims to contribute to the knowledge of patterns of genetic diversity of plants in the European-wide scale.

1.2. The aims and scope of the present study

The aim of this study is to analyze the pattern of genetic diversity of *Pulsatilla vernalis* as a model of European alpine plants with marked (relict) lowland distributions (Ronikier 2001b). Due to its distribution type involving isolated areas of the European mountain system, as well as lowland areas in temperate and (to a small extent) boreal zone (cf. chapter 2.2), this species is particularly suitable to seek information to answer several phytogeographical questions.

The study involves a detailed analysis of a distribution-wide sample set of populations, with all important parts of the range represented (adequately to the species' frequency in respective areas). The chloroplast DNA markers are used as the main source of information. Additionally, the population genetic structure is studied (applying the AFLP fingerprinting) in a case study in the Polish lowlands, to provide insights into the level of genetic diversity and its distribution within and among populations of *Pulsatila vernalis*.

Molecular tools are used to try to answer the following specific questions:

1) Is it still possible to identify the primary centre of differentiation of the species, based on the pattern of its genetic diversity, and if so, where was it located?
2) What are the relationships between the isolated parts of the species' range in the Central European mountain system (*sensu* Ozenda 1985), and particularly:
    a) what are the genetic relationships between the populations from the Alps and from the Pyrenees,
    b) do populations from the French Central Massif show detectable affinities to the neighbouring big mountain ranges (the Alps and/or the Pyrenees),
    c) are the populations from the Tatra Mountains a result of recent (postglacial) immigration from the Alps, or are they an older element,
    d) what are the genetic connections of the Balkan populations (do they show affinities to the Carpathian or Alpine parts of the range),
    e) what are the genetic affinities of the populations from the Sudety Mts.?
3) What is the history of the lowland part of the species' distribution range? Is it homogeneous or does it display traces of several independent colonization events? What are the genetic origins of the lowland populations? When did the lowland migrations take place?
4) Was Fennoscandia colonized in the Holocene by one genetic lineage of *Pulsatilla vernalis*, or can independent migration histories be found for eastern and western parts of this area?
5) Are the distributions of genetic lineages across Europe concordant with those of infraspecific taxa sometimes distinguished in *Pulsatilla vernalis*?
6) What is the level of genetic diversity and its structure in the lowland populations undergoing recently a dramatic decline? Can traces of strong isolation of populations be detected?

PULSATILLA VERNALIS AS A SUBJECT OF PHYLOGEOGRAPHICAL STUDY

2. *PULSATILLA VERNALIS* AS A SUBJECT OF PHYLOGEOGRAPHICAL STUDY

2.1. *PULSATILLA VERNALIS* (L.) MILL. – GENERAL CHARACTERISTICS

2.1.1. Taxonomy

The genus *Pulsatilla* was extracted from the Linnean genus *Anemone*, based mainly on the presence of long and feather-like hairy styles in the fruit; the division of the old genus *Anemone* in Europe further included *Hepatica* and *Anemone* s. str. (Hantula et al. 1989). Monophyly of this entire group is well supported by molecular studies (Johansson 1995) but the phylogenetic relationships within the group are still debated. Extensive phylogenetic analyses taking advantage of cpDNA analysis unraveled the complexity of relations within *Anemone* s. l. and nesting of *Pulsatilla* within *Anemone* s. str. (Hoot 1995). This suggests the inclusion of *Pulsatilla* within *Anemone* again, or – probably more plausibly – division and redefinition of the traditional multisectional genus *Anemone* s. str. according to monophyletic clades. Regardless the higher-rank relationships, the genus *Pulsatilla*, counting ca. 50 species, shows to be monophyletic based on both morphological and molecular characters. The centre of differentiation for this group is located in the middle- and eastern Asia (Scharfetter 1953). In Europe representatives of two out of five sections of the genus are present: section *Preonanthus* (Ehrh.) DC. containing the *Pulsatilla alpina* complex and section *Pulsatilla* (DC.) Aichele et Schwegler including remaining European species (Baumberger 1971, Damboldt & Zimmermann 1974). An ancient division separates *Pulsatilla vernalis* from other European representatives of the section *Pulsatilla.* Its primary differentiation can be dated back even to the early Tertiary (Zāmels & Paegle 1927, Scharfetter 1953) and could occur still before migrations to Europe; it would be supported not only by strong morphological and biological differences, but also by the fact, that the supposed closest relatives of *Pulsatilla vernalis* are seen in the eastern Asia, as *Pulsatilla ajanensis* (Zāmels & Paegle 1927, Scharfetter 1953, Heß et al. 1977). According to the infrageneric taxonomy proposed by Aichele & Schwegler (1957) *Pulsatilla vernalis* belongs to the subsection *Vernales* of the section *Pulsatilla* and it constitutes the type of this subsection. It is a species occurring only in Europe (for detailed description of the distribution range see chapter 2.2). Based on detailed morphological analyses of the material from the entire range of the species, Aichele & Schwegler (1957) proposed four intraspecific taxa in the rank of variety: var. *vernalis* (containing the type of the species), var. *bidgostiana*, var. *alpina* and var. *pyrenaica*. All these taxa but the last one are geographically intermingled. The variety *alpina* is consistently attached to subalpine and alpine habitats in most mountain ranges from the Alps to the

Scandes, it is only replaced in the Pyrenees and the Cordillera Cantábrica by the local var. *pyrenaica* occurring in similar habitats. The varieties *vernalis* and *bidgostiana* are confined to the lowlands. The relatively large intraspecific variability of this species is widely recognized, but most of differing characters are of purely quantitative character (Aichele & Schwegler 1957). Moreover, they are usually correlated with habitat conditions at large scale, as alpine vs. lowland habitats, or small scale, as local growth conditions, shading, etc. (pers. obs.). Only the pyrenean variety is supported by clearly smaller, more incised and hairy leaves (Aichele & Schwegler 1957, Laínz 2001). Several authors doubt in the value of taxonomic subdivision in *P. vernalis* (Jalas 1950, Uotila 2001).

2.1.2. Morphology and biology

Pulsatilla vernalis (Fig. 2.1) is a hemicryptophyte 3–20 cm high when in flower (but the pedicel having a tendency to elongate at fruiting up to 35 cm) (Kucowa 1985, Damboldt & Zimmermann 1974, Uotila 2001). Its thick, dark rhizome can divide and serve inefficient vegetative propagation. The most striking character of the species differing it from other representatives of the genus are overwintering, only one-imparipinnate, thick, subcoriaceous basal leaves with relatively large leaflets variously incised and either rounded or acute at apex. Basal leaves can have long petioles (up to 15 cm) and usually have a prostrate aspect (especially older ones). Seasonal cauline leaves are short, narrow, violet-brown, covered with silky, whitish to bronze-coloured hairs, alike the pedicel. These seasonal leaves form an involucre sheltering the flower bud during its development (it develops in the year preceding the flowering; Jonsson et al. 1991). Campanulate flowers are erect to nodding, with six sepals broadly lanceolate to obovate, creamy, violetish and covered with bronze-coloured hairs outside, and white, turning violetish when older inside. Fruits are single-embryo achenes with long (3–4 cm), feathery styles allowing a limited wind-mediated dispersal. Detailed dimensions of plant elements can vary strongly depending especially on habitat type; eg. length of pedicel at flowering is 5–24 cm for lowland plants and 2–11 cm for alpine plants, mean length of leaf blade is 2.5 cm and 5.5 cm respectively (Aichele & Schwegler 1957).
Pulsatilla vernalis is the earliest flowering species of the genus in Europe (Jonsson et al. 1991), probably due to presence of overwintering leaves. In the lowlands it already flowers in March–April (Wójtowicz 2001). In the mountains usually a shift by 1–2 months is observed (Ronikier et al., in prep.), but in the subalpine belt flowers can also be observed as early as beginning of April (pers. obs.). Self-compatibility was observed in *Pulsatilla vernalis* at least

Fig. 2.1. *Pulsatilla vernalis*. A – early flowering plants frozen by late spring snow; B – young flowers; C – early flowering aspect, opening flowers, flower buds covered by involucral leaves and old overwintering leaves are visible (young leaves not yet developed); D – full flowering aspect; E – fruiting specimens; F – sterile specimens with developed fresh leaves; G – example of pronounced differences in habit, plants from one population growing in open pasture (left) or among shrubs (right) at the Plateau des Bouillouses (Eastern Pyrenees). A, B, C, D, G – mountain populations; E, F – lowland populations.

Photo: A – Y.-M. Yuan, B to G – M. Ronikier

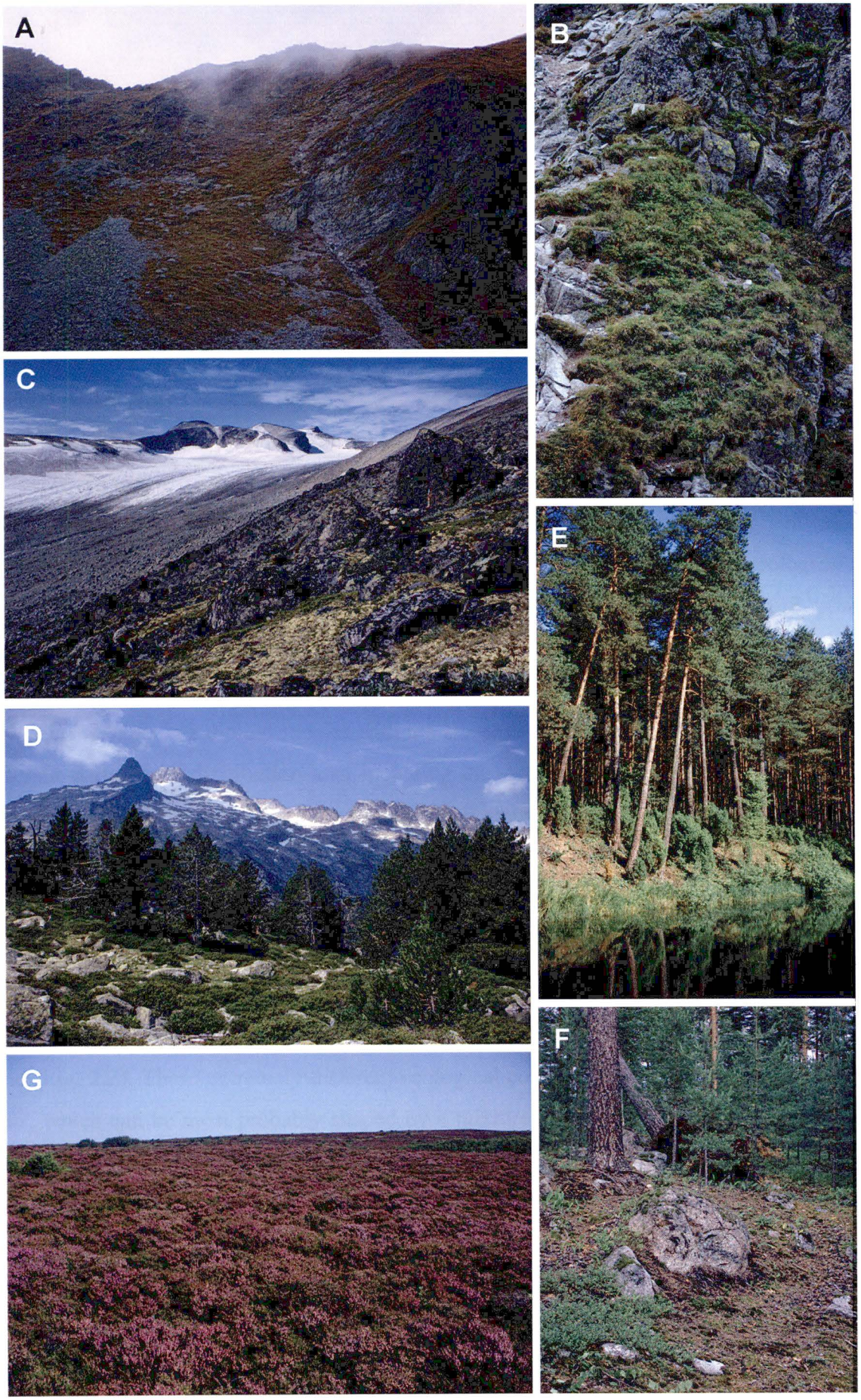


Fig. 2.2. Habitats of *Pulsatilla vernalis*. A, B – grasslands in the alpine belt (Vyšne Koprové Sedlo pass and Wrota Chałubińskiego pass, Tatra Mts.); C – sparse, lichen-rich alpine tundra close to the upper vegetation limit in the Jotunheimen Mts.; D – subalpine forests of *Pinus uncinata* and shrubs of *Rhododendron ferrugineum* (Massif de Néouvielle, Central Pyrenees); E, F – bright stands in lowland pine forests (Bory Tucholskie forests in Poland, southern Finnish lakelands); G – heathlands (Vind Hede, Denmark).

Photo: M. Ronikier

in parts of the distribution area (Uotila 2001), but in general, self-fertilization is supposed to be restricted in the genus *Pulsatilla*. The breeding system of *Pulsatilla vernalis* is characterized by partial dichogamy (protogynous flower development), strongly restricting probability of fertilization within flower (Jonsson et al. 1991). Pollination experiments demonstrated that the seed set is mainly pollen-dependent. Marked protogyny, together with observed overlap of the female phase with the beginning of the male phase could suggest favoured cross-pollination with self-pollination as a security mechanism in case of lack of external pollen (Jonsson et al. 1991).

Hybrids of *Pulsatilla vernalis* with other species of the section *Pulsatilla* (*P. patens, P. pratensis, P. vulgaris*) were observed (Zāmels & Paegle 1927, Damboldt & Zimmermann 1974). Usually, however, hybridization is restricted by at least partial geographical (eg. altitudinal ranges, as in *P. vernalis* vs. *P. montana* in the Alps; pers. obs.) or ecological and phenological barriers (habitat conditions, flowering periods, eg. *P. vernalis* vs. *P. patens*; Uotila 1980). Decreased fertility of F1 generations suggests also presence of genetic barriers (Uotila 1980).

Pulsatilla vernalis is a diploid species with the chromosome number 2n=16, as reported from different parts of its distribution area including the Alps, the Pyrenees, the Tatra Mts. and Scandinavia (eg. Aichele & Schwegler 1957, Baumberger 1971, Löve 1980, Lauber & Wagner 2000, Uotila 2001).

2.1.3. Ecology and habitats

Two distinct and very different kinds of habitats are characteristic for *Pulsatilla vernalis* in its range-wide distribution; these are high mountains on the one hand and lowlands on the other hand (Fig. 2.2). These extremely different ecological conditions certainly have also common traits, which can be most probably shaped by a mixture of edaphic and light parameters and selected climatic characters as suboceanic influence (Jäger 1970). The mountain area can be presumably treated as primary area; Jalas (1950) states, that the species could have evolved as a high-mountain taxon already before the Quaternary glaciations. Throughout the entire mountain area it is considered to be a siliceous or lime indifferent species with preference to nutrient-poor soils of pH 4,5(–6,5). However, it happens to occur, although rarely, even in typical calcareous areas (Allgäuer Alpen – M. Sheuerer, pers. inf.)

In most high-mountain areas it is a genuine alpine species, but with a pronounced tendency to inhabit subalpine belt and sometimes to descending even into lower belts (Aeschimann et al.

2004). It usually grows between 1700 and 3000 m in the Pyrénées (Laínz 2001), and between 1500 and 3100 m in the Alps (Damboldt & Zimmermann 1974). In the Carpathians and the Rila Mts. it grows uniquely in the alpine belt (pers. obs.).

In the mountains *Pulsatilla vernalis* is usually confined to alpine grasslands representing *Caricetalia curvulae*, developing on acidic soils. In the mountain ranges where it is common (mainly Alps and Pyrenees), it often grows abundantly also in subalpine belt among shrub communities, eg. *Vaccinio-Rhododendretum ferruginei.* Such shrub communities, especially at the transition zone to subalpine *Pinus uncinata* forests in the Pyrenees, form a habitat inspiring comparisons with lowland poor heaths and open-canopy pine forests and linking to some extent the two ecological aspects of the species (pers. obs.). In the Tatra Mts. the species occurs in the siliceous massifs but usually in peculiar mylonitic habitats with more neutral than acidic soil reaction (Komárková 1974), where it is a locally characteristic species for the endemic community *Festuco versicoloris-Agrostietum alpinae* (Pawłowski et al. 1929). In the lowlands *Pulsatilla vernalis* inhabits pine forests (of the type *Peucedano-Pinetum*) and heaths (*Calluno-Ulicetalia*) on acidic (pH 4,3–5,0), sandy soils (Wójtowicz 2001). In pine forests a clear affinity to more open places as clearings or forest road edges can be observed (pers. obs.).

In the centre of distribution (mainly the Alps) populations are usually numerous and abundant, often covering large areas, while populations in the lowland part of the range are very sparse and usually count few individuals (rarely exceeding a few dozen plants – Wójtowicz 2001, Piękoś-Mirkowa & Mirek 2003, M. Ronikier pers. obs.). Mountain populations at the edge of the range (Tatra Mts., Rila Mts.) are also small, but apparently of stable size; the largest ones exceed 100 plants (Piękoś-Mirkowa & Miechówka 1999, Ronikier et al., in prep.). Additionally, the temporary number of effective plants in the population as well as and percentage of flowering plants and seedlings development seems to strongly depend on yearly climatic fluctuations, with importance of atlantic-like set of conditions (Wójtowicz 2001). Extreme variations in flowering and fruiting are also observed in the populations in the Tatra Mts. across years (Ronikier et al., in prep.).

2.1.4. Conservation status

Two types of areas can be delimited with respect to contemporary abundance and condition of *Pulsatilla vernalis* populations. One type comprises central-western European mountains (mainly the Alps and the Pyrénées) and the Scandinavian mountains, where the species is

abundant and belongs to (at least locally) common species with stable populations not subjected to any regular threat. The second type of area includes central-eastern and northern European parts of the distribution range, mainly the whole lowland area; lowland populations everywhere are decreasing and this part of the distribution is on the way of dramatic reduction. This situation is well reflected by the conservation status attributed to the species in different parts of its area. *Pulsatilla vernalis* is protected and qualified as endangered or critically endangered in all parts of its lowland area (eg. Bielousova 1984, Korneck & Sukopp 1988, Wind et al. 1998, Čeřovský 1999, Gärdenfors 2000, Wójtowicz 2001, Piękoś-Mirkowa & Mirek 2003). Eastern mountain populations in the Carpathians and the Rila Mts. are not numerous, small and scattered, but relatively stable. Because of their scarcity and resulting susceptibility to the influence of various stochastic processes, they are also estimated to be endangered (Koeva 1984, Ronikier et al., in prep.)

2.2. Analysis of the contemporary distribution

Pulsatilla vernalis is an European endemic species (Jalas & Suominen 1989). It belongs to the Central-European subelement of the Holarctic geographic element (Pawłowska 1977). Its distribution range (Fig. 2.3) comprises large part of the continent and includes isolated areas in most of the European alpine system' mountains on the one hand, and a lowland part of the distribution – mainly in Germany, Poland and southern Scandinavia – on the other hand (Meusel et al. 1965, Jalas & Suominen 1989). This particular type of distribution with two well marked centres (mountains and lowlands) was defined by Pawłowski (1928) as a separate, unispecific pattern (*Pulsatilla vernalis*-type) within the "alpine-middle European" group of species.

In the south-westernmost part of its area, *Pulsatilla vernalis* enters the Iberian peninsula. It is relatively common in the siliceous massifs of the Eastern and Central Pyrenees, it also occurs in the siliceous Southern Pre-pyrenean chains and in the central part of the Cordillera Cantábrica (Montserrat Martí 1982, Dupont 1990, Laínz 2001). Several works cite occurrence of the species also in the interior of the Peninsula including the Sistéma Central and the Sierra Nevada (Pawłowski 1928, Hultén & Fries 1986, Wójtowicz 2001), but these data are surely erroneous and probably based on an 19th-century misinterpretation (Laínz 1984). North of the Pyrenees, isolated populations exist in the highest mountains of the French Central Massif (Dupont 1990). The Alps are a well-pronounced centre of distribution of *Pulsatilla vernalis.* The species is present throughout the whole Alpine arch, it is only markedly absent from the

southern part of the Maritime Alps (Bonnier 1922, Aeschimann et al. 2004). It is relatively common in most siliceous massifs, only in easternmost parts of the Austrian Alps (Niedere Tauern) populations become very sparse. Several populations were known in the Sudety Mountains, but nowadays only one, strongly declining population exists in the Czech Krkonoše Mts. (Špatenková 1996), while the species is extinct on the Polish side of the massif (Fabiszewski & Kwiatkowski 2002).

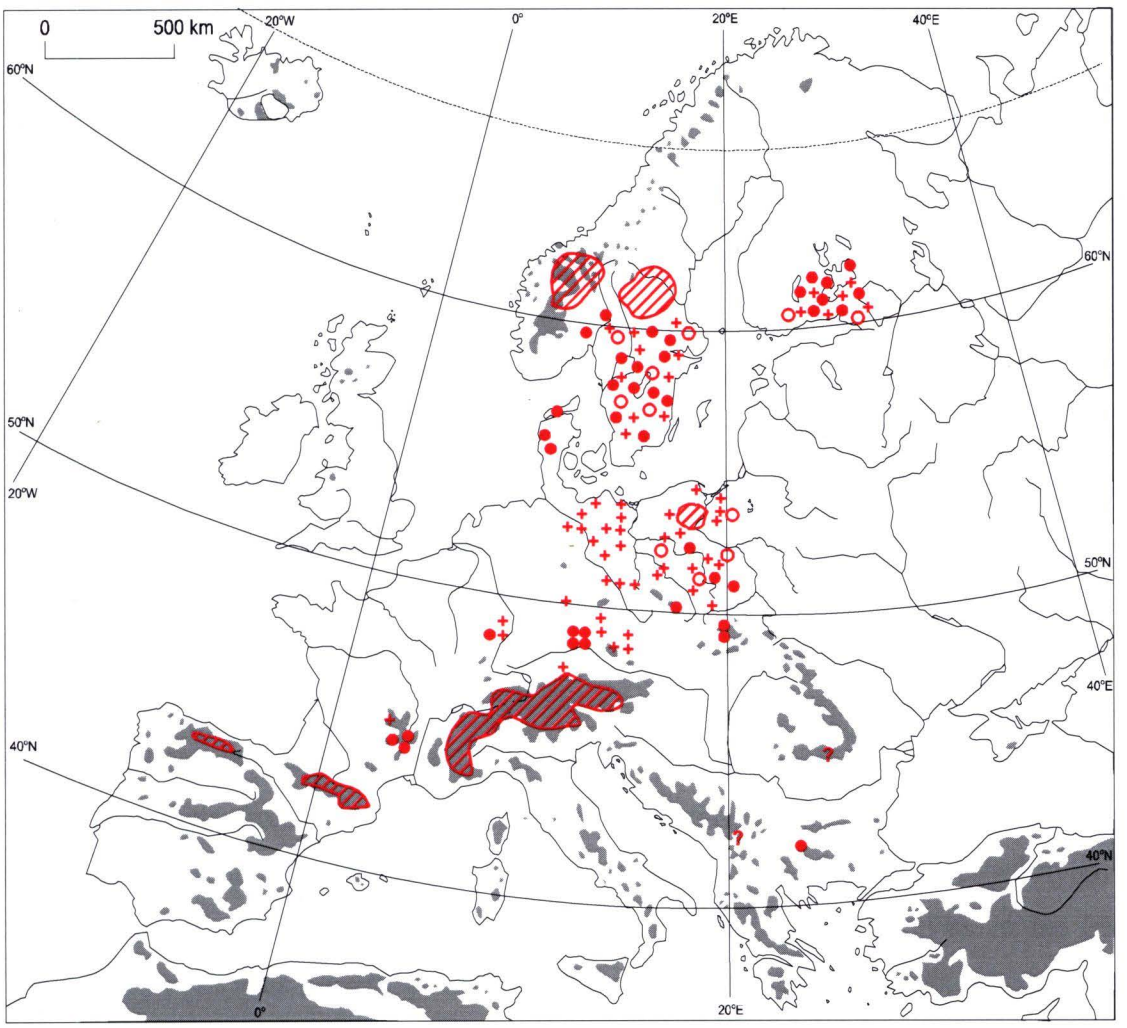


Fig. 2.3. Distribution of *Pulsatilla vernalis* (based on Jalas & Suominen 1989, modified according to different sources cited in the text). Hatched areas represent continuous range fragments; filled circles – existing isolated populations; crosses and open circles show areas with many populations extinct or with uncertain existence respectively. Question marks signalize stands mentioned in literature and probable but not surely documented.

In the Carpathians, the only confirmed populations occur in the Tatra Mountains, where the species was reported for the first time by Pawłowski et al. (1929) and then on new site by Pawłowski (1930). The species is currently known from a dozen or so very small populations in the whole Tatras (gathered in Ronikier et al., in prep.) The occurrence of *Pulsatilla vernalis* was also mentioned from the Southern Carpathians (Munţii Bucegi) by J. C. G. Baumgarten,

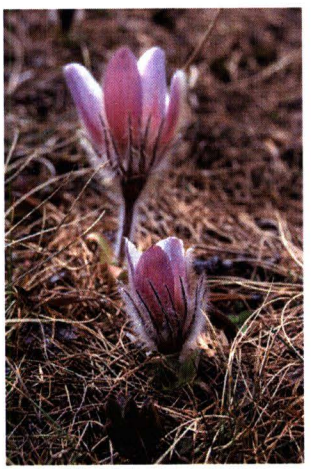
but no herbarium materials are available or further confirmations known (Nyárády 1953). Probable presence of the spring anemone in this area is highly supported by presence of convenient habitats (pers. obs.) and confirmed occurrence of the species in the south-eastern Europe. In the Balkans, a few isolated populations occur in the Rila Mountains (Jordanov & Kožuharov 1970), but not in the Pirin Mts, as mentioned in several works (eg. Kucowa 1985, Wójtowicz 2001). The occurrence of the species was also mentioned from the Dinaric Alps (Damboldt & Zimmermann 1974), but no further data concerning this area were found.

To the north of the (Central-)European alpine system, extensive lowland areas covered by boreal-type pine forests or heathlands constitute the Central European lowland part of the *P. vernalis* distribution. Historical botanical data indicate the relative commonness of this species in its lowland area in the past; for last 100–150 years, however, dramatic decline of these lowland populations has been observed. The westernmost lowland populations were reported from heathlands in the French Alsace-Lorraine region and its German vicinity by Schultz (1846); in the beginning of XXth century it was still mentioned without notion of rarity (eg. Bonnier 1922). Recently, only one still existing population was reported there in the region of Bitche (Muller 1997). In Germany few of once more numerous lowland populations exist until present, mainly in the eastern Bavaria (Niederbayern and Oberpfalz regions); they are critically endangered (Scheuerer et al. 1991, Scheuerer 1996). All populations located more to the north, in the Saxony, are extinct – last individuals were observed in 1922 (Hempel 1976, Otto 2004). Also lowland populations in the Lower Austria (Niederösterreich) are totally extinct (Adler et al. 1994) although it is still represented in herbarium materials from the beginning of the 20th century (pers. obs.) Only one out of several dozens neighbouring populations in the southern Czech Republic has survived, but with single, dispersed individuals likely to disappear soon (Čeřovský 1999). In the Polish lowlands *P. vernalis* was present in scattered but relatively common populations in western and central Poland from Silesia to Pomerania (eg. Ascherson & Graebner 1898–1899, Schube 1904), locally more abundantly, especially in the region of Bory Tucholskie (Zając A. & Zając M. 2001). From the Polish perspective the lowland distribution dominated the mountain one represented only by few populations in the Tatras, unlike all other mountain plants having also lowland distribution (Zając M. 1996). As in other lowland regions, however, a dramatic decline has been observed for last century and only few localities are confirmed according to recent data (Wójtowicz 2001, Ronikier, unpublished data). The region of the Bory Tucholskie forests harbours most of the existing local populations, single and isolated stations exist also

eg. in the Central Poland (Hereźniak et al. 2001) and in the region of the Góry Świętokrzyskie Mountains (E. Bróż, pers. inf.)

The northernmost edge of the distribution area in Scandinavia comprises two isolated parts (Hultén 1950). The eastern part, of purely lowland character, involves southern Finland's lakelands (Uotila 1998) and extends to the Russian Karelia (Bielousova 1984). In both areas the plant once more abundant is currently rare and endangered. The western part of the Scandinavian distribution comprises Denmark, Sweden and Norway (Hultén 1950). In Denmark, southern Sweden and southern Norway the species occurs in lowland habitats and suffers from a strong decrease of its populations. In Denmark only 6 out of a few dozens of historical populations in the Jylland peninsula were recently confirmed (Wind 1993), only one, in Vind Hede, being still fairly abundant (Moeslund 1987). Likewise, the historical abundance of lowland populations in Sweden amounting to several hundreds of localities (Hultén 1950) is continuously decreasing with half of populations still observed in 1965 now extinct, a current negative trend in more than 90% of existing ones and population number not exceeding 5 individuals in more than 60% of currently known sites (Åström & Stridh 2003). The highest concentration of existing populations in Sweden can be observed in the northern part of the local distribution area, in the northern parts of the Värmland and Dalarna regions, as well as in the Härjedalen region (Uotila 2001). The only Scandinavian area where the species is locally common and not threatened includes a few massifs in the southern part of the Scandinavian Mountains – Jotunheimen and Dovrefjell, where the spring anemone occurs in the alpine habitats (Uotila 2001).

METHODS –
A THEORETICAL BACKGROUND



3. METHODS – A THEORETICAL BACKGROUND

3.1. CHOICE OF METHODS FOR THE ANALYSIS OF DNA VARIABILITY

The methods applied in the study were chosen with respect to main aspects of the project: (i) a reliable analysis of historically relevant markers, (ii) a detailed analysis of selected populations to obtain information on genetic diversity in varied case studies and refining the picture of geographical relationships if needed. The analysis of chloroplast DNA was used as a method of choice for inference of geographical patterns (eg. Comes & Kadereit 1998), because of basically uniparental (maternal in Angiosperms) inheritance mode (Reboud & Zeyl 1994), and therefore haplotypic genome and presumable lack of recombination (McCauley 1995, Comes & Kadereit 1998). Thanks to these properties it is assumed, that haplotypic cpDNA genomes gradually accumulate mutations and polymorphisms gained in source populations can be detected in derived lineages as autapomorphies. Additionally, the effective population size for uniparentally inherited genome is expected to be much lower than in autosomal loci (only females have it and only in single copies) and, as a consequence, haplotypes are more likely to reflect different processes as isolation, range fragmentations etc. (Halliburton 2004) and these historical patterns are less obscured by current gene flows (Comes & Kadereit 1998).

Apart from chloroplast DNA, also selected nuclear DNA regions were used for analysis, in order to have comparison between DNA fraction distributed only by seeds (cpDNA) and the DNA fraction distributed by both seeds and pollen (nDNA), across the whole distribution range.

PCR-RFLP method was used as a basic method for screening of the cpDNA variability. This relatively cheap method, used in several comparable studies (eg. Holderegger et al. 2002, Petit et al. 2002, Stehlik 2002), allows for analysis of many regions of cpDNA and indirect inference of mutations which differentiate respective haplotypes (see below for details). The reliability of PCR-RFLP results was checked by application of direct DNA sequencing to two regions of cpDNA. This control experiment was designed in a way to allow: (i) direct check of reliability of PCR-RFLP data, (ii) independent analysis of sequences' dataset in a typical way applied in published works, and methodological comparison of the two methods.

The DNA sequencing was also applied to obtain sequences of nuclear DNA regions.

The AFLP analysis (Vos et al. 1995) was chosen for complementary studies using the whole genomic DNA as a source of the information. This method is considered to be the most reliable and highly resolving universal method of complex DNA fingerprinting (Mueller &

Wolfenbarger 1999), to be applied with two purposes: (i) fine-scale analyses of phylogeographical relationships in case where cpDNA and ITS data would not show enough resolution, (ii) analyses of genetic diversity of selected populations, with focus on endangered and declining part of the distribution range. This method proved to be very efficient in a series of recent fine-scale phylogeographical studies (eg. Stehlik et al. 2001, Schönswetter et al. 2004b).

3.2. PCR-BASED RESTRICTION FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP)

The Polymerase Chain Reaction(-based) Restriction Fragment Length Polymorphism (PCR-RFLP) analysis consists of two main parts: (i) amplification of a DNA region in a standard Polymerase Chain Reaction, (ii) digestion of the amplicon by single or combined restriction enzymes cutting strictly determined target DNA sequences (Dowling et al. 1996). Products of

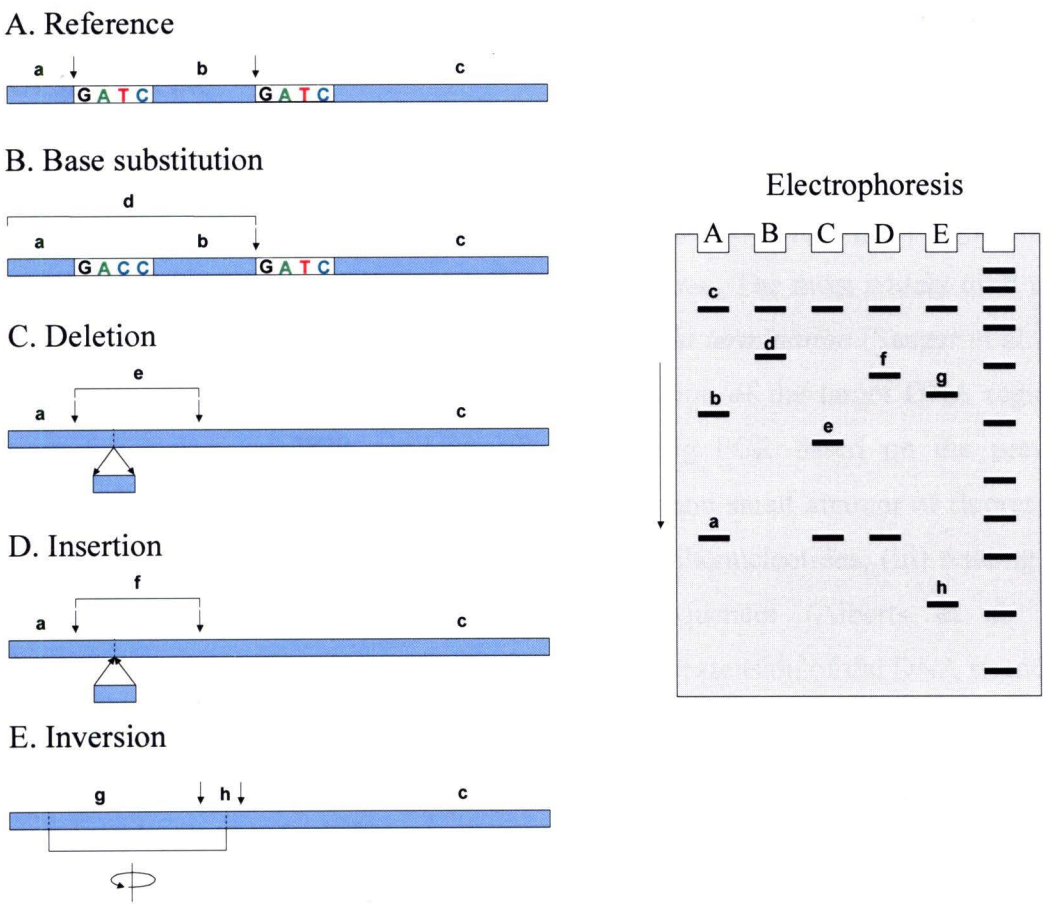


Fig. 3.1. Types of mutations detected in PCR-RFLP and their influence on patterns of restriction fragments. On the left – DNA fragment with different mutations; on the right, electrophoresis gel with resulting band patterns in respective lanes (last lane contains a DNA size marker). A – initial fragment; B – example of modification in enzyme restriction site (here: GATC) causing lack of restriction and one fragment (d) instead of two (a + b); C, D – fragment length polymorphisms ("indels"); E – change of restriction site location due to a local inversion (based on Dowling et al. 1996, modified).

digestion are separated by means of a gel electrophoresis. It results in a multiband restriction profile depending on the number of the enzyme target sequences in the analysed region.
Analysis of restriction profiles permits an indirect inference of mutations (Fig. 3.1). Two main kinds of genetic polymorphisms can be detected here: (i) restriction site polymorphism – when the target sequence of an enzyme appears or disappears, usually due to a point nucleotide mutation; it is manifested by lack of cutting or additional cutting of the DNA fragment, respectively, (ii) band length polymorphism – when an insertion or deletion of a sequence occurs in the DNA fragment (so-called "indel"), but without generating change in the number of restriction sites; it is manifested by differences in the migration speed of bands in the electrophoresis gel. Often it is not possible to state exactly what kind of mutation caused change in the band pattern (insertion, deletion, inversion etc.), therefore polymorphisms are usually recorded as phenotypic characters without exact notion of their molecular basis.

3.3. DNA SEQUENCING

The sequencing of DNA allows for the most direct analysis of the genetic polymorphism. The application of this method results in a sequence of nucleotides forming a given region of DNA and gives insight into all changes that occurred in this region. The most widely used method of sequencing is the *dideoxy-method* or the *method of chain termination* (Sanger et al. 1977). It includes three main steps: (i) standard PCR amplification of the target DNA region and subsequent purification of PCR product, (ii) sequencing PCR based on the previously obtained amplicon, with one primer (forward or reverse) and small amount of fluorescently-labelled dideoxynucleotides in addition to normal deoxyribonucleotides, (iii) reading of the DNA sequence by electrophoresis in automated sequencer (Alberts et al. 2002). Dideoxynucleotides in the sequencing reaction prevent the extension of the DNA strand at the 3' end (due to lack of the -OH group). Each of the four dideoxynucleotide types are labelled with different fluorescent dye. In the course of reaction a rare, random incorporation of dideoxynucleotide occurs in different moments of DNA strand elongation, and causes termination of the process. At the end of the sequencing PCR, a mixture of single strand DNA fragments of different lengths is obtained, with a step of one base pair, each with a fluorescently-labelled dideoxynucleotide. Subsequent electrophoresis and detection of laser-stimulated fluorescent signal by the sequencer's camera in the order of fragment size gives the sequence of nucleotides which constitutes the analysed DNA fragment.

3.4. Amplified Fragment Length Polymorphism (AFLP)

The AFLP (Amplified Fragment Length Polymorphism) method (Vos et al. 1995) allows for obtaining robust genetic fingerprinting useful for fine-scale intraspecific studies (eg. Stehlik et al. 2001) as well as for solving phylogenetic problems at above-species level (eg. Despres et al. 2004). This multilocus marker is based on digestion with restriction enzymes and amplification in PCR of resulting fragments (Fig. 3.2). Hundreds of highly reproducible bands can be assessed without prior knowledge of sequences and design of specific primers (Mueller & Wolfenbarger 1999). The procedure involves an ingenious combination of several

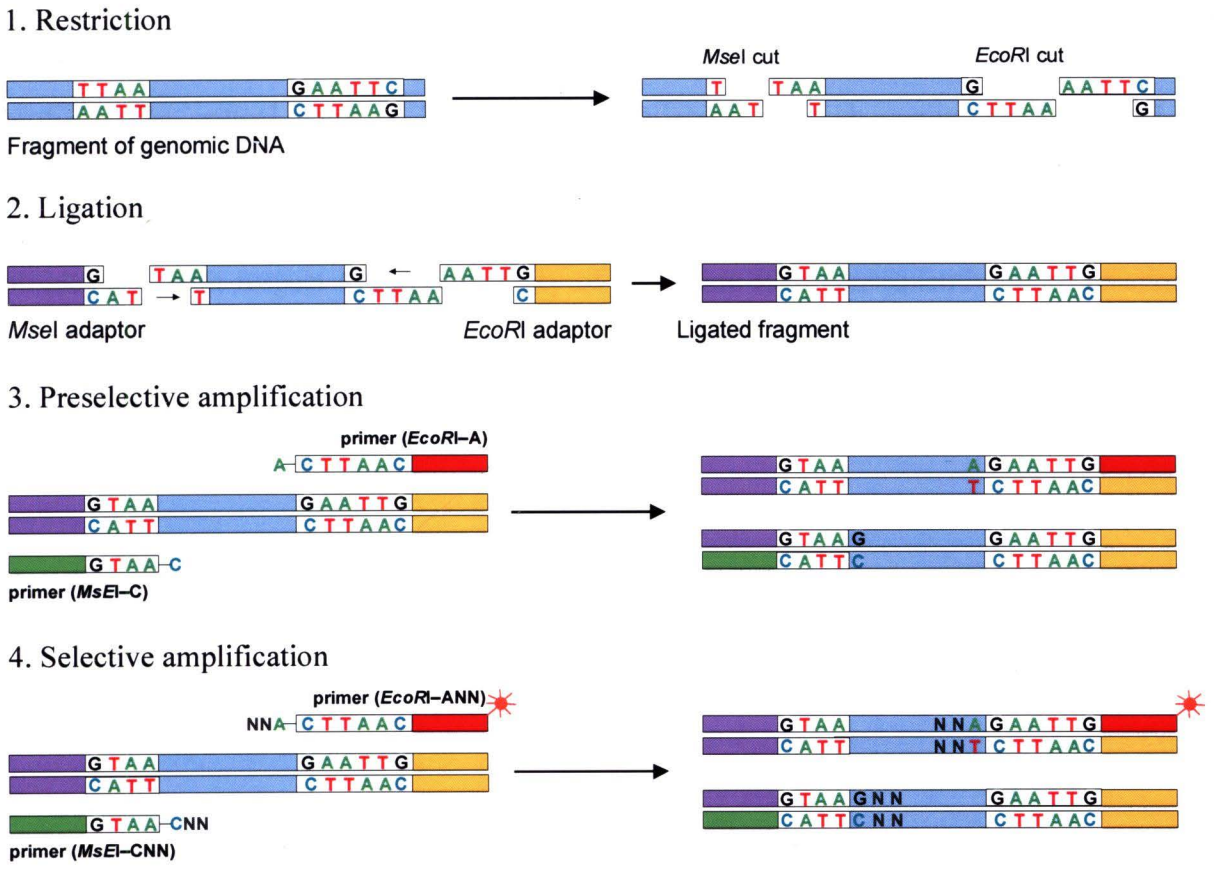


Fig. 3.2. Principle of AFLP analysis (see text for detailed explanations). Fluorescently labelled *Eco* RI primer used in selective amplification is marked by orange light.

enzymatic reactions: (i) digestion of the whole genomic DNA with two restriction enzymes generating "sticky ends" in digest products; typically *Eco* RI and *Mse* I are used; (ii) enzymatic ligation of two-strand adapters – synthesized oligonucleotide fragments with "sticky ends" complementary to those resulting from restriction digestion, using the T4 DNA Ligase enzyme; (iii) first PCR ("preselective PCR") amplifying a subset of ligated digested

fragments using primers complementary to the adapter sequence and having 1–2 randomly chosen nucleotides at the 3'-end (allowing for the first selection of subset of fragments); (iv) second PCR ("selective PCR") using product of previous PCR as a template and amplifying a subset of previously amplified fragments by use of primers from preselective PCR elongated by further 2–3 selective nucleotides at the 3'-end (Vos et al. 1995, Mueller & Wolfenbarger 1999). Many combinations of "selective" nucleotides at the end of fixed primer sequences can be tested resulting in amplification of different subsets of restriction fragments. Choice of primers can also be adjusted for eg. genome size (typically, three selective nucleotides are used for the second PCR, but two or four nucleotides can be used for small or large genomes respectively, to regulate the complexity of the final band pattern).

Small amounts of genomic DNA are needed for the analysis (typically ca. 50 ng/µl). Stringent conditions of PCR ensure highly reproducible amplification and generation of reliable profiles. The critical point is, however, the availability of high quality DNA, devoid of inhibitors influencing enzymatic digestion and ligation of DNA (Mueller & Wolfenbarger 1999); plants with high concentrations of secondary phenolic compounds etc. may be problematic with respect to reproducibility of restriction (and consequently entire reaction). Nevertheless, AFLP is certainly the most robust method of genomic DNA analysis without knowledge on sequence of target region.

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. SAMPLING OF MATERIAL

4.1.1. Methods of field sampling and subsequent storage of plant material

Sampling was done during three years, since 2001 until 2003; samples were collected throughout whole vegetation periods, since May until September (most samples were collected in July–August). One to three leaves per individual were cautiously removed without damaging the whole plant. Preferably, young but well developed leaf blades were chosen for collections. In rare cases when new leaves were not yet well developed (early in the season), last year-leaves as well as pieces of young flowers were collected. If collecting took place late in the season, the youngest available leaves were taken, with special attention paid to avoid possible traces of fungal infections, necroses, etc.

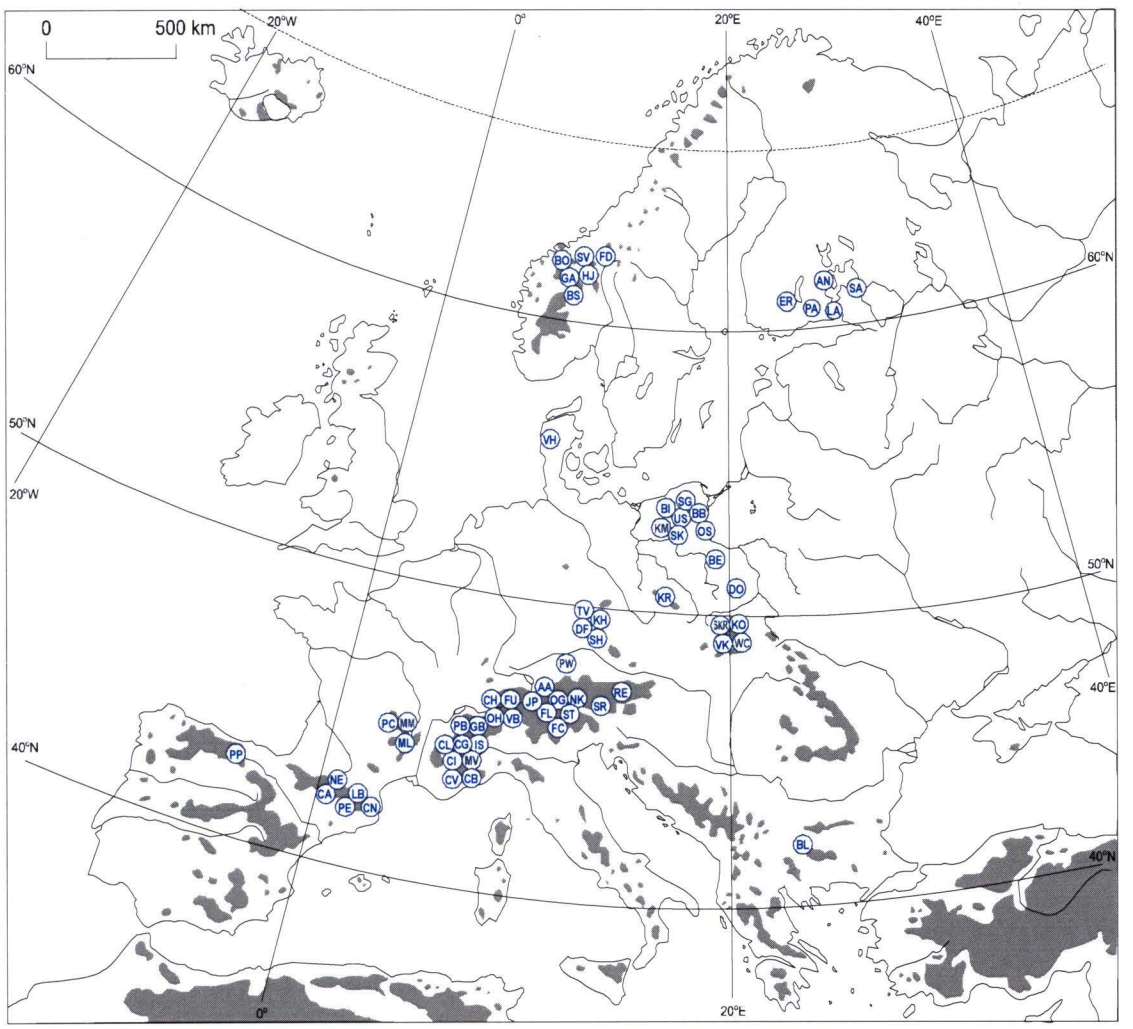


Fig. 4.1. Location of sampled populations in Europe. Two-letter codes correspond to populations' acronyms used in the Table 4.1.

Plant material from each individual was immediately put to separate, airy paper bags. Material from each population was put together into strip-closed plastic bags with silica gel for quick-drying (Chase & Hill 1991).

For long-term storage in the laboratory, the materials were also kept in hermetic bags in presence of silica gel, at room temperature (with periodical exchange of silica gel if necessary).

Herbarium specimens were collected as vouchers for all populations sampled in areas where *P. vernalis* is not a rare and endangered species. In regions where the plant is rare and protected, no entire plants were taken. Herbarium vouchers were deposited in the Herbarium of the Institute of Botany, Polish Academy of Sciences, in Kraków (KRAM).

4.1.2. Strategy for sampling of populations

Selected populations from the entire range of the species' distribution were sampled for this study. Representative populations from all distinct parts of the distribution area were chosen (Fig. 4.1), in order to avoid gaps in sampling of the contemporary distribution range. The areas with potentially more complex patterns expected (especially the Alps) were more densely covered by sampling. In the Alps efforts were put to sample populations in most important massifs. An additional, hypothesis-driven sampling was included in the analysis after initial screening of variability (among others populations from German Prealps and lowlands, or Danish Jylland).

In general, special effort was put to sample many populations. Five individuals per population were sampled for the phylogeographic study. According to the strategy adopted, this relatively low within-population sampling was balanced by including many populations, which was estimated to be more important and informative for wide-range geographical studies than rich sampling of few populations (eg. Pons & Petit 1995).

In the field, first the population size and extension were estimated in order to choose individuals for sampling throughout the whole population instead of one patch. In most cases geographical coordinates were obtained on place using a GPS utility (Garmin e-Trex Vista).

In total, samples from 63 populations were gathered (Table 4.1), amounting to 330 samples used for analyses in total.

Table 4.1. List of populations used in the study with their distribution details.

No	Pop. code	Date of collection	Country	Locality	Altitude	Latitude/ Longitude[1]	Collector[2]	Sample size[3]
1.	AA	09.09.1999	Germany	Allgäuer Alpen; Jöchlspitze (2226 m)	1970 m	[47°17' N] [10°22' E]	MS	5*
2.	AN	12.07.2003	Finland	Region of Mikkeli; Veeravuori, East from Anttola	90 m	61°34'40" N 27°39'44" E	MR & AR	5*
3.	BB	22.06.2001	Poland	Northern Poland, Pojezierze Kaszubskie, Bory Tucholskie; vicinity of the village Brzozowe Błota	-	53°39'50" N 18°08'00" E	MR & AR	5♦
4.	BE	12.05.2000	Poland	Central Poland, region of Łódź; vicinity of village Rogowiec, S of Bełchatów	-	51°16'40" N 19°16'00" E	JH, MR & AR	5* (20♦)
5.	BI	24.06.2001	Poland	Northern Poland, Pojezierze Kaszubskie, Bory Tucholskie; vicinity of the village Biała	-	53°40'40" N 17°59'20" E	MR & AR	5* (15♦)
6.	BL	16.08.2001	Bulgaria	Rila Mts.; S ridge of Goliam Blizniak (2779 m)	2765 m	42°09'39" N 23°35'14" E	MR & PK	5*
7.	BO	05.08.2003	Norway	Oppland; Jotunheimen, Bøverkinn, SW slopes of Leirho (1178 m)	1050 m	61°39'34" N 08°09'30" E	MR & AR	5*
8.	BS	07.08.2003	Norway	Oppland; Jotunheimen, Beitostølen, S slopes of Grønekinnkampen (1151 m)	1090 m	61°17'13" N 08°49'14" E	MR & AR	5*
9.	CA	17.07.2001	Spain	Pirineo Occidental, Aragón, Huesca; Sierra de Chía, N slopes of Casania (2372 m)	1988 m	42°34'05" N 00°24'48" E	MR & AR	5*
10.	CB	05.07.2001	France	Western Alps, Alpes Maritimes; Mercantour, massif of Cime de la Bonette (2802 m), SW slopes of Cime de Voga (2777 m)	2620 m	45°20'26" N 06°49'58" E	MR	5*
11.	CG	07.07.2001	France	Western Alps, Alpes de Savoie; N of Col du Gallibier (2646 m)	2478 m	45°04'13" N 06°24'38" E	MR	5*
12.	CH	24.06.2001	Switzer-land	Western Alps, Valais, Alpes Valaisannes; massif of Grand Chavalard (2899 m), vicinity of Lac Inférieur de Sully	2056 m	46°10'01" N 07°06'07" E	MR	5*
13.	CI	06.07.2001	France	Western Alps, Briançonnais, Queyras; Col d'Izoard (2360 m)	2360 m	44°49'11" N 06°44'09" E	MR	5*
14.	CL	07.07.2001	France	Western Alps, Alpes de Savoie; above Col de Lautaret (2058 m)	2075 m	45°02'09" N 06°24'10" E	MR	5*
15.	CN	14.07.2001	France	Pyrénées Orientales; Massif de Canigou (2784 m), Plas de Cady	2300 m	42°30'30" N 02°26'00" E	PS & AT	4*

16.	CV	06.07.2001	France	Western Alps, Alpes de Haute-Provence; Chaîne de Parpaillon, N of Col de Vars (2111 m)	2139 m	44°32'29" N 06°41'59" E	MR	5*
17.	DF	17.05.1999	Germany	Niederbayern; Siegenburg-Daßfeld	-	[48°43' N] [11°53' E]	MS	3*
18.	DO	16.06.2000	Poland	Southern Poland, Góry Świętokrzyskie Mts.; valley of Wierna Rzeka, vicinity of the villages Bocheniec and Dołki, W of Chęciny	-	50°48'20" N 20°18'30" E	MR	5* (10♦)
19.	ER	11.07.2003	Finland	Region of Helsinki; Erkyla, East from Riihimäki	130 m	60°42'58" N 24°53'10" E	MR & AR	5*
20.	FC	08.07.2001	Italy	Central Alps, Trentino, Dolomiti; Lagorai, Malga di Valmaggiore, vicinity of Forcola di Coldose	2200 m	46°15'31" N 11°37'34" E	PS & AT	5*
21.	FD	08.2001	Norway	Hedmark; Folldal	-	[62°08' N] [10°06' E]	AS	5*
22.	FL	03.08.2001	Switzer-land	Central Alps, Graubünden, Rätische Alpen; E of Fluelapass (2388 m)	1980 m	46°47'11" N 09°55'16" E	MR & AR	5*
23.	FU	02.08.2001	Switzer-land	Central Alps, Wallis/Uri; N of Furkapass (2431 m)	2451 m	46°34'27" N 08°25'01" E	MR & AR	5*
24.	GA	06.08.2003	Norway	Oppland; Jotunheimen, Spiterstulen, E slopes of Galdhøpiggen (2469 m)	1715 m	61°38'49" N 08°23'22" E	MR & AR	5*
25.	GB	09.07.2001	Italy	Western Alps, Alpi Pennine; S of Colle di Gran San Bernardo (2469 m)	2170 m	45°51'47" N 07°09'19" E	MR	5*
26.	HJ	08.2001	Norway	Oppland; Dovrefjell, N of Hjerkinn	-	[62°14' N] [09°33' E]	TB & TM	5*
27.	IS	08.07.2001	France	Western Alps, Alpes de Savoie; Col d'Iseran (2764 m)	2425 m	45°23'37" N 07°02'40" E	MR	5*
28.	JP	23.09.2001	Switzer-land	Central Alps, Graubünden; W of Julierpass (2284 m)	2210 m	46°28'29" N 09°43'17" E	MR	5*
29.	KH	13.06.1999	Germany	Oberpfalz; Köferinger Heide bei Amberg	-	[49°22' N] [11°55' E]	MS	4*
30.	KM	15.05.2000	Poland	Northern Poland, Pojezierze Kaszubskie, Bory Tucholskie; vicinity of the village Kiełpiński Most	-	53°19'20" N 17°59'20" E	MR & AR	2♦
31.	KO	12.09.2000	Poland	Western Carpathians, High Tatra Mts.; ridge between Koszysta (2192 m) and Mała Koszysta (2013 m)	2050 m	49°14'35" N 20°03'05" E	MR	5*
32.	KR	07.10.2003	Czech Republic	Krkonoše; Obří důl, Čertová zahrádka (samples come from plants put in culture in the garden of KrNaP administration in Vrchlabi)	[1100 m]	[50°44' N] [15°43' E]	JZ	5*

33.	LA	13.07.2003	Finland	Region of Lappeenranta; Lappeenranta, Pontus, Pontuksenkaivanto	95 m	61°04'44" N 28°18'15" E	MR & AR	5*
34.	LB	19.07.2001	France	Pyrénées Orientales; massif of Puig Carlit (2921 m), plateau du Lac des Bouillouses	2163 m	42°33'49" N 01°59'07" E	MR & AR	5*
35.	ML	23.07.2001	France	Massif Central; Mont Lozère (1699 m), near Col de Finiels	1526 m	44°25'56" N 03°45'43" E	MR & AR	5*
36.	MM	24.07.2001	France	Massif Central, Monts du Vivarais; Mont Mézenc, pass between two culminations	1716 m	44°54'45" N 04°11'23" E	MR & AR	5*
37.	MV	03.07.2001	Italy	Western Alps, Alpi Cozie; massif of Monte Viso (3841 m), E slopes of Punta Barraco (3237 m)	2672 m	44°39'22" N 07°06'14" E	MR, PS & AT	5*
38.	NE	21.07.2001	France	Pyrénées Centrales; massif de Néouvielle (3091 m), vicinity of Lac d'Aubert	2170 m	42°50'23" N 00°08'38" E	MR & AR	5*
39.	NK	30.08.2001	Austria	Eastern Alps, Osttirol, Hohe Tauern; SW ridge of Nussingkogel (2991 m) above Matrei in Osttirol	1995 m	47°01'55" N 12°32'48" E	MR & PS	5*
40.	OG	05.09.2001	Austria	Eastern Alps, Tirol, Oetztaler Alpen; Oetztal, above Obergurgl	2016 m	46°51'55" N 11°01'32" E	MR	5*
41.	OH	29.04.2001	Switzer-land	Western Alps, Wallis, Walliser Alpen; environs of Visp, vicinity of Bürchen-Zeneggen, Oberi Hellela	1600 m	46°16'25" N 07°49'57" E	MR & AR	5*
42.	OS	23.06.2001	Poland	Northern Poland, Pojezierze Kaszubskie, Bory Tucholskie; vicinity of the village Osie	-	53°37'45" N 18°20'55" E	MR & AR	5* (6♦)
43.	PA	13.07.2003	Finland	Region of Lappeenranta; Pajari, between Kouvola and Luumäki	110 m	60°54'19" N 27°15'49" E	MR & AR	5*
44.	PB	08.07.2001	Italy	Western Alps, Alpi Graie; E of Colle di Piccolo San Bernardo (2188 m)	2116 m	45°41'31" N 06°53'22" E	MR	5*
45.	PC	25.07.2001	France	Massif Central, Monts du Cantal; massif of Plomb du Cantal, E of Prat-de-Bouc	1429 m	45°03'11" N 02°48'00" E	MR & AR	5*
46.	PE	18.07.2001	Andorra	Pyrénées Orientales; NE of Port d'Envalira (2430 m)	2469 m	42°32'35" N 01°43'15" E	MR & AR	5*
47.	PP	14.07.2001	Spain	Cordillera Cantàbrica, Cantabria; massif of Peña Prieta (2536 m), ridge between Peña Prieta and Puerto de San Glorio	2192 m	43°02'58" N 04°44'14" W	MR & AR	5*
48.	PW	07.1999	Germany	Oberbayern; Pupplinger Au/Wolfratshausen	-	[47°52' N] [11°26' E]	MS	5*

49.	RE	25.08.2001	Austria	Eastern Alps, Steiermark, Wölzer Alpen; SE ridge of Rettlkirchspitze (2475 m)	2415 m	47°15'21" N 14°07'35" E	PS	5*
50.	SA	14.07.2003	Finland	Region of Savonlinna; Savonlinna, Inkerinkylä	95 m	61°51'13" N 28°56'53" E	MR & AR	5*
51.	SG	19.05.2000	Poland	Northern Poland, Pojezierze Kaszubskie, Bory Tucholskie; vicinity of the village Struga	-	53°46'45" E 18°02'50" N	MR & AR	2*
52.	SH	31.05.1999	Germany	Niederbayern; Sandharlandener Heide	-	[48°47' N] [11°55' E]	MS	2*
53.	SK	21.07.2002	Poland	Northern Poland, Pojezierze Kaszubskie, Bory Tucholskie; vicinity of the village Sokole-Kuźnica	-	53°24'50" N 17°56'20" E	MR & AR	5*
54.	SKR	15.09.2000	Poland	Western Carpathians, High Tatra Mts.; N slopes of Skrajna Turnia (2096 m)	1880 m	49°13'25" N 19°59'55" E	MR	5*
55.	SR	01.09.2001	Austria	Eastern Alps, Kärnten, Gurktaler Alpen; NW ridge of Schoberriegel (2150 m) above Turracher Höhe (1783 m)	2115 m	46°54'50" N 13°53'19" E	MR, PS & AT	5*
56.	ST	29.09.2001	Austria	Eastern Alps, Osttirol, Defereggen Gebirge; N of Stallersattel (2052 m)	2150 m	46°53'32" N 12°11'52" E	MR	5*
57.	SV	08.2001	Norway	Oppland; Dovrefjell, S of Svåni	-	[62°17' N] [09°21' E]	EF	5*
58.	TV	13.06.1999	Germany	Oberpfalz; Trasgschieß bei Vohenstrauß	-	[49°37' N] [12°23' E]	MS	4*
59.	US	16.05.2000	Poland	Northern Poland, Pojezierze Kaszubskie, Bory Tucholskie; vicinity of the village Ustroń	-	53°45'30" N 18°01'20" E	MR & AR	5* ♦
60.	VB	30.07.2001	Switzer-land	Central Alps, Ticino, Alpi Ticinese; Val Bedretto, below Nufenenpass (2478 m)	2022 m	46°28'23" N 08°25'30" E	MR & AR	5*
61.	VH	10.08.2001	Denmark	Jylland; S of Holstebro,Vind Hede	90 m	56°15'45" N 08°30'31" E	MR & AR	5*
62.	VK	19.09.2000	Slovakia	Western Carpathians, High Tatra Mts.; E slope of Vyšne Koprovské Sedlo (2180 m)	2080 m	49°10'22" N 20°03'08" E	MR & AR	5*
63.	WC	17.09.2000	Poland/ Slovakia	Western Carpathians, High Tatra Mts.; S of Wrota Chałubińskiego pass (2022 m)	2025 m	49°11'32" N 20°02'48" E	MR & AR	5*

[1] Most coordinates were taken directly in the field using GPS system. Coordinates taken from maps are given in square brackets.

[2] Collectors: AR – Anna Ronikier; AS – Anne-Cathrine Scheen; AT – Andreas Tribsch; EF – Eli Fremstad; JH – Janusz Hereźniak; JZ – Jitka Zahradníková; MR – Michał Ronikier; MS – Martin Scheuerer; PK – Philippe Küpfer; PS – Peter Schönswetter; TB – Tore Berg; TM – Thomas Marcussen.

[3] Symbols after numbers of samples indicate the kind of analysis performed: * – PCR-RFLP and sequencing; ♦ – AFLP.

4.2. DNA EXTRACTION FROM PLANT MATERIAL

Approximately 10 mg of plant tissue (dry weight) per sample was taken for isolation of DNA. The material was first mechanically disrupted either manually or automatically. In the first case the tissue was deep-frozen by submerging in liquid nitrogen and grinded in a cold ceramic mortar and pestle. In the latter case tissue was put into 1.5 ml Eppendorf tubes together with 3-mm tungsten balls and disrupted using an automated mill (Mixer Mill 200, Retsch) during 2 minutes. Special care was taken to grind the tissue into a fine powder, as effective disruption is one of key steps for a high yield of DNA. The whole genomic and organellar DNA was extracted from the tissue powder using the Qiagen Dneasy Plant Mini Kit (Qiagen 2001). Extraction was performed according to a slightly modified manufacturer's protocol (proportions of chemicals given for 1 sample):

1. The grinded material was incubated 20 min. at 65°C with 400 µl of the detergent lysis buffer (buffer AP1) and 4 µl of RNase A, in order to disintegrate cell membranes (and release DNA), degrade RNA and block oxydation of secondary compounds.
2. 130 µl of the precipitation buffer (buffer AP2) was added to the lysate and the mixture was incubated 5–10 min. on ice, in order to precipitate proteines and polysaccharides. After this time the mixture was centrifuged for 5 min. at maximum 13 000 rpm to spin down the precipitate.
3. Supernatant from the previous step was applied to a QIAshredder spin column with a microsieve and centrifuged for 2 min. at 13 000 rpm, in order to remove remaining cell debris from the lysate.
4. The flow-through from the previous step was transferred to a new 1.5 ml Eppendorf tube and mixed with 1.5 volume of the binding buffer (buffer AP3); usually 400–450 µl of the lysate was mixed with 675 µl of the buffer. The binding buffer contains a high concentration of chaotropic salts and ensures an environment in which DNA has a reversible affinity to siliceous membrane.
5. The mixture from the previous step was applied (in two rounds) onto the DNeasy mini spin column with a siliceous membrane and centrifuged for 1 min. at 8 000 rpm; DNA was bound on the membrane.
6. The DNA bound on the membrane was washed by applying 500 µl of the ethanol-based washing buffer (buffer AW), incubating for 5 min. at room temperature and centrifuging for 1 min. at 8 000 rpm. This step was repeated twice. After last washing

membranes with DNA were air-dried from ethanol residues by centrifuging 2 min. at 13 000 rpm.

7. 50 µl of the elution buffer (buffer AE) preheated at 65°C was placed onto the centre of the siliceous membrane; after 10–15 minutes of subsequent incubation at the room temperature the columns were centrifuged for 1 min. at 8 000 rpm. The elution step was then repeated with another 50 µl of the elution buffer in order to obtain 100 µl of the final DNA extract.

8. Quality and quantity of DNA extract was routinely checked in electrophoresis in 1% agarose gels (containing Ethidium Bromide for DNA visualisation) and compared with a 1 kb DNA Ladder (Fermentas) and a 2.5–100 ng dilution series of λ-DNA.

The extracted DNA was stored frozen at -20°C.

For use in AFLP experiments requiring very clean DNA, different procedures of additional purification were tested. Finally, extracts were purified using the Wizard DNA Clean-up system (Promega). The procedure exactly followed the manufacturer's protocol and included mixing sample with 1 ml of the DNA Clean-up resin, applying the mix on the Wizard Minicolumn, washing the DNA bound in the minicolumn with 2 ml of 80% isopropanol, drying the resin by 2 min. of maximum-speed centrifugation and elution of DNA with 50 µl of prewarmed (65°C) elution buffer, by centrifugating 20 sec. after 1 min. incubation at room temperature.

4.3. PCR-RFLP METHODOLOGY

4.3.1. Choice of cpDNA regions and parameters of PCR

A complex set of published universal primers was tested for their amplification ability in the cpDNA genome of *Pulsatilla vernalis*, in order to analyse regions from different parts of the circular chloroplast genome (Grivet et al. 2001). Classical universal primers amplifying the non-coding trnL–trnF regions (Taberlet et al. 1991) being the base of most phylogenetic analyses so far (Shaw et al. 2005) and known to give also a high intraspecific variability in some species (eg. *Draba* – Widmer & Baltisberger 1999) were first used. All primer pairs designed by Demesure et al. (1995) and used with success in a number of intraspecific phylogeographic works were tested. Two primer pairs designed by Dumolin-Lapegue et al. (1997) were also assessed. The screening set was completed by an additional primer pair reported by Shaw et al. (2005) to be statistically the most variable among different groups of

plants and thus the most appropriate for intraspecific studies. In total, 13 universal primer pairs amplifying different regions were tested (Table 4.2).

Table 4.2. Primer pairs used for tests of amplification of cpDNA regions in *Pulsatilla vernalis*.

No	Name[1]	Primer forward sequence (5'→3')	Primer reverse sequence (5'→3')	Bp[2]	Ref.[3]
1	*trn*H–*trn*K	ACGGGAATTGAACCCGCGCA	CCGACTAGTTCCGGGTTCGA	1690	A
2	*trn*K–*trn*K	GGGTTGCCCGGGACTCGAAC	CAACGGTAGAGTACTCGGCTTTTA	2580	A
3	*trn*C–*trn*D	CCAGTTCAAATCTGGGTGTC	GGGATTGTAGTTCAATTGGT	3000	A
4	*trn*D–*trn*T	ACCAATTGAACTACAATCCC	CTACCACTGAGTTAAAAGGG	1800	A
5	*psb*C–*trn*S	GGTCGTGACCAAGAAACCAC	GGTTCGAATCCCTCTCTCTC	1680	A
6	*trn*S–*trn*fM	GAGAGAGAGGGATTCGAACC	CATAACCTTGAGGTCACGGG	1700	A
7	*psa*A–*trn*S	ACTTCTGGTTCCGGCGAACGAA	AACCACTCGGCCATCTCTCCTA	3700	A
8	*trn*S–*trn*T	CGAGGGTTCGAATCCCTCTC	AGAGCATCGCATTTGTAATG	1500	A
9	*trn*M–*rbc*L	TGCTTTCATACGGCGGGAGT	GCTTTAGTCTCTGTTTGTGG	2900	A
10	*trn*L–*trn*F	CGAAATCGGTAGACGCTACG	ATTTGAACTGGTGACACGAG	1015	B
11	*trn*K2–*trn*Q	TAAAAGCCGAGTACTCTACCGTTG	CTATTCGGAGGTTCGAATCCT	3075	C
12	*trn*Q–*trn*R	GGGACGGAAGGATTCGAACC	ATTGCGTCCAATAGGATTTGAA	3086	C
13	*rpo*B–*trn*C	CKACAAAAYCCYTCRAATTG	CACCCRGATTYGAACTGGGG	1280	D

[1] Forward/reverse primer anchoring region.
[2] Approximate expected length, based on data published for other species in cited works.
[3] A – Demesure et al. (1995); B – Taberlet et al. (1991); C – Dumolin-Lapegue et al. (1997); D – Shaw et al. (2005).

Amplification of regions was tested on 4-sample screening sets composed of single samples from four populations. Different conditions of both chemical composition of the PCR mix and thermal parameters of cycling were tested. PCR conditions described by Demesure et al. (1995) were taken as a basic procedure. If necessary, modifications were introduced. The following items were modified in PCR mix: (i) concentration of nucleotides (dNTPs), (ii) concentration of primers, (iii) concentration of Taq DNA polymerase, (iv) concentration of DNA template in the reaction, (v) addition of different concentrations of chemicals enhancing the reaction or stabilizing the polymerase, as BSA (Bovine Serum Albumine), Qiagen's Q-solution, DMSO (Dimethyl sulphoxide). In the PCR cycling conditions the optimal annealing temperature (T_A) was empirically tested for each DNA fragment using the gradient option of thermal cycler (MJ Research PTC-200 Gradient Thermal Cycler), in a gradient of 10°C around T_A published in original papers, with a 2°C step. The elongation time was always adapted to the expected length of the fragment (1 min./1000 bp on average). A special procedure was applied for amplification of longer PCR products (>2500 bp) in case the standard protocol was not successful (Qiagen 2000). This approach was based on two main

features. First was lowering the time of exposition of mix to the high temperatures, in order to limit the degradation of the DNA template in the long PCR reaction by depurination, which is negligeable in short products but can become a serious problem for longer products (Lindahl & Nyberg 1972), and also to limit the heat-desactivation of the polymerase. Second was a gradual extension of the elongation time, to compensate for decrease of dNTPs in the reaction mix in the later cycles of the amplification.

The composition of the basic PCR reaction mix (per sample) included:

ddH_2O	9.80 µl
PCR buffer (10 x)	1.25 µl
$MgCl_2$ (25 mM)	1.00 µl
dNTP (10 mM each)	0.10 µl
Primer 1 (25 µM)	0.10 µl
Primer 2 (25 µM)	0.10 µl
BSA (10 ng/µl)	0.05 µl
Taq DNA polymerase (5 U/µl)	0.10 µl
Σ	12.50 µl
+ DNA extract (1–3 ng/µl)	0.50–1.00 µl

For longer PCR products a mix enriched in dNTPs, BSA and Taq polymerase was used, to ensure the appropriate amount of material for reaction and to compensate the heat-inactivation of enzyme in long reaction:

ddH_2O	9.40 µl
PCR buffer (10 x)	1.25 µl
$MgCl_2$ (25 mM)	1.00 µl
dNTP (10 mM each)	0.25 µl
Primer 1 (25 µM)	0.10 µl
Primer 2 (25 µM)	0.10 µl
BSA (10 ng/µl)	0.10 µl
Taq DNA polymerase (5 U/µl)	0.30 µl
Σ	12.50 µl
+ DNA extract (1–3 ng/µl)	0.50–1.00 µl

In difficult amplifications different concentrations of the initial amount of DNA template were tested, aiming at reducing the influence of PCR inhibitors present in the DNA extract. In some cases also trials of chemical amelioration of the amplification mix were done, in order to facilitate the amplification of templates potentially having complex secondary structure or GC-rich sequence (eg. Choi et al 1999). Q-solution (Qiagen) was applied at one 20% final concentration, according to the manufacturer's suggestions. DMSO was tested in a concentration gradient: 1%–2.5%–5%.

General conditions of thermal cycling described by Demesure et al. (1995) were applied as a basic protocol:

4'	/	94°C	
45"	/	92°C	35×
45"	/	T_A*	
E**	/	72°C	
10'	/	72°C	
∞	/	4°C	

* Annealing temperature empirically tested for each primer pair.
** Elongation time depending on the length of the amplicon (1 min. for each 1000 bp).

For longer PCR products the cycling conditions were applied as follows:

1'	/	94°C	
10"	/	94°C	10×
1'	/	T_A	
E	/	68°C	
10"	/	94°C	25×
1'	/	T_A	
E*	/	68°C	
10'	/	68°C	
∞	/	4°C	

* Increasing by 10 sec. per cycle.

Results of all amplifications were routinely tested by electrophoresis in 1% agarose gels, with 1 kb DNA Ladder (Fermentas) as reference.

4.3.2. Digestion of amplicons with restriction enzymes and electrophoretic separation of restricted fragments

Fragments of cpDNA amplified in PCR reactions were subsequently digested with restriction enzymes. The first stage of work involved screening of different restriction enzymes in order to find appropriate enzymes for analysis of genetic polymorphism in each DNA fragment. The screening was done on a subset of samples including 13 individuals from most divergent populations, covering the entire distribution range of *Pulsatilla vernalis* (populations: BL, CA, CB, CG, FD, KO, MM, MV, PP, RE, SR, SV, VK).

First, presence or absence of cutting by different enzymes were checked, then potential polymorphism of restriction profiles was analysed. Ten enzymes (from Amersham

Biosciences or NE Biolabs) recognizing different four- five- or six-nucleotide sequences were tested:

a) "four-cutters": *Afa* I (=*Rsa* I), *Alu* I, *Hae* III, *Hap* II (=*Hpa* II), *Hha* I, *Mse* I

b) "five-cutters": *Hinf* I

c) "six-cutters": *Bam* H I, *Eco* R I, *Hin*d III

Approximately 50 ng of PCR product were used for digestion. The reaction was carried out according to conditions recommended by enzyme manufacturer. The reaction mix (per sample) was as follows:

ddH2O	11.10 µl
Enzyme-specific buffer*	2.00 µl
BSA	0.60 µl
Restriction enzyme (10 U/ µl)**	0.30 µl
Σ	14 µl
+ DNA fragment	3 µl (~50 ng)

* Provided by manufacturer.
** Adjusted accordingly for other enzyme concentrations (8 U/µl, 15 U/µl) and balanced by amount of H_2O.

Reaction mix was incubated at least 4 hours (but usually overnight) at 37°C, optimal temperature for activity of all enzymes used. Products of digestions were separated in electrophoresis on 8% polyacrylamide gels, performed in the dual cooled vertical electrophoresis system Hoefer SE 600 coupled with the MultiTemp III Thermostatic Circulator and EPS 3501 XL Power Supply (all from Amersham Biosciences). The mix for one set of four gels included:

dH_2O	61.00 ml
Acrylamide (40% solution)	17.10 ml
Bisacrylamide (2% solution)	9.40 ml
TBE buffer (5x)	22.00 ml
Glycerine	6.00 ml

The solution was mixed in a vacuum-filter recipient and degazed using a pump for 10 min. After degazing 528 µl of 10% Ammonium Persulfate (APS) and 88 µl of TEMED, factors initiating the polymerization, were added, after which gels were immediately poured into glass sandwiches (Weising et al. 1994). 28-well Teflon combs were used and gels were polymerizing during 1 hour in presence of light (lamps on both sides).

In order to ensure comparable migration conditions across runs and eveness of migration across gels, electrophoresis was always carried out at a constant temperature of 4°C. The electrophoresis included an initial step of 30 min./250 V and second step of 3,5–5 h/400 V

(time depending on length of fragments). In each gel, lanes with GeneRuler 100 bp DNA Ladder and 1 kb DNA Ladder (Fermentas) were added as size reference. Loading dye for electrophoresis included mixture of 1% Bromophenol blue and Xylene cyanole. After electrophoresis, gels were stained in baths with Ethidium Bromide (0,8 µl/ml) during 10 min. and visualized on UV-tray in a GeneGenius system (Syngene).

4.3.3. Analysis of PCR-RFLP data

Digital pictures of UV-visualised gels from the GeneGenius system camera were archivized in the GeneSnap ver. 6.00.26 software (Syngene) as computer files. Length of uncut PCR products and of restricted fragments were estimated against the DNA ladders using the GeneTools ver. 3.02.00 software (Syngene). Special care in analysis of restriction profiles was made to detect and exclude potential "partial digests" being major source of potential artefacts (Dowling et al. 1996). Restriction polymorphisms and length polymorphisms (indels) were detected across all the data set and all samples were analysed with respect to each polymorphism using the GeneTools (Syngene) and CorelDraw ver. 11 (Corel Corp.) softwares. Polymorphisms were scored as present (1) or absent (0) and an overall presence/absence matrix was created. A minimum spanning tree was calculated from the data matrix using the NTSYS-pc software (Rohlf 1997). Due to the simple pattern of genetic diversity, no particular statistical treatment was applied in the phylogeographical analysis.
Basic estimates of the genetic variability of populations (within- and among populational distribution of cpDNA haplotypes) and their differentiation were calculated. Values of the haplotype frequency, average within-population gene diversity (h_S) and the total genetic diversity in the pooled populations (h_T) were calculated according to Pons & Petit (1995). Based on these values, the statistic G_{st} was calculated, allowing an estimation of genetic differentiation over all populations (Pons & Petit 1995, Culley et al. 2002). The software Haplodiv (Petit 1995) was used for calculation of these values.

4.4. DNA SEQUENCING METHODOLOGY

4.4.1. Choice of DNA regions and PCR parameters

DNA sequencing method was applied to obtain data from chloroplast DNA regions (complementary data to those obtained for cpDNA in PCR-RFLP) as well as data from nuclear DNA regions.

Three chloroplast DNA regions were tested, to supplement the data set obtained from PCR-RFLP and to make comparative analysis between both methods (cf. chapter 3). Regions belonging to the most widely used were chosen. One region constituted the fragment of the *trn*L–*trn*F region, namely the intergenic spacer between *trn*L (UAA) 3' exon and *trn*F (GAA) (Taberlet et al. 1991), included in the fragment amplified also for PCR-RFLP to directly compare this region. Additionally, the region *psb*A–*trn*H (being a part of *trn*H–*trn*K used for PCR-RFLP) and *trn*S–*trn*G (Hamilton 1999) were used (Table 4.3).

In case of the nuclear DNA, the internal transcribed spacer (ITS) region of ribosomal nuclear DNA (the 18S–5.8S–26S nuclear ribosomal cistron) was taken as the main source of information. Ribosomal DNA is by far the most often used nuclear region for inference of phylogenetic relationships, due to universality of available primers and presence of a high number of copies of this region in the genome. The evolution of this region is not yet well known and has several unpredictable features; it has recently been pointed out that it might not always be reliable for inference of evolutionary relationships (Álvarez & Wendel 2003). This risk is lower, however, when a low taxonomical level is involved, and especially for intraspecific analyses. Here, nevertheless, the degree of polymorphism can be not satisfactory in many groups of plants (Soltis & Soltis 1998). The primer ITS1A (= primer ITS5 from White et al. [1991] slightly modified as described by Fuertes Aguilar et al. [1999]) located in the 18S rDNA region and primer ITS4 (White et al. 1991) located in the 26S rDNA region, were used for amplification of the entire ITS1–5.8S–ITS2 sequence (Table 4.3).

Table 4.3. Primer pairs used for amplification of DNA regions for sequencing.

No	Name	Primer forward sequence (5'→3')	Primer reverse sequence (5'→3')	Bp[1]	Ref.[2]
Nuclear regions					
1	ITS	GGAAGGAGAAGTCGTAACAAGG	TCCTCCGCTTATTGATATGC	650	A, B
2	*G3pdh*	GATAGATTTGGAATTGTTGAGG	AAGCAATTCCAGCCTTGG	1000	C
Chloroplast regions					
3	*trn*L–*trn*F	GGTTCAAGTCCCTCTATCCC	ATTTGAACTGGTGACACGAG	438	D
4	*trn*H–*psb*A	ACTGCCTTGATCCACTTGGC	CGAAGCTCCATCTACAAATGG	495	E
5	*trn*S-*trn*G	GCCGCTTTAGTCCACTCAGC	GAACGAATCACACTTTTACCAC	844	E

[1] Approximate expected length, based on data published for other species in cited works.
[2] A – Fuertes Aguilar et al. (1999), B – White et al. (1991); C – Strand et al. (1997); D – Taberlet et al. (1991); E – Hamilton (1999).

Additionally to the ITS region, attempts were made to obtain sequences of single-copy nuclear genes. They are more difficult to amplify but can give the most robust information

from biparentally inherited DNA genome, as they don't share potential ambiguities of the ITS (Álvarez & Wendel 2003). The gene of Glyceraldehyde 3-phosphate dehydrogenase (*G3pdh*) was choosed, as it was successfully amplified in all species from different systematic groups used in initial tests of primers, including *Aquilegia*, a member of Ranunculaceae (Strand et al. 1997).

PuRE Taq Ready-To-Go PCR beads (Amersham Biosciences) were used for preparation of PCR mix. 22 µl of ddH_2O, 0,5 µl of each primer (10 µM), 1 µl DMSO and 0,5–1 µl of the DNA extract were added to the beads and reaction mix was applied to thermocycling after 5 min. incubation at room temperature.

Basic cycling program was based on general recommendation of PCR Beads manufacturer:

5'	/	95°C	
45"	/	95°C	35–40×
1'	/	T_A*	
E**	/	72°C	
10'	/	72°C	
∞	/	4°C	

* Annealing temperature empirically tested for each primer pair.
** Elongation time depending on the length of the amplicon (1 min. for each 1000 bp).

In case of each fragment, annealing temperature, elongation time and number of PCR cycles were individually adapted.

In case of the ITS region a touchdown PCR procedure (Weising et al. 1994) was applied to increase specificity of amplification:

5'	/	95°C	
30"	/	95°C	
30"	/	58°C*	20×
1'	/	72°C	
30"	/	95°C	
30"	/	52°C	20×
1'	/	72°C	
10'	/	72°C	
∞	/	4°C	

* Decrease by 0.5°C per cycle.

Results of amplifications were routinely checked in electrophoresis on 1% agarose gels. After successful PCR, amplicons were always purified using Qiaquick PCR Purification Kit (Qiagen) prior to sequencing reaction. The purification procedure was carried our according

to slightly modified manufacturer's protocol and included mixing the PCR product with 5 volumes (500 µl) of binding buffer (PB buffer), binding DNA on columns with silicaceous membrane by centrifigation 1 min. at maximum speed, washing twice with 750 µl of ethanol-based PE buffer (with subsequent 1 min. centrifugation), drying the membrane by additional 1 min. of centrifugation, and elution with 50 µl of the elution buffer (buffer EB) prewarmed at 65°C, after 5 min. of buffer incubation on membrane.

4.4.2. Sequencing PCR and detection of sequences in the automated sequencer

Purified amplicons served as templates for the subsequent sequencing reaction performed using the dideoxy-chain termination method (Sanger et al. 1977). Sequencing was performed using the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems). The reaction mix per one sample, with use of the sequencing buffer, contained: 4 µl of Reaction Premix (2,5 x), 2 µl of BigDye Sequencing Buffer (5 x), 6,5 pmol of primer (0,65 µl of 10 µM solution), 5 µl of the DNA template, and ddH_2O up to 20 µl. PCR was carried out in the GeneAmp PCR System 9700 (Applied Biosystems), using the ramping speed of 1°C/sec. The program of thermal cycling followed suggestions of manufacturer:

1'	/	96°C	
10"	/	96°C	25×
5"	/	50°C	
4'	/	60°C	
∞	/	4°C	

Products of the cycle sequencing were purified in order to remove unincorporated dye terminators from the reaction mix prior to electrophoresis. Purification was performed using the ethanol/sodium acetate precipitation method (Sambrook & Russell 2001). 20 µl of sequencing reaction product was mixed with 2 µl 3M NaOAc pH 4.6 and 50 µl 95% ethanol, incubated 20 min. at room temperature and centrifuged 20 min. at 13 000 rpm, after which the solution was drained off using a pipette. The DNA pellet was washed with 250 µl 70% ethanol with subsequent centrifugation (5 min. at 13 000 rpm) and draining off the ethanol. The clean DNA pellet was dried under fume hood (30 min.) and stored frozen before resuspension in formamide for electrophoresis.

Electrophoresis was carried out on the ABI Prism 3100-*Avant* Genetic Analyzer (Applied Biosystems) using 36-cm capillary arrays with the POP-4 polymer or 50-cm capillary array with POP-6 polymer.

Five-sample sets were used to test amplification and sequencing of DNA regions. Thirteen-sample sets were used to test the polymorphism among sequences (see chapter 4.3.2. for the composition of the screening set). At the stage of screening all fragments were sequenced in both forward and reverse directions. For the overall analyses, in case of clear sequences only one strand was sequenced.

4.4.3. Analysis of DNA sequence data

Sequences were analysed and aligned in the BioEdit 5.0.9 software (Hall 1999). When two strands were sequenced, forward and reverse sequences were compared (after complement transformation of reverse sequence) in order to obtain a consensus sequence. All sequences were aligned manually to seek for mutations. Polymorphisms (point mutations and indels) were scored in the 0/1 way and incorporated into a presence-absence matrix and further analysed in the same way as the PCR-RFLP data. Sequences of all obtained genotypes were submitted to the NCBI GenBank database.

4.5. AFLP METHODOLOGY

4.5.1. AFLP procedure

The general procedure of AFLP analysis followed Vos et al. (1995). The whole genomic extract of DNA (approximately 50 ng/µl) was used for digestion with restriction enzymes *Eco* RI and *Tru* 9I (=*Mse* I). The reaction mix was prepared as follows:

ddH_2O	33.50 µl
Enzyme-specific buffer (10x)	5.00 µl
Tru 9I (10 U/µl)	0.50 µl
Eco RI (40 U/µl)	0.50 µl
BSA (10 mg/ml)	0.50 µl
Σ	40 µl
+ DNA extract	10 µl

The reaction mix was incubated for 3 hours at 37°C, with following desactivation of enzymes (15 min. at 70°C), in a thermal cycler.

Eco RI and *Mse* I double strand adapters for subsequent ligation (Table 4.4) were prepared by mixing respective pairs of oligonucleotides (40 µM each) at equal rates and diluting twice with ddH_2O to obtain a final concentration of 10 µM. Mix was then submitted to a denaturation/gradual renaturation to ensure correct oligonucleotide pairing, using a

thermocycler program: 2'/95°C > 5'/65°C > 5'/37°C > 5'/25°C > ∞ /4°C. Working solutions of adapters were prepared in aliquots of 50 µl.

Ligation of the adapters to the products of the genomic DNA enzymatic restriction was prepared as follows:

ddH_2O	2.00 µl
T4 DNA Ligase buffer (10x)	5.00 µl
Eco RI Adapter (10 µM)	1.00 µl
Mse I (*Tru* I) Adapter (10 µM)	1.00 µl
T4 DNA Ligase (1 U/µl)	1.00 µl
Σ	10.00 µl
+ *Eco* RI/*Mse* I (*Tru* I) digested genomic DNA	40.00 µl

The reaction mix was incubated at room temperature for 3 hours (according to enzyme supplier's guidelines). Products of ligation were used as templates (Table 4.4) for two rounds of PCR reactions amplifying subsets of restriction fragments.

Preselective amplification using primers *Eco* RI-**A** and *Mse* I-**C(A)** (Table 4.4) and the Promega Taq Polymerase (Promega) was prepared as follows:

ddH_2O	12.80 µl
PCR buffer without Mg^{++} (10 x)	2.00 µl
$MgCl_2$ (25 mM)	1.60 µl
dNTP (10 mM each)	0.50 µl
Eco RI-**A** preselective primer (10 µM)	0.50 µl
Mse I-**C** preselective primer (10 µM)	0.50 µl
Taq DNA polymerase (5 U/µl)	0.10 µl
Σ	18.00 µl
+ Ligated genomic DNA digest	2.00 µl

PCR was performed in the thermocycler using the cycling conditions:

2'	/ 94°C	
45"	/ 94°C	28×
45"	/ 56°C	
1'	/ 72°C	
10'	/ 72°C	
∞	/ 4°C	

Results of the preselective amplification were routinely checked by running electrophoresis of 5 µl of the product in a 1,5% agarose gel. Products of successful amplifications (presence of a smear or faint bands in gel) were 20-fold diluted with ddH_2O prior to second amplification.

Products of the preselective amplification were further used as templates for the selective PCR. The selective amplification using primers *Eco* RI-**ANN** and *Mse* I-**CNN(N)** (Table 4.4) and the Promega Taq Polymerase (Promega) was prepared as follows:

ddH_2O	11.70 µl
PCR buffer without Mg^{++} (10 x)	2.00 µl
$MgCl_2$ (25 mM)	1.60 µl
dNTP (10 mM each)	0.50 µl
Eco RI-**ANN** selective primer (10 µM)	0.50 µl
Mse I-**CNN(N)** selective primer (10 µM)	0.60 µl
Taq DNA polymerase (5 U/µl)	0.10 µl
Σ	17.00 µl
+ Product of preselective PCR	3.00 µl

Eco RI selective primers were 5'-labelled with fluorescent dyes (FAM, HEX or NED) in order to allow further detection in the automated sequencer.

PCR was performed in thermocycler using the cycling conditions:

2'	/	94°C	
30"	/	94°C	13×
30"	/	65°C*	
1'	/	72°C	
30"	/	94°C	20×
30"	/	56°C	
1'	/	72°C	
5'	/	72°C	
∞	/	4°C	

* Decrease by 0,7°C per cycle.

Products of the selective amplification were separated in electrophoresis on the ABI Prism 310 Genetic Analyser (Applied Biosystems). The sample (0.50 µl if labelled with FAM, 0.80 µl and 1.00 µl if labelled with NED and HEX, respectively) was mixed with 12 µl of deionized formamide and 0.50 µl of the GeneScan ROX-500 size standard (Applied Biosystems), heated 2 min. at 95°C for DNA denaturation and immediately cooled on ice. Electrophoresis was performed using 36-cm capillary and the POP-4 polymer (Applied Biosystems). For selective amplification, 32 different primer combinations were tested on a subset of 6 individuals with respect to the number of fragments generated, clarity of profiles and polymorphism of markers. *Eco* RI-selective primers with 3 selective nucleotides only were used, while *Mse* I-selective primers were used with 3 or 4 selective nucleotides. All oligonucleotide sequences are given in the Table 4.4.

Table 4.4. Oligonucleotides used in the AFLP analysis.

Name	Sequence
Adapter *Eco* RI – 1	CTCGTAGACTGCGTACC
Adapter *Eco* RI – 2	AATTGGTACGCAGTCTAC
Adapter *Mse* I – 1	GACGATGAGTCCTGAG
Adapter *Mse* I – 2	TACTCAGGACTCAT
Preselective primer *Eco* RI-A	GACTGCGTACCAATTC**A**
Preselective primer *Mse* I-C(A)*	GATGAGTCCTGAGTAA**C(A)**
Selective primers *Eco* RI-ANN**	E-**AGA**; E-**AGT**; E-**ATC**; E-**ATG**
Selective primers *Mse* I-CNN(N)**	M-**CAA**; M-**CAC**; M-**CAG**; M-**CAT**; M-**CTA**; M-**CTC**; M-**CTG**; M-**CTT**; M-**CAAC**; M-**CACA**; M-**CACG**; M-**CTAC**

* 2-preselective nucleotide primer was used when 4-selective nucleotide primers were subsequently used.
** Sequence as in preselective primers, with additional 2–3 selective bases. Whole set of selective nucleotides given (including those from preselective primers); E- =GACTGCGTACCAATT, M- = GATGAGTCCTGAGTAA

4.5.2. AFLP data analysis

AFLP patterns were visualised and preliminarily assessed using the GeneScan Analysis software 3.1 (Applied Biosystems). The AFLP bands in the whole dataset were scored using the Genographer software (Benham 2001) and binary values were incorporated into the presence/absence matrix. For each population proportions of mono- and polymorphic loci were calculated to check the overall genetic diversity. Private fragments, unique for respective populations, were also noted. The level of genetic diversity was calculated using the average gene diversity index (Nei 1987). Analysis of the molecular variance – AMOVA (Excoffier et al. 1992) was performed to estimate the distribution of genetic diversity within and between populations; these data were obtained using the Arlequin 2.00 software (Schneider et al. 2000). An UPGMA clustering of individuals, based on similarity matrix calculated with the Jaccard's coefficient, was constructed using the NTSYS-pc software (Rohlf 1997) to show relationships between all individuals and populations. One well-structured population from the Central Poland (BE) was used for an additional detailed analysis of within-population genetic structure (relationships between subpopulations). In this case, genetic relationships between individuals, assessed using UPGMA clustering, were analysed in the context of their spatial distribution. A permanent observation plot covering the population and divided into hierarchical squares of 2 × 2 m and 1 × 1 m, designed by J. Hereźniak (Hereźniak et al. 2001), was used to record the location of individuals in the field.

PHYLOGEOGRAPHY OF *PULSATILLA VERNALIS*

5. PHYLOGEOGRAPHY OF *PULSATILLA VERNALIS*

5.1. ANALYSIS OF THE CHLOROPLAST DNA OF *PULSATILLA VERNALIS*

5.1.1. PCR-RFLP analysis of cpDNA

Seven out of thirteen cpDNA fragments tested were successfully amplified: *trn*H–*trn*K, *trn*K–*trn*K, *trn*C–*trn*D, *psb*C–*trn*S, *psa*A–*trn*S, *trn*L–*trn*F, *rpo*B–*trn*C. In case of the *trn*S–*trn*fM region a weak and inconsistent amplification was obtained, but it was not possible to improve it, therefore this region was not further used. Remaining five primer pairs did not yield any amplification. The successfully amplified fragments were evenly distributed in different parts of the chloroplast genome, therefore allowing a test of the variability across all the part of the genome for which universal primers were available from published sources during the analysis (Fig. 5.1). No correlation of amplification failure with fragment length or location in the chloroplast genome was observed. Short fragments (<2000 bp) were successfully amplified with the standard PCR protocol adopted. In case of three longer fragments a special protocol for long PCR products proved to be very useful and yielded the best outcome (Table 5.1). In two cases addition of empirically tested amounts of DMSO improved the reaction efficiency, but in all cases tested an excess of DMSO had a strong inhibitory effect on the amplification. Q-solution (Qiagen) did not improve the PCR in any case tested.

Table 5.1. cpDNA fragments successfully amplified in *Pulsatilla vernalis*. Empirically chosen annealing temperature (T_A) is given for each fragment together with information on amplification details (cf. chapter 4.3.1), as well as the approximate fragment length.

No	Name	T_A	PCR mix/program	DMSO	Length (bp)
1	*trn*H–*trn*K	60°C	Standard	–	1730
2	*trn*K–*trn*K	53.5°C	Long	2,5%	2700
3	*trn*C–*trn*D	58°C	Long	–	3500
4	*psb*C–*trn*S	57°C	Standard	–	1700
5	*psa*A–*trn*S	57°C	Long	2,5%	3700
6	*trn*L–*trn*F	55°C	Standard	–	1050
7	*rpo*B–*trn*C	53°C	Standard	–	1300

All successfully amplified DNA fragments were used for polymorphism screening by restriction cutting of a subset of samples with all ten enzymes. In total, seventy DNA region/restriction enzyme combinations were tested. Seventeen combinations did not result in any restriction. Remaining combinations produced 181 restriction bands in total. No polymorphism was detected in only one fragment – *rpo*B–*trn*C – although the same number

of restriction bands was generated as in other fragments of comparable size. All other regions were polymorphic; between one and four polymorphisms per fragment were found. Interestingly, the degree of genetic polymorphism observed was not correlated with the fragment size. Two most variable regions were among the shortest ones (*trn*H–*trn*K, *trn*L–*trn*F), while the longest fragment (*psa*A–*trn*S) yielded only one polymorphic feature. The number of restriction bands generated, however, was correlated with fragment length as it was intuitively expected. Four fragments smaller than 2000 bp had 15–16 bands, while larger fragments had 20–29 bands.

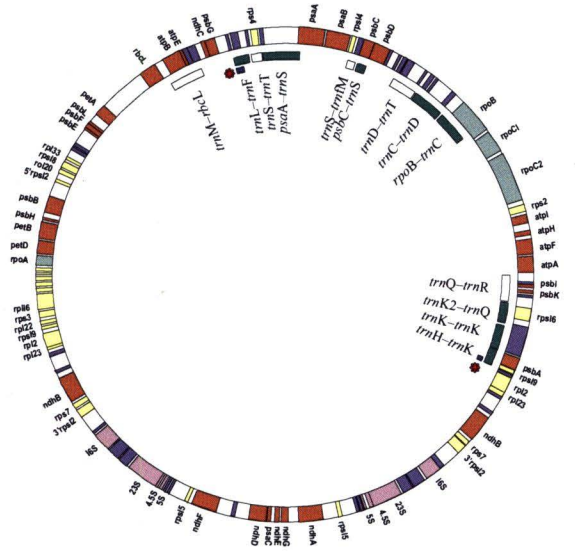


Fig. 5.1. Schematic representation of the 155 kb-circular cpDNA genome of *Nicotiana tabacum* (based on Grivet et al. 2001, modified), with approximate location of regions tested for amplification given inside the circle; successfully amplified fragments used for the PCR-RFLP are marked in green, rejected fragments in white. Two regions used for sequencing analysis are marked in blue and indicated by asterisks; *trn*H–*psb*A is a sequenced part of *trn*H–*trn*K, and *trn*L^{E}-*trn*F is a sequenced part of *trn*L–*trn*F.

Fourteen fragment/enzyme combinations were chosen for the analysis of the entire dataset: *trn*H–*trn*K/*Hae* III, *trn*H–*trn*K/*Hap* II, *trn*H–*trn*K/*Mse* I, *trn*K–*trn*K/*Afa* I, *trn*K–*trn*K/*Bam* HI, *trn*K–*trn*K/*Mse* I, *trn*C–*trn*D/*Hae* III, *psb*C–*trn*S/*Alu* I, *psb*C–*trn*S/*Hae* III, *psb*C–*trn*S/*Mse* I, *psa*A–*trn*S/*Hha* I, *trn*L–*trn*F/*Eco* RI, *trn*L–*trn*F/*Hap* II, *trn*L–*trn*F/*Hha* I. Twelve polymorphisms were detected altogether, among which there were seven different length polymorphisms ("indels") and five restriction site polymorphisms (cf. Fig. 5.2). The analysis of polymorphisms allowed detection of seven haplotypes across all populations of *Pulsatilla vernalis* (Table 5.2). Two main groups can be distinguished among these seven haplotypes after their arrangement in the Minimum Spanning Tree (Fig. 5.3), these groups are separated by as many as five polymorphisms (out of all twelve detected). This major split accounts for 72% of variability, as explained by the first axis of the Principal Coordinates Analysis (data not shown). One group is composed of the most common haplotype of largest geographical distribution (A), and its local derivatives, while the second one seems to be based on the area of the South-Western Alps and Eastern Pyrenees, with a secondary extension to the north.

Table 5.2. Variability of the cpDNA in *Pulsatilla vernalis* based on the PCR-RFLP analysis. Polymorphisms detected in different cpDNA regions are presented in relation to the most common haplotype ($\mathbf{H_C}$= haplotype A) taken as reference. The length polymorphisms ("indels") are presented in terms of length difference between states (in base pairs – **bp**). In case of restriction site polymorphisms the enzyme generating the polymorphism is given along with its recognition sequence, followed by the character state in H_C (number of restriction sites – **res**). Polymorphisms are presented as difference in number of restriction sites. * – the restriction polymorphisms generated by the two enzymes are due to one point mutation (as revealed by analysis of the sequence *trn*H–*psb*A), therefore these two polymorphisms are treated as one. Total numbers of polymorphisms differentiating haplotypes from the H_C are given at the bottom of the table (**Σ**).

cpDNA region	**Character state in the common haplotype (H_C)**	**Polymorphism**	**Haplotypes**						
			A	**B**	**C**	**D**	**E**	**F**	**G**
*trn*H–*trn*K	Length: 1730 bp	+ 10 bp	0	0	0	1	1	1	1
		+ 40 bp	0	0	0	0	0	0	1
		– 5 bp	0	0	1	0	0	0	0
	Hae III: 1 res [GG↓CC]	+ 1 res*	0	0	0	1	1	1	1
	Hap II: no cut [CC↓GG]								
*trn*K–*trn*K	Length: 2700 bp	+ 25 bp	0	0	0	1	1	0	1
	Mse I: 13 res [TT↓AA]	+ 1 res	0	0	0	1	1	1	1
*trn*C–*trn*D	Length: 3500 bp	+ 10 bp	0	0	1	0	0	0	0
*psb*C–*trn*S	*Mse* I: 5 res [TT↓AA]	+ 1 res	0	0	0	1	1	1	1
*psa*A–*trn*S	Length: 3700 bp	+ 20 bp	0	1	0	0	0	0	0
*trn*L–*trn*F	Length: 1050 bp	+ 50 bp	0	0	0	1	1	1	1
	Hap II: no cut [CC↓GG]	+ 1 res	0	0	0	1	0	0	0
	Eco RI: 2 res [G↓AATTC]	– 1 res	0	1	0	0	0	0	0
Polymorphism (Σ)			**0**	**2**	**2**	**7**	**6**	**5**	**7**

The haplotype A is by far the most frequent one (frequency 0.53 in the total dataset), while two most common haplotypes from the second group, D and E, had frequencies of 0.13 and 0.20 respectively. Remaining haplotypes were rare and very restricted spatially. Two of them were present in few neighbouring populations: four (haplotype C – frequency 0.07) or three (haplotype B – frequency 0.05). Haplotypes F and G were found in single populations only, in

five and two individuals respectively (frequencies 0.02 and 0.007). The total gene diversity value h_T was 0.663 (s.e. 0.04930).

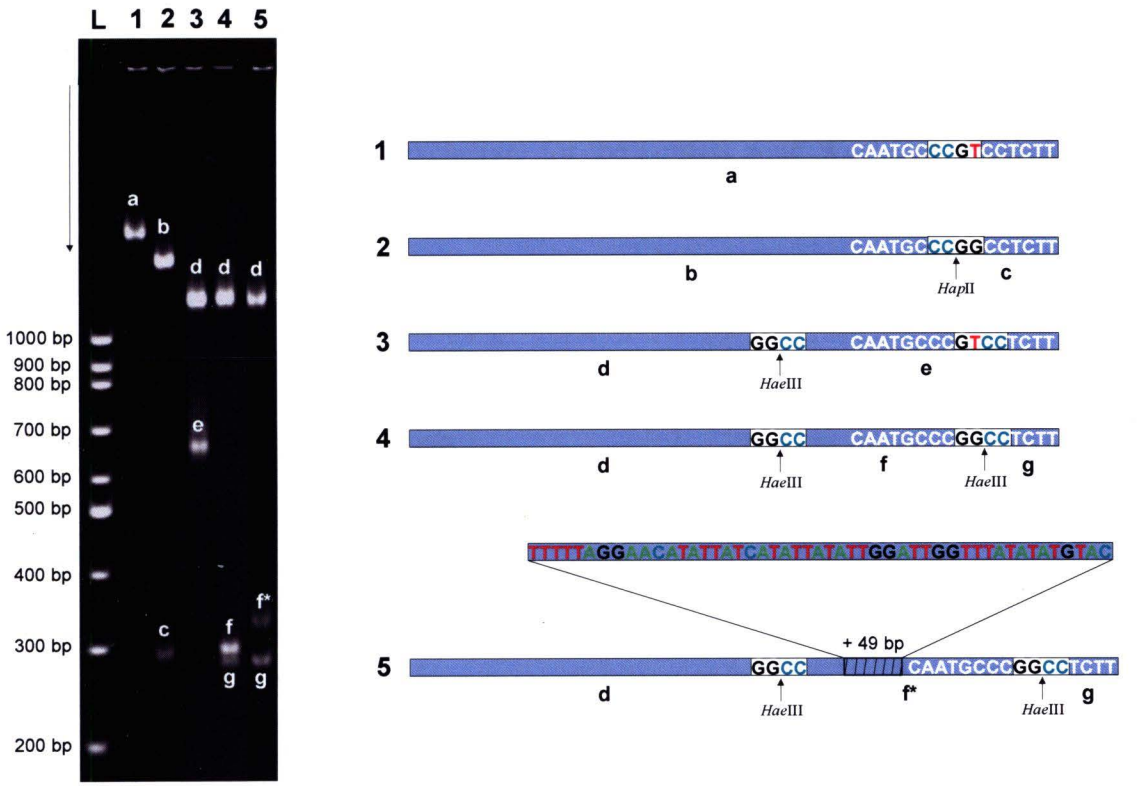


Fig. 5.2. Examples of cpDNA polymorphisms detected in *Pulsatilla vernalis* using PCR-RFLP (the *trn*H–*trn*K region, for which a partial sequence (*trn*H–*psb*A) is available allowing analysis of causes of polymorphism). On the left – selected lanes of a gel with different restriction patterns, on the right, schematic representations of the *trn*H–*trn*K region with mutations responsible for the variability in restriction patterns unravelled by the sequence analysis. The lanes 1 and 2 contain restriction patterns after digestion with the enzyme *Hap* II (recognition sequence: CCGG); lane 1 – lack of digestion due to a point mutation in restriction site, lane 2 – restriction site exists and the region is digested (result: two bands, where b+c = a from the lane 1). The lanes 3, 4 and 5 contain restriction patterns after digestion with the enzyme *Hae* III (recognition sequence: GGCC). One restriction site is present in all samples, and the region is digested (result: two bands, d and e); only this site is present in sample from lane 3. Samples from lanes 4 and 5 have additional restriction site (partly overlapping with that for *Hap* II) in the fragment e and therefore it is further digested into fragments f and g. In sample from lane 3 there is a point mutation supressing this site. Sample from lane 5 additionally has a mutation of "indel" type – insertion of 49 bp within the fragment f, therefore this fragment migrates slower (f*). The restriction site mutation differentiates haplotypes A, B, C (lack of digestion) from D, E, F, G (presence of digestion). The insertion differentiates the haplotype G from all the rest. L – the 100 bp DNA size marker (ladder) with weights of bands given on the left. For exact location and sequence of variable sites cf. also Fig. 5.5.

Pulsatilla vernalis shows a high level of population differentiation. Most populations (approx. 75%) were monomorphic in respect of chloroplast DNA (harbouring only one of cpDNA haplotypes). Only nine populations harboured two different haplotypes (Fig. 5.3). The average within-population gene diversity h_S equalled 0.079 (s.e. 0.02486). The overall G_{st} value, measuring the gene differentiation over all populations, was 0,881 (s.e. 0.03832), which proves the poor genetic exchange between genetic lineages (Pons & Petit 1995). Nevertheless,

in the major contact zones of two main haplotypes some mixt populations were also observed. Such intermediate zones were particularly noticeable at the border between the South-western Alps and the rest of the Alps, in the Eastern Pyrenees as well as in the Polish lowland area. They accounted for the total within-population variability, while in other areas all populations were fixed for single haplotypes. G_{st} values in these contact zones were lower, but still showing relatively small genetic exchange; for the contact zone Western/Central Alps G_{st}= 0.71 and for the lowland area G_{st}=0.83.

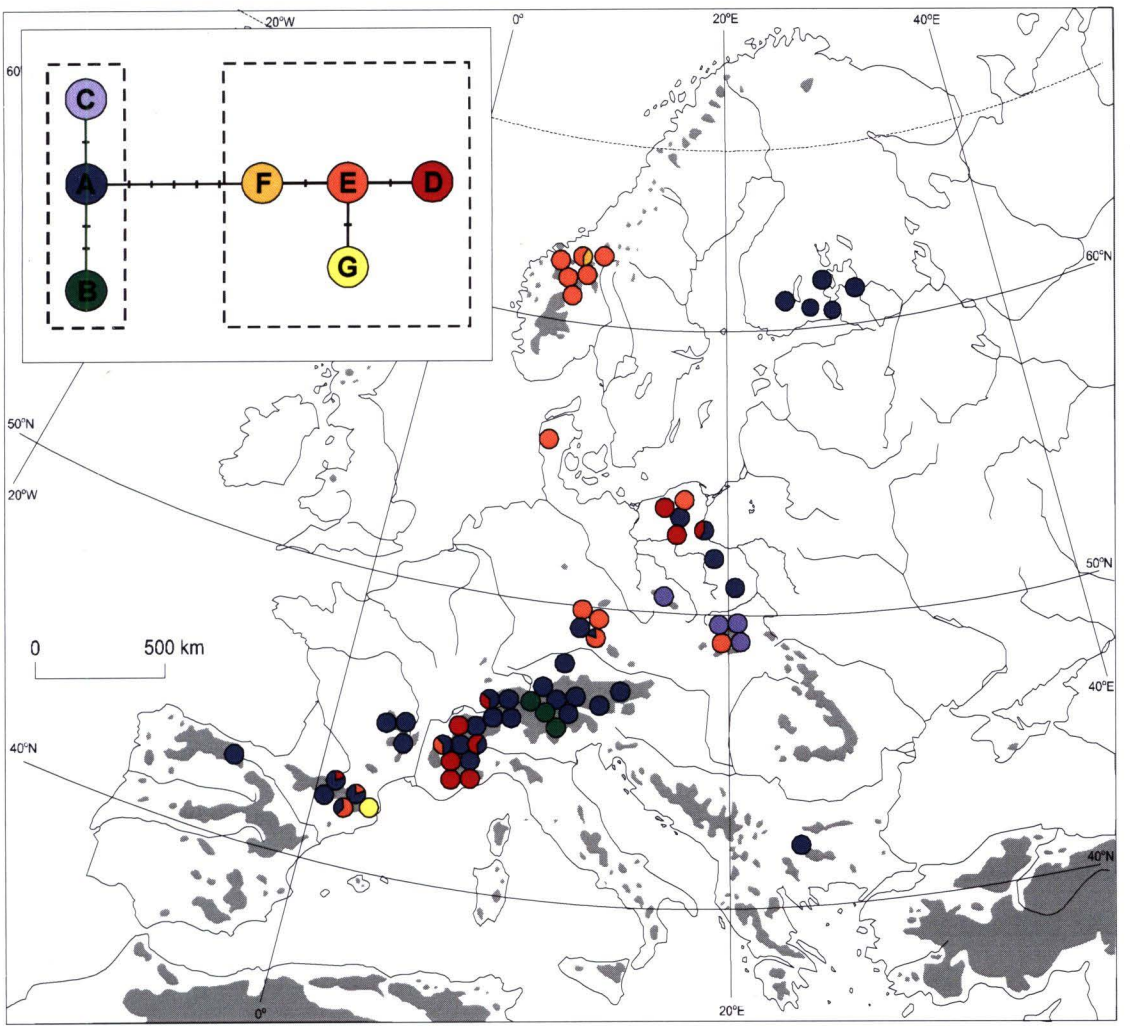


Fig. 5.3. Geographical distribution of cpDNA haplotypes of *Pulsatilla vernalis* revealed by the PCR-RFLP analysis, and the Minimum Spanning Tree of haplotypes (inset) with two major groups of haplotypes embedded with dashed lines. Colours of haplotypes on the map correspond with those indicated in the tree. In case of mixed populations proportion of colours reflects frequency of haplotypes.

5.1.2. Sequencing of cpDNA

Selected regions of the chloroplast DNA (Fig. 5.1) were additionally submitted to sequencing analysis in order to check the reliability and robustness of the PCR-RFLP indirect mutation inference. Two cpDNA regions out of three regions tested were efficiently amplified for the

sequencing analysis: *trn*L^{E}-*trn*F and *trn*H-*psb*A. Their optimized annealing temperatures (T_A) were 50°C and 53°C respectively. These regions belong to the most popular ones in the phylogenetic analyses, therefore they are appropriate for the "model" sequence analysis. On the other hand, these regions are also included in the PCR-RFLP analysis, which allows the direct comparison of the results. The *trn*L^{E}-*trn*F region used for sequencing constitutes the intergenic spacer between *trn*L(UAA) 3'-exon and *trn*F. It is included in the region *trn*L^{C}-*trn*F used for PCR-RFLP, which is longer and additionally contains the *trn*L(UAA) intron. The *trn*H-*psb*A region constitutes a part of the *trn*H-*trn*K region used for PCR-RFLP.

It was not possible to obtain specific amplification of the third cpDNA region: *trn*S-*trn*G. The universal primers used always yielded a set of many bands of comparable (weak) intensity. Therefore, this primer pair was not further used.

The sequencing reaction was successful in case of both amplified chloroplast DNA regions. Clear sequences were obtained in all cases. As the regions were short enough to obtain entire sequence, only screening on a subset of samples was made using two strand sequences, to detect reliably the polymorphisms. Afterwards, most samples were sequenced in one direction only, with random controls of second strand.

The sequence of *trn*L^{E}-*trn*F region (Fig. 5.4) had the length of 485 bp in all samples (no length variation detected). It contained two single nucleotide polymorphisms (point mutations), both between cytosine and guanine. One point mutation was located in the recognition site of the *Hap* II enzyme (CC↓GG), in the position 132–136 of the sequence. The second point mutation was located in the recognition sequence of the *Eco* RI enzyme (G↓AATTC) in the position 233–239 of the sequence. Both mutations were also reliably detected during the polymorphism screening in the PCR-RFLP method.

Most sequences of *trn*H-*psb*A region had the length of 333–337 bp; two individuals from a Scandinavian population had a 49 bp-insertion and therefore their sequences counted 386 bp (Fig. 5.5). In total, three polymorphic sites were found in the sequence alignment. One of them was the already mentioned conspicuous large insertion, present only in one population and probably of recent origin, hence of no phylogeographical value. The second polymorphism involved a single nucleotide mutation between guanine and thymine at – or close to – the position 270 of the sequence. It is located in overlapping recognition sites of two enzymes: *Hap* II (CC↓GG) and *Hae* III (GG↓CC) and it was reliably detected in the PCR-RFLP analysis (see Fig. 5.2).

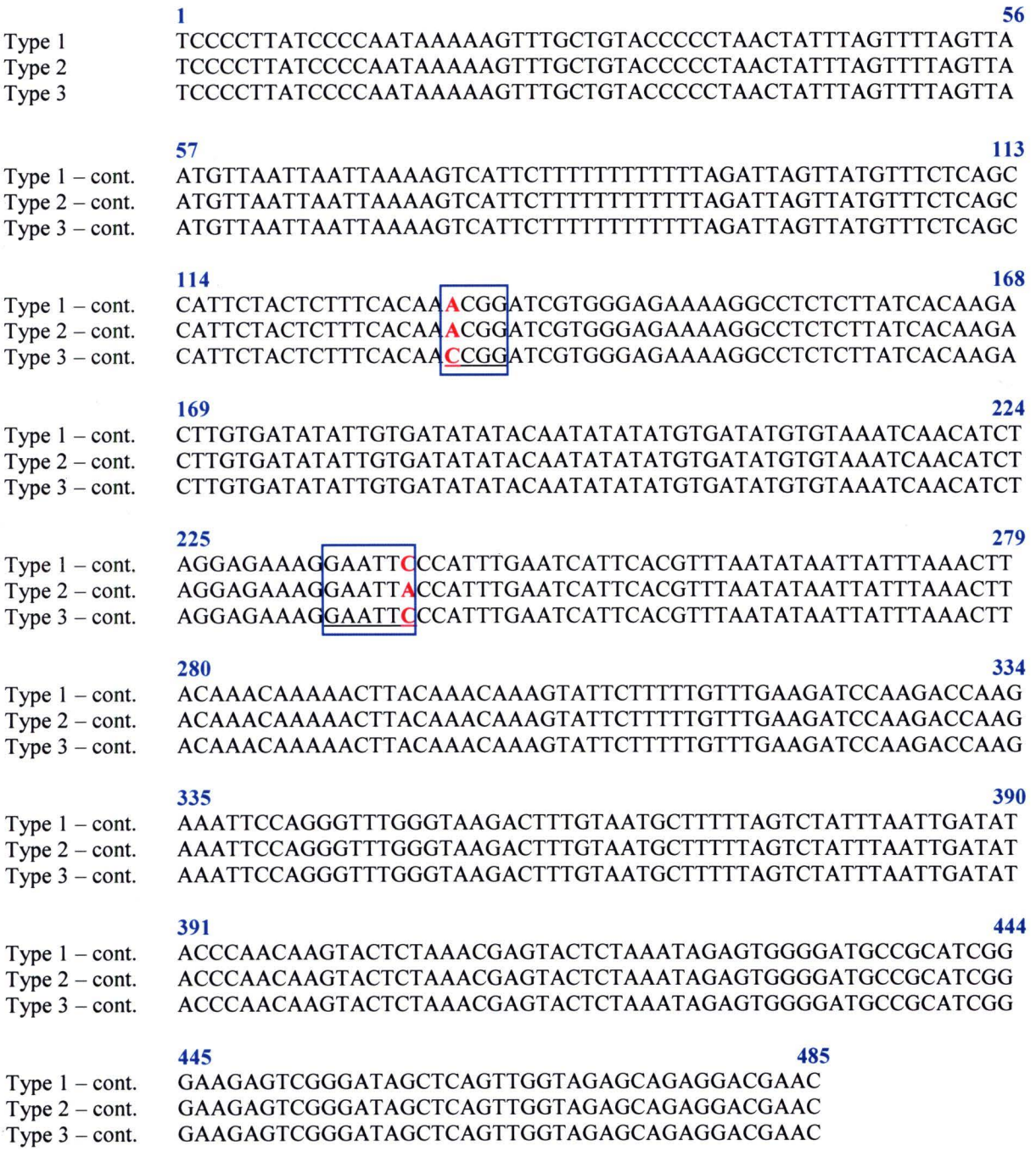


Fig. 5.4. The alignment of three different types of the *trn*L-*trn*F sequence in *Pulsatilla vernalis*. Two single nucleotide mutations are marked in red boldface. Recognition sequences of two enzymes used in the PCR-RFLP analysis, modified by these mutations, are marked by blue frames. Substitution of C by A in the position 132 suppresses restriction by *Hap* II; substitution of C by A in the position 239 suppresses restriction by *Eco* RI.

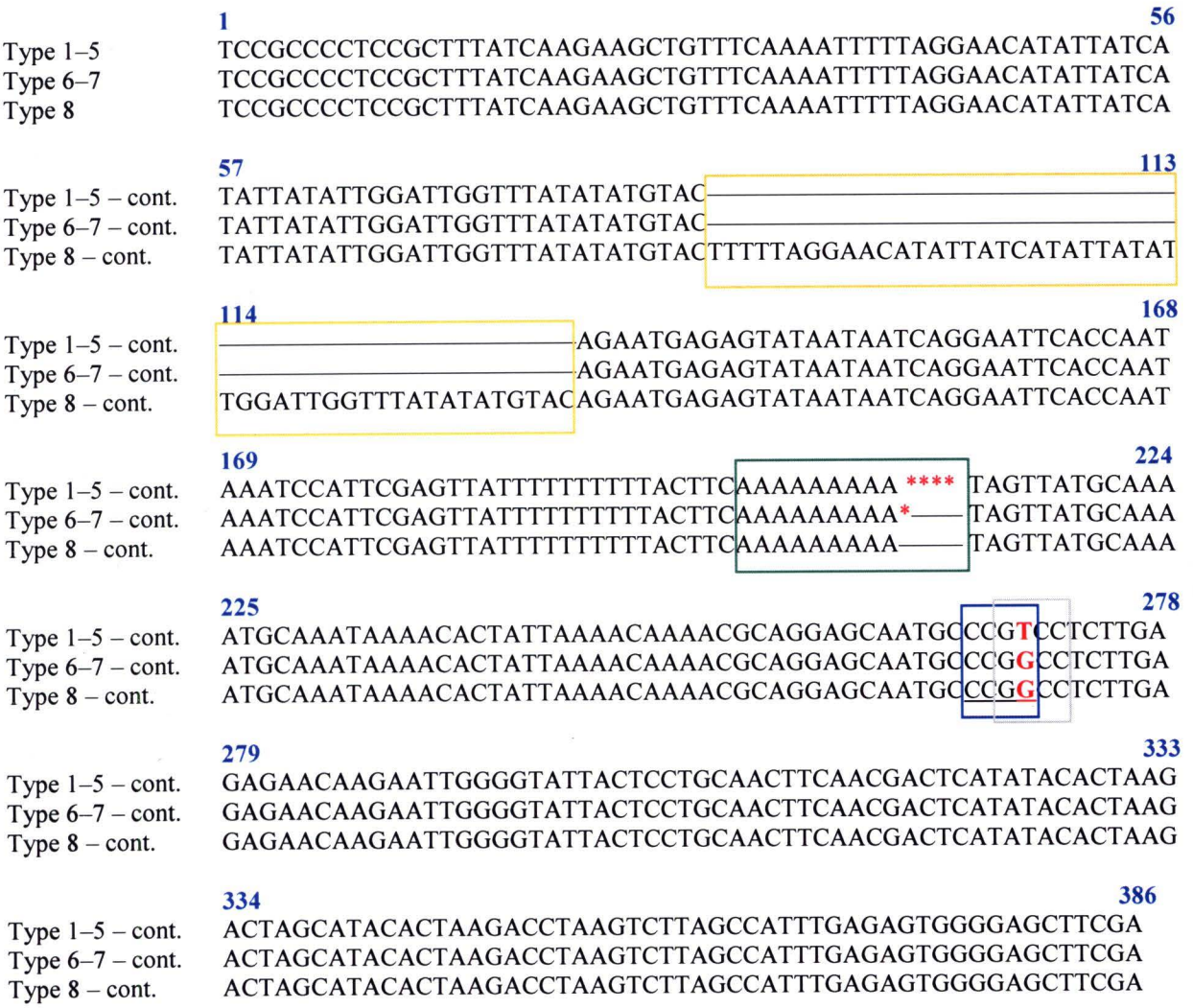


Fig. 5.5. The alignment of different types of the trnH-*psb*A sequence in *Pulsatilla vernalis*. The single nucleotide mutation is marked in red boldface. Recognition sequences of the enzymes used in the PCR-RFLP analysis, modified by this mutation, are marked by blue frame (for *Hap* II) and grey frame (for *Hae* III); substitution of G by T in the position 270 suppresses restriction by *Hap* II and *Hae* III. The 49 bp indel differentiating the type 8 of the sequence is marked by yellow frame. The poly-A repeat is marked by green frame. Types 1–5 all contain T in the position 270 and differ by the number of A (type 1 – 9 A, type 2 – 10 A, type 3 – 11 A, type 4 – 12 A, type 5 – 13 A). Types 6–7 contain G in the position 270; they have 9 and 10 A-repeats respectively. Adenines present in only subset of types are marked by asterisks.

The third polymorphic site was a poly-A repeat at the position 200–213 of the sequence. Five different states of this site were found across all populations (a succession of: 9, 10, 11, 12 or 13 adenines). Such a small length difference was not visible in the restriction analysis. Single nucleotide repeats or short tandem nucleotide repeats (microsatellite-like variation) can quickly evolve because of relatively frequent polymerase errors during DNA replication. Hence, they are considered to be inappropriate for phylogenetic or phylogeographic analyses due to high risk of polytomic polymorphisms (Salemi & Vandamme 2003). This poly-A site of *trn*H-*psb*A was therefore excluded from the formal analysis of cpDNA haplotypes. It was, nevertheless, cautiously analysed separately in order to seek for patterns at local scales. In this

analysis, each state (number of A) was treated as a separate character, as evolution of such region does not necesserily occur gradually.

The Minimum Spanning Tree of haplotypes detected in the sequencing analysis perfectly confirms the trends of diversity in the cpDNA of *Pulsatilla vernalis* as revealed by the PCR-RFLP analysis (Fig. 5.6), however the number of characters detected is lower and only one-step point mutations differentiate respective haplotypes. All the most important haplotypes were detected in both PCR-RFLP and sequencing analyses. Only two rare haplotypes C and F, supported only by single characters in the PCR-RFLP analysis, were not detected in the sequencing analysis; the larger number of haplotypes and more pronounced level of variability detected in the PCR-RFLP analysis was due to involvment of more cpDNA region.

5.1.3. Variability level of the chloroplast DNA in *Pulsatilla vernalis*

Two main evolutionary groups of cpDNA haplotypes were recognized in the dataset of *Pulsatilla vernalis*, counting altogether seven haplotypes in the entire species' range, based on twelve polymorphisms (cf. chapter 5.1.1). Low number of polymorphisms shows that mutation rate in the studied species and the process of population differentiation are slow. Based on comparison of data for different species it can be stated, that genetic differentiation rates are very variable across taxa and it is not possible (or only exceptionally possible) to apply constant assumptions for molecular evolution. In a spatially well comparable area of the Central Alps, an extensive genetic differentiation was observed eg. in *Draba aizoides* (Widmer & Baltisberger 1999) or *Rumex nivalis* (Stehlik 2002). Several haplotypes were also detected in *Eritrichium nanum* (Stehlik et al. 2002a). In contrast, no genetic variation was found in the subalpine *Erinus alpinus*, despite its presumably complex Quaternary history in the area (Stehlik et al. 2002b). It should be underlined, however, that in some cases the low diversity can be attributed rather to historical events, as in *Saxifraga oppositifolia*, where few haplotypes with no geographical structure across its Alpine populations could be caused by relatively recent (postglacial) colonization of this area from the Arctic (Holderegger et al. 2002); this species displayed anyway a low level of cpDNA diversity all over its distribution area (Holderegger & Abbott 2003). No genetic diversification at all was found in the sequences of the chloroplast *trn*L–*trn*F region in a European-wide study of *Juniperus communis* (Vargas 2003). Interestingly, very large differences in genetic diversity are found not only in the chloroplast DNA, which is supposed to have a relatively slow mutation rate, but also in such whole-genome markers as AFLP; for instance, no differentiation was found using this technique in case of *Veronica bellidioides* across Europe (A. Tribsch, pers. comm.).

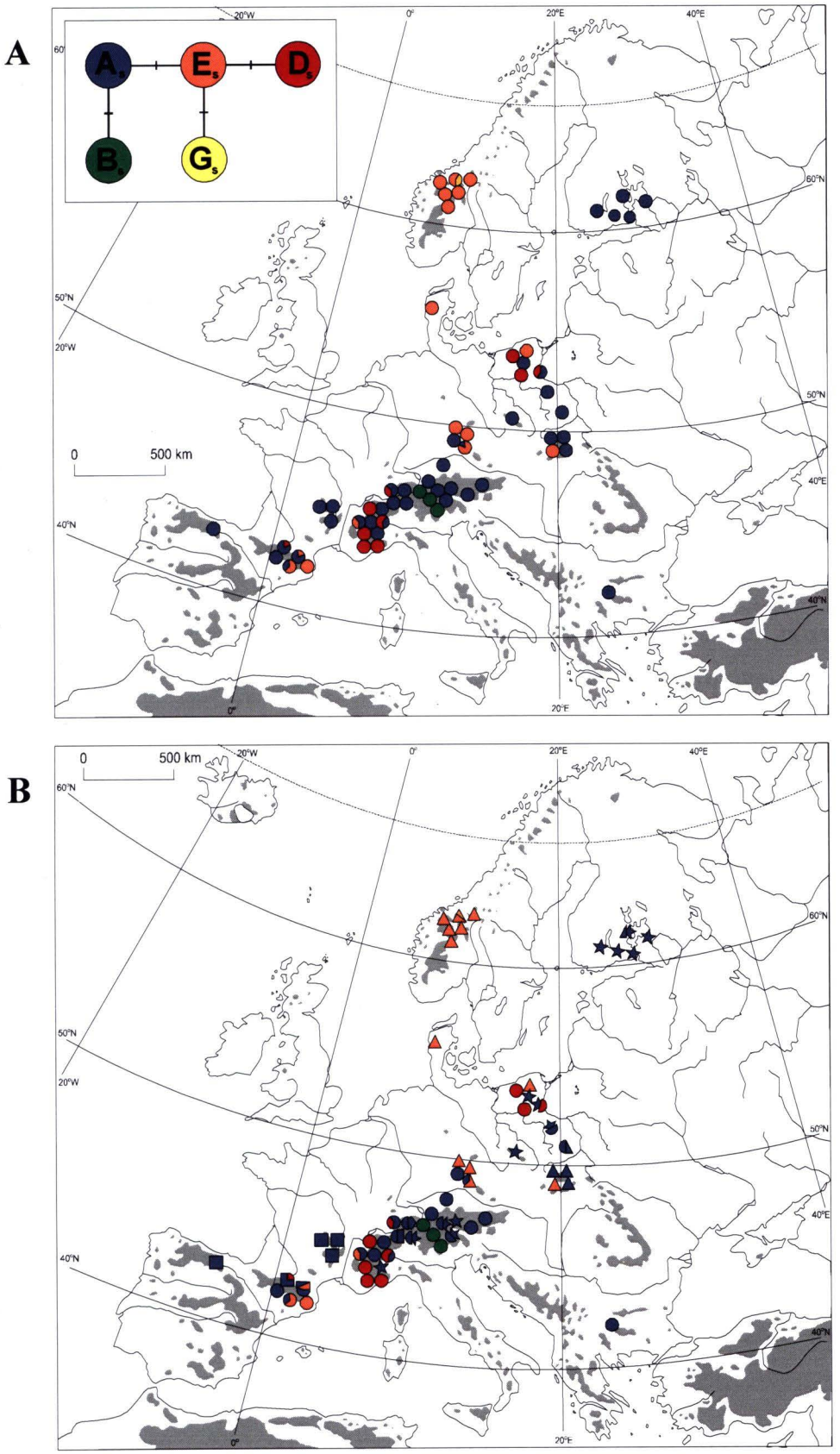


Fig. 5.6. Geographical distribution of cpDNA haplotypes of *Pulsatilla vernalis* revealed by the sequencing analysis (A), and the Minimum Spanning Tree of haplotypes (inset). Colours of haplotypes on the map correspond with those indicated in the tree; letters and colours assigned to haplotypes correspond to homologous haplotypes revealed by PCR-RFLP (Fig. 5.3). In case of mixed populations proportion of colours reflects frequency of haplotypes. Second map (B) presents the same haplotypes but including data from the poly-A repeat in the *trn*H–*psb*A region (see text for details): triangle – 9A, circle – 10A, square – 11A, star – 12A, rhomb – 13A.; mixed populations have parts of different symbols.

At the molecular level, no correlation was found in *Pulsatilla vernalis* between the degree of polymorphism and the length of DNA fragments confirming, that differentiation does not occur in a random way even along the non-coding cpDNA sequences (Kelchner 2000) and some regions are more appropriate for biogeographical inferences than other. Nevertheless, choice of regions for analysis is far from being straightforward and always necessitates a thorough preliminary screening. Noteworthily, in case of *Pulsatilla vernalis* the only fragment used for screening and not yielding any variation (*rpo*B–*trn*C) was statistically ranked in the literature as one of two most variable cpDNA regions in vascular plants (Shaw et al. 2005).
Apart from the low overall diversity, a strong differentiation of populations was observed in the entire range of *Pulsatilla vernalis*, what was reflected by a very high G_{st} value (cf. chapter 5.1.1), which indicates almost no within-population variability (Culley et al. 2002). In the spatial distribution of the cpDNA diversity, only the contact zones between putative migrational lineages exhibited higher population diversity as some mixed-haplotype populations occur there (although no more than two haplotypes per population were found in any case), while in most areas populations are monomorphic. It is important in the context of a general assumption of refugial areas as reservoirs of the species' genetic diversity. This seems to be true in the scale of the entire range's diversity, but at regional scales (eg. for designation of particular populations to preserve the most of the diversity) contact zones between lineages coming from different refugial areas are of utmost importance as they can harbour combined large fractions of genetic diversity. Hence, in species with European-wide distribution the richest populations are likely to be located in the contact zones between different lineages from separate refugia. Few works are available to test this intuitively simple hypothesis, but it was well supported already by comparative analysis on European trees and shrubs (Petit et al. 2003). Also in American *Pinus resinosa* a stable transition zone between descendants of two distinct refugia was marked by much greater chloroplast DNA diversity (Walter & Epperson 2005).

5.1.4. Comparison of methods for assessing cpDNA variability – reliability of the PCR-RFLP analysis

As mentioned before, two regions (fragments) of the chloroplast DNA (*trn*L^E-*trn*F, *trn*H–*psb*A) were sequenced to compare the results from direct analysis of sequence with the indirect inference of variation using the PCR-RFLP method. These two fragments are among

the most popular used for inter- and intraspecific plant analyses (reviewed eg. by Kelchner 2000). They also make parts of two fragments used for analysis by PCR-RFLPs (cf. Fig. 5.1). Without considering the variable poly A-repeat in the *trn*H–*psb*A region (cf. chapter 5.1.2), which is too short to be detected as polymorphism of restriction fragments (but it can be very instable and usually is anyway excluded from analyses; Kelchner 2000), the comparative analysis of the two sequenced cpDNA regions with relative regions submitted to the PCR-RFLP analysis allows to state, that 100% of the variability existing in these fragments was detected during a rigorous standard PCR-RFLP approach. Moreover, no false variability or convergence was obtained in the PCR-RFLP results.

The whole picture of the DNA variability obtained with restriction analysis is more complex than results of sequencing, due to the use of more regions in the former analysis, but both analyses perfectly unraveled the major geographical pattern (Fig. 5.3, 5.6). Comparison of a typical PCR-RFLP vs. typical sequencing approach allows the following conclusions: (i) PCR-RFLP proved to be a very reliable method in indirect detecting of DNA variability; (ii) economic aspect of both methods favours the first one, as for the same or lower costs more regions can be examined resulting in obtaining a more complex picture of variability.

5.2. Use of nuclear and whole-genomic DNA markers

Several approaches were adopted in order to explore the nuclear DNA variability of *Pulsatilla vernalis*. The Amplified Fragment Length Polymorphism (AFLP) method was tested to infer geographical relationships among populations as well as to carry out several fine-scale ecological studies. Unfortunately, this method showed to be reliable only in a very restricted sample set, and it was not possible to obtain reliable and reproducible results for a large part of samples (for detailed discussion see chapter 6.1). The failure of the AFLP analysis in the genus *Pulsatilla* discouraged trying much less robust random PCR-based genomic markers such as RAPDs or ISSRs.

As the general DNA fingerprinting was not possible due to presence of inhibitors of enzymatic reactions, a more direct approach was applied to analyze specific DNA regions. The study of the genetic diversity in the range-wide geographical context could be supported by a specific study of particular nuclear DNA regions. Such a specific region-approach based on amplification of a definite region allows a strict control of results. Therefore, trials of sequencing analysis were performed targeting the most universally used ITS region of nuclear ribosomal DNA; possibility of use of coding "simple-copy" genes was also explored.

In case of the ITS nrDNA region, it was possible to amplify and sequence this fragment in the whole screening set of samples. The sequence of the entire ITS1–5.8S–ITS2 had a length of 633 bp (Fig. 5.7). The results from the initial screening set did not allow to detect polymorphic sites. Despite further enlarging the screening set by including more populations, it was still not possible to find any polymorphisms in the ITS region and therefore it proved to be not useful for phylogenetic analysis. It is most probably the first ITS sequence obtained in the genus *Pulsatilla* and it can be interesting for future phylogenetic analyses in the genus, nevertheless this region showed to be of no interest for infraspecific phylogeographical study.

	1	55
ITS	TCGTAGGTGACCTGCGGAGGATCATTGTCGATGCCTGCTCAGCAGAACGACCCGC	
	56	109
ITS – cont.	GAACAAGTGAAAACAACAACTCACGCCGGGGAACAGGACGCCGGACAGCCTCAC	
	110	163
ITS – cont.	CGCTGCCCCCGACCTGCGACCCAGCACACCACAAAAAATCCGGCGCAACTGGCG	
	164	217
ITS – cont.	CCAAGGAATACTTACCGGAAACAACGGGTCGACACGTCGACGCCGTGGATCCGA	
	218	272
ITS – cont.	ATACTCAAACGACTCTCGGCAACGGATATCTCGGCTCTTGCATCGATGAAGAACG	
	273	327
ITS – cont.	TAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTT	
	328	382
ITS – cont.	TGAACGCAAGTTGCGCCCGAAACCTTTCTGGTCGAGGGCACGTCTGCCTGGGCGT	
	383	436
ITS – cont.	CACACACAGCGTCGCCCCCACCAAAGCATTTGGATGGGGGCGGAAATTGGCCCC	
	437	490
ITS – cont.	CCGAGCCCCCCGGGGCACGGTCGGCACAAATGTTGGCCCTCGGCGGCGAGCGTC	
	491	545
ITS – cont.	GCGGTCAGCGGTGGTTGTACTCTCATCCTCCAAAGACAAAATGACGCGTCCGCTT	
	546	600
ITS – cont.	CGTCGCCCGCTGGACGAAGATGACCCAAGGAGTCCCCCCTACCGGAGACTTCCAC	
	601	633
ITS – cont.	CTGCGACCCCAGTCAGGCGGGATACCCGCAGTA	

Fig. 5.7. The consensus sequence of the ITS nrDNA region (ITS1–5.8S–ITS2) in *Pulsatilla vernalis*.

The amplification trials of the glyceraldehyde-3-phosphate dehydrogenase gene (*G3pdh*), a low copy nuclear region, resulted in obtaining one strong band of approximate length of 1200 bp, accompanied by a smeared set of weak unspecific bands. The specific amplicon was

extracted by means of separation on concentrated agarose gel with subsequent cutting the band in gel, solubilisation and purification. This extracted DNA band was used as a template for the cycle sequencing reaction. Despite several attempts it was not possible to obtain a clear sequence of this region and it had to be abandoned.

5.3. PHYLOGEOGRAPHY OF *PULSATILLA VERNALIS* BASED ON DISTRIBUTION OF CPDNA HAPLOTYPES

5.3.1. General survey of cpDNA variability in the context of its geographical distribution

Two distinct groups of haplotypes can be distinguished in the analysis of cpDNA variation (Fig. 5.3). The separation of these groups involves at least five mutational steps and accounts for explanation of as much as 70% of variability of cpDNA, as analyzed by Principal Coordinates Analysis. Analysis of genetic distance between haplotypes together with their geographical distribution does not allow an unambiguous reconstruction of historical processes which originally caused this differentiation. The distribution of the two lineages is complicated and difficult to interprete, especially in the western edge of the distribution area (the Pyrenees). Two main scenarios are admissible. Taking into account the genetic distance between two main haplotype groups on the one hand, and conservative character of the chloroplast DNA and its generally low mutation rate (McCauley 1995) on the other hand, presence of two old evolutionary lineages resulting from an old isolation dating back to periods before the last glaciation can be suspected. In such a case primary patterns and causes of this genetic differentiation would have been swept away by further glacial isolations, migrations, altitudinal shifts etc. In consequence, the contemporary data would be helpful for analyzing the last glacial history and postglacial range expansion patterns, but not the evolutionary processes responsible for the development of observed haplotypes. Main assumptions leading to the above explanations are: (i) the slow evolution of the chloroplast DNA, (ii) stable and constant mutation rate for the analysed taxon. This would fit the general view of Merxmüller (1952) who stated, that in most cases the differentiations of plant lineages have preglacial roots, while glaciations played major role in shaping their contemporary distributions. It would also be the simplest explanation for a large geographical distribution of both lineages and their complicated spatial sequence eg. in the Pyrenees (Fig. 5.3, 5.6; see chapter 5.3.3 for details). Contribution of old, historically separated lineages later co-existing in a sympatric distribution, was invoked in case of similar patterns in populations of *Alnus*

glutinosa in Turkey (King & Ferris 1998) or in Alpine and Pyrenean populations of *Saxifraga oppositifolia* (Vargas 2003).

However, as the examples of cpDNA variation in diverse groups of plants become more and more available allowing insights into mechanisms of its evolution, the conviction grows that the assumptions of random and stable evolution in the chloroplast DNA are far from being universal and clear, even in the non-coding cpDNA sequences, which are actually the most widely used for phylogenetic purposes. Kelchner (2000) points out several mechanisms which are responsible for disturbances in the stability of cpDNA evolution. He argues that the accumulation of mutations in the non-coding cpDNA sequences is usually far from being random. Due to molecular mechanisms during replication, some sequences are particularly susceptible for occurrence of mutations (indels, inversions as well as point mutations); moreover, the type of mutations to occur (eg. transitions or transversions) can be strictly determined by neighbouring sequences. Some regions can act as "triggers" which launch subsequent mutational steps in a non-random way. Although such processes are not possible to be proven when applying standard methods estimating the DNA variation, it can be stated that assumption of low and constant mutation rate in cpDNA does not necessarily have to be true, because of probability of accelerated non-random accumulation of mutations. In fact, in the genome, with the extreme complexity of its functioning, constant rates of molecular evolution can be an exception rather than a rule (Welch & Bromham 2005). In the consequence of such an assumption, a higher differentiation of a lineage in one region would not necessarily imply a substantially longer isolation. This point of view is also, to some extent, supported by a complete lack of variability detected in the nuclear ITS region in *Pulsatilla vernalis*, which would be strange (although not improbable) in old lineages. Also the contemporary spatial distribution of haplotypes (excluding areas covered by glaciers during last glacial maximum) suggest that the region of differentiation of one lineage can still be detected, and therefore spatial isolation in late Pleistocene played an important role in this differentiation process. A strict separation of the two lineages in the Alps would be somehow difficult to explain with the assumption, that they are very old.

The dilemma of discerning between results of evolutionary processes and of distribution patterns has probably been underestimated in works dealing with the cpDNA variation. Automatic assumption of differentiation of recognized haplotypes directly in the last glacial refugia where they were detected was admitted (eg. Petit et al. 2002). As a consequence, presence of the same haplotypes in different refugia was interpreted as a recent exchange,

while in fact differentiation of given haplotypes could very well precede the isolation in the refugia (R. J. Petit, pers. comm.).

The hypothesis of old lineages in case of *Pulsatilla vernalis* is more parsimonious in the sense that it necessitates less theoretical assumptions to be accepted; nevertheless, its main basis, i.e. that of stable molecular clock, is highly debatable. Application of more strongly resolving (whole-genomic) molecular markers could help to discern between the two abovementioned hypotheses. In any case, most of the conclusions inferred from the detected distribution of haplotypes remain valid in the light of both assumptions (with exception of exact relationships between the South-Western Alps and the Pyrenees), only time of lineages' evolutionary divergence is arguable.

When describing the geographical distribution of the genetic diversity it is worth mentioning, that the location of genetic lineages does not coincide with the occurrence of infraspecific taxa (varieties) as presented by Aichele & Schwegler (1957). It can be clearly stated for the alpine varieties (*alpina* and *pyrenaica*), as populations belonging to these taxa are characterized by all kinds of haplotypes detected in the analysis. This conclusion can be also transferred to the lowland varieties (*vernalis* and *bidgostiana*), although with less direct evidence in some areas because of a general character of the map published by Aichele & Schwegler (1957). Described varieties of *Pulsatilla vernalis* do not represent monophyletic evolutionary lineages. Their (mainly quantitative) morphological differentiation can be rather attributed to the habitat-induced variability.

The detailed discussion of particular phytogeographical questions, presented below is based both on data from PCR-RFLP and sequencing (Fig. 5.3, 5.6) analyzed in concert, and anyway congruent in most cases. The main relationships between populations of *Pulsatilla vernalis* in different parts of the range, as discussed in detail below, are presented in Fig. 5.9.

5.3.2. The Alps

The pattern of chloroplast DNA variation in the Alpine populations of *Pulsatilla vernalis* is by far dominated by a clear diversification between the South-Western Alps and the Central/Eastern Alps (Fig. 5.8). Populations from the former are dominated by the haplotype D, while all the rest is characterized by the haplotype A or its close derivative. The border of occurrence of the haplotype D in the east involves the Val d'Aosta region (area of the Petit-Saint-Bernard pass where the sampled population contained uniquely this haplotype) and the western Valais region, where the sampled population in the massif of Grand Chavalard was

mixed with respect to cpDNA haplotypes. The haplotypes from the Western Alps ("red" group, cf. Fig. 5.3, 5.8) and Central/Eastern Alps ("blue" group) are separated by seven mutational steps (PCR-RFLP polymorphisms). Additionally, a distinct haplotype derives from the haplotype A in the central part of the Alps with two differing mutational steps (haplotype B). It is geographically restricted and was detected in three neighbouring sampled populations in the Dolomites and in the Rhaetic Alps (eastern Switzerland).

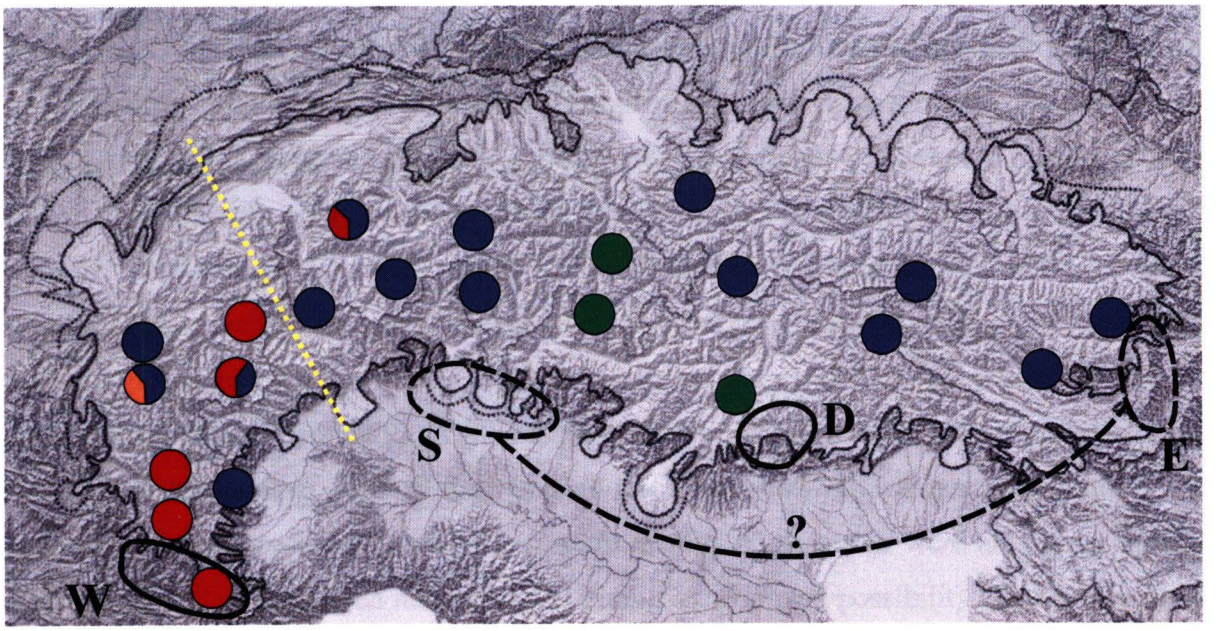


Fig. 5.8. Detailed distribution of cpDNA haplotypes in populations of *Pulsatilla vernalis* in the Alps. Colours of haplotypes correspond to those presented on general maps. Thin lines indicate the extension of alpine glaciers during the Würm (continuous line) and Riss (dotted line) glaciations. General location of last glacial refugia is showed: W – Western-Alpine refugium; S – Southern-Alpine refugium; E – Eastern-Alpine refugium D – refugium in the Dolomites. S and E are marked with dashed line as no certain location of refugial area is possible; the dashed line indicates possibility of survival in the neighbouring plains. Yellow dotted line shows the approximate major genetic division between the South-Western and Central/Eastern Alps.
Background map from Aeschimann et al. (2004).

Regardless the exact time of initial divergence of the two main lineages (was it entirely caused by the last glacial maximum isolation or occurred already earlier?), this split belongs to the strongest intraspecific examples of diversification between the Western an Eastern Alpine floras so far demonstrated at the molecular level. The substantial differentiation between floras of the Western and Eastern Alps has been remarked and studied since long time. It is well reflected on the specific (floristic) level by the existence of many species with vicariant distributions as well as by importance of regional endemics (Ozenda 1985, Favarger 1995, Aeschimann et al. 2004). The probable centre of diversification of the Western Alpine haplotype of *Pulsatilla vernalis* corresponds with the area of highest degree of endemism in the Alps, as stated by Pawłowski (1970), with several remarkable local palaeoendemic species

as *Saxifraga florulenta* or *Berardia subacaulis*. The Western/Eastern Alpine split was also observed in cytological studies (eg. Küpfer 1974, also recapitulated by Ozenda 1985 and Favarger 1995); eg., a case study of the distribution of chromosomic races of *Sempervivum arachnoideum* (Welter 1979, after Ozenda 1985) shows an almost identical borderline between South-Western and Central Alps as do haplotypes of *Pulsatilla vernalis*.

For a few last years, considerable efforts have been launched in order to study the genetic diversity of alpine plants and elucidate history of survival and expansion of alpine flora during the Quaternary glaciations and after the retreat of ice sheet (Gugerli & Holderegger 2001). Thanks to that, in contrast to other European mountain ranges, complex datasets from the Alps, based on sampling of many populations, were already published for a few species allowing preliminary comparative analyses. Several species of (mainly or uniquely) siliceous bedrock were analyzed, which makes a comparison with *Pulsatilla vernalis* even more straightforward thanks to similar habitats (and therefore also potential refugial areas). During the cold glacial periods the Alpine ice sheet was extending and covering almost completely the mountain area (Mojski 1993). Only restricted zones of some peripheral massifs constituted available alpine habitats between the glaciated areas and relatively warmer areas south of the Alps and cold steppe north of the Alps. Additionally, single peaks were emerging directly among the glaciers (so-called nunataks) and giving a theoretical possibility of *in situ* survival for high-alpine plant species. Importance of peripheral refugia and possibilities of plant survival on nunataks have been subject for debate for more than a century (discussed in detail by Stehlik 2000). Potential peripheral refugia for plants during the last glacial maximum (approximately 20 000 years b.p.) can be deduced based on the bedrock, topography of peripheral massifs, knowledge on extension of glaciers and approximate location of snowline (Tribsch & Schönswetter 2003). As most of Alpine peripheral massifs are built of limestone, potential refugia for "siliceous" plants are much more restricted; they include above all large siliceous massifs at borders of the Eastern Alps which remained unglaciated (Mojski 1993, Tribsch & Schönswetter 2003) and smaller areas at the south-western limit of the range as well as at the southern edge of the mountains. Most species analyzed using molecular markers revealed a strong division into main Western Alpine and Eastern Alpine clades, which were dividing further on to form four more or less distinct subgroups across the Alps: south-western, western, central and eastern group; such remarkably congruent patterns were exhibited by three different high-alpine plants of siliceous bedrock: *Androsace alpina*, *Phyteuma globulariifolium* and *Ranunculus glacialis* (Schönswetter et al. 2002, 2003b, 2004c). A clear differentiation of the Western Alpine group was also detected in *Comastoma*

tenellum (Schönswetter et al. 2004a), but with little diversification in the Central/Eastern Alpine group. The geographical extent of the Western Alpine cpDNA haplotype of *Pulsatilla vernalis* (haplotype D) is greatly congruent with distributions of populations from the south-western clade in case of all the aforementioned taxa. Altogether, all these data clearly indicate a strong differentiating role of the South-Western Alps, due to presence of highly isolated south-western peripheral refugium in this area which suffered relatively little from the glaciation, but was to a high extent separated by the glaciers from the area of Piemont (and also Italian plains) and its potential further refugial areas. Eastern limits of the area influenced by the south-western peripheral refugium are located generally along the Val d'Aosta region.

The refugial area for the populations of *Pulsatilla vernalis* bearing the haplotype A could be located at both the Eastern and the Southern edge of the Alps and it is not possible to give more precise indication. It cannot be either excluded that the main refugium for haplotype A was not highly isolated but interconnected by populations in the plains. Below is given the rationale for such an assumption.

The pattern of genetic diversity of *Pulsatilla vernalis* is the most congruent with that of *Comastoma tenellum*, where only two strongly differentiated groups are present in the Alps, with markedly unequal spatial distribution: one limited to the south-western area as mentioned above, and the second one covering the majority of the Alps (Schönswetter et al. 2004a). The only important difference between the patterns of these two species is caused by presence of an additional small centre of variability in the Central Alps in case of *Pulsatilla vernalis*. *Comastoma tenellum*, alike *Pulsatilla vernalis*, is a predominantly alpine species but has a tendency to grow in subalpine belt (Aeschimann et al. 2004) and even descend down to alluvial terraces; moreover, Central Asian populations were reported to often occur in steppe-like communities (Schönswetter et al. 2004a). Similar division of the South-Western Alps from remaining area of the Alps was found in a thermophilous (submediterranean) orophyte *Anthyllis montana* having today a large altitudinal amplitude (200–2700 m) in the Alps (Kropf et al. 2002). Lack of phylogeographical structure was found in *Oxytropis campestris* which has a tendency to grow in subalpine belt and lower (Schönswetter et al. 2004b). The abovementioned species have much larger ecological amplitudes than remaining alpine species subjected to detailed analysis over the Alps: *Androsace alpina*, *Phyteuma globulariifolium* and *Ranunculus glacialis*, which all are stenoecious high-mountain taxa strongly attached to a specific bedrock and altitudinal conditions (Aeschimann et al. 2004). They don't descend today below limits of 2000–2200 m. All these high-mountain taxa are characterized by much more highly structured genetic diversity across Alps. The presence of

such tendencies, although still based on small set of data, could suggest significantly different histories of glacial survival, with high-alpine plants strongly restricted to small unglaciated alpine refugia (between alpine ice sheets and relatively warm climate in plains south of the Alps), and alpine plants with more flexible ecology surviving in refugia with weaker isolation and therefore higher possibility of contact and genetic exchanges.

In the three cited high-alpine taxa which have several genetic groups delimited across the Alps it is important to point out, however, that two western groups, as well as the central and eastern groups, form above all two clades of higher rank: the western and the eastern clade, respectively. These species were studied using AFLPs. Information on the chloroplast DNA variation is mentioned only in the case of *Phyteuma globulariifolium* and allows a more direct comparison with the cpDNA dataset in *Pulsatilla vernalis* than AFLP data. The difference between two major clades in the cpDNA of *Phyteuma globulariifolium* amounts to seven nucleotide substitutions in the *trn*L-F region (Schönswetter et al. 2002), hence it is even higher than that found in *Pulsatilla vernalis*. This could suggest, that the main split between the two major clades (eastern and western), for all or most species, occurred already before the last glacial isolation, while survival of the Würm glaciation in isolated refugia caused in many cases further structuring of the genetic diversity.

Such an additional genetic differentiation, and surely dating back to the isolation due to the Würm glaciation, was discovered in *Pulsatilla vernalis* in the Central Alps (haplotype B). It can be linked with a high probability with a survival in a small and isolated refugium in the region of the Dolomites, probably in a restricted area between the borders of alpine glaciers and the permanent snow line. These data further support results from several other taxa as *Androsace alpina* (Schönswetter et al. 2003b) and *Androsace wulfeniana* (Schönswetter et al. 2003c), *Eritrichium nanum* (Stehlik et al. 2001, 2002a), *Phyteuma globulariifolium* (Schönswetter et al. 2002), *Saponaria pumila* (Tribsch et al. 2002), suggesting the existence of a glacial refugium in this area. In total, the region of Dolomites is supported as a glacial refugium by data from the highest number of species, together with the South-Western and Eastern Alpine refugia.

In summary, the phylogeographic pattern of *Pulsatilla vernalis* in the Alps gives a clear indication for existence of at least three peripheral refugia: the highly isolated south-western refugium, the eastern refugium (probably extending along southern margins of the mountains further west), and a small and visibly isolated refugial area located in the Dolomites or their vicinity (Fig. 5.8).

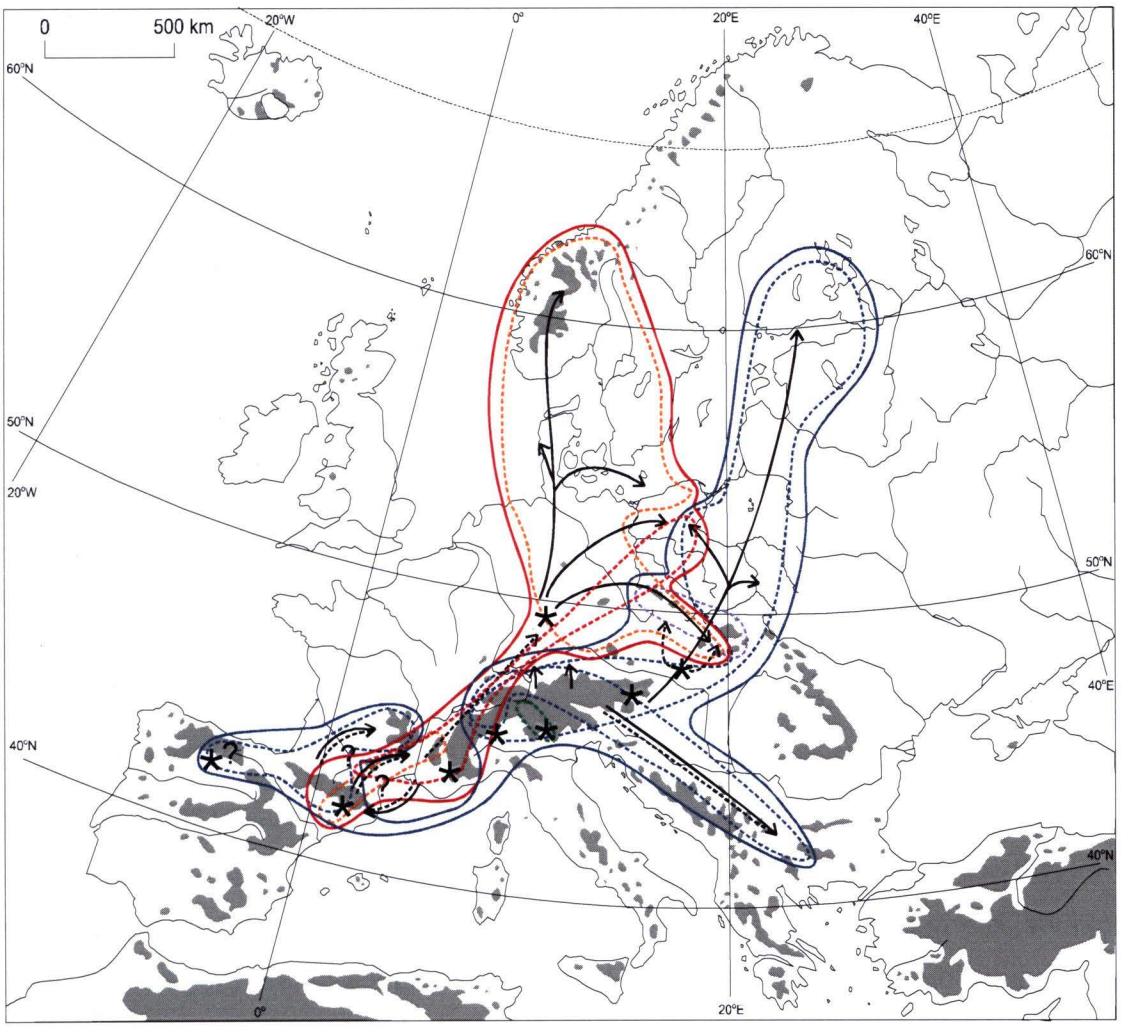


Fig. 5.9. The synopsis map showing relationships between populations of *Pulsatilla vernalis* in different parts of its distribution range, possible to infer from cpDNA haplotypes. Colour lines are embedding areas linked by genetic lineages (coulours correspond to those earlier used for haplotype designations). Dashed lines correspond with populations with identical haplotypes (considering both PCR-RFLP and sequencing data). Continuous lines (blue and red) correspond with two major groups of cpDNA haplotypes. Asterisks represent assumed refugial areas during last glacial period. Continuous-line arrows indicate probable postglacial migration events, and dashed lines indicate earlier migrations. When dating of events is not possible or several distinct in time migration events are supposed, combined arrows are used. Question marks show particularly little resolved problems.

5.3.3. The Pyreneo-Cantabrian chain and its relationships with the South-Western Alps

The relationships between alpine floras of the Alps and the Pyrenees were extensively studied by Küpfer (1974) based on cytological characteristics of populations and distributions of diploid and polyploid lineages across these mountain ranges. The author stated the high importance of the old Tertiary common flora, reflected by common distribution of diploid populations across the area, and also presence of numerous true vicariant taxa. An important role, however, has been demonstrated also for more recent, Quaternary exchanges of floras involving both directions: from the Alps to the Pyrenees (eg. *Minuartia sedoides, Oxytropis*

halleri, Tanacetum alpinum) and *vice versa* (eg. tetraploid race of *Bupleurum ranunculoides, Senecio doronicum*) (Küpfer 1974). The cpDNA haplotype data from *Pulsatilla vernalis* show clear relationships between the Pyrenees and the South-Western Alps, reflected by the localized presence of haplotypes from the "red" group in the south-westernmost Alpine populations and in the eastern part of the Pyreneo-Cantabrian range of the species (Fig. 5.3, 5.6). The exact history of this pattern, however, cannot be elucidated with certainty based on these data. Clear relationships between Pyrenean and Western Alpine populations were demonstrated in other species, eg. in siliceous *Phyteuma globulariifolium*, for which AFLP data suggested a relatively recent immigration to the Eastern Pyrenees from the Western Alps (Schönswetter et al. 2002). Traces of longer-term isolation was detected in another high alpine siliceous species, *Ranunculus glacialis* in the Central Pyrenees (Schönswetter et al. 2003a). In case of *Pulsatilla vernalis* there is a clear spatial sequence of haplotypes in the Pyreneo-Cantabrian chain, where westernmost populations including those from Cordillera Cantábrica and central Pyrenean ranges harbour uniquely, or almost so, the haplotype A (with a specific 11-A repeat in the *trn*H–*psb*A fragment, but with no other private characters against the Alpine populations with this haplotype), while the eastern Pyrenean populations are increasingly characterized by presence of the "red group" haplotypes. Also, the Eastern Pyrenees constitute the only area where all three haplotypes from the "red group" are present (D, E, F). Several hypotheses are possible to explain this pattern and their probability depends on the weight given to particular theoretical assumptions.

1) Several migration waves could have occurred from the Alps to the Pyrenees, with haplotype A (the most common in the total distribution range) being the oldest one. It is necessary to assume here, that the mutation rate does not have to be constant and particular molecular events can cause an acceleration of diversification (see the discussion in the chapter 5.3.1). The "red" group of haplotypes could have originated in the Alps and colonized the Pyrenees in several waves distinct in time, therefore not only the last one but also less differentiated haplotypes are present there. Based on the data available it cannot be excluded, however, that the "red" haplotypes originated in the Pyrenees and migrated eastwards (representation of all three haplotypes from this group would support this).

2) The diversification of the two major groups of haplotypes is much older than patterns which can be detected and contemporary distribution of cpDNA variation can only serve to analyze migrations of populations from last glacial and postglacial periods but not to retrace original evolutionary processes. A very complex pattern of diversity between the Alps and the Pyrenees, indicating at long-distance dispersal or sequence of several colonization and

recolonization events with redistribution of ancient genotypes, was found as well in *Soldanella alpina* (Zhang et al. 2001, Vargas 2003) and in *Saxifraga oppositifolia* (Vargas 2003) suggesting the often complicated relationships between alpine floras of the two regions. A fine-scale analysis using more complex method of screening of the genomic DNA variation could probably help to discern between the above hypotheses and a lack of possibility to obtain reliable AFLP data for *Pulsatilla vernalis* is particularly disappointing here.

5.3.4. The Central Massif – between the Alps and the Pyrenees

The French Central Massif, despite its moderate altitude, reaching 1886 m a.s.l. (Puy de Sancy), has relatively low limits of forest vegetation belts (approx. 1550 m a.s.l.), most probably due to the atlantic climatic impact and high pluviosity, and isolated stands of its highest peaks (Puy de Sancy, Plomb de Cantal, Mont Mézenc, Mont Lozère) harbour considerable relict alpine flora (Braun-Blanquet 1923). These mountains are located between the two main sources of alpine flora in Europe – the Alps and the Pyrenees – and origins of this element there have been debated for long time. Analysis of the species composition reveals that relationships with both neighbouring mountain ranges are true because of presence of plants absent from the Alps but present in the Pyrenees, and the other way round (Küpfer 1974). As Braun-Blanquet (1923) points out, there are no taxa suggesting survival of an old, tertiary autochthonous alpine flora in the region. On the other hand, a recent long-distance migration is highly improbable to be at the origin of such a complex group of species and last (Würm) and before-last (Riss) glaciations should be considered as main periods of formation of stands of the alpine flora in this area.

In all analyzed stands of *Pulsatilla vernalis* in the Central Massif (covering all distinct massifs where the species occurs there) the species was characterized by the common haplotype A, present in both Alps and Pyrenees, therefore no high resolution data are available to discuss the origin of these populations. Nevertheless, an attention should be paid to the poly-A sequence in the *trn*H–*psb*A fragment. All populations from the Central Massif have the 11-A repeat, which also dominates in the Pyrenean and Cantabrian populations harbouring the haplotype A (Fig. 5.6 B), while the 11-A repeat was found in the Alps only in one distant population in the Central Swiss Alps (most probably of a polytomic origin). As it was already stated, single-nucleotide repeats are to be treated cautiously in phylogeographic considerations, because of their potential instability and frequent mutations (Kelchner 2000, Salemi & Vandamme 2003). Nevertheless, an indirect proof of relative stability of this poly-A

repeat in *Pulsatilla vernalis* – at least at the postglacial time scale – is given by northern, Fennoscandian populations characterized by high stability of this sequence (cf. Fig. 5.6 B). Also, a recent European-wide study dealing with *Fraxinus excelsior*, entirely based on similar chloroplast microsatellite sequence polymorphisms, showed that phylogeographical inferences based on them can be reliable, despite a drawback of possible homoplasy (Heuertz et al. 2004). In case of *Pulsatilla vernalis* this molecular evidence, relatively weak itself, corroborates the floristic data, as the number of Pyrenean orophytes largely surpasses the number of Alpine orophytes; moreover, although no intermediate localities can be stated for alpine plants, a succession of such intermediate stations between the Pyrenees and the Central Massif can be observed in case of several montane or subalpine species till present (Braun-Blanquet 1923). The migration from the Pyrenees has been demonstrated for several siliceous species, and for many of them, as *Jasione humilis* or *Silene ciliata*, calcareous habitats of the Jura massif was presumably a notable obstacle which did not allow them to reach the Alps (Küpfer 1974). The data do not suggest old history of *Pulsatilla vernalis* in the Central Massif, in contrast to *Calluna vulgaris* which exhibited the highest cpDNA variability in the Cévennes and this area was proposed as a major refugium for this main species building heath communities (Rendell & Ennos 2002), in which *Pulsatilla vernalis* nowadays also occurs. Unfortunately, despite increasing interest in fine-scale analysis of history and evolution of alpine plants, the Central Massif was usually not included in datasets of published papers and no other molecular data are available to discuss the origins of alpine flora in this area; extending a comparative analysis into other species would be of high value to continue retracing its history.

5.3.5. The Carpathians, the Sudety Mts. and Balkans – eastern edge of the Central European mountain distribution range

The only Balkan accession of *Pulsatilla vernalis* (population from the Rila Mts.) was characterized by the most common haplotype A with the 10-A repetition in the *trn*H-*psb*A region (characteristic for most populations in the Alps). A recent, postglacial migration from the Alps to the Dinaric Alps and Balkans can be therefore suspected here, although it remains speculative and older time of this migration could not be excluded, if a potential high stability of this haplotype is considered. In any case, its affinity to the Alps rather than to the Western Carpathians (Tatra Mts.) is clear. The close relationship of the Balkan alpine flora with the

Eastern Alps is in line of other data, eg. for *Anthyllis montana* or *Soldanella alpina* (Zhang et al. 2001, Kropf et al. 2002, Vargas 2003) showing the importance of these migrations.

In the Tatra Mountains, two haplotypes belonging to the two major groups were found. Each population was monomorphic with respect to its cpDNA haplotypes, three of them had the haplotype C ("blue" group) and one population had the haplotype E ("red" group). The haplotype C, inferred from the PCR-RFLP analysis, differs by one mutational step from the common haplotype A and occurs only in the Tatras and in the Sudety Mts. It was not found in any of the Eastern Alpine populations. The relatively small diversification of the haplotype C suggests that it appeared in an isolated refugial area previously colonised by the haplotype A (most probably from the Eastern Alps). As this haplotype has not spread further, it can be hypothesized that its origin is prior to the last glaciation and it survived the last glacial period already in refugium/-a in- or close to the Tatras and the Sudety Mts. Already other – the few they are – available datasets showed a high isolation of the Tatrean populations of alpine plants with respect to the populations from the Alps. Such genetic isolation was particularly strong eg. in case of *Ranunculus glacialis* (Schönswetter et al. 2003a). Separate lineages were discovered also in case of *Comastoma tenellum* (Schönswetter et al. 2004a), where a recent long-distance colonization from the Tatras to the Eastern Alps was suggested. All these data show the old age of the alpine flora of the Tatra Mts. which was surely shaped in a large part earlier than last glacial exchanges; it also confirms results of investigations on distribution of diploids and polyploids in the European mountains (Skalińska 1963, Küpfer 1974, Favarger 1991). A close clustering of North-Eastern Alpine populations with those of the Carpathians (including the Western and Southern Carpathians) was only showed for *Pritzelago alpina* (Kropf et al. 2002), in a dataset suggesting a gradual colonisation of the European mountain system from the Cantabria and the Mediterranean area.

The population of *Pulsatilla vernalis* characterized by the haplotype E is most probably of a relatively recent origin and results from a long-distance dispersal from the lowland lineage (it is identical, also in respect of the poly-A repeat of the *trn*H–*psb*A region, with the haplotype in the western part of the lowland range – see chapter 5.3.6). Such long-distance dispersal events are discovered to occur relatively often (eg. Schönswetter et al. 2004a, or discussed for northern flora by Brochmann et al. 2003) and to have sometimes a large impact on shaping the genetic diversity of plant populations (Trakhtenbrot et al. 2005). These data suggest a very interesting conclusion, that the presence of *Pulsatilla vernalis* in the Tatra Mountains is due to at least two independent colonization events.

Also, an unexpected genetic relationship between the Tatra Mts. and the Sudety Mts. was demonstrated showing that, although floristically rather distinct in many aspects (mainly in the relative poverty of the flora of the Sudety in relation to that of the Carpathians – Pawłowski 1977), plants from these two ranges can have linked histories. Pawłowski (1977) deduced from the floristic data, that historical migrations shaping the flora of the Sudety Mts. must evidently have been different in a large part from that of the Carpathians, but he also stated that this fact remains very surprising in the context of small distance between the two mountain systems. Still, it should be underlined, that the data concerning the population of *Pulsatilla vernalis* from the Sudety Mts. are not clear and cannot be unambiguously interpreted. If results from PCR-RFLPs are considered (as discussed above), the Tatrean haplotype C is found there. If results from sequencing are considered, however, both Tatra Mts. and Sudety Mts. have the haplotype A (haplotype C was not seen in sequencing data), but with different states of the poly-A repeat of the *trn*H–*psb*A region. Such difference in the poly-A repeat could have very well evolved since separation of haplotype C lineages between the two mountain ranges (a pre-Würm isolation for haplotype C was suggested anyway); nevertheless, the sequencing haplotype A in the Sudety Mts. is identical with the haplotype A in Finland (common 12-A repeat); if the haplotype C from the PCR-RFLP were not considered, a direct Eastern-Alpine origin of the Sudetean population could be suggested. Both scenarios are supported by single characters and are comparatively parsimonious, if the relative stability of the poly-A repeat is considered (as already discussed above).

5.3.6. The lowland distribution range and Fennoscandia

The lowland part of the distribution area of *Pulsatilla vernalis*, located to the north of the European alpine system, belongs to the regions characterized by the highest variability of the chloroplast DNA in populations of *Pulsatilla vernalis*. This is due to a complex history of formation of this part of the range, well reflected by the contemporary spatial pattern of cpDNA haplotypes. It can be surely stated, that the lowland populations did not arise in consequence of a simple process of postglacial descending of species from the source populations in the Alps. The lowlands were colonized by both of the main genetic lineages, one of presumably Central/Eastern Alpine origin (haplotype A) and the other one of presumably Western Alpine–Pyrenean origin (haplotypes D and E). The lowland populations were at origins of further migrations northwards after the retreat of the Weichselian ice sheet from the south of the Fennoscandia. The present distribution of the haplotypes clearly

suggests, that the colonization of lowlands by "blue" and "red" lineages occurred independently, by two separate migrational waves which had contact zones in the German and Polish lowlands. Distinction of these lineages is suggested especially by completely separate colonization of western (Denmark/Norway) and eastern (Finland) Scandinavia. The western part of the lowland range was built by a lineage which migrated from the Western Alps/Eastern Pyrenees. Noteworthily, all lowland and Scandinavian populations bearing the haplotype E have a constant 9 poly-A repeat in the *trn*H–*psb*A region, while contemporary populations with this haplotype in the Alps and the Pyrenees have a 10 poly-A repeat. The distinctiveness of the haplotype E in this part of distribution and its predominance over the haplotype D (nowadays dominating in the Western Alps) could suggest that the lowland area was not subjected to one migrational event in the last late glacial/postglacial period with the western European mountain area as direct source. This pattern indicates rather an earlier migration from the mountains to the lowland area (although it is difficult to date: could it be earlier glacial period or beginning of the last glaciation?) and allows to involve a northern periglacial refugium, localized between the Alpine ice sheet in the south and the Scandinavian glacier in the north, in the large area covered by tundra and cold steppe vegetation (Mojski 1993). It is more difficult to explain the presence of the haplotype D (at present dominating in the Western Alps), in separate stands in the Polish lowlands. This haplotype is very close to haplotype E and differs from it only by one character (cf. Fig. 5.3, 5.6). It can be supposed that it migrated paralelly to haplotype E, or that it represents a separate (more recent) migration wave.

In fact, *Pulsatilla vernalis* – taking into account its ecology – could have been present in the lowlands during even long periods of climatic oscillations, probably apart from the warm interglacial maximum. Expansion from the periglacial refugium would also explain better the purity of the western Scandinavian lineage. The hypothesis of northern lowland refugium might also concern the "blue", Central/Eastern Alpine haplotype, but no suggestions for that can be found in the data available and a direct more recent (postglacial) colonization from the Eastern Alps should be considered here more plausibly. Also the complete lack of the haplotype A in the western-Scandinavian lineage suggests, that only haplotypes from the "red" lineage, which probably migrated through the depressions of the Rhone and Saone valleys, were present in that refugium, and therefore present populations in the northern foothills of the German Alps (characterized by the haplotype A) are of more recent, postglacial origin. Possibility of alpine plant survival in the area between the major ice sheets has been rarely invoked so far, and yet the importance of such "cryptic" refugia (Stewart &

Lister 2001) in more northerly latitudes could be high for further colonization processes. Stewart and Lister (2001), based on macrofossil data, argue for the presence of several northern refugia even for relatively thermophilous trees during the last glacial maximum. Remains of plants and mammals found in different areas and their assemblages "nonanalogue" to present communities show that a mosaic character of the vegetation in unglaciated Middle Europe was much more pronounced than expected from generalistic models. Therefore, refugia for different groups of organisms, including temperate deciduous forest species, could exist there due to sets of local topographic and climatic conditions. The concept of cryptic northern refugia would also explain arrival of such trees and shrubs as *Quercus*, *Ulmus* or *Corylus* to the Scandinavian Mountains immediately after deglaciation, as suggested by fossils from there dating back to 8 500 – 8 000 years b.p., without necessity to invoke a rapid, leptokurtic dispersal uniquely from South-European refugia. Glacial survival in diffuse refugia at the edges of Scandinavian ice sheet was proposed for *Calluna vulgaris* (Rendell & Ennos 2002), based on relatively high diversity of cpDNA in Scandinavian populations of this shrub and lack of a clear south-based colonization lineage. The case of *Pulsatilla vernalis* (identity of Western-Scandinavian lineage with more southerly lowland populations, but some difference with respect to the mountain populations of the same lineage) brings an even stronger support to such a possibility.

After the Holocene vegetation change, in the temperate lowland regions *Pulsatilla vernalis* survived in open-canopy pine forests and heathlands. It is considered as a preboreal (approx. 10 000 years b.p.) relict in the lowland areas (Szafer 1977, Muller 1997), connected to establishment of pine forests, but in fact the venue of spring anemone to this area could be older. As it was already stated, the ecology of *Pulsatilla vernalis* has to be taken into account when discussing its historical fate and shaping its present distribution range. Ecological demands and the amplitude of tolerance for bedrock or climatic parameters obviously play an important role in ability to migrate between isolated optimal alpine habitats (eg. Küpfer 1974). The spring anemone, being a clearly alpine species having large populations much above 3000 m a.s.l. in the Alps, has a marked tendency to occupy also subalpine belt or even dry steppe-like habitats (Favarger 1995). In the contemporary distribution range, its commonness and stability of populations in most mountain areas, being important features in detecting the centres of distribution for taxa (Cain 1974), clearly points to the alpine belt as a primary habitat of this species. The lowland part of the distribution area was historically much more abundant than currently, and a strong decline has been observed especially during the last century. This massive decline of populations can be obviously correlated with

anthropogenic disturbance of habitats and changes of land use more than by natural (climatic) factors; it can be suspected that *Pulsatilla vernalis* persisted in these areas due to its large ecological amplitude, but at the edge of this amplitude, and therefore breaking the stability of these ecological conditions, would it be due to anthropogenic impact or minor climatic changes, may have launched a process of erasing this relict range, as it was much earlier the case of other, more stenoecious, arctic and alpine taxa. Noteworthily, the "*Pulsatilla vernalis* distribution type" after erasing its lowland range will present a model type of "alpine-north European distribution type" as defined by Pawłowska (1977). The distribution range of *Pulsatilla vernalis* demonstrates a somehow intermediate – or rather linking – character between the non-mountain group and the high-mountain group (Pawłowska 1977) with the presumed provenance affinity to the latter one. Jäger (1970) emphasizes the importance of the South – North extension of the distribution range in the suboceanic region in case of some species of presumed high-mountain origin. Apart from edaphic conditions, the "Ozeanitätsamplitude" showing some similarities between southern mountain areas and northern lowland areas could play an important role in shaping these distribution patterns. Based on such an assumption, Jäger (1970) gathers together a few species (sometimes much ecologically heterogenous), as eg. *Sesleria uliginosa, Pulsatilla vernalis* or *Schoenus ferrugineus.*

The pattern of genetic diversity of *Pulsatilla vernalis* detected in the Scandinavia brings the clearest ever proof for independent colonization histories experienced by the western and eastern Scandinavia. Despite a much easier than now land contact due to lowered sea level still in the beginning of Holocene, a hypothesis of a common and subsequently isolated migration wave is not supported in case of the studied species. As already mentioned above, all populations from the western Scandinavia (Denmark and Norway) had identical cpDNA pattern belonging to the Western Alpine–Eastern Pyrenean lineage, while all populations from the eastern Scandinavia (Finland) had the Central/Eastern Alpine haplotype. The few studies on alpine species with disjunct populations in the north, together with the present one, once more testify that colonization histories, especially in large spatial scales, had no clear common line and that the genetic links between populations are complex (Taberlet et al. 1998). A very localized colonization event from Eastern Alps was demonstrated to be at the origin of the northern European populations of *Ranunculus glacialis* (Schönswetter et al. 2003). Close links between northern populations and those from the eastern edge of the Central European Alpine System (the Carpathians) were found in *Trollius europaeus* (Després et al. 2002) and *Comastoma tenellum* (Schönswetter et al. 2004a). Apart from that, relatively complex datasets

on plant genetic diversity, covering the whole Europe, are available almost exclusively for trees and shrubs, thanks to an extensive European project dedicated to studies of European forests' history and genetic resources (CYTOFOR). Only these data allow a larger comparison of cpDNA data across Europe. It is not very useful to conclude about refugial areas of mountain species, as ecological and climatical conditions avoided by trees are exactly those ensuring survival of mountain plants, but it can serve to retrace major tendencies in postglacial re-immigrations to temperate and northerly lowland areas. In case of most deciduous tree species palaeobotanical, pollen-based predictions of glacial survival in southern refugia localized mainly in the peninsulas of the Southern Europe (eg. Huntley & Birks 1983) was confirmed. Their postglacial recolonisation was often due to single genetic lineages (predominantly from the Balcans), causing marked bottleneck effects nowadays reflected by presence of single cpDNA haplotypes in all the Central and Western Europe, in contrast to higher allelic richness in the South, as in case of *Carpinus betulus* (Grivet & Petit 2003) or *Fagus sylvatica* (Demesure et al. 1996). Marked contrast between the highly diversified Southern European populations and those north of the Alps fixed for only one haplotype was also detected in *Frangula alnus* (Hampe et al. 2003). In *Alnus glutinosa* (King & Ferris 1998) a single haplotype originating from a Carpathian refuge was responsible for colonization of the whole Fennoscandia. There are trees, however, for which re-immigration from all major southern refugia was documented, as e.g. *Quercus robur* and *Q. petraea* (Ferris et al. 1998, Petit et al. 2002) or *Abies alba* (Konnert & Bergmann 1995). Apart from ecology and dispersal abilities such different histories were surely shaped by stochastic events or chronological sequences of colonization (possibility of efficient filling open habitat gaps for earlier immigrants) and such differences can be retraced for all interglacial periods (Grivet & Petit 2003). The situation is further complicated by the already invoked above assumption of temperate, "northern" refugia deduced mainly from macrofossils (Stewart & Lister 2001). Molecular support for this concept was not found so far among thermophilous tree species, but it can be hypothesized to have played an important role for less thermophilous trees and shrubs with more markedly boreal distributions as *Calluna vulgaris* (Rendell & Ennos 2002) or *Betula pendula* (Palmé et al. 2003), which would be reflected by their genetic patterns not conforming to the overall patterns of the "southern refugia" paradigm. The northern survival is particularly suggested also for *Pulsatilla vernalis*. On average, importance of Central and South-Eastern European refugia (Balkans, Carpathians, Eastern Alps) and their genetic lineages for colonization of Central and Northern Europe should be underlined in case of trees (eg. Demesure et al. 1996, King & Ferris 1998, Grivet & Petit 2003) as well as disjunct

mountain(-arctic) herbaceous species (eg. Després et al. 2002, Schönswetter et al. 2003, 2004a). Such a tendency is also confirmed by the colonization of Finland (eastern Scandinavia) by the Central/Eastern Alpine lineage of *Pulsatilla vernalis*, although it should be mentioned that no trace of influence of the Carpathian populations (haplotype C) was found in this migration wave. Nevertheless, the eastern lineage was not responsible for the build-up process of all Scandinavian range. The distinctiveness of colonization history of western and eastern Scandinavia was never as clearly demonstrated as in case of the present study. Mixed genetic character was already proposed for Scandinavian populations of *Quercus* (Ferris et al. 1998) and afterwards confirmed in other detailed studies (Jensen et al. 2002, Petit et al. 2002) but genetic lineages were not so spatially independent and contact between the rest of the peninsula and the Finnish territory occurred. Independent genetic lineages were also proposed to be responsible for colonization of Scandinavia also in case of forest understorey herbs, *Carex digitata* and *Melica nutans,* but with parallel genetic intermingling (Tyler et al. 2002), too.

GENETIC STRUCTURE OF POPULATIONS – INSIGHTS FROM A CASE STUDY IN THE POLISH LOWLANDS

6. GENETIC STRUCTURE OF POPULATIONS – INSIGHTS FROM A CASE STUDY IN THE POLISH LOWLANDS

6.1. AFLP ANALYSIS OF *PULSATILLA VERNALIS* POPULATIONS IN THE POLISH LOWLANDS

A detailed study of genetic diversity in populations of *Pulsatilla vernalis* was performed in several populations from the endangered and rapidly decreasing part of lowland distribution range, using the AFLP method allowing a complex genomic fingerprinting (Ronikier 2002). It was planned as a part of a more complex study involving selected populations from all regions, with special emphasis on comparison of ecologically divergent lowland and mountain populations as well as of declining populations and stable populations in the distribution centre. AFLP analysis of the genomic DNA was also planned as a tool for refining regional scale geographic patterns in the phylogeographic analysis. In a laboratory study the possibility of using AFLPs in *Pulsatilla vernalis* appeared to be very limited due to methodological problems. It was possible to obtain reliable and repeatable fingerprinting only for a part of individuals from the Polish lowlands, while analysis of most other populations was rejected due to insufficient repeatibility. AFLP is a very efficient fingerprinting method under condition of having clean DNA devoid of high concentrations of cellular compounds which could interfere with DNA and inhibit enzymatic reactions (restriction, amplification). In the AFLP specifically, the presence of inhibitors can cause inconsistent enzymatic restriction which inevitably influences all further steps of reaction (Mueller & Wolfenbarger 1999). As composition and concentration of secondary compounds is species dependent, the method is inequally useful among groups of taxa. In case of *Pulsatilla vernalis* the usefulness of the DNA extract for AFLPs seems to be correlated with phenological phases of development. It was possible to obtain repeatable results from a part of plant samples collected in the spring, while the analysis of those collected later in the year was problematic, and finally some of the autumn samples were hardly susceptible to any amplification. It can be most probably correlated with changes in concentration and composition of cytoplasmatic substances (accumulation of inhibitors, such as secondary phenolic compounds, during the vegetation season).

Obtaining generally stable AFLP profiles in *Pulsatilla vernalis* failed also in an independent laboratory test (kindly done by P. Schönswetter, Vienna). The similar methodological problems with application of the AFLP method occurred also in other works dealing with the genus *Pulsatilla*: another experiment of analysis of the DNA variability in local populations

of *Pulsatilla vernalis* in Bayern (Jehle 2000), as well as a pilot study on *Pulsatilla alpina* (Zetzsche & Blattner 2001, H. Zetzsche, pers. comm.). For fine-scale ecological investigations a microsatellite study would be adviseable. It was not possible, however, to undertake it within the framework of the present study due to a long and expensive process of development of species-specific microsatellite primers (eg. Powell et al. 1996).

Reproducible AFLP results obtained in the spring samples permitted a preliminary analysis of genetic diversity in the populations from the Polish lowlands (analyzed in chapter 6.2). For this case study, it was finally possible to include 63 samples in the analysis, ranging from two to twenty per population, from seven populations in the total (Table 6.1). This sample set, although fragmentary, allowed an approximate analysis of the amount and structure of genetic variability in these endangered and declining populations.

Table 6.1. Populations studied (acronyms as in the Table 4.1), estimated population size (S), number of individuals analysed (N), main or unique cpDNA haplotype found in the population – if determined (cpDNA); number and proportion of polymorphic loci in the population for five individuals per population (except for population 5) (P), number and proportion of polymorphic loci in the total population sampling (P_T), number of unique ("private") fragments, confined to only one population (U), average gene diversity in the population calculated for five individuals per population (except for population 5) (D). For the population BE number of samples used for general analysis is followed by the number of samples used for the population analysis (in parentheses).

No	Population	S	N	cpDNA	P	P_T	U	D
1	BB	10	5	–	117 (26%)	117 (26%)	0	0.145
2	BE	25	12 (20)	A	163 (36%)	228 (51%)	0	0.182
3	BI	80–100	15	D	204 (46%)	291 (65%)	3	0.214
4	DO	30	10	A	155 (35%)	191 (43%)	1	0.167
5	KM	2	2	–	62 (14%)	62 (14%)	0	0.139
6	OS	40	6	D	148 (33%)	156 (35%)	0	0.162
7	US	6	5	A	159 (36%)	159 (36%)	2	0.180

Five primer combinations were selected based on clarity of produced fragment patterns: *Eco*-ATC/*Mse*-CTAC, *Eco*-AGT/ *Mse*-CACG, *Eco*-AGA/ *Mse*-CACA, *Eco*-AGA/ *Mse*-CACG, *Eco*-AGA/ *Mse*-CAAC. In total, 446 scorable fragments (bands) were obtained, out of which 169 (37.9%) were monomorphic across all populations investigated. Nevertheless, all individuals revealed their own AFLP phenotypes. Three populations possessed single "private", unique fragments. At the intra-population level the number of polymorphic loci varied between 14% (in the two-individual population) and 46% (mean value 32.3%) in a

normalized number of samples fixed as five per population (the common number for all populations except the abovementioned two-individual one).

The mean index of average gene diversity in populations was 0.170 and varied from 0.139 to 0.204 (Table 6.1). The genetic diversity of populations appeared to be moderately correlated with population size, and it was conceivable only when most differing populations were compared.

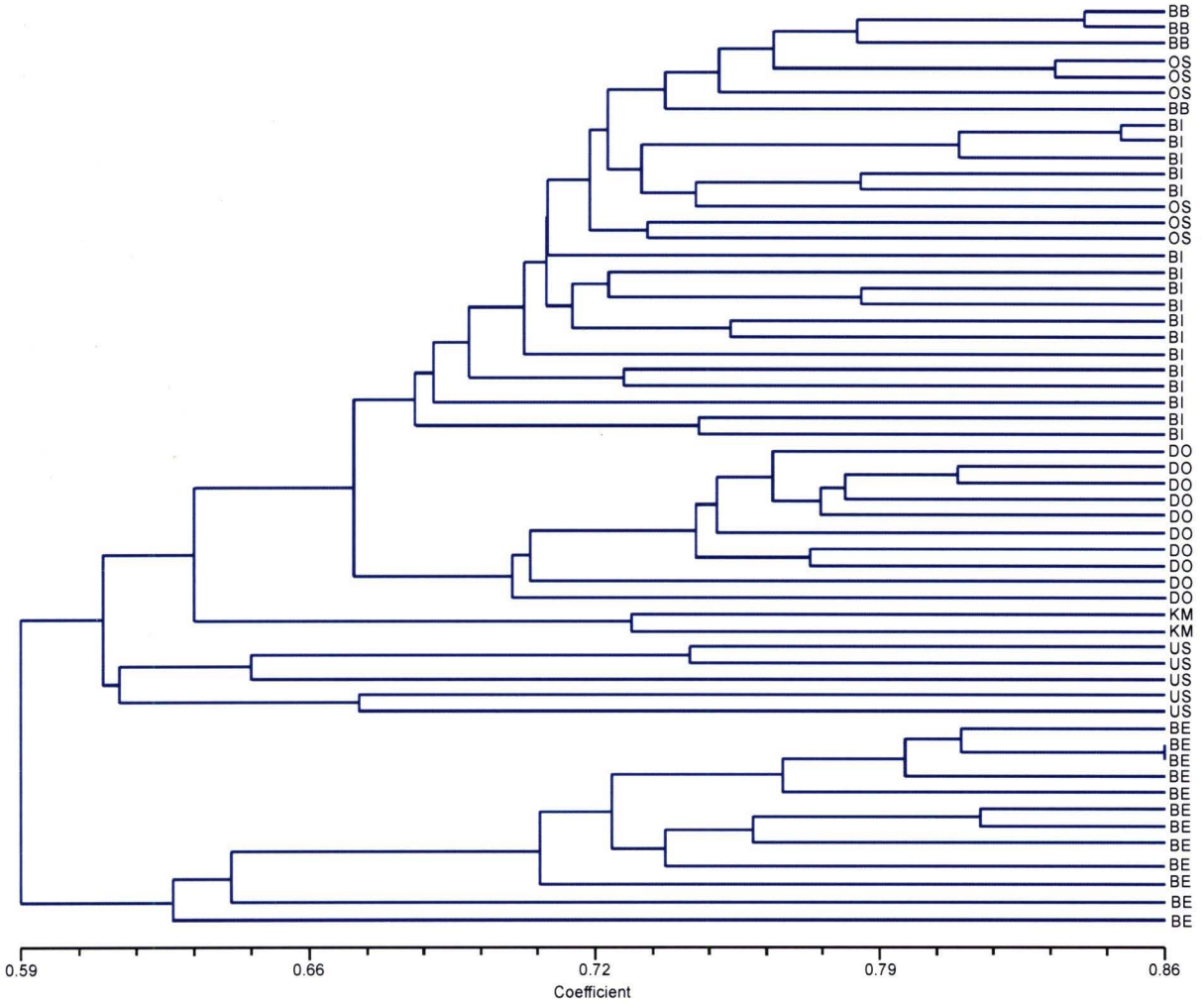


Fig. 6.1. UPGMA clustering of 56 individuals of *Pulsatilla vernalis* from seven populations (respective individuals are marked with two-letter acronyms of populations), based on 446 AFLP markers and computed using a similarity matrix based on Jaccard's coefficient.

Genetic similarity between all individuals was assessed using the UPGMA clustering method (Fig. 6.1; see chapter 6.2 for detailed discussion). The AMOVA analysis (Table 6.2) did not reveal strong between-population variability and accounted most of the diversity (approx. 72%) to the within-population component, which generally confirms the picture obtained with UPGMA analysis.

Table 6.2. Results of the Analysis of Molecular Variance (AMOVA) for 58 individuals of *P. vernalis* from 7 populations, using 446 AFLP fragments. Levels of significance are based on 1023 random permutations. Fixation index (FST) amounts to 0.2822.

Source of variation	d.f.	Variance component	Percentage of variation	P
Among populations	6	14.90	28.22	<0.001
Within populations	52	37.91	71.78	<0.001

A separate fine-scale analysis of spatial distribution of genetic diversity was performed for the population from Central Poland (BE). Clustering of all samples available (20) made using the UPGMA method was compared with the spatial distribution of sampled individuals in the populations based on the system of permanent observation plots established by J. Hereźniak (Hereźniak et al. 2001). The genetic relationship between individuals in most cases corresponded exactly to their spatial distributions. In some cases, however, examples of longer distance gene flow were also found (Fig. 6.2).

6.2. Level of genetic diversity and genetic structure of populations

The analysis revealed a moderate genetic diversity in populations of *Pulsatilla vernalis* studied using AFLPs (Ronikier 2002), with the average percentage of polymorphic loci slightly exceeding 30% and the mean average gene diversity index amounting 0,170. A comparison of these values with literature data for various species, as gathered eg. by Gaudeul et al. (2000), allows a general statement that populations of *Pulsatilla vernalis* in the studied area have an intermediate level of diversity. When comparing a normalized data set of 5 individuals per population, the variability was higher in the largest population (BI) in comparison to other populations (cf. Table 6.1). This difference was significant, however, only in case of this largest population exceeding 2–3 times the second largest population in the analysis (OS). In populations not exceeding approx. 40 individuals, the diversity values were mostly comparable and not correlated with the population size. It should be underlined, however, that the relationship between population size and genetic diversity should be interpreted with caution, as it can be biased to some extent by potential high annual fluctuations in development of plants (Wójtowicz 2001); as a consequence, a part of population can be cryptic and present only as underground rhizomes. Clearly, the largest populations (as BI) have considerably higher levels of genetic diversity and special efforts should be paid to protect them as the main depositaries of species' gene pool; this confirms

the general view, that big populations are more likely to maintain a high level of diversity (Frankham 1996).

The largest part of the genetic variation detected was the within-population variability. It was well demonstrated by both high value of the AMOVA analysis for this parameter (more than 70%) and long individual branches (corresponding to lower values of similarity index) in the UPGMA tree (Table 6.2, Fig. 6.1). Such a situation can be interpreted as a result of a relatively recent history of strong decrease in *Pulsatilla vernalis* populations, as it was suggested for some other rare species with recently increasing isolation of populations (Rossetto et al. 1995, Zawko et al. 2001). If this is true, it can be expected that with time the diversity of populations of *Pulsatilla vernalis* will respond to such isolation in a stronger way (as showed e.g. in a study of *Saxifraga aizoides*; Lutz et al. 2000). This interpretation (suggested by Ronikier 2002) has a possibility to be further verified when AFLP data (from biparentally inherited genomic DNA) can be analyzed in concert with available data from the chloroplast DNA (uniparentally inherited and dispersed by seeds).

Despite a relatively low between-population variability, populations were mostly grouped in population-specific clusters in the UPGMA tree (Fig. 6.1). Nevertheless, some individuals from populations BI, OS and BB from the Bory Tucholskie region were intermingled. Interestingly, these populations form altogether a separate clade in the tree based on AFLP data, and both populations OS and BI are characterized by the haplotype D belonging to the "red" (cf. Fig. 5.3) group of chloroplast DNA haplotypes (BB was not included in the analysis of cpDNA), while all populations characterized by the haplotype A ("blue" group of haplotypes), i.e. BE, US and DO, are located outside this clade (KM was not included in the analysis of cpDNA). In populations from the Bory Tucholskie region (BB, BI, KM, OS, US) groups representing "red" and "blue" lineages were well separated. Nevertheless, populations DO and BE (located in the Central Poland, hence most distant geographically from the abovementioned group) were more distant with respect to all populations from the Bory Tucholskie region. The population DO was placed in the UPGMA tree next to populations bearing cpDNA haplotype A from the Bory Tucholskie region, while BE (also bearing haplotype A) had a separate position to all remaining populations.

This separation of the two historical lineages – revealed by the cpDNA analysis – also in the AFLP analysis (involving information from the entire genome) indicates, that genetic exchanges between populations by means of pollen were probably not large enough to erase the identity of lineages showed by chloroplast DNA (only reflecting gene flow by seeds). These results suggest, that: (i) short distance gene flow and dispersal dominates via both seeds

and pollen, as traces of historical lineages detected using cpDNA can be also found when studying whole-genomic DNA, even among relatively spatially close populations; (ii) nevertheless, genetic exchanges between close populations must have surely occurred, as larger-scale geographical structure dominated the old lineage differentiation and even though haplotype lineages were well separated within the Bory Tucholskie region, overall, two most distant geographically populations (BE and DO) were also the most distant genetically in the entire sample set.

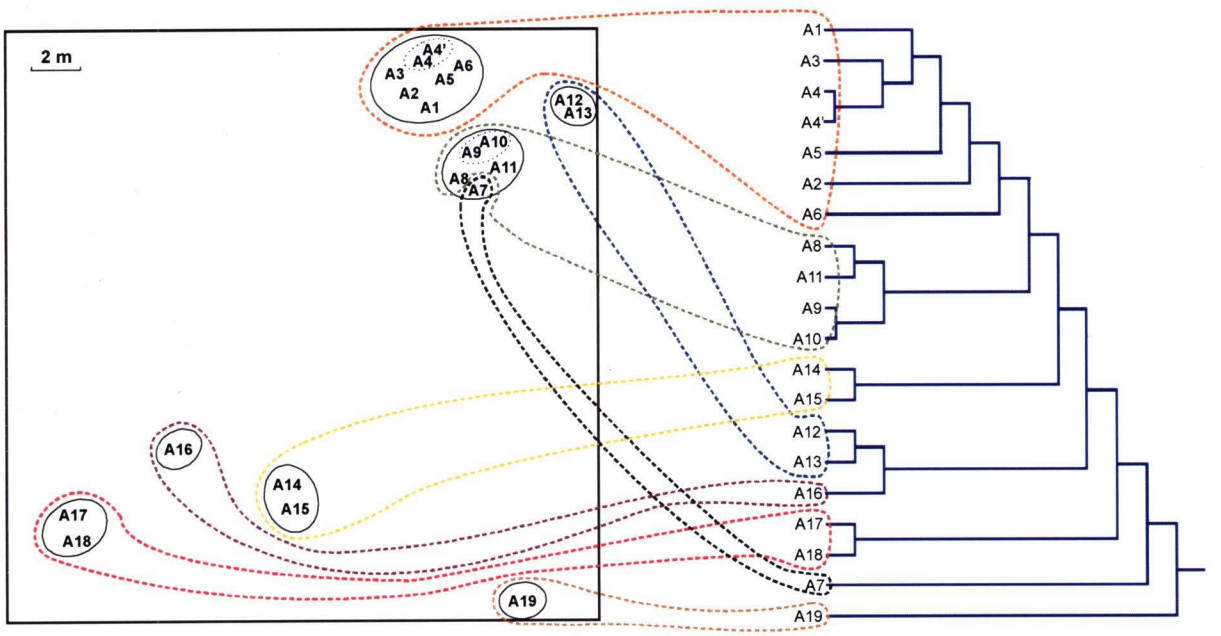
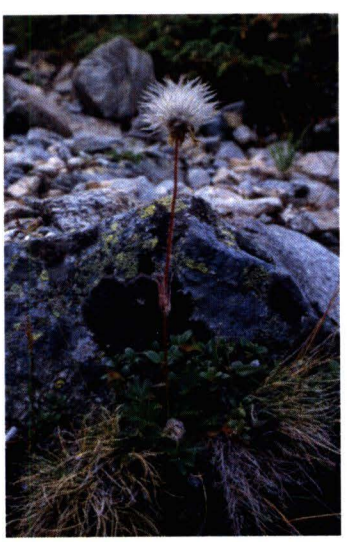


Fig. 6.2. Analysis of correlation between genetic similarity of individuals and their spatial relationships. Genetic similarity was assessed by the UPGMA clustering of 18 individuals from all groups composing the population; spatial structure of the population is represented on a plot. Relationships between genetic clusters of the tree and groups of individuals on the plot are showed by dashed lines. Pairs of individuals growing very close one to another are marked by dotted lines.

A more detailed analysis of spatial and genetic relationships in populations of *Pulsatilla vernalis* was possible using the population BE (Central Poland) as a model. This population was used as it was well structured, well described spatially thanks to monitoring plots previously established by J. Hereźniak (Hereźniak et al. 2001) and as samples from approx. 75% of individuals, from all spatial groups, were collected. Twenty samples were subjected to a separate UPGMA clustering and their genetic relationships, as reflected by the topography of the tree, were compared with the spatial location of respective individuals (Fig. 6.2). Most spatially defined groups of individuals were perfectly clustered together also based on genetic data. This is true especially for the group of individuals A1–A6 and its neighbouring group A8–A11, as well as for the groups A16 and A17–A18. Nevertheless, in some individuals or groups their spatial location was not correlated with position on the tree, as in the case of

A12–A13, genetically paired with A16 but spatially located in the opposite part of the plot, or A7, genetically closest to a separate individual A19 but spatially located within the group A8–A11. Noteworthily, some spatially close (or very close) pairs of individuals exhibited very small genetic differentiation, as e.g. in case of A4 and A4' or A9 and A10. A similar picture of spatial/genetic relationships was observed in another large population – BI (data not shown in detail). The results of this fine-scale analysis corroborate to a large extent the conclusions drawn from the general analysis: (i) relatively high congruence between spatial and genetic structuring of the population shows that the short-distance gene flow prevails in the populations, moreover, (ii) very close genetic affinities (genetic distance within 5% of the overall diversity) of several pairs of individuals very close in the space suggests, that such mechanisms as vegetative spread by rhizome split, or autogamous reproduction occur in the population; (iii) longer distance gene flow between groups – although to a minor degree – also occurs, and it has also a considerable role in shaping the population structure.

MAIN RESULTS AND CONCLUSIONS OF THE STUDY



7. MAIN RESULTS AND CONCLUSIONS OF THE STUDY

1) The study was based on the plant material collected from sixty-three populations of *Pulsatilla vernalis* sampled in all parts of the species' distribution range. Sixty-one populations were analyzed in a cpDNA-based phylogeographical study (304 individuals in total). Seven populations were used for a supplementary study of genetic structure of endangered populations in the Polish lowlands using the AFLP fingerprinting method (63 individuals in total).
2) The PCR-RFLP analysis of the chloroplast DNA involved seven cpDNA regions; after preliminary screening, fourteen fragment/restriction enzyme combinations were selected for the full-range study. The analysis revealed seven cpDNA haplotypes in the entire range of *Pulsatilla vernalis*, belonging to two well differentiated genetic lineages (haplotypes A, B, C versus D, E, F, G).
3) The sequencing analysis of two cpDNA regions (which were also involved in the PCR-RFLP) was also performed for all studied populations, yielding one of the largest sets of intraspecific sequences published so far. It was confronted with the PCR-RFLP analysis, unraveling the same major pattern of the chloroplast DNA diversity as did the latter analysis. All important mutations found in the sequences were also detected as PCR-RFLP polymorphisms. This methodological comparison indicates a high reliability of a rigorously conducted analysis of restriction patterns.
4) Among the cpDNA haplotypes detected, the haplotype A was characterized by the highest frequency in the dataset (0.53); two other haplotypes (B, C) were close derivatives of A, but they were rare and local. Haplotypes D and E from the second lineage were relatively abundant, and two other haplotypes from that lineage (F, G) were present only in single populations. Haplotype A was present in all parts of the distribution range. Haplotypes D and E were also widely distributed across Europe. The distribution of haplotypes does not allow a reasonable attempt to identify the primary centre of differentiation. The differentiation of the two main lineages can be explained as: (i) an old (at least pre-Würmian) evolutionary split, origins of which were subsequently obscured by migrations, shifts etc. during following glacial periods, or (ii) a localized differentiation of one lineage from the most widely distributed and stable haplotype (A) and explained by an assumption of possible uneven mutation rate in non-coding cpDNA regions.

5) The phylogeographical information obtained was much clearer in the areas where a total postglacial recolonization occurred (Scandinavia, Alps) than in areas where a more continuous genetic exchange could have occurred (eg. the Pyrenees).
6) A clear spatial pattern of cpDNA variability was found in the Alps, where two strongly differentiated areas were detected: the South-Western Alps (haplotypes D + E) and the Central and Eastern Alps (dominated by haplotype A). In the latter region, a separate small area characterized uniquely by the haplotype B (derivative of A) was detected close to the Dolomites. These results show a very strong intraspecific split separating the South-Western Alps from the rest of the range; an indication for at least three peripheral refugia during the last glaciations can also be inferred, with possible survival of populations in interconnected lowland areas close to the mountains. These data confirm the conclusions drawn for a few other alpine species and contribute to a knowledge on the Pleistocene fate of alpine plants.
7) The populations from the Pyrenees show close genetic relationships with those from the South-Western Alps; nevertheless, the complicated spatial distribution of haplotypes in the Pyrenees does not allow simple conclusions on history of this part of the species' range.
8) A weak evidence for relationships between the populations from the Central Massif with those from he Pyrenees, rather than with those from the Alps, was found.
9) The lowland part of the distribution range did not evolve as result of a simple postglacial migration down from the mountains to adjacent lowland areas; at least two independent migration events occurred involving two Alpine genetic lineages (with Western and Eastern alpine populations as sources); additionally, at least in case of the Western part of the lowland area, existence of northern periglacial refugia north of the Alps is suggested, based on sequence polymorphisms, for the last glacial period. These data indicate an older than usually expected presence of *Pulsatilla vernalis* in the lowlands.
10) Two independent migration lineages responsible for the creation of the Central European lowland range were also responsible for colonization of the Scandinavia. Western and eastern Scandinavian areas (Denmark/Norway and Finland respectively) were colonized independently by two genetic lineages of *Pulsatilla vernalis*. Such independent histories in plant colonization of these areas were never so clearly demonstrated before.
11) The Tatra Mts. were characterized by a separate haplotype (C), differing by one mutation from A and suggesting a certain degree of isolation, and not a recent immigration, from

the Alps. Furthermore, the data indicated a second, independent colonization event from the lowland populations.

12) In the population from the Sudety Mts. the Tatrean haplotype was identified, but with a small difference in the sequence analysis, which suggests some isolation and not a recent long-dispersal. Nevertheless, the resolution of the data is poor here and precludes strong conclusions.

13) The isolated populations in the Balkan mountains harboured identical haplotype as that most widespread in the Alps, hence demonstrating close relationships with the Alps and not with the Carpathians (Tatra Mts.)

14) The distribution of the cpDNA haplotypes was not congruent with that of described infraspecific taxa (varieties) proving, that these taxa do not represent monophyletic evolutionary units but rather expression of ecotypic variation.

15) The supplementary AFLP analysis of the Polish lowland populations revealed an intermediate level of genetic diversity, and presumed existence of between-population gene flow responsible for relatively low between-population variability. Nevertheless, this genetic exchange is limited, as traces of old genetic lineages can still be detected in the genetic relationships between individuals.

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