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Comparative European phylogeography of mountain forest and peatland species using the example of two plant and two butterfly species

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To Walter Bujnoch,

who never loses his faith in science!

"There are some sharks around, but people who are very aggressive and personally ambitious miss the point:

they don't influence others to do things, they don't have the combined effects of lots of people working together."

Godfrey M. Hewitt (1940 – 2013)

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1 General Introduction

During the quaternary, glacial-interglacial oscillations led to numerous range expansions and regressions of many plant and animal species (Hewitt 2000; Zhang *et al.* 2001; Vargas 2003; Schmitt 2007). During glacial periods, most of Northern Europe and large parts of the high mountain systems in Southern Europe were covered by glaciers, while arctic tundra and cold steppe ecosystems marked major parts of Central Europe (Webb & Bartlein 1992; Dansgaard *et al.* 1993; Hewitt 2004; Schmitt 2007).

Many phylogeographic studies have already shown that climatic changes during the Quaternary had significant implications on population genetic structures through these impacts on the distribution of species by range shifts in latitude and altitude (e.g. Comes & Kadereit 1998; Taberlet *et al.* 1998; Hewitt 2004; Schmitt 2007). One of the main challenges in phylogeographic research is to identify quaternary glacial refugia, which may represent long-term reservoirs of genetic variation, where evolution has produced unique genotypes and high levels of diversity (Willis & Whittaker 2000; Liepelt *et al.* 2002; Taberlet & Cheddadi 2002; Hampe & Petit 2005).

In this context, three major biogeographic types can be distinguished in Europe (Schmitt 2007): (i) Mediterranean, (ii) arctic-alpine and (iii) continental. Still in the second half of the 20th century, the most common hypothesis of glacial survival and postglacial colonisation postulated the complete extinction of most species from glaciated areas of Central Europe – the so called *tabula rasa* hypothesis. This hypothesis could be proved for many species with a present day distribution reaching Central and sometimes, Northern Europe (Birks 1993; Dumolin-Lapègue *et al.* 1997; Petit *et al.* 2002, 2003; Grivet & Petit 2003). Consequently, warm-adapted organisms re-colonised Central and Northern Europe postglacially from the classical Mediterranean refugia (de Lattin 1949), mostly from the Iberian Peninsula, Italy and the Balkans as well as areas south of the Caucasus (i.e. Anatolia) (Comes & Kadereit 1998; Taberlet *et al.* 1998; Hewitt 1999, 2004), as evidenced by many genetic studies (reviews in Hewitt 2004; Schmitt 2007; Terrab *et al.* 2008; Aguilar *et al.* 2011).

In the group of arctic-alpine species, the typical high mountain species are relatively well studied (review in Schmitt 2009; Borer *et al.* 2010; Lohse *et al.* 2011; Alvarez *et al.* 2012; Winkler *et al.* 2012; Theissinger *et al.* 2013). During glacial periods, their current areas were covered by glaciers (Holdhaus 1954; Varga & Schmitt 2008). The species had to retreat into suitable refugial areas in the vicinity of these mountains, involving the formation of genetic lineages mostly linked to the respective mountain systems due to

longterm isolation and evolution. Due to retreat into several geographically isolated refugia and postglacial re-colonisation into the mountains systems, different genetic lineages in one area could occur next to each other (Schmitt 2009).

Mountain forests have an intermediate position between lowland and oreal biomes. Phylogeographic studies about species belonging to this specific habitat are still underrepresented. Nevertheless, there are few studies on boreo-montane species (e.g. Despres *et al.* 2002; Alsos *et al.* 2005; Kramp *et al.* 2009; Mardulyn *et al.* 2009; Habel *et al.* 2010, 2011c; Michl *et al.* 2010), but the glacial history about montane forests species is still not investigated satisfactorily.

As far as tree species are concerned, some studies already reveal their biogeographic structures (for some examples, see Table 1.1). However, only few studies have analysed the phylogeographic structure of boreo-montane herbaceous plants of the mountain forest biome, e.g. *Polygonatum verticillatum* (Kramp *et al.* 2009), *Cyclamen purpurascens* (Slovák *et al.* 2012) or the moss *Rhytidium rugosum* (Sabovljević & Frahm 2011), and also phylogeographic studies on mountain forest animal species are still underrepresented, e.g. *Nucifraga caryocatactes* (Haring *et al.* 2007) or *Lycaena helle* (Habel *et al.* 2011a).

Table 1.1: Examples for molecular studies on European tree species growing in mountains.

Tree species	Molecular marker analysed	References
Abies alba	isozymes	Konnert & Bergmann 1995; Breitenbach-Dorfer *et al.* 1997
Carpinus betulus	cpDNA microsatellites, PCR-RFLPs, sequencing; AFLP	Grivet & Petit 2003; Coart *et al.* 2005
Castanea sativa	microsatellites	Mattioni *et al.* 2013
Fagus sylvatica	cpDNA, nDNA sequencing	Demesure *et al.* 1996; Magri *et al.* 2006
Fraxinus excelsior	cpDNA & nuclear microsatellites, PCR-RFLPs	Heuertz *et al.* 2004a, 2004b
Picea abies	RAPD	Collignon & Favre 2000; Ravazzi *et al.* 2006
Pinus mugo	microsatellites	Heuertz *et al.* 2010
Pinus sylvestris	allozymes, mtDNA sequencing	Sinclair *et al.* 1999; Cheddadi *et al.* 2006; Sannikov & Petrova 2012

cpDNA = chloroplast DNA; mtDNA = mitochondrial DNA; nDNA = nuclear DNA; PCR-RFLPs = polymerase chain reaction-restriction fragment length polymorphisms; RAPD = randomly amplified polymorphic DNA.

As well as the boreal-montane biome, Peatlands, which often are essential parts within boreal and montane forests, are an important component of European landscapes, but phylogeographic studies of such wetland species in Europe are also not adequately represented. Nevertheless, there are some studies on plants adapted to bog habitats, e.g. *Vaccinium uliginosum* (Alsos *et al.* 2005) or *Carex nigra* (Jiménez-Mejías *et al.* 2012) and some animal species like *Rana arvalis* (Babik *et al.* 2004) or *Nehalennia speciosa* (Bernard *et al.* 2011). Due to the lack of phylogeographic studies on peatland species it is necessary to close this gap for a better understanding of their biogeography. With the knowledge of the location of potential glacial refugia and the geographical distribution of unique genetic lineages, the remaining peatlands and their flora and fauna worthy for protection could be better understand and will ease the development of potential conservation actions.

1.1 Mountain forests

Forests are the richest and most widely spread climax association. They cover 29 % of the total land area on earth and represent the relatively most natural ecosystems remaining on the mainland (Müller 1991).

Various forest communities that occur in mountain ranges are grouped together in the term mountain forests. They extend over three different vegetation zones: the transition stage (submontane), the mountain forest zone (montane) and the Krummholz level (subalpine). The timberline, from which no typical forest can occur anymore, is at about 2,200 – 2,400 m in the Central Alps and at about 1,900 m in the marginal regions of the Alps. The alpine timberline represents the upper limit of possible forest growth (Wieser *et al.* 2009). Although, the timberline does not function as an abrupt boundary but can be seen as a transition zone between forest and tree line, with the Krummholz zone appearing as an intermediate zone (Wieser & Stör 2005; Holtmeier 2009). It separates the subalpine vegetation zone from the treeless alpine zone (Franz 1979).

About 70,000 years ago, the last ice age (Würm) began. This glacial period was interrupted by several warmer periods (interstadials) during which coniferous forests also including some *Corylus*, *Quercus* and *Fagus* could expand. The Last Glacial Maximum began about 30,000 years BP and ended 16,000 years BP. At the end of the late glacial, the colonisation of the Alpine region first started by tundra vegetation. Gradually, the forest trees re-migrated from their retreats. The pines (*Pinus*) already appeared about 12,000 years ago when the great Alpine valleys became ice-free. The Holocene (14,000 until today) is also influenced by climate fluctuations between warmer and colder periods

(Birks & Ammann 2000). During the late glacial and early Holocene, the ice melted back to small remnants and forests began to spread across Europe. However, this re-forestation was not characterized by a common rapid advance of all recently existing forest tree species in the different regions, but these re-immigrated from their glacial refugia from different directions and at different speeds; consequently, different species came to dominance one after another (Lang 1994). Thus, sparse pine forests were followed by *Corylus* and *Picea*. In the interglacial, mixed oak forests spread out, which were later replaced by fir and beech (Reisigl & Keller 1989). Recently, *Fagus sylvatica* and *Picea abies* are the main stand-forming tree species in Central European mountain areas (Fig. 1.2).

The present European mountain forest vegetation represents more or less a vertical reflection of this postglacial forestation history. The submontaneous vegetation is primarily dominated by *Quercus*, *Fagus* and *Castanea*. In the montane level, *Quercus* is substituted by *Abies* and by *Picea*, especially in the central mountain areas. In the transitional region between the montane and alpine level, the vegetation mainly consists of *Pinus* and *Larix* (Ozenda 1988) (Fig. 1.1).

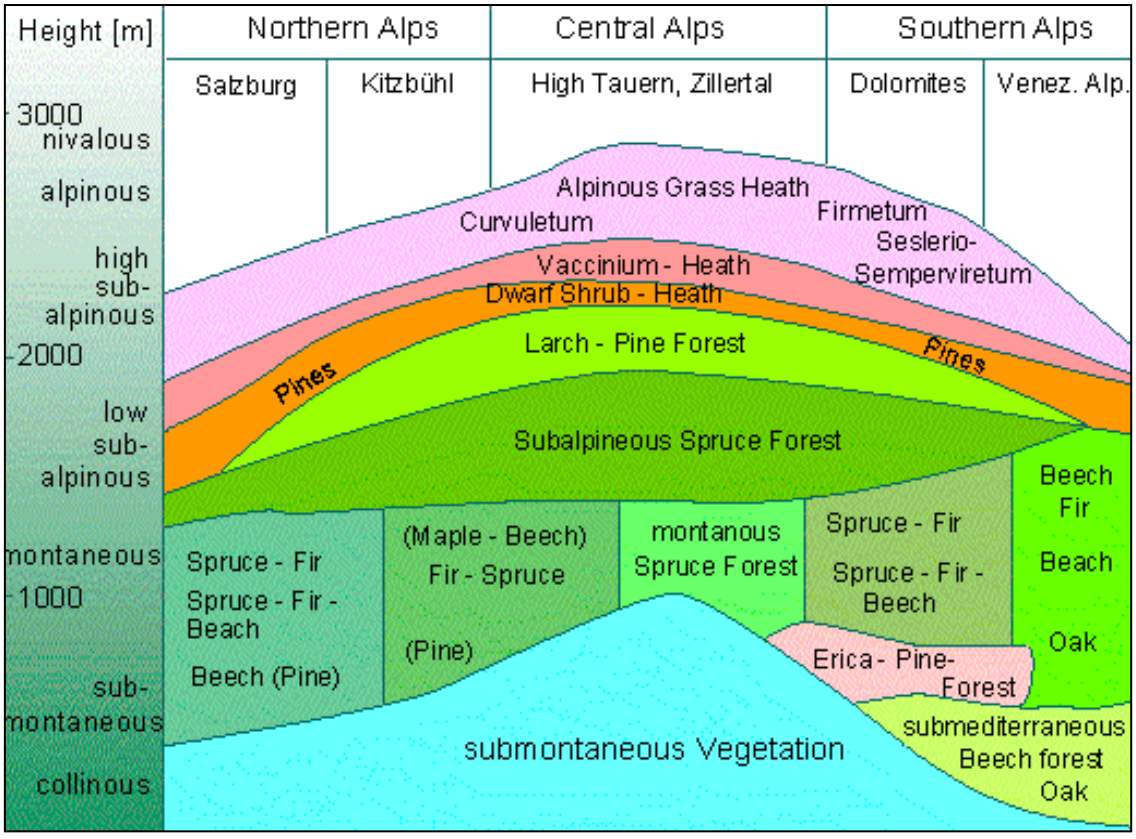

Fig. 1.1: Distribution of the altitudinal zones in the Alps and their respective vegetation (Source: Uni Hamburg, Botany online 1996-2004)

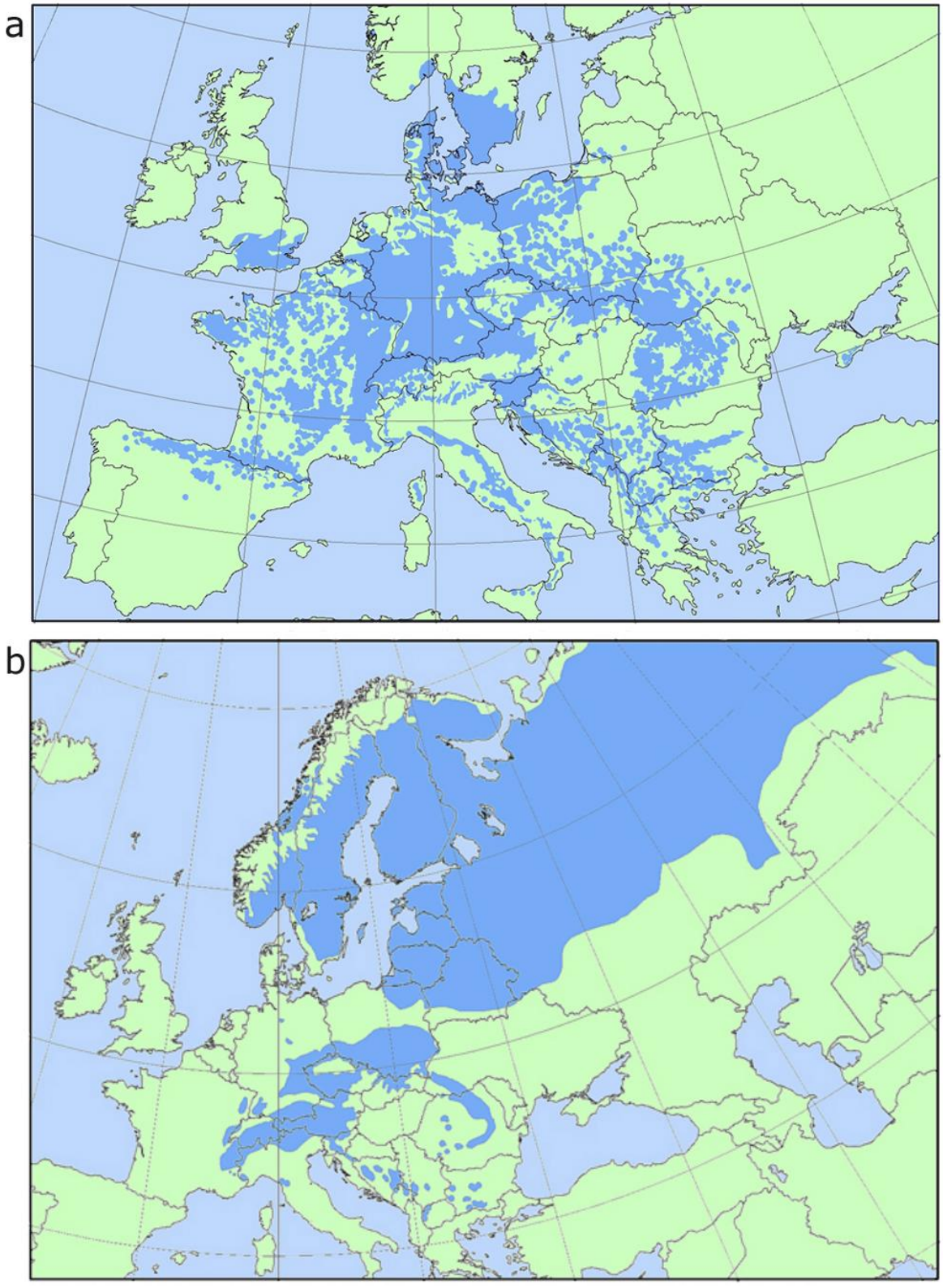

Fig. 1.2: Distribution map of (a) Beech (*Fagus sylvatica*) and (b) Norway spruce (*Picea abies*) (slightly modified from EUFORGEN 2009).

1.2 Peatlands

Due to their intermediate position between water and dryland, bogs play an important key role in the landscape. They catch the melting waters and heavy rainfalls in spring and they provide ground water in drier periods. They filter and retain or metabolize organic material as well as nutrients and pollutants. Bogs both have a high ecological and economic value and provide habitats for many endangered species (Rydin & Jeglum 2006).

As well as forests, bogs underwent profound changes during the Holocene. During the last glacial, rather little standing waters existed in the unglaciated areas of Europe. In contrast, during the ice melting, numerous lakes formed in the areas of retreat of glaciers both in Northern Europe and in the vicinity of the southern mountains. In the beginning, they only had aquatic vegetation with low demands, but during the Holocene, when the water temperatures rose and nutrient supply was increased, they were colonised by plants with higher and more complex ecological demands. In the course of succession, they gradually silted up. Depending on size, numerous lakes were sooner or later converted into bogs (Lang 1994).

The increasing population density in Europe caused a generally strong pressure on landscapes and thus also on peatlands. Therefore, intact and extended bogs have almost vanished from Europe. Compared to all other continents, Europe experienced the most severe losses. 57 % of all former bogs do not show any peat growth today, and in many European countries, the bogs were even reduced to less than 10 % of their original range (Joosten 2012).

In Germany, Great Britain, Iceland, Denmark, the Netherlands and Switzerland, the losses are mainly due to the high demand for land for agricultural use. In Finland, Ireland and Sweden, the main reason is the extensive peat extraction for energy production. In other states, such as the Baltic States, Belarus and Russia, peat cutting plays a decisive role in substrate reclamation for horticulture and private gardens (especially in Western Europe). While in many countries almost all bogs are recently degraded, there are still some regions in Europe where bogs are under less pressure, e.g. in Norway, where 80 % of the original peatlands are still preserved (Fig. 1.3).

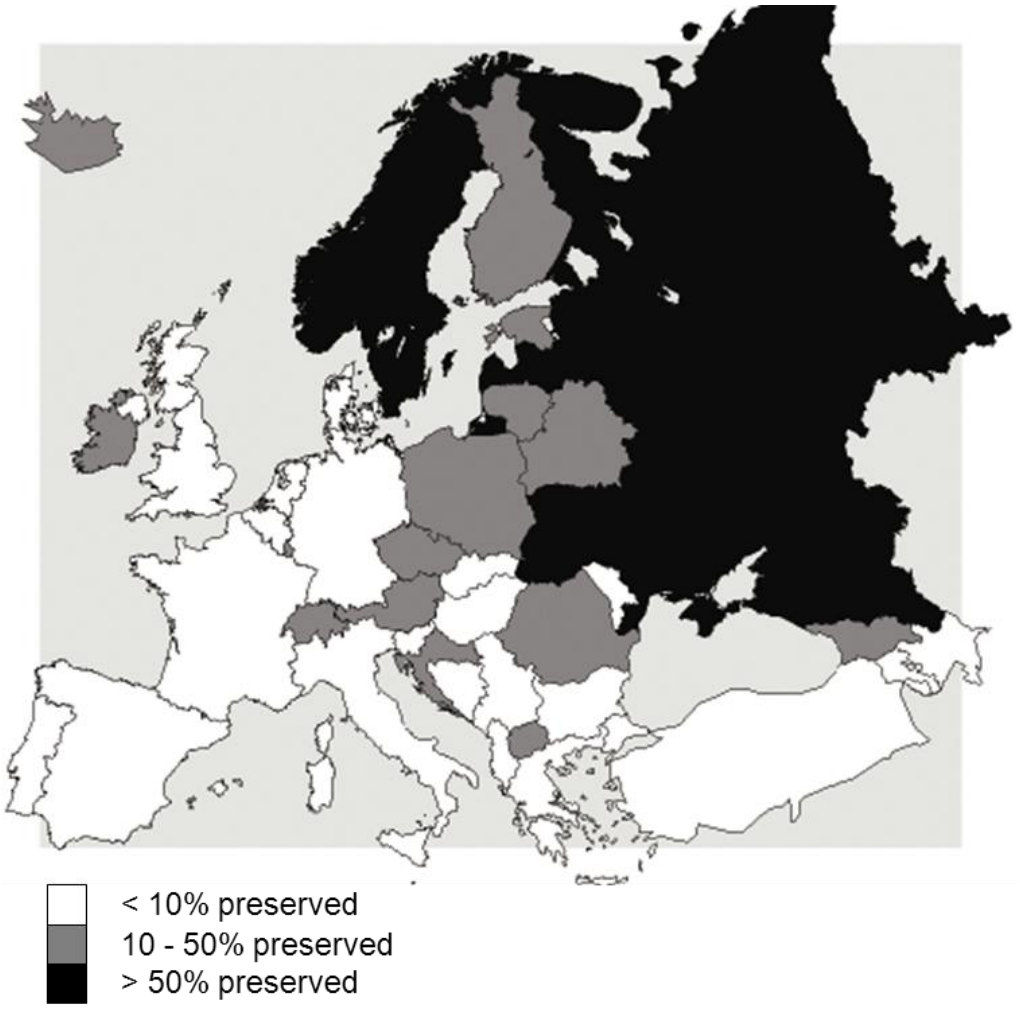

Fig. 1.3: Remaining peat bog areas in Europe given in % of the original area. Within countries, no further differentiation was made (slightly modified from Joosten 2006).

According to the German Red Data Book on endangered habitats, all bog habitat types are already endangered or under threat of complete destruction. Therefore, most of the bog habitat types are under special protection (Riecken *et al.* 2006). However, bogs located in protected areas are also subjected to massive degradation. Intact peat accumulating bogs in Germany have already been pushed back to 1 % (140 km²) of their former extent (Joosten 2012).

1.3 Objectives and model species of the study

Because of their important function in the European landscape, a better understanding about the biogeography of mountain forest and peatlands is essential. The species studied in this thesis have been chosen to further enlarge insights gained so far about glacial history and postglacial migration routes of boreo-montane and peatland species and also to provide new knowledge on the past and present of these two habitat types in order to understand better the complexity of quaternary floral and faunal history. Furthermore, this study represents fundamental research, whereby the main focus lays on the mountain forests, and the results should be the basis for future projects.

For a comprehensive insight into the phylogeographic structure of the mountain forests and peatlands, four model species have been selected; two herbaceous plants as representatives for sessile species and two butterflies as more mobile species. Also, the study species cover different habitat requirements:

Aposeris foetida (Odorous Pig Salad) is a calcicole species and is described as a representative associated species of *Fagus sylvatica* (Hegi 1987). The distribution area of the species includes most of the calcareous regions of the Alps, scattered occurrences across the Carpathians and the north-western Balkans. The phylogeographic results should reveal the questions if the two major disjunct areas in the Northern and Southern Alps represent different genetic lineages and thus evidence multiple glacial refugia and consequently for the existence of Würm glacial forest refugia along the northern foothills of the Alps. Furthermore the question arises whether evidence can be gained for glacial survival of *A. foetida* also in the Northern Carpathians. Additionally, it has to be revealed whether the present continuous distribution of the Southern Alps and the Northern Dinaric Alps and the more scattered distribution in the southern Dinaric Alps represent one or multiple genetic lineages of *A. foetida* being translated in a single or multiple glacial forest refugia in this region. Finally, the question arises whether the putative refugia north of the Alps show further genetic substructures, which might evidence secondary glacial fragmentation of such forest stands.

Melampyrum sylvaticum (Small Cow Wheat) grows on shallow soils with low available nitrogen where humidity remains high (Ellenberg 1988). It is a widespread element of the European montane and subalpine flora, and its geographic range covers mountain ranges from Western Europe to the Urals (Meusel & Jäger 1992). To foster the understanding of the glacial distribution of European forest biomes apart from the classical Mediterranean refugia in Southern Europe, *M. sylvaticum* was selected as a further model species for

montane forests. Also with this species, the aim is to detect potential glacial refugia as well as the postglacial range shifts and (re)colonisation routes in Europe for montane woodland plants. It has to be revealed where the main genetic lineages are located for this species in Europe, reflecting possible glacial refugia. A main focus will be on the Pyrenean population, since the peripheral areas of the Pyrenees are already postulated as a glacial retreat for various mountain species. Additionally it should be clarified whether Southeastern Europe, especially the Balkans has played a role in glacial and postglacial history for *M. sylvaticum*. Finally, the possibility of a refugial area in the vicinity of the Tatra Mountains and the Alps has to be exposed.

Erebia euryale (Large Ringlet) inhabits mainly grassy, flowery places in pine and spruce forest clearings and grassy slopes above treeline. Larval host plants include several Poaceae as well as few Cyperaceae species. The butterfly is distributed over several European mountain systems (Cantabrian Mountains, Pyrenees, Massif Central, Jura Mountains, Alps, mountains along the Czech-German border, Apennines, Carpathians, and Balkans (Tolman *et al.* 1998; Kudrna 2002). Preliminary studies about the phylogeography of *E. euryale* based on a rather limited number of populations are already published (Schmitt & Haubrich 2008; Vila *et al.* 2011). With an increased data set, previously postulated hypotheses about genetic lineages and glacial refugia have to be evaluated. The relation of the Apennines populations to the neighbouring genetic lineages in the Western and Eastern Alps also has to be revealed. Furthermore, the relevance of the Slovakian Tatra and the Massif Central as putative independent glacial centres of survival for *E. euryale* has to be discussed, and finally, the biogeographic coherence between the Cantabrian Mountains, the Pyrenees and the Massif Central needs to be reconsidered.

Colias palaeno (Moorland Clouded Yellow) is a characteristic species of bogs and comparable wetlands in the montane regions (Erhardt 1985). The species is monophagous and larvae only feed on *Vaccinium uliginosum*. All over its holarctic distribution, the species has a montane to subalpine distribution at its southern distribution borders, where it is mostly found from 1800 to 2200 m asl. (Huemer 2004), whereas it is a lowland species in the North (Henriksen & Kreutzer 1982). In this study, it is discussed whether *C. palaeno* is differentiated into several genetic lineages (i.e. ESUs) within Europe, and if so, how these lineages are geographically distributed. Furthermore, it has to be revealed whether *C. palaeno* survived the last glaciation in a single or multiple refugia in the western Palaearctic with independent postglacial expansions. Finally, conservation implications should be given based on the results on the phylogeographic study of this peatland species.

2 Material and Methods

2.1 DNA extraction and AFLP fingerprinting of plants

Silicagel-dried leaves were deep-frozen with liquid nitrogen and pulverised with the TissueLyser LT (Qiagen, Hilden, Germany). Genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions with the following minor modifications: to increase the DNA concentration in the solution, purified DNA was diluted first in 50 µl and then in another tube in 100 µl ultrapure water (instead of AE buffer). The 100 µl dilution was used for further analyses.

AFLP procedures followed Vos *et al.* (1995) also with minor modifications. AFLPs were made with fluorescence labelled primers (6-FAM, Tamra, HEX; Biomers, Ulm, Germany). For each sample, genomic DNA concentration was determined with the Qubit Fluorometer (Invitrogen, Carlsbad, California, USA) and standardized to 20 ng/µl. For digestion, 100 ng of genomic DNA was used. The initial restriction-ligation step was performed for 16 h at 21°C using a thermocycler (Biometra, Göttingen, Germany). Genomic DNA was digested simultaneously with EcoRI and MseI. Double-stranded EcoRI and MseI adapters were ligated to the sticky ends of the fragments. In the following two-step amplification, pre-selective primers with one selective base (EcoRI primer E, MseI primer M) and selective primers with two additional selective bases (EcoRI primer E+2, MseI primer M+2) were used. For the selective amplification, we used the fluorescence-labelled primer combinations. For *Aposeris foetida*, two primer combinations were used and for *Melampyrum sylvaticum* we used three combinations (see Table 2.1 and Table 2.2).

Table 2.1: Adapter, primer and fluorescent primer combinations used for the AFLP analysis of *Aposeris foetida*.

Adapter	**Sequence**
EcoRI	5'-CTCGTAGACTGCGTACC-3'
	3'-CATCTGACGCATGGTTAA-5'
MseI	5'-GACGATGAGTCCTGAG-3'
	3'-TACTCAGGACTCAT-5'
Primer +1	**Sequence 5' - 3'**
E+A	GACTGCGTACCAATTCA
M+C	GATGAGTCCTGAGTAAC
Primer +3	**Sequence 5' - 3'**
E+AAT	GACTGCGTACCAATTCAAT
E+ACA	GACTGCGTACCAATTCACA
M+CTA	GATGAGTCCTGAGTAACTA
M+CAC	GATGAGTCCTGAGTAACAC
Fluorescent dye colour	**Primer combination (labelled)**
6-FAM	E+AAT / M+CTA (blue)
HEX	E+ACA / M+CAC (green)

Table 2.2: Adapter, primer and fluorescent primer combinations used for the AFLP analysis of *Melampyrum sylvaticum*.

Adapter	**Sequence**
EcoRI	5'-CTCGTAGACTGCGTACC-3'
	3'-CATCTGACGCATGGTTAA-5'
MseI	5'-GACGATGAGTCCTGAG-3'
	3'-TACTCAGGACTCAT-5'
Primer +1	**Sequence 5' - 3'**
E+A	GACTGCGTACCAATTCA
M+C	GATGAGTCCTGAGTAAC
Primer +3	**Sequence 5' - 3'**
E+AAT	GACTGCGTACCAATTCAAT
E+ACG	GACTGCGTACCAATTCACG
E+ATG	GACTGCGTACCAATTCATG
M+CCT	GATGAGTCCTGAGTAACCT
M+CTG	GATGAGTCCTGAGTAACTG
M+CTG	GATGAGTCCTGAGTAACTG
Fluorescent dye colour	**Primer combination (labelled)**
6-FAM	E+AAT / M+CCT (blue)
Tamra	E+ACG / M+CTG (yellow)
HEX	E+ATG / M+CTG (green)

Multiplex products were run for 75 min on a GE Healthcare 96-capillary-sequencer (MegaBACE 1000) to separate fragments together with an internal size standard (MegaBACE ET550-R; GE Healthcare, Munich, Germany). The raw data were aligned with the analysis software MegaBACE Fragment Profiler 1.2 (Amersham Biosciences, Munich, Germany). Fragments were scored as present when the appropriate peak height exceeded the standard parameter-setting thresholds (blue: 60; yellow: 60; green: 60; red: 60). In the finally assembled binary matrix, the presence of a band was scored as '1', whereas the absence of a band was scored as '0' (Bensch & Åkesson 2005). All electropherograms were re-examined visually in order to check for possible misinterpretations of the automated Fragment Profiler analysis. The resulting binary matrix was used for statistical analyses.

We checked the reproducibility of the AFLP amplification by randomly selecting five samples each from *A. foetida* and *M. sylvaticum* and amplifying the DNA extracts twice. The error rate was calculated as the ratio between all differences and all fragment comparisons in these five duplicated AFLP profiles (Bonin *et al.* 2004).

2.2 Allozyme electrophoresis of butterflies

Half of the abdomen of each butterfly individual was homogenized in PGM-buffer (Harris & Hopkinson 1976) by ultrasound and centrifuged at 8,000 g for 5 min. Electrophoresis was run on cellulose acetate plates (Hebert & Beaton 1993). For *Erebia euryale*, we analysed 12 enzyme systems representing 15 loci and for *Colias palaeno* we analysed 16 enzyme systems representing 20 loci. The analysed loci and electrophoresis conditions for each species are given in Table 2.3 and Table 2.4.

Table 2.3: Analysed enzyme systems and electrophoresis conditions for *Erebia euryale*.

Enzyme	No. of loci	Buffer	Homogenate applications	Running time (min)
6PGDH	1	TM	3	50
AAT	2	TM	3	40
FUM	1	TC	3	45
G6PDH	1	TM	2	40
GAPDH	1	TC	3	45
GPDH	1	TM	4	45
IDH	2	TM	3	50
MDH	2	TC	2	40
ME	1	TG	3	30
$PEP_{LEU-GLY-GLY}$	1	TC	2	30
PGI	1	TG	2	30
PK	1	TC	2	30

TC: Tris–citrate pH 8.2 (Richardson *et al.* 1986); TG: Tris–glycine pH 8.5 (Hebert & Beaton 1993); TM: Tris–maleic acid pH 7.0 (adjusted from TM pH 7.8 (Richardson *et al.* 1986)). All buffers were run at 200 V.

Table 2.4: Analysed enzyme systems and electrophoresis conditions for *Colias palaeno*.

Enzyme	No. of loci	Buffer	Homogenate applications	Running time (min)
6PGDH	1	TM	3	50
AAT	2	TC	3	40
ACON	2	TG	3	40
FUM	1	TC	4	45
G6PDH	1	TM	2	45
GAPDH	1	TC	3	45
GPDH	1	TM	3	40
HBDH	1	TM	3	45
IDH	2	TM	3	50
MDH	2	TC	3	45
ME	1	TG	2	40
MPI	1	TG	3	30
$PEP_{PHE-PRO}$	1	TC	2	30
PGI	1	TG	1	45
PGM	1	TG	1	45
PK	1	TC	3	45

TC: Tris–citrate pH 8.2 (Richardson *et al.* 1986); TG: Tris–glycine pH 8.5 (Hebert & Beaton 1993); TM: Tris–maleic acid pH 7.0 (adjusted from TM pH 7.8 (Richardson *et al.* 1986)). All buffers were run at 200 V.

2.3 mtDNA sequencing of *Colias palaeno*

For mtDNA analyses, DNA was extracted from the heads of the samples using the standard Chelex 100 protocol (Walsh *et al.* 1991). Partial Cytochrome oxidase I (COI) gene was amplified by polymerase chain reaction (PCR) with the forward primer LCO1490 (5' - GGTCAACAAATCATAAAGATATTGGC - 3') and reverse primer HCO2198 (5' - TAAACTTCAGGGTGACCAAAAAATCA - 3') (Folmer *et al.* 1994). PCR amplifications were performed in 20 µl total volume, using 5 µl 5X PCR Colourless Buffer (pH 8.5), 2 mM (of a 2 µl 25 mM $MgCl_2$ solution), 0.2 mM (0.5 µl of a 20 mM dNTP stock), 0.2 µl 5 u/µl 1 U GoTaq DNA polymerase Promega (Madison, USA) and 0.2 µM (0.4 µl of a 10 µM stock) of each primer.

The COI PCR profile consisted of 2 min at 95ºC, 35 cycles of 30 sec at 94ºC, 30 sec at 53ºC followed by an extension for 1 min at 72ºC and a final one with 5 min. Negative controls were included in each set of reactions. The PCR results were checked afterwards by electrophoresis in a 1 % GelRed stained agarose gel and purified by ethanol precipitation (Sambrook & Russell 2001).

Sequencing was performed on an ABI 3130xl (Applied Biosystems) capillary sequencer using the forward primer employed in the PCR at facilities of the Centre of Marine Sciences (CCMAR), located on the Gambelas campus belonging to the University of Algarve in Portugal.

2.4 Statistical analyses

2.4.1 AFLP

Within-population genetic diversity was assessed using the percentage of polymorphic loci *(%Ppop*), Shannon diversity index H_{SH} with $H_{SH} = -\sum_i p_i \times \ln p_i$, where p_i is the relative frequency of the *i*-th fragment based on all AFLP fragments of the dataset (Legendre & Legendre 1998) and Nei's gene diversity H_N (Nei 1973). Nei's gene diversity and the percentage of polymorphic loci were calculated for each population with the R script AFLPDAT (Ehrich 2006). The same script was used to calculate frequency down-weighted marker values (*DW*; Schönswetter & Tribsch 2005) with modifications given in (Winkler *et al.* 2010).

The number of private and fixed private fragments (if present) per population was extracted from the binary matrix. Private fragments are confined to a single population. Fixed private fragments appear in every individual of a population (e.g. Stehlik *et al.* 2001; Tribsch *et al.* 2002; Huck *et al.* 2009; Kramp *et al.* 2009).

For *Aposeris foetida*, Kruskal-Wallis one-way ANOVAs and Mann–Whitney U-tests were carried out with SPSS STATISTICS 20 (IBM, Armonk, NY, USA) to evaluate potential differences between three predefined geographic groups.

From the binary matrix of the AFLP profiles, genetic distances (D) among individuals were calculated using a complementary value (SC) based on Nei & Li (1979) similarity coefficient ($\hat{S}$) ($D = 1 - SC = 1 - \left[2n_{xy} \div (n_x + n_y)\right]$, see Kropf *et al.* 2002). Based on the resulting distance matrix, relationships between all individuals were constructed using Neighbour Joining (NJ) analysis (Saitou & Nei 1987) as implemented in the program NEIGHBOUR and CONSENSE of the software PHYLIP 3.69 (Felsenstein 2005). Node support for the phenogram was estimated by a bootstrap analysis using the program CONSENSE with 10,000 replicates. Additionally, the relationships between the sampled populations were estimated based on Nei's pair-wise genetic distances after Lynch & Milligan (1994), calculated by the program AFLP-SURV 1.0 (Vekemans 2002).

To examine similarities among populations, a matrix of pair-wise genetic distances among all populations was subjected to subsequent Principal Coordinates Analysis (PCoA). This analysis was performed using the Excel Add-in GENALEX 6.5b5 (Peakall & Smouse 2012). Genetic distance values were plotted against pair-wise geographic distances, and correlation between genetic and geographic distances (isolation by distance) among

populations was estimated by linear regression (Wright 1943). Significance of the linear regression was evaluated by a Mantel test (Mantel 1967), both also computed with GENALEX.

To study the underlying genetic structure in more detail, we used the multilocus assignment method of Pritchard *et al.* (2000) as implemented in the software STRUCTURE 2.3.4 (Hubisz *et al.* 2009). The program assumes that all individuals come from one or multiple unknown populations, each of them characterized by a set of allele frequencies at each locus. To perform inference, the method uses the Markov chain Monte Carlo (MCMC) method. STRUCTURE attempts to assign individuals to populations on the basis of their genotypes, while simultaneously estimating population allele frequencies (Pritchard *et al.* 2000). In our analysis, values of *K* (i.e. assumed populations) ranged from 1 to the number of the studied populations for every species. We used ten replicates for every *K* with a length of burnin period of 100,000 and a number of MCMC replicates after burnin of 500,000.

An analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) was conducted with the program ARLEQUIN 3.5.1.3 (Excoffier & Lischer 2010). AMOVA values were calculated for the entire dataset and also for different genetic groups, in order to compare levels of among-population differentiation between these groups. Significance levels of variance components were computed by a non-parametric permutation approach with 10,000 replicates.

2.4.2 Allozyme electrophoresis

Allozyme alleles were labelled according to their relative mobility, starting with '1' for the slowest. Allele frequencies and parameters of genetic diversity (mean number of alleles per locus (*A*), expected and observed heterozygosity (H_E, H_O), total percentage of polymorphic loci (P_{tot}) and percentage of polymorphic loci with the most common allele not exceeding 95 % (P_{95})) were computed with GSTAT 3.2 (Siegismund 1993). Weighted F-statistics were calculated using the estimators described by Weir & Cockerham (1984). Their values and significance were estimated in FSTAT 2.9.3 (Goudet 1995) after 10,000 randomizations. To level out differences in population sizes, allelic richness (A_R) was calculated with the software HP-RARE 1.1 (Kalinowski 2005), which uses the technique of rarefaction, so the number of alleles in large samples can be compared with the number of alleles in smaller samples (Hurlbert 1971). Estimator of actual differentiation (D_{est}; Jost 2008) was calculated with the software SMOGD 1.2.5 (Crawford 2010), available online at www.ngcrawford.com/django/jost/.

Conventional *F* statistics, AMOVAS, hierarchical genetic variance analyses, tests of Hardy-Weinberg equilibrium and linkage disequilibrium were calculated with ARLEQUIN 3.5.1.2 (Excoffier & Lischer 2010). Significance was obtained after 10,000 permutations. Sequential Bonferroni corrections were performed as described in Rice (1989). Additionally, we determined the number of private alleles per population. Nei's standard genetic distances (Nei 1972) were calculated and Neighbour joining phenograms were constructed with PHYLIP 3.6.7 (Felsenstein 2005), including bootstrap values (based on 10,000 iterations) with the programs NEIGHBOUR and CONSENSE and drawn with the software SPLITSTREE 4.6 (Huson & Bryant 2005).

For *Erebia euryale*, Φ_{ST} values were plotted against pair-wise geographic distances, and correlation between genetic and geographic distances (isolation by distance) among populations was estimated by linear regression (Wright 1943). Significance of the linear regression was evaluated by a Mantel test (Mantel 1967).

To disentangle the population structure, we used also the program STRUCTURE 2.3.4 (Pritchard *et al.* 2000) with the same settings as described in section 2.4.1.

Additionally, we used the Bayesian multilocus assignment method of Corander & Marttinen (2006) as implemented in the software BAPS 5.4. The program needs an expectation for the maximum number of groups (*K*) as a prior. However, the program allows multiple inputs of *K*. For each *K* value, BAPS tries to determine the optimal partitions, stores these internally, and, after all runs have been processed, it merges the stored results according to the log-likelihood values. In our analysis, values of *K* ranging from 1 to 21 (the number of studied populations) were explored. The optimization algorithm of BAPS is stochastic and consequently different results can be obtained for the same value of *K*. Hence, we used 20 replicates for each value of *K*.

For *Colias palaeno*, we supplementary tested the validity of the assumed genetic groups with a population assignment test implemented in the software GENALEX.

2.4.3 mtDNA

All COI sequences were aligned using the software CLUSTAL X 2.0.3 (Larkin *et al.* 2007) with default settings, implemented in GENEIOUS 5.4 (Drummond *et al.* 2011) and double-checked manually. To reduce sequences to haplotypes we used the software COLLAPSE 1.2 (Posada 2004). Number of individuals (*N*), frequency (*f*), number of haplotypes (*n*), number of private haplotypes (n_P), and haplotype (*h*) and nucleotide diversities (Θ) for each location were calculated in ARLEQUIN 3.5.1.3 (Excoffier & Lischer 2010).

An mtDNA haplotype network was constructed with the median-joining algorithm (MJ) using the software NETWORK 4.5 (Bandelt *et al.* 1999). Corrected genetic distances between clades were estimated in MEGA 5.05 (Tamura *et al.* 2011).

Alignments of nucleotide sequences were constructed using the software CLUSTAL X 1.83 (Thompson *et al.* 1997) and verified visually in order to maximize positional homology. Partial nucleotide sequences of the COI gene of 83 taxa (600 bp) representing 74 species of *Colias* and four outgroup sequences (*Luehdorfia puziloi, Catopsilia pomona, Likoma apicalis* and *Oleria paula)*. The Akaike information criterion (Akaike 1974), implemented in the software MODELTEST 3.7 (Posada & Crandall 1998), selected the GTR + I + Γ (I = 0.54; Γ = 0.68) as the evolutionary model that fitted best for the data set. The selected model and model parameters were used in the Maximum Likelihood analysis performed with PHYML 2.4.4 (Guindon & Gascuel 2003). The robustness of the inferred trees was tested by nonparametric bootstrapping using 1,000 pseudoreplicates. ML analyses were carried out on the freely available Bioportal (http://www.bioportal.uio.no).

All COI sequences were queried against the NCBI public database GenBank with the BLASTn algorithm (Altschul *et al.* 1997) to compare our results with other sequences and in order to verify that the investigated sequences really belong to the target group.

3 Odorous Pig Salad - *Aposeris foetida*

3.1 Introduction – *Aposeris foetida*

The glacial and interglacial oscillations of the Quaternary and further complex interactions between tectonic and orbital forces have been responsible for the environmental instability that strongly affected the living conditions of biota over major parts of Europe (Webb & Bartlein 1992), causing highly dynamic distribution fluctuations (Hewitt 2000; Zhang *et al.* 2001; Vargas 2003; Schmitt 2007). The resulting consequences of these climatic fluctuations are still imprinted in the community composition and the genetic make-up of extant natural populations (Bennett 1990). During the glacial periods, arctic tundra and cold steppe ecosystems covered major parts of Central Europe, while most of Northern Europe and large parts of the high mountain systems in the South of Europe were covered by glaciers (Webb & Bartlein 1992; Dansgaard *et al.* 1993; Hewitt 2004; Schmitt 2007).

One common hypothesis of glacial survival and postglacial colonisation applied for many species in Europe has postulated the complete extinction of most species from these cold and dry periglacial areas of Central Europe – the so called *tabula rasa* hypothesis. This hypothesis could be proved for many species today existing in Central and Northern Europe (Petit *et al.* 2002, 2003; Grivet & Petit 2003). In these cases, warm-adapted organisms re-colonised the more northern parts of Europe postglacially from the classical Mediterranean refugia (de Lattin 1949), as evidenced by many genetic studies (reviews in Hewitt 2004; Schmitt 2007).

However, this traditional view of European refugia has been focused on temperate and warm-adapted taxa whose populations were believed to have been pushed by the glacial climatic conditions into the Mediterranean refugia of Iberia, Italy and the Balkans (Hewitt 2000, 2004; Hänfling *et al.* 2002; Schmitt 2007). This view for warm-adapted species has been questioned for more cold-tolerant taxa, and the hypothesis of additional cryptic northern refugia for non-tundra species supplementing the southern ones has emerged (Stewart & Lister 2001; Stewart 2003; Stewart *et al.* 2010; Schmitt & Varga 2012). This hypothesis has recently been supported by various phylogeographic studies including animals (e.g. Fink *et al.* 2004; Deffontaine *et al.* 2005; Pauls *et al.* 2006; Ursenbacher *et al.* 2006), trees (e.g. Willis *et al.* 2000; Magri *et al.* 2006; Boratyński *et al.* 2007) and herbaceous plant species (e.g. Tyler 2002a, 2002b; Kramp *et al.* 2009; Michl *et al.* 2010; Huck *et al.* 2012). However, the locations of cryptic extra-Mediterranean forest refugia,

especially along the northern foothills of the Alps, are still controversially debated (Muster 2002; Magri *et al.* 2006; Magri 2008).

To foster the understanding of the glacial distribution of forests outside the classical Mediterranean refugia in southern Europe, we selected a montane woodland plant as a model species for montane forests: *Aposeris foetida* (L.) Less. is a calcicole plant species with a disjunct distribution in Europe covering the calcareous parts of the Northern and Southern Alps, parts of the Dinaric Alps and some isolated areas of the Northern, Central and Southern Carpathians (Meusel & Jäger 1992). Analysing the spatial genetic structure of *A. foetida* across its entire present range allowed addressing the following questions:

(i) Do the two major disjunct areas in the Northern and Southern Alps represent different genetic lineages and thus evidence for multiple glacial refugia and consequently for the existence of Würm glacial forest refugia along the northern foothills of the Alps?

(ii) If so, the question arises whether evidence can be gained for glacial survival of *A. foetida* also in the Northern Carpathians.

(iii) Does the present continuous distribution of the southern Alps and the Northern Dinaric Alps and the more scattered distribution in the southern Dinaric Alps represent one or multiple genetic lineages of *A. foetida* being translated in a single or multiple forest refugia in this region?

(iv) Finally, the question arises whether the putative refugia north of the Alps show further genetic substructures, which might evidence secondary glacial fragmentation of such forest stands.

3.2 Study species – *Aposeris foetida*

The odorous pig salad *Aposeris foetida* (L.) Less. (Asteraceae) is the only species of the genus *Aposeris* (Necker ex Cass.) Less. (*Lactuceae*). A diploid chromosome number of 2n = 16 has been reported (Oberdorfer 1983) for the species. It is a perennial deciduous hemicryptophyte with a basal rhizome-rosette, hygro-mesomorphic leaves, malodorous milk and just one day flowering capitula (Eberle 1962). *A. foetida* usually flowers from June to August. The species is hermaphroditic and the reproduction is effected either via autogamy or via pollination by insects (Knuth 1898). The achenes do not have a pappus and the fruit might be carried by ants (Hegi 1987).

The calcicole species is mainly found in mesophilic, summer-green deciduous forests (*Carpino-Fagetea*) and occurs less frequently in thermophilic and mesophilic forest-grassland ecotones (*Trifolio-Geranietea sanguinei*) in mountain areas (Eberle 1962). *A. foetida* is described as a distinctive accessory species of *Fagus sylvatica* (Hegi 1987). The distribution area of the species includes most of the calcareous regions of the Alps, scattered occurrences across the Carpathians and the north-western Balkans. The local isolated area in the northern coastal Apennines (Meusel & Jäger 1992) seems to be a misidentification and confusion with *Hyoseris* spec.

3.3 Sampling – *Aposeris foetida*

To reveal the genetic variation of *A. foetida* and its range dynamics, we analysed 265 individuals from 25 populations across almost the entire distribution of the species (Table 3.1, Fig. 3.1). Leaf material was collected *in situ* across the 25 populations from at least seven different plants and dried in plastic zip-lock bags filled with silica gel. To avoid sampling clones, plants were collected at distances of at least 10 m. The maximum geographic distance of the analysed samples is 1,180 km between the Swiss population in the Northern Alps (CH-Ja) and the population from southern Poland (PL-Wo). The shortest distance (9 km) is between two populations from the Alps (CH-Ob and CH-Ja). Finally, seven to 12 individuals per population were included in the Amplified Fragment Length Polymorphisms (AFLP) analysis.

Table 3.1: Geographically ordered codes of populations, sample locations, mountain ranges, coordinates, elevation and number of individuals of the 25 *Aposeris foetida* populations analysed.

No.	pop. code	location	mountain range	country	coordinates	altitude (m asl.)	*N*
1	CH-Ja	Jaunpass	Alps	Switzerland	07° 20' E 46° 35' N	1500	12
2	CH-Ob	Oberwil	Alps	Switzerland	07° 24' E 46° 39' N	1200	12
3	CH-Sc	Schuders	Central Alps	Switzerland	09° 44' E 46° 59' N	1300	12
4	D-Aq	Ammerquelle	Ammergauer Alps	Germany	11° 03' E 47° 34' N	900	10
5	D-Pf	Pfaffenwinkel	Lower Alps	Germany	11° 00' E 47° 52' N	800	11
6	A-Lo	Lofer	Alps	Austria	12° 42' E 47° 35' N	700	12
7	A-Ga	Gaisberg	Lower Alps	Austria	13° 06' E 47° 48' N	800	12
8	A-Ra	Ramsau am Dachstein	Alps	Austria	13° 39' E 47° 26' N	1300	11
9	I-Fo	Foppolo	Southeastern Alps	Italy	09° 45' E 46° 03' N	1500	10
10	I-Pc	Passo di Croce Domini	Southeastern Alps	Italy	10° 22' E 45° 55' N	1500	11
11	I-Mb	Monte Baldo	Southeastern Alps	Italy	10° 50' E 45° 41' N	2000	12
12	I-Ve	Passo di Vézzena	Southeastern Alps	Italy	11° 23' E 45° 57' N	1300	8
13	I-An	Andraz	Alps	Italy	11° 59' E 46° 30' N	1600	9
14	I-Cl	Claut	Southern Alps	Italy	12° 34' E 46° 20' N	1000	9
15	SLO-Ma	Bovec	Julian Alps	Slovenia	13° 36' E 46° 20' N	1000	12
16	SLO-Lo	Loiblpass	Julian Alps	Slovenia	14° 16' E 46° 26' N	1300	11
17	SLO-Kr	Krvavec	Julian Alps	Slovenia	14° 29' E 46° 18' N	800	9
18	SLO-Cv	Črni Vrh	Dinaric Alps	Slovenia	14° 02' E 45° 55' N	900	12
19	PL-Wo	Wolosate	North Carpathian Mountains	Poland	22° 38' E 49° 04' N	1000	12
20	SRB-Mg	Mt. Gučevo	Dinaric Alps	Serbia	19° 19' E 44° 29' N	700	12
21	SRB-Mt	Mt. Tara	Dinaric Alps	Serbia	19° 32' E 43° 56' N	1100	8
22	BIH-Mm	Mt. Maglic	Dinaric Alps	Bosnia and Herzegovina	18° 43' E 43° 17' N	1800	11
23	HR-Mv	Mt. Velebit	Dinaric Alps	Croatia	14° 58' E 44° 48' N	1500	9
24	HR-Pl	Plitvička Jezera	Dinaric Alps	Croatia	15° 36' E 44° 53' N	600	11
25	HR-Uc	Učka	Istrian Mountains	Croatia	14° 12' E 45° 18' N	1000	7

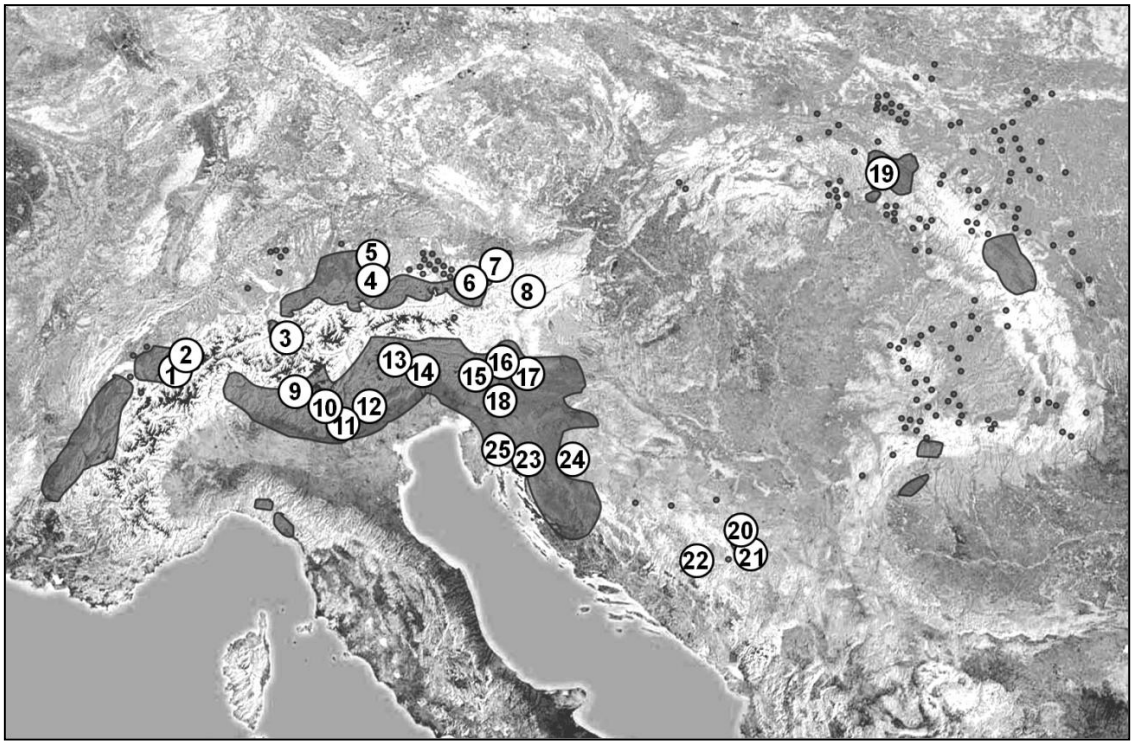

Fig. 3.1: Geographic distribution of *Aposeris foetida* and collection sites. The distribution area is based on Meusel & Jäger (1992). See Table 3.1 for further details on populations and regions. The local isolated area in the northern coastal Apennines seems to be a misidentification and confusion with *Hyoseris* spec.

3.4 Results – *Aposeris foetida*

The two selective AFLP primer combinations amplified a total of 433 fragments ranging from 60 to 549 bp for the 265 individuals of *A. foetida*. The primer combination E+AAT / M+CTA yielded 243, E+ACA / M+CAC 190 fragments. In total, 98.56 % of the fragments were polymorphic, and only 1.44 % were scored in all individuals from all populations. No identical AFLP profiles were shared by any two individuals. The number of fragments in a single individual ranged from 53 (I-Ve04) to 98 (CH-Ja08). The average number of fragments per individual in a population ranged from 64 (I-Ve) to 89 (SLO-Cv), with a mean of 78 ± 7 SD. Calculation of the technical error rate during the scoring and labelling of the AFLP fragments resulted in five differences in the 847 comparisons, resulting in an error rate of 0.59 %.

The 25 analysed *A. foetida* populations showed varying levels of intrapopulation genetic diversity (Table 3.2, Fig. 3.2). The average percentage of polymorphic loci (*%Ppop*) was 21.6 % (SD: 3.9 %), the average Shannon–Wiener diversity index (H_{SH}) was 0.098 (SD: 0.016) and the average gene diversity (H_N) was 0.064 (SD: 0.011). The highest diversity was detected for one of the Austrian populations from the Northern Alps (A-Ra: *%Ppop*: 27.6 %; H_{SH}: 0.123; H_N: 0.080) and the lowest for one of the Croatian populations (HR-Uc: *%Ppop*: 12.4 %; H_{SH}: 0.040; H_N: 0.061) (Fig. 3.2a). The *DW* values showed strong differences among populations with an average of 29.5 (SD: 11.8) ranging from 13.7 in Italy (I-Cl) to 59.1 in the Swiss population (CH-Ja). The other Swiss population from the Western Alps also had a high *DW* value of 54.4 (Fig. 3.2b).

Table 3.2: Genetic diversity, frequency-down-weighted marker values (*DW*) and number of private fragments for the 25 *Aposeris foetida* populations analysed.

No.	pop code	*N*	Mean number of loci	% pol loci	Nei's gene diversity (H_N)	Shannon Index (H_{SH})	*DW*	Private fragments
1	CH-Ja	12	86	26.13	0.077	0.118	59.1	9
2	CH-Ob	12	82	24.49	0.073	0.112	54.4	5
3	CH-Sc	12	82	23.87	0.068	0.105	34.6	4
4	D-Aq	10	84	22.22	0.066	0.101	30.5	7
5	D-Pf	11	77	25.10	0.075	0.115	42.0	13
6	A-Lo	12	80	27.16	0.083	0.126	22.5	3
7	A-Ga	12	82	24.90	0.066	0.102	34.1	4
8	A-Ra	11	79	27.57	0.080	0.123	31.7	5
9	I-Fo	10	72	19.34	0.055	0.085	16.9	2
10	I-Pc	11	68	21.60	0.061	0.094	21.1	3
11	I-Mb	12	67	24.49	0.070	0.108	20.6	2
12	I-Ve	8	64	18.11	0.050	0.079	16.2	3
13	I-An	9	74	17.49	0.054	0.082	20.4	3
14	I-Cl	9	71	19.75	0.060	0.092	13.7	2
15	SLO-Ma	12	74	17.90	0.058	0.088	14.1	0
16	SLO-Lo	11	79	23.46	0.069	0.106	30.2	6
17	SLO-Kr	9	71	19.75	0.057	0.089	17.0	5
18	SLO-Cv	12	89	22.22	0.068	0.105	33.2	4
19	PL-Wo	12	72	24.69	0.064	0.101	32.2	8
20	SRB-Mg	12	74	22.22	0.066	0.101	48.0	6
21	SRB-Mt	8	85	20.99	0.065	0.099	28.6	3
22	BIH-Mm	11	88	19.55	0.058	0.090	31.9	3
23	HR-Mv	9	73	21.60	0.069	0.104	27.8	4
24	HR-Pl	11	84	13.17	0.039	0.061	25.2	0
25	HR-Uc	7	87	12.35	0.040	0.061	31.1	7
	Mean	10.6	77.9	21.610	0.064	0.098	29.5	4.4
	SD	1.6	7.1	3.880	0.011	0.016	11.8	2.9
	SE	0.3	1.4	0.780	0.002	0.003	2.4	0.6

SD = standard deviation; SE = standard error

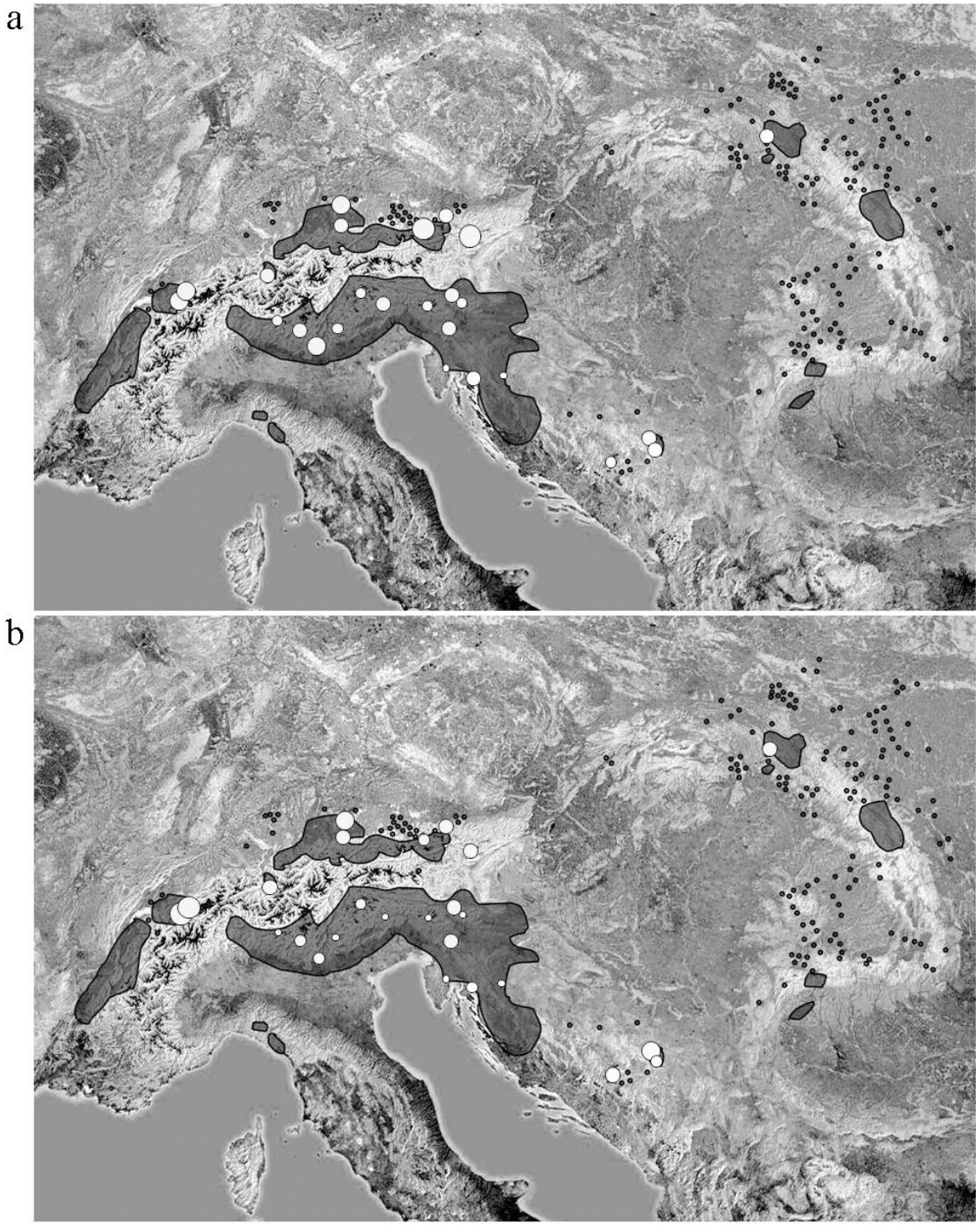

Fig. 3.2: Nei's gene diversity (a) and DW values (b) in the 25 investigated populations of *Aposeris foetida*. The circle size is proportional to the index values. For more details see Table 3.2.

Not all populations contained private fragments. The German population D-Pf contained with 13 the highest number of private fragments, followed by the Swiss population CH-Ja with nine private fragments. On the other extreme, HR-Pl and I-Cl had no private fragments.

All pair-wise Φ_{ST} values were highly significant ($p < 0.001$). The average Φ_{ST} over all examined samples was high (0.412) and the maximum pair-wise Φ_{ST} reached 0.590 between one of the Serbian populations (SRB-Mg) and one of the Croatian populations HR-Pl, followed by 0.588 between HR-Pl and the South-eastern Alps population I-Ve. The lowest Φ_{ST} was 0.169 between the two Slovenian populations SLO-Lo and SLO-Kr, followed by 0.198 between the two western Swiss populations CH-Ja and CH-Ob. Isolation by distance analysis yielded no significant correlation.

Principle Coordinate Analysis (PCoA) based on Nei's genetic distances distinguished four geographic groups (Fig. 3.3): The three Croatian populations were distinguished along the first axis; just as it happened with the two populations from Western Switzerland. The second axis separated the Northern Alps populations from Germany and Austria, whereas the combination of the second and the third axis distinguished the Slovenian population SLO-Cv together with the Balkan populations and the population from Poland. Axis 1 explained 22.87 %, axis 2 20.65 % and axis 3 16.65 % of the total variation.

Fig. 3.3: PCoA of the 25 *Aposeris foetida* populations analysed based on Nei's genetic distances. (a) axis 1 and 2, (b) axis 2 and 3.

A Neighbour Joining (NJ) phenogram based on Nei's genetic distances modified after Lynch & Milligan (1994) underlined the structure shown in the PCoA (Fig. 3.4). Six groups were detected, partly supported by bootstrapping: (i) the Croatian populations, (ii) the Slovenian populations, (iii) the German and Austrian populations from the Northern Alps, (iv) the Swiss populations, (v) the Serbian populations together with Poland and the Slovenian population SLO-Cv, (vi) almost all the Italian populations from the Southern Alps, except of I-Fo, which clustered together with the population from Bosnia-Herzegovina.

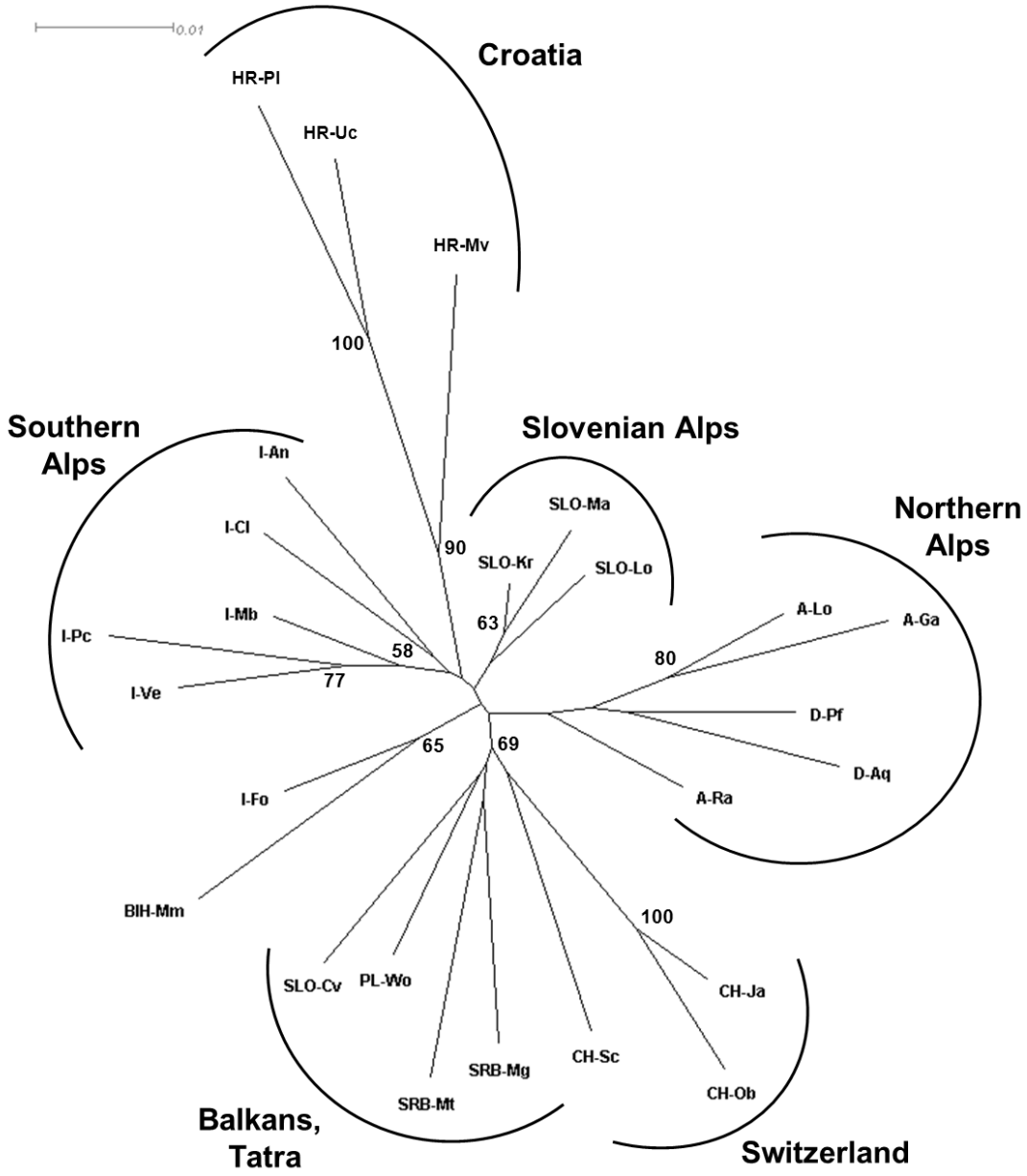

Fig. 3.4: NJ phenogram based on the Nei's genetic distances of the AFLP-phenotypes of the 265 individuals from the 25 investigated populations of *Aposeris foetida*. Bootstraps > 50% are given. For abbreviations see Table 3.1.

A more detailed genetic structuring was revealed by the model-based Bayesian cluster analysis performed with STRUCTURE. The Evanno *et al.* (2005) method, implemented in the program STRUCTURE HARVESTER 0.6.93 (Earl & vonHoldt 2012), obtained the best resolution for $K = 5$. Regarding the different STRUCTURE plots, a successive separation of genetic groups was observable. For $K = 2$, STRUCTURE identified a Northern Alps group with the populations from Switzerland, Germany and Austria distinguished from all other samples. For $K = 4$, the northern group is split into the two samples from western Switzerland, the sample from eastern Switzerland and the samples from Austria and Germany; in the remaining samples, the Croatian samples are distinguished from the other south-eastern samples. However, the latter are not distinguished from the eastern Swiss sample. $K = 5$ discriminates the southern and south-eastern Alps' populations from the ones of the Pannonian flank of the western Balkan mountains and the Tatra Mountains (Fig. 3.5). Only $K = 6$ separated the eastern Swiss population from this latter group. For all STRUCTURE plots from $K = 2$ to $K = 5$ see Fig. 3.6.

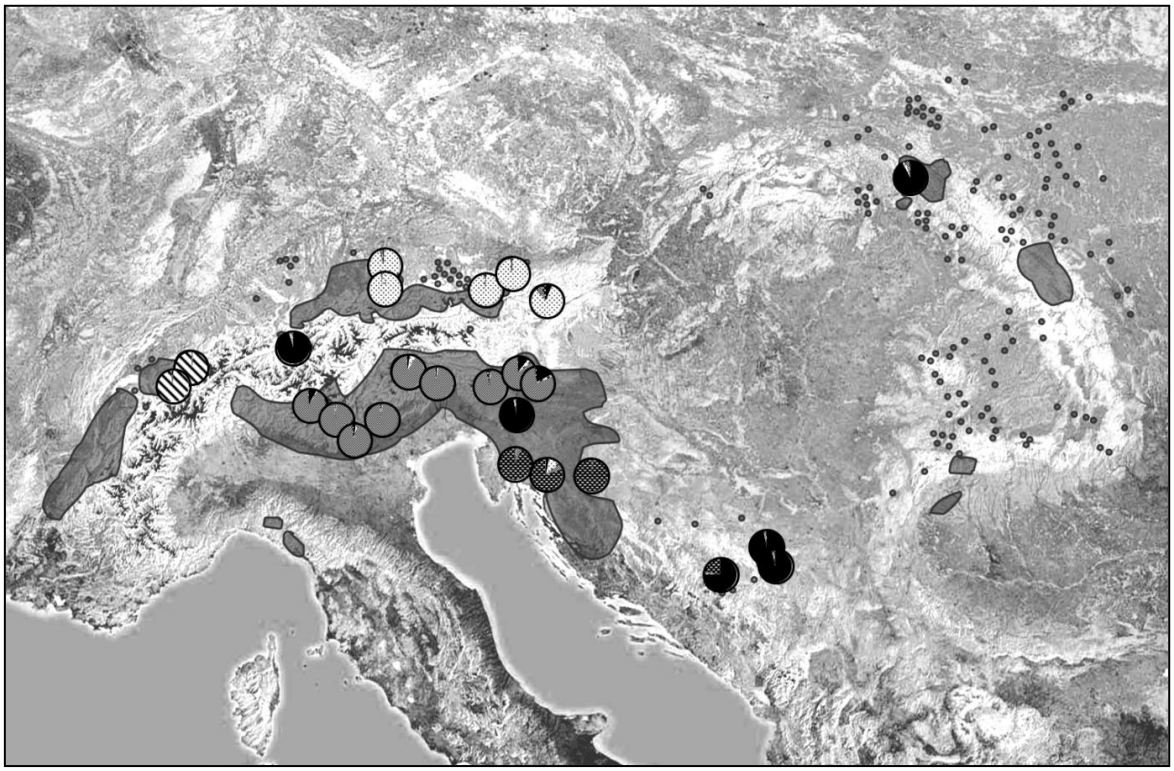

Fig. 3.5: Genetic clustering analyses of *Aposeris foetida* genotypes. Different colours represent proportional memberships of a population to the respective genetic clusters, averaged over individual membership proportions for $K = 5$ as calculated by STRUCTURE.

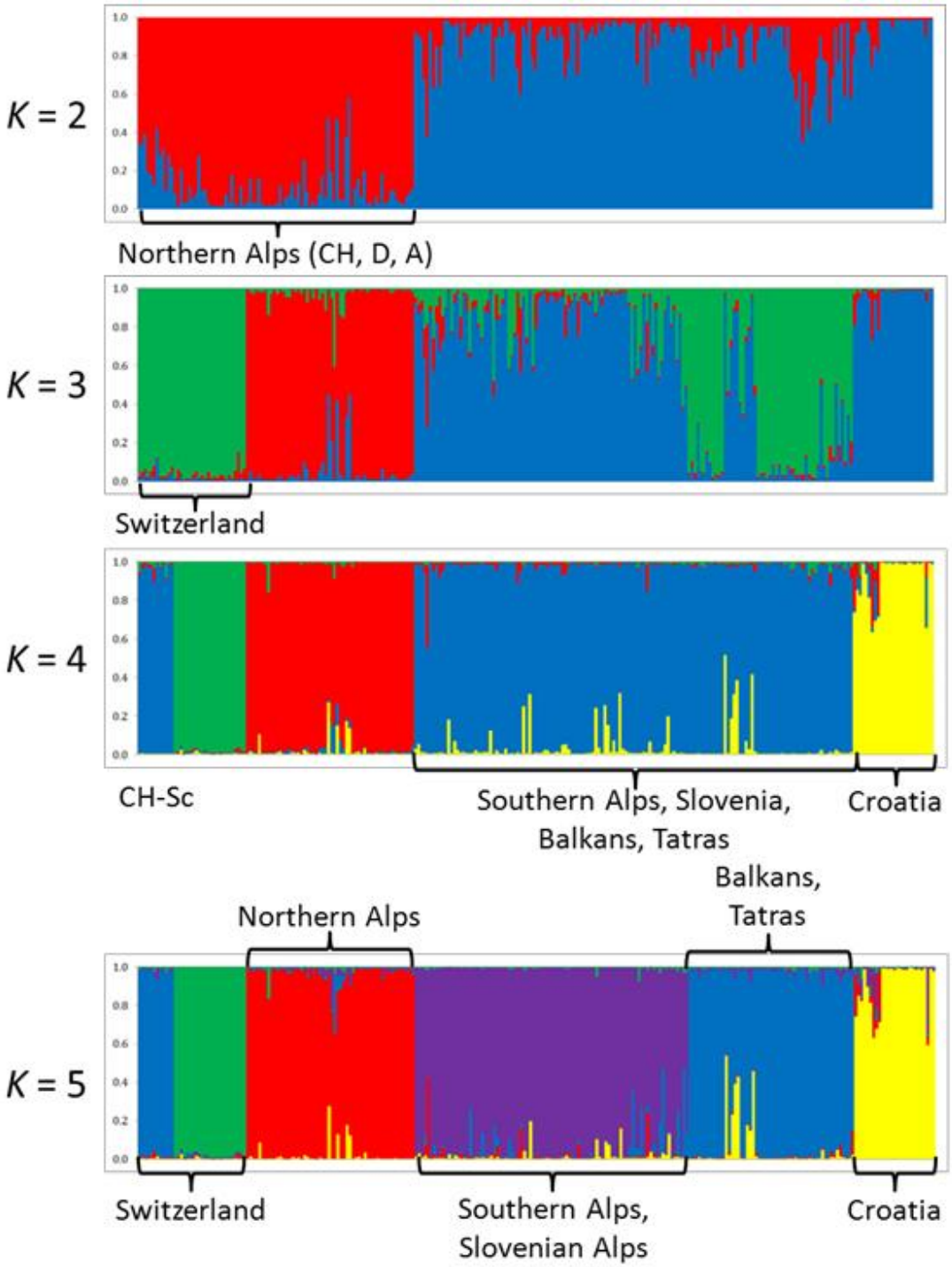

Fig. 3.6: STRUCTURE plots from *K* = 2 to *K* = 5.

A hierarchical AMOVA testing the five detected STRUCTURE groups revealed the stepwise changes of the levels of variation among groups, among populations in groups and within populations. Calculating the total variation gained 41.2 % of variation among populations and 58.8 % within populations (Table 3.3). For the five genetic groups (Switzerland, Northern Alps, Southern Alps, Croatia and southern Balkans, including Poland, SLO-Cv and CH-Sc), the variation among groups was 12.4 %, 30.4 % among populations within groups and 57.3 % within populations.

Table 3.3: Analysis of molecular variance (AMOVA) of AFLP genotypes. The total data set contained 265 individuals from 25 *Aposeris foetida* populations. Hierarchical AMOVA for K = 5 as calculated by STRUCTURE.

	Source of variation	df	SS	CV	% total
total	Among Pops	24	3629.232	12.582	41.19%
	Within Pops	240	4311.508	17.965	58.81%
K = 5	Among groups	4	1262.684	3.888	12.39%
	Among Pops within groups	20	2366.548	9.522	30.35%
	Within Pops	240	4311.508	17.965	57.26%

df = degrees of freedom; SS = sum of squares; CV = coefficient of variation; % total = percentage of total variance.

We tested for significant differences in six genetic diversity values among three major geographic regions. For all parameters, the Northern and the Southern Alps were significantly different. The western Balkan populations had an intermediate position of their genetic diversities (Table 3.4).

Table 3.4: Kruskal–Wallis ANOVAS testing for significant differences of genetic diversity values among the three major geographic groups of *Aposeris foetida* (Poland was excluded from the analysis). Differences between all pairs of groups were tested by a posterior U-test; significant differences were indicated by different letters.

Variables	**Geographic groups**						
	group 1		group 2		group 3		*P*
	Mean	SD	Mean	SD	Mean	SD	
Mean number of loci	82^{a}	2.8	73^{b}	7.1	82^{a}	6.4	0.005
% pol loci	25.2^{a}	1.8	20.4^{b}	2.4	18.3^{b}	4.4	0.006
H_N	0.73^{a}	0.01	0.60^{b}	0.01	0.56^{b}	0.01	0.015
H_{SH}	0.11^{a}	0.01	0.09^{b}	0.01	0.09^{b}	0.02	0.010
DW	20.7^{a}	3.3	13.8^{b}	2.3	18.0^{a}	2.4	0.002
Private fragments	6.3^{a}	3.3	3.0^{b}	1.7	$3.8^{a,b}$	2.5	0.036

Group 1 = Northern Alps (A, CH, D)
Group 2 = Southern Alps (I, SLO)
Group 3 = South-eastern Europe (BIH, HR, SRB)

3.5 Discussion – *Aposeris foetida*

Compared to other AFLP studies on the phylogeography of mountain plants, we obtained an intermediate value for the global differentiation among all populations (Φ_{ST}: 0.41). This value is comparable to respective findings in *Meum athamanticum* (0.47) (Huck *et al.* 2009), *Trollius europaeus* (0.39) (Despres *et al.* 2002) or *Ranunculus glacialis* (0.51) (Schönswetter *et al.* 2003). While lower values were revealed for some species, e.g. *Veratrum album* (0.13) (Treier & Müller-Schärer 2011), far higher differentiation values were shown for *Polygonatum verticillatum* (0.73) (Kramp *et al.* 2009).

The model-based clustering method implemented in the software STRUCTURE and the PCoA of all individual AFLP profiles revealed at least three major genetic groups: (i) Northern Alps, (ii) Croatia as well as (iii) Southern Alps and central Balkans, with further putative substructures in the first and the third group. Based on all these analyses on genetic structures and the interpretation of various genetic diversity indices, we unravelled the evolutionary history of this species, with special focus on differentiation centres, glacial refugia and postglacial range changes.

3.5.1 Glacial survival north of the Alps

Chronologically, the first split separates the Northern Alps populations from all the remaining ones (Southern Alps, Balkans, and Tatras). Both STRUCTURE analyses and PCoA support a clear distinctiveness of this Northern Alps group. The high genetic differentiation of this group and the higher *DW* values (especially in the western and eastern populations of the Northern Alps), support glacial survival at the northern foothills of the Alps at least during the last glacial period. This assumption is further supported by the relatively high number of private fragments within populations from the Northern Alps, compared to the considerably lower numbers in populations from the Southern Alps. Finally, all the average values of all investigated genetic diversity were significantly higher in the Northern than in the Southern Alps (Table 3.4).

A similar biogeographic structure was also found for *Polygonatum verticillatum* (Kramp *et al.* 2009). In contrast to our results, the distribution of genetic diversity observed in other studies on alpine plants (Tribsch *et al.* 2002; Schönswetter *et al.* 2003, Schönswetter *et al.* 2004, Schönswetter *et al.* 2005; Albach *et al.* 2006; Michl *et al.* 2010) showed an almost reversed pattern with a decrease from the genetically richer Southern to the genetically poorer Northern Alps. These results support the idea that the majority of alpine organisms

had their glacial retreats mostly south of the glaciated Alps, or that northern retreats, if existing at all, were small and adding little to the postglacial recolonisation of these mountains. However, plants like *A. foetida* or *P. verticillatum*, but also animals like the butterflies *Erebia epiphron* (Schmitt *et al.* 2006a) or *E. sudetica* (Haubrich & Schmitt 2007), strongly underline the great importance of more northern retreats for at least some alpine organisms.

Furthermore, these glacial refugia of *A. foetida* provide strong evidence for the existence of forest ecosystems or at least habitats with some forest characteristics in the northern foothills of the Alps during the last glacial maximum. As already evidenced over the last few years, forest ecosystems are highly likely to have persisted in the easternmost Alps and around the south-western Alps (Magri *et al.* 2006; Magri 2008; Schmitt & Haubrich 2008). However, our data show that these ecosystems were even more widespread than previously thought.

3.5.2 Two parallel vicariance events north of the Alps and at the Balkans

Apart from the above mentioned remarkable genetic splits, two other but less pronounced splits indicate two further vicariance events. The genetic structures revealed by all applied analytical methods supported the idea that both splits have taken place at the same time horizon. As these vicariance events are apparently rather young, they could have been triggered by the Last Glacial Maximum as e.g. evidenced for genetic differentiation in *Erebia medusa* around the Alps (Hammouti *et al.* 2010).

Thus, the Northern Alps group consisted of at least two subgroups, one in the West including the three Swiss populations and one in the central and eastern part of the Northern Alps embracing the populations from Austria and Southern Germany, most probably resulting from the Würm glacial vicariance in two centres of survival north of the Alps. These genetic subgroups are also reflected in the recent disjunction of *A. foetida* in the northern calcareous Alps (Meusel & Jäger 1992), maybe representing still incomplete postglacial range expansion out of these centres.

For $K = 4$, the STRUCTURE analyses distinguished the eastern Swiss population in the Central Alps (CH-Sc) from all other Northern Alps populations, and for $K = 6$ and higher, this populations is ranked as an independent group from all other populations. This pattern might be the result of recent (most probably secondary) differentiation in this

particular region or even indicate a third independent late Würm glacial refugium along the northern border of the Alps.

At the same hierarchical level as the populations from the Northern Alps, the populations from Croatia (i.e. the south-western slopes of the Dinaric Alps facing the Adriatic) are distinguished from the other Balkan populations as well as the ones from Slovenia, the south-eastern Alps and the Tatras. This pattern supports the idea of survival of *A. foetida* at both flanks of the Dinaric Alps, the Adriatic and the Pannonian side with a vicariance event maybe triggered by the remarkable drop of temperature around the Last Glacial Maximum. This assumption is well supported by the high number of private fragments (e.g. seven private fragments in the Učka population) and the high genetic diversity values (e.g. H_N: 0.069 in HR-Mv). A similar pattern was also communicated by Magri *et al.* (2006) and Magri (2008), who assumed the Dinaric Alps as a refugial area for *Fagus sylvatica* or *Edraianthus serpyllifolius* (Surina *et al.* 2011), which also support a possible refugium of these species along the north-eastern Adriatic Sea.

3.5.3 The northern Balkans, most important postglacial expansion centre for *A. foetida*

The Balkan Peninsula is topographically the most diverse landscape in Europe (Reed *et al.* 2004), and such variability could have provided a suitable environment for altitudinal shifts in response to climatic change during glacial–interglacial oscillations as well as for small-scale allopatric isolation (Hewitt 2000; Kryštufek *et al.* 2007). As already pointed out above, the Balkans served as an important refugial area (Reinig 1938, 1950; de Lattin 1967; Dennis 1993). This is not only true for tree and shrub species such as *Fagus sylvatica* (Magri *et al.* 2006) and *Carpinus betulus* (Grivet & Petit 2003), but also for a multiplicity of other plant and animal species, including warm-adapted taxa like *Podarcis melisellensis* (Podnar *et al.* 2004) and *Melanargia galathea* (Habel *et al.* 2005; Schmitt *et al.* 2006b), as well as cold-adapted taxa like *Vipera berus* (Ursenbacher *et al.* 2006), *Polygonatum verticillatum* (Kramp *et al.* 2009) or *Cicerbita alpina* (Michl *et al.* 2010).

Our data also support the great importance of the Balkans as expansion centre. This fact is well known for warm-adapted species (e.g. *Erinaceus concolor*, Seddon *et al.* 2001; *Maniola jurtina*, Habel *et al.* 2009a; *Melanargia galathea*, Habel *et al.* 2011b). The genetic structures of *A. foetida* support the idea that the Balkans also served as postglacial expansion centre for cold-adapted species. The order of groups distinguished in the STRUCTURE analyses and the genetic diversity and uniqueness of populations strongly

support postglacial expansion from Würm glacial refugia at the Pannonian flank of the Dinaric Alps (see above) via the Slovenian mountain areas and throughout the southern calcareous Alps in western direction and to the calcareous parts of the Tatra Mountains in north-eastern direction.

3.6 Conclusions – *Aposeris foetida*

The montane plant species *Aposeris foetida* most probably survived the last ice age in multiple independent periglacial refugia. It is plausible that *A. foetida* survived those periods of unsuitable climatic conditions in areas bordering the water-donating high-mountain systems like the Alps and the high-mountain systems of the western Balkans (Schmitt 2007). All our analyses indicated two main centres of genetic diversity for *A. foetida*. High genetic diversity values and a higher number of private fragments in the Northern Alps populations indicated at least two potential glacial refugia at the northern foothills of the Alps for *A. foetida*. These glacial refugia of *A. foetida* give strong evidence for forest ecosystems or at least habitats with some forest characteristics in the northern pre-Alps. The second main centre is represented by the Dinaric Alps region, which is considered to be a glacial centre of persistence for *A. foetida* at both flanks of the mountain range, as well as an important expansion centre of postglacial recolonisation of the Southern Alps and the Tatra region.

4 Small Cow Wheat – *Melampyrum sylvaticum*

4.1 Introduction – *Melampyrum sylvaticum*

The complex actual distribution patterns of animal and plant species in Europe are closely related to the climate changes of the Quaternary (Hewitt 1996, 2000; Comes & Kadereit 1998; Grivet & Petit 2003). The enormous climate changes during the ice ages have led to migration, isolation, mixing and extinction of plant and animal populations (Hewitt 1996; Bennett 1997; Taberlet *et al.* 1998; Avise 2000; Stehlik 2003). Consequently, phylogeographic studies for many taxa have shown that the climate changes during the Quaternary had significant impacts in the population genetic structures through these impacts on the distribution of species by range shifts in latitude and altitude (e.g. Comes & Kadereit 1998; Taberlet *et al.* 1998; Hewitt 2004; Schmitt 2007).

The beginning of the reconstruction of the Quaternary vegetation history was established by palynological investigations. However, the quaternary distribution history of most herbaceous plants cannot be reconstructed by pollen profiles (Bonn & Poschlod 1998; Hewitt 1999; Honnay *et al.* 2005). Therefore, the development of molecular techniques and the opportunity to elucidate the genetic relationships at the intra-specific level provided the potential to evaluate and refine palynological evidence for glacial refugia and postglacial dispersal routes (reviewed in Bhagwat & Willis 2008) and also to reconstruct these biogeographical aspects for species without fossil evidence (Hewitt 1996, 2000; Comes & Kadereit 1998; Taberlet *et al.* 1998).

In this biogeographical content, the so called *tabula rasa* hypothesis is one common hypothesis of glacial survival and postglacial colonisation applied to many species in Europe, postulating the total extinction of most species from the periglacial areas of Central Europe. This hypothesis could be proved for many species recently living in Central and Northern Europe (Petit *et al.* 2002, 2003; Grivet & Petit 2003). In these cases, warm-adapted organisms re-colonised the more northern parts of Europe postglacially from the classical Mediterranean refugia in Iberia, Italy and the Balkans (de Lattin 1949), as recently evidenced by many genetic studies (reviews in Hewitt 2004; Schmitt 2007).

Recently, this view has been questioned for more cold-tolerant taxa, and the hypothesis of additional cryptic northern refugia for non-tundra species supplementing the southern ones came up (reviews in Stewart & Lister 2001; Stewart *et al.* 2010; Schmitt & Varga 2012). In the last few years, this hypothesis has been supported by diverse phylogeographic studies including animals (e.g. Pauls *et al.* 2006; Ursenbacher *et al.*

2006; Mardulyn *et al.* 2011; Theissinger *et al.* 2011), trees (e.g. Willis *et al.* 2000; Boratyński *et al.* 2007; Magri 2008) and herbaceous plant species (e.g. Tyler 2002a, 2002b; Kramp *et al.* 2009; Michl *et al.* 2010; Huck *et al.* 2012). However, the locations of cryptic extra-Mediterranean forest refugia, especially along the northern foothills of the Alps, are still controversially debated (Muster 2002; Magri *et al.* 2006; Magri 2008).

To foster the understanding of the glacial distribution of European forest biomes apart from the classical Mediterranean refugia in Southern Europe, we selected a montane woodland plant as a model species for montane forests: *Melampyrum sylvaticum* (L.), which is a widespread element of the European montane and subalpine flora. The species' geographic range covers mountain ranges from Western Europe to the Urals and lowlands in the boreal zone with focus in Northern Europe extending into north-western Russia (Meusel & Jäger 1992). Our aim is to detect potential glacial refugia for this species as well as the postglacial range shifts and (re)colonisation routes in Europe. We analysed AFLPs of 445 individual samples from 44 populations across the species' European distribution and studied differences in molecular diversity and differentiation among these populations. The dataset may allow us to obtain a better understanding of the distribution of montane forests in Europe during the last ice age and foster the knowledge of the range dynamics of forest dwelling plant species over the late Quaternary.

4.2 Study species – *Melampyrum sylvaticum*

The genus *Melampyrum* (L.) includes approximately 30 species spread across most of the temperate and boreal areas of the northern hemisphere (Dalrymple 2007). *Melampyrum sylvaticum* (L.) (Scrophulariaceae or Orobanchaceae, as redefined by Olmstead *et al.* 2001) is an annual, hemiparasitic therophyte. A diploid chromosome number of 2n = 18 has been reported for the species (Oberdorfer 1983). As a hemiparasite, the plants are obtaining some nutrition from the roots of a wide range of plants like *Picea abies*, *Salix aurita*, *Briza media*, *Calluna vulgaris, Carex sempervirens* and also on living roots of *M. pratense* (Weber 1976a, 1976b), but it can also survive independently. The height of the individuals reaches from 5 to 18 cm; the plant has narrow leaves and produces deep yellow tubular flowers from June to August (Broome 2003). The species is hermaphroditic and the flowers are pollinated by bumble bees, but it also self-pollinates if cross pollination fails (Knuth 1898; Rumsey 1994). The fruits are two-seeded, ridged, dark brown capsulae, which are as long as the calyx and are dispersed by ants (Gibson 1993).

M. sylvaticum mainly inhabits light, open, broad-leaved woodlands, scrubs and meadows on shallow soils with low available nitrogen where humidity remains high (Ellenberg 1988). The species is assigned to the boreo-montane floral element (Preston *et al.* 1994; Preston & Hill 1997) and is mainly found in mesophilous, summer-green deciduous forests (*Carpino-Fagetea*) and boreal-continental coniferous forests with dwarf scrubs (*Vaccinio-Piceetea*), but also in alpine calcareous dwarf scrub communities (*Rhododendro hirsuti-Ericetea carneae*) and arctic-alpine wind-exposed heathlands (*Loiseleurio-Vaccinietea*). Thus, *M. sylvaticum* is a widespread element of the European montane and subalpine flora, and its geographic range covers mountain ranges from Western Europe to the Urals (e.g. Alps, Pyrenees, Balkan, Carpathians, Scottish Highlands and some lower mountain ranges of Central Europe) and lowlands in the boreal zone. However, the major extent of the species' range is confined to Northern Europe and north-western Russia (Meusel & Jäger 1992).

4.3 Sampling – *Melampyrum sylvaticum*

To reveal the genetic variation of *M. sylvaticum* and its range dynamics, we analysed 445 individuals from 44 populations across its distribution except for occurrences in north-eastern Europe (Fig. 4.1, Table 4.1).

Leaf material was collected in situ across all the populations from at least five different plants and dried in plastic zip-lock bags filled with silica gel. To avoid sampling of clones, plants were collected at distances of at least 10 m. The maximum geographic distance of the analysed samples is about 5,800 km between the Bulgarian population in the Rhodope Mountains (BG-Pa) and the population from Island (IS-Ho). The shortest distance (43 km) is between the other two populations from Bulgaria (BG-Gr and BG-Vh). Finally, five to twelve individuals per population have been included in the Amplified Fragment Length Polymorphisms (AFLP) analysis.

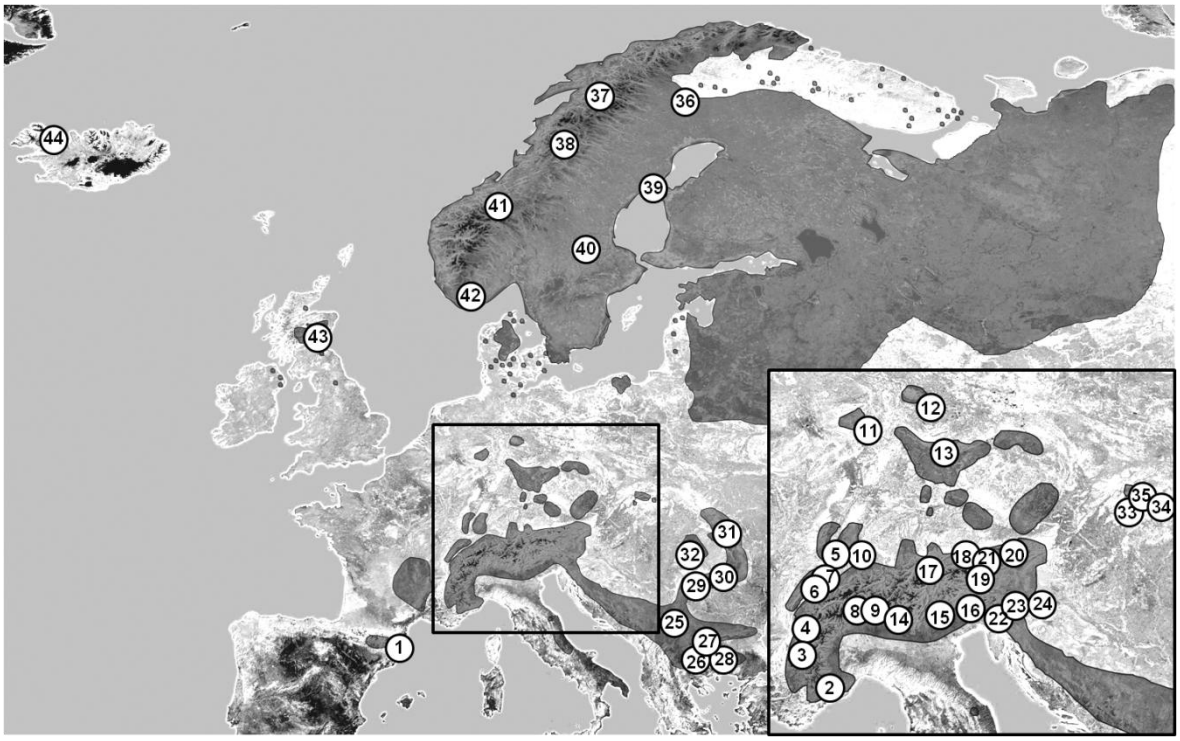

Fig. 4.1: Geographic distribution of the collection sites of the 44 populations of *Melampyrum sylvaticum* analysed. Distribution area is based on Meusel & Jäger (1992). For abbreviations and further details see Table 4.1

Table 4.1: Geographically ordered codes of populations, collection sites, mountain ranges, coordinates and altitude (m asl) of the 44 *Melampyrum sylvaticum* populations surveyed for Amplified Fragment Length Polymorphism (AFLP).

No.	Pop. code	Location	Mountain range	Country	Coordinates	Altitude (m asl)	*N*
1	E-Bo	Bosc de la Mare	Pyrenees	Spain	N 42°23' E 02°09'	2100	14
2	F-Va	Val d l'Urbaye	Alps	France	N 44°40' E 06°55'	1900	7
3	F-Ri	Le Rivier d'Allemont	Alps	France	N 45°12' E 06°02'	2100	12
4	F-An	Annecy	Alps	France	N 45°49' E 06°06'	1400	12
5	F-Gb	Grand Ballon	Vosges Mountains	France	N 47°54' E 07°05'	1200	12
6	CH-Vu	Vue des Alpes	Jura Mountains	Switzerland	N 47°04' E 06°52'	1300	6
7	CH-Gj	Le Chasseron	Jura Mountains	Switzerland	N 46°51' E 06°32'	1300	6
8	CH-Ai	Airolo	Alps	Switzerland	N 46°33' E 08°35'	1300	10
9	CH-Sb	San Bernardino	Alps	Switzerland	N 46°26' E 09°12'	1500	14
10	D-To	Feldberg	Black Forest	Germany	N 47°52' E 08°00'	1400	16
11	D-Ro	Ahretal	Rothaargebirge	Germany	N 51°08' E 08°32'	540	12
12	D-Ha	Stiege	Harz Mountains	Germany	N 51°38' E 10°49'	520	12
13	D-Tw	Neuhaus am Rennweg	Thuringian Forest	Germany	N 50°31' E 11°08'	730	12
14	I-Sm	Passo San Marco	Alps	Italy	N 46°04' E 09°36'	2000	11
15	I-Pa	Passo del Manghen	Alps	Italy	N 46°10' E 11°25'	1600	11
16	I-Cl	Claut	Alps / Friaul	Italy	N 46°21' E 12°32'	1000	11
17	A-Ho	Holzleitner Sattel	Alps	Austria	N 47°19' E 10°54'	1100	7
18	A-Bi	Bichl / Wilder Kaiser	Alps	Austria	N 47°33' E 12°15'	1000	11
19	A-Ko	Kolm-Saigurn	Alps	Austria	N 47°05' E 13°00'	1600	11
20	A-Lo	Loser	Alps	Austria	N 47°40' E 13°47'	1300	11
21	A-Hk	Hochkönig	Alps	Austria	N 47°25' E 15°17'	1300	10
22	SLO-Kr	Krvavec	Steiner Alps	Slovenia	N 46°02' E 13°43'	1200	12
23	SLO-Me	Medvodje	Karavanke	Slovenia	N 46°25' E 14°24'	1000	11
24	SLO-Ma	Mangart	Julian Alps	Slovenia	N 46°21' E 15°35'	1500	12
25	SER-Ko	Mt Kopaonik	Balkan Mountains	Serbia	N 43°16' E 20°48'	1800	12
26	BG-Vh	Vihren-Hütte	Pirin Mountains	Bulgaria	N 41°45' E 23°24'	2000	12
27	BG-Gr	Granchar Lake	Rila	Bulgaria	N 42°07' E 23°35'	2200	12
28	BG-Pa	Pamporovo	Rhodope Mountains	Bulgaria	N 41°39' E 24°41'	1600	8
29	RO-Io	Iorgovan	Retezat Mountains	Romania	N 45°17' E 22°55'	1100	6
30	RO-Ca	Cârnic	Făgăraş Mountains	Romania	N 45°20' E 24°47'	1300	12
31	RO-Lr	Lacu Roşu	Carpathians	Romania	N 46°47' E 25°47'	1000	12
32	RO-Ar	Arieşeni	Apuseni	Romania	N 46°29' E 22°42'	1000	12
33	SK-Ko	Kosodrevina	Mala Fatra	Slovakia	N 48°55' E 19°37'	1400	11
34	SK-Sb	Sucha Bela	Lower Tatras	Slovakia	N 48°56' E 20°24'	900	12
35	PL-Mo	Morskie-Oko	High Tatras	Poland	N 49°13' E 20°05'	1300	14
36	FIN-Mu	Muonio	Scandes	Finland	N 68°03' E 24°03'	470	7
37	S-Bj	Björk	Scandes	Sweden	N 68°23' E 18°37'	450	10
38	S-St	Strima	Scandes	Sweden	N 66°05' E 14°52'	550	6
39	S-Sb	Stridbäck	Scandes	Sweden	N 63°33' E 19°43'	10	7
40	S-Ge	Gesund	Scandes	Sweden	N 60°51' E 14°30'	400	5
41	N-Ge	Geiranger	Scandes	Norway	N 62°45' E 10°00	800	9
42	N-Ev	Evje	Scandes	Norway	N 58°30' E 07°35'	450	6
43	SCO-Bi	Birks of Aberfeldy	Grampian Mountains	Scotland	N 56°36' W 03°52'	250	13
44	IS-Ho	Holmavik		Iceland	N 65°42' W 21°41'	30	12

4.4 Results – *Melampyrum sylvaticum*

The three selective AFLP primer combinations amplified a total of 983 fragments ranging from 60 to 550 bp for the 445 individuals of *M. sylvaticum*. The primer combination EcoRI-AAT (6-FAM) – MseI-CCT yielded 326, EcoRI-ACG (Tamra) – MseI-CTG 367 and EcoRI-ATG (HEX) – MseI-CTG 290 fragments. In total, 99.8 % of the fragments were polymorphic and only 0.2 % were scored in all individuals from all populations. No identical AFLP profiles were shared by any two individuals. The individual number of fragments was from 151 (N-Ge04) to 270 (F-Gb09) (Table 4.2, Fig. 4.2). The average number of fragments per individual in a population ranged from 163 (N-Ge) to 257 (F-Gb), with a mean of 199 (SD: 24). Calculation of the technical error rate during the scoring and labelling of the AFLP fragments resulted in 12 differences in 2143 comparisons, resulting in an error rate of 0.56 %.

The 44 analysed *M. sylvaticum* populations showed varying levels of intrapopulation genetic diversity. The average percentage of polymorphic loci (*%Ppop*) was 23.8 % (SD: 6.7), the average Shannon index (H_{SH}) was 0.174 (SD: 0.043) and the average gene diversity (H_N) was 0.089 (SD: 0.019). The highest diversity was detected for two populations from France, one SW-Alpine population (F-An) and one from the Vosges Mountains (F-Gb) (F-An: *%Ppop*: 38.9 %; H_{SH}: 0.277; H_N: 0.137; F-Gb: *%Ppop*: 38.2 %; H_{SH}: 0.266; H_N: 0.131); the lowest for the population from the German Rothaargebirge (D-Ro: *%Ppop*: 14.5 %; H_{SH}: 0.107; H_N: 0.053) (Fig. 4.2a). The *DW* values showed strong differences among populations with an average of 2.12 (SD: 1.11) ranging from 0.90 in Western Norway (N-Ge) to 5.81 in the population from the Thuringian Forest (D-Tw) (Fig. 4.2b).

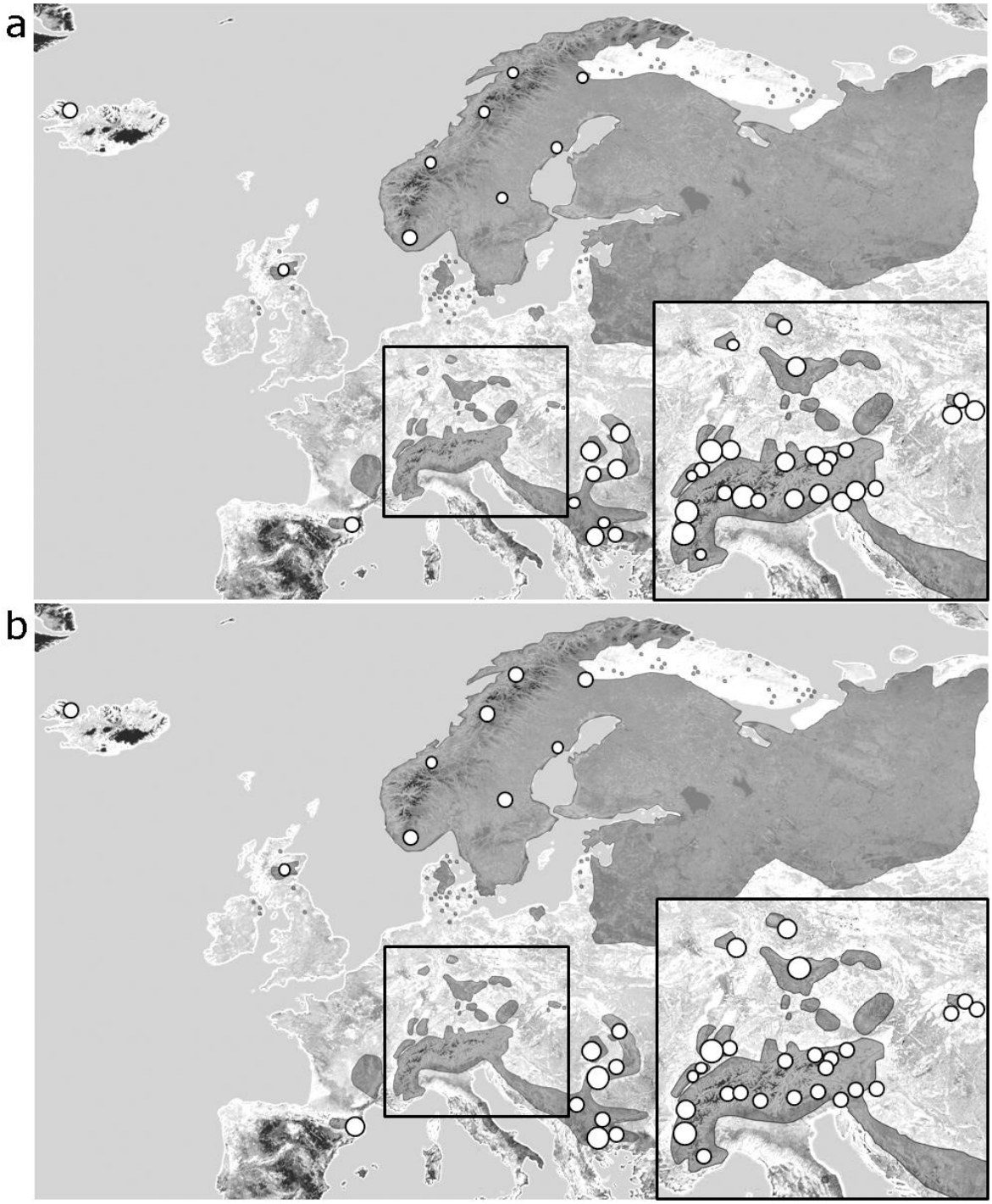

Fig. 4.2: Nei's gene diversity (a) and *DW* values (b) in the 44 investigated populations of *Melampyrum sylvaticum*. The circle size is proportional to the index values. For more details see Table 4.2.

Table 4.2: Genetic diversity, frequency-down-weighted marker values (*DW*) and the number of private fragments of the 44 populations of *Melampyrum sylvaticum* analysed.

No.	pop code	*N*	number of loci	% pol loci	*DW*	private fragments	Nei's gene diversity (H_N)	Shannon Index (H_{SH})
1	E-Bo	12	175	21.97	3.15	3	0.079	0.158
2	F-Va	6	196	14.85	1.60	1	0.068	0.121
3	F-Ri	12	220	36.52	4.65	2	0.126	0.257
4	F-An	12	221	38.86	3.16	4	0.137	0.277
5	F-Gb	12	257	38.15	4.31	4	0.131	0.266
6	CH-Vu	6	185	15.56	1.02	-	0.072	0.128
7	CH-Gj	6	170	17.70	1.17	-	0.080	0.143
8	CH-Ai	10	169	22.08	1.40	1	0.081	0.161
9	CH-Sb	12	197	33.88	2.04	6	0.119	0.241
10	D-To	12	182	32.66	1.56	2	0.106	0.220
11	D-Ro	12	239	14.45	2.49	-	0.053	0.107
12	D-Ha	12	229	23.40	2.61	4	0.080	0.164
13	D-Tw	12	247	28.48	5.81	1	0.105	0.211
14	I-Sm	8	200	19.53	1.82	-	0.078	0.148
15	I-Pa	11	185	28.28	1.53	-	0.095	0.194
16	I-Cl	11	206	27.77	2.22	3	0.101	0.201
17	A-Ho	7	192	22.69	1.40	1	0.096	0.178
18	A-Bi	11	194	28.38	1.57	-	0.104	0.207
19	A-Ko	11	167	23.50	1.32	1	0.086	0.172
20	A-Lo	11	187	27.26	1.68	2	0.092	0.187
21	A-Hk	10	176	22.48	1.36	-	0.086	0.168
22	SLO-Kr	12	214	27.77	1.98	1	0.102	0.204
23	SLO-Me	11	196	26.45	1.37	-	0.094	0.189
24	SLO-Ma	12	227	23.09	2.17	3	0.086	0.172
25	SER-Ko	11	177	20.14	1.53	-	0.069	0.140
26	BG-Vh	12	237	26.96	4.51	2	0.100	0.199
27	BG-Gr	12	185	18.92	1.55	-	0.066	0.134
28	BG-Pa	8	167	19.74	1.93	-	0.075	0.145
29	RO-Io	6	232	20.14	4.29	2	0.092	0.164
30	RO-Ca	12	194	29.81	2.22	2	0.107	0.216
31	RO-Lr	12	213	32.45	2.42	1	0.110	0.224
32	RO-Ar	12	199	29.40	2.86	2	0.098	0.200
33	SK-Ko	11	230	27.37	2.29	3	0.100	0.200
34	SK-Sb	12	201	28.89	2.18	4	0.101	0.204
35	PL-Mo	12	175	26.65	1.24	-	0.089	0.182
36	FIN-Mu	7	192	16.28	1.32	-	0.067	0.125
37	S-Bj	10	191	18.72	1.26	1	0.068	0.135
38	S-St	6	207	15.56	1.41	-	0.071	0.127
39	S-Sb	7	171	14.95	1.00	-	0.063	0.117
40	S-Ge	5	199	13.43	2.00	4	0.064	0.110
41	N-Ge	9	163	18.41	0.90	-	0.073	0.142
42	N-Ev	6	187	16.58	1.55	1	0.078	0.138
43	SCO-Bi	12	187	16.17	1.05	-	0.062	0.122
44	IS-Ho	12	240	22.69	2.37	1	0.081	0.164
		M	**199.40**	**23.84**	**2.12**	**1.14**	**0.089**	**0.174**
		SD	**24.20**	**6.70**	**1.11**	**1.51**	**0.019**	**0.043**
		SE	**3.65**	**1.01**	**0.17**	**0.23**	**0.003**	**0.006**

M = mean; SD = standard deviation; SE = standard error.

Not all populations contained private and no population contained fixed private fragments. The southern Switzerland population (San Bernadino CH-Sb) comprised with six the highest number of private fragments.

All pair-wise Φ_{ST} values were highly significant ($p < 0.001$). The average Φ_{ST} over all examined samples was rather high (0.528), and the maximum pair-wise Φ_{ST} reached 0.774 between the Spanish population E-Bo and the population from the Rothaargebirge D-To. The lowest Φ_{ST} was 0.274 between the two Austrian populations A-Bi and A-Ho. Isolation by distance analysis yielded a significant correlation ($p = 0.008$), but R^2 was 0.10 so that the explained variance was low (data not shown).

PCoA based on Φ_{ST} values distinguished five geographic groups: the population from Spain (E-Bo) and the one from the Thuringian Forest (D-Tw) were clearly separated along the first and the second axis from all other populations (Fig. 4.3). The combination of the first and the second axis distinguished the Alps including the Slovenian populations from the northern populations from Sweden, Finland, Norway, Scotland and Iceland. The Balkan populations were distinguished along the second axis. The combination of the second and the third axis separated the German uplands (D-Ha, D-Ro) and the population from the Vosges Mountains (F-Gb) from all other populations. Axis 1 explained 26.9 %, axis 2 18.1 % and axis 3 16.0 % of the variance.

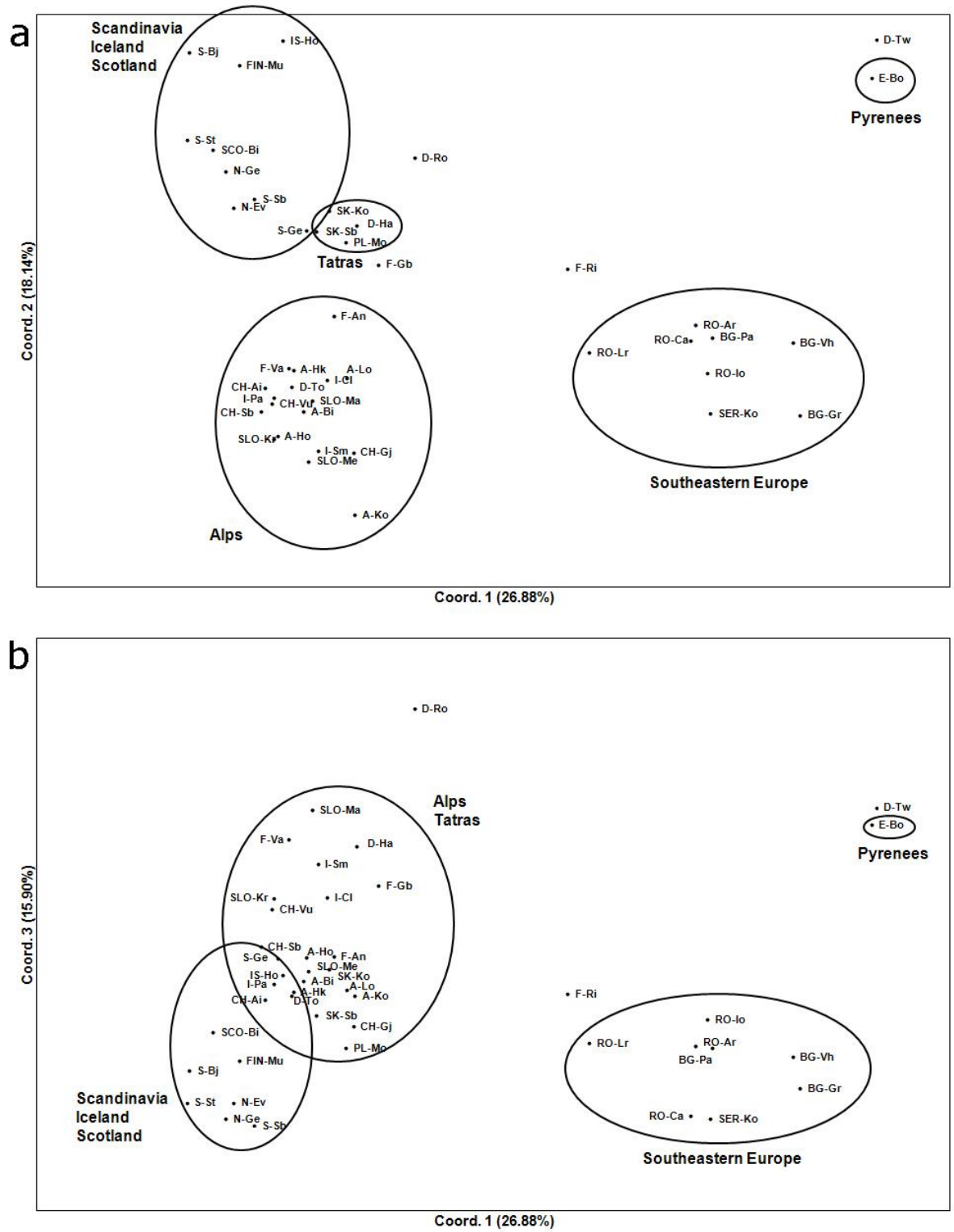

Fig. 4.3: Principal coordinates analysis of the 44 *Melampyrum sylvaticum* populations analysed based on pairwise Φ_{ST} values.

A NJ phenogram of the 44 populations (Fig. 4.4) based on Nei's genetic distances modified after Lynch & Milligan (1994) distinguished four partly supported clusters of populations: (i) a cluster containing the population from the Pyrenees and the Thuringian Forest, (ii) a group containing all populations from Southeastern Europe (Bulgaria, Romania, Serbia), (iii) an Alpine group, including the populations from the Julian Alps and (iv) a northern group with some substructure, however scarcely supported by bootstrapping. One of these subgroups contained all the populations from Scandinavia, Iceland and Scotland, another subgroup included the Tatra populations and finally a subgroup was built by the German uplands.

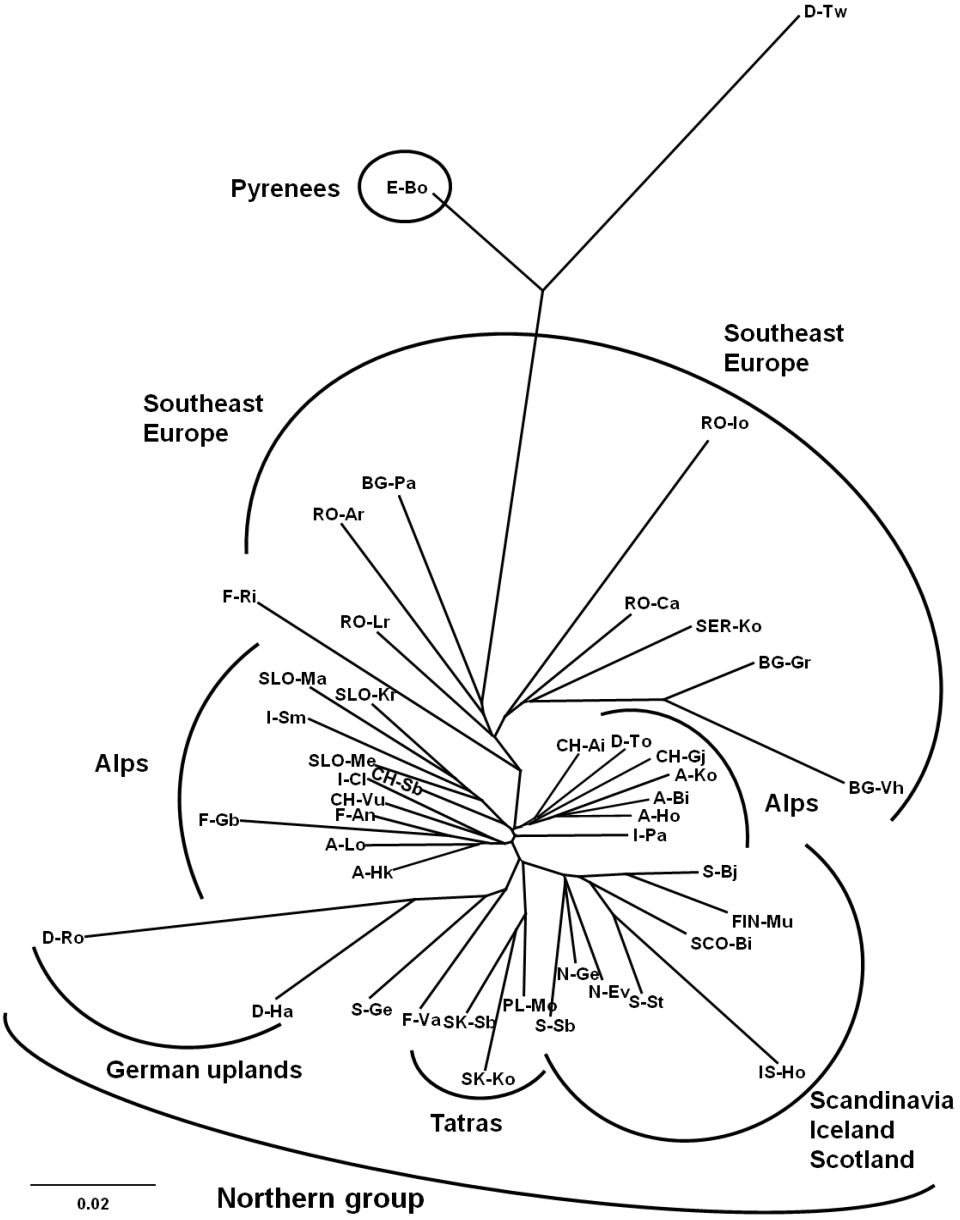

Fig. 4.4: Neighbour joining phenogram of the AFLP-phenotypes of the 445 individuals from the 44 investigated populations of *Melampyrum sylvaticum*. For abbreviations see Table 4.1.

A more refined genetic structuring was revealed by the model-based Bayesian cluster analysis performed with STRUCTURE. The Evanno *et al.* (2005) method obtained the best resolution for $K = 5$ (Fig. 4.5). Regarding the different STRUCTURE plots, a successive separation of genetic groups was observable. For $K = 3$, the population from Spain and the one from the Thuringian Forest were clearly separated from a Southeastern Europe cluster, including one population from the Southwestern Alps (F-Ri); the third cluster contained all other populations. In the next step ($K = 4$), STRUCTURE identified all the northern populations (German uplands, Tatra Mountains, Scandinavia, Scotland and Iceland) as a distinct group. The last split ($K = 5$) detached the populations from the German uplands together with the Vosges population (F-Gb) from the group of the Tatras and northern populations.

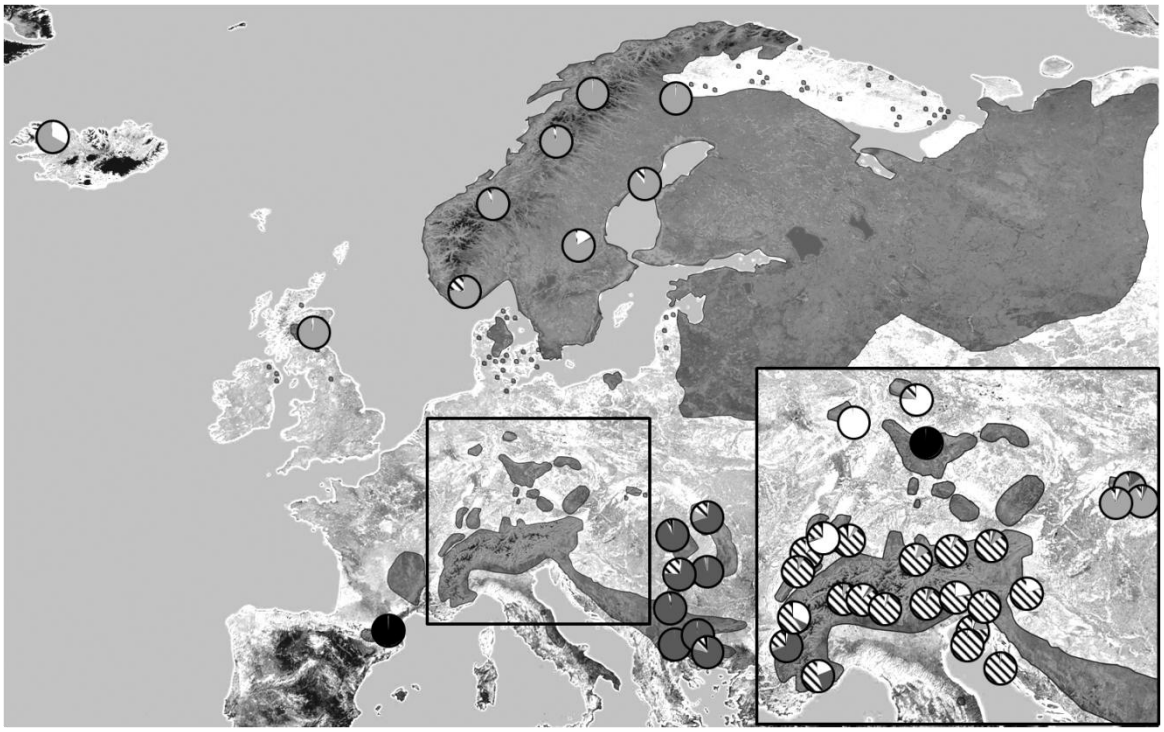

Fig. 4.5: Genetic clustering analyses of *Melampyrum sylvaticum* genotypes. Different colours represent proportional memberships of a population to the respective genetic clusters, averaged over individual membership proportions for $K = 5$ as calculated by STRUCTURE.

Calculating the total variation with AMOVA revealed a percentage of variation among populations of 52.8 % and within populations of 47.2 % (Table 4.3). Hierarchical AMOVA testing (i) the two highly distinct groups Spain and Thuringian Forest versus all other populations and (ii) the five detected STRUCTURE groups revealed the levels of variation among groups, among populations within groups and within populations. For the two genetic groups, the variation among groups was 34.8 %, 32.7 % among populations within groups and 32.5 % within populations. The variation among the five STRUCTURE groups

was 16.5 % among groups, 38.2 % among populations within groups and 45.3 % within populations.

Table 4.3: Analysis of molecular variance (AMOVA) based on Amplified Fragment Length Polymorphism (AFLP) data for *Melampyrum sylvaticum*. In the hierarchical AMOVA, populations were grouped according to the two main genetic groups (Spain and Thuringian Forest against all other populations) and for $K = 5$ as calculated by the software STRUCTURE (see text for detailed information).

Source of variation	SSD	CV	% total	*p*
Total				
Among populations	23619.295	49.95853 Va	**52.8**	< 0.001
Within populations	17909.339	44.66169 Vb	**47.2**	< 0.001
two groups (E-Bo & D-Tw vs. rest)				
Among groups	2751.393	47.79934 Va	**34.8**	<0.001
Among populations within groups	20867.901	44.96097 Vb	**32.7**	<0.001
Within populations	17909.339	44.66169 Vc	**32.5**	< 0.001
five groups (STRUCTURE)				
Among groups	7196.415	16.26212 Va	**16.5**	<0.001
Among populations within groups	16422.88	37.67730 Vb	**38.2**	<0.001
Within populations	17909.339	44.66169 Vc	**45.3**	< 0.001

SSD = sum of squares; CV = variance component estimates; % total = percentage of total variance; *p* = *p*-value.

4.5 Discussion – *Melampyrum sylvaticum*

Compared to other AFLP studies on the phylogeography of mountain plants, we obtained an intermediate value for the global differentiation among all populations (Φ_{ST}: 0.53). Thus, this value is comparable to *Ranunculus glacialis* (0.51) (Schönswetter *et al.* 2003), *Meum athamanticum* (0.47) (Huck *et al.* 2009) or *Dryas octopetala* (0.47) (Skrede *et al.* 2006), but on the one hand is considerably higher than in e.g. *Veratrum album* (0.13) (Treier & Müller-Schärer 2011), or *Trollius europaeus* (0.39) (Despres *et al.* 2002), and on the other hand remarkably lower than in *Polygonatum verticillatum* (0.73) (Kramp *et al.* 2009).

The bulk of phylogeographies of plant and animal species refer to the glacial refugia during the last glacial period (i.e. the Würm) and the postglacial re-colonisation routes after the ice finally had retreated. However, *M. sylvaticum* shows a hierarchical structure of splits, a first level distinguishing three groups (Pyrenees, Southeastern Europe and all other populations). This third group is subdivided at the second level into three to four other groups (Alps, Central German uplands, Tatras, Scandinavia with Scottish highlands and Iceland, with the latter two groups not being separated unambiguously). These two levels of differentiation call for a more extended evolutionary time than Würm ice age processes and might therefore best be explained by subsequent evolutionary events during the last two full glacial-interglacial cycles.

4.5.1 Riss glaciation: separation and survival in three southern refugia

During the Riss glacial (200,000-130,000 years bp), some glaciers in Europe reached their largest extent during the entire Pleistocene (Penck & Brückner 1909; van Husen 1999, 2004). Within these hostile conditions, *M. sylvaticum* had to retreat to areas where it could survive. STRUCTURE analyses, neighbour-joining phenograms and PCoA support a first separation into three genetic groups, which might be partially viewed as potential refugia during the Riss glaciation. STRUCTURE separates first the population from the Pyrenees (including the Thuringian forest) as a discrete genetic group. Furthermore, all Balkan and Carpathian (except Tatras) populations were summarized into the second group. The third and largest group contained the Alps, the central German uplands, the Tatras and Scandinavia. As it is generally accepted that the rather extreme conditions during the Riss ice age enhanced the *tabula rasa* in Central Europe in relation to Würm ice age conditions (Habel *et al.* 2011b), one may also assume relatively southern Riss

refugia for *M. sylvaticum*. In this context and reflecting the observed genetic structuring, it has to be assumed that areas in the vicinity of the Pyrenees and in the Balkan Peninsula served as refugial areas for *M. sylvaticum* during the Riss glacial.

The great importance of the Pyrenees and its adjoining areas as glacial refugia for many mountain taxa is largely accepted since long and recently has been supported by genetic studies for various plant and animal species (e.g. Muster & Berendonk 2006; Pauls *et al.* 2006; Muller *et al.* 2007; Schmitt & Haubrich 2008; Huck *et al.* 2009; Michl *et al.* 2010; Vila *et al.* 2011).

The Balkan Peninsula with its important topographical variability in general is providing a suitable environment for altitudinal shifts in response to climatic change during glacial–interglacial oscillations and also for small-scale allopatric isolation (Varga 1975; Hewitt 1996, 1999, 2000; Taberlet *et al.* 1998; Kryštufek *et al.* 2007). Consequently, the area served as an important refugial area (Reinig 1938, 1950; de Lattin 1967; Dennis 1993; Hewitt 1996), for trees such as *Fagus sylvatica* (Magri *et al.* 2006) and *Carpinus betulus* (Grivet & Petit 2003), and for many other plant and animal species, including numerous warm-adapted taxa (Seddon *et al.* 2001; Podnar *et al.* 2004; Schmitt *et al.* 2006b; Habel *et al.* 2011b), but also more cold-tolerant taxa like the adder *Vipera berus* (Ursenbacher *et al.* 2006) and mountain species like *Erebia euryale* (Schmitt & Haubrich 2008), *Polygonatum verticillatum* (Kramp *et al.* 2009) and *Cicerbita alpina* (Michl *et al.* 2010). The importance of the Balkan Peninsula for the latter group is further supported by a high number of Balkan endemic mountain taxa at the species and subspecies level (Varga 1975). The importance of the Balkans and adjoining regions in Southeastern Europe for the glacial survival of *M. sylvaticum* is also underlined by our results e.g. by high rarity indices (i.e. *DW* values) in the Balkans where the highest value was found in the Bulgarian population in the Pirin Mountains (BG-Vh) and by high genetic diversity especially in the Romanian population. All these results are indicators for an area of glacial persistence in Southeastern Europe, most likely for more than just one glacial period.

The most likely Riss glacial refugium for the large third genetic group should have been located south of the intensively glaciated Alps. Almost 100 years ago, (Brockmann-Jerosch & Brockmann-Jerosch 1926) postulated the area of the southernmost Alps as a suitable refuge area for alpine plants supported by the lower degree of glaciations of the southern peripheral Alps as compared with the northern peripheral or central Alps. Recent phylogeographic studies have supported this assumption and have underlined the great importance of the southernmost Alps as refugia for many alpine species (e.g. Tribsch *et al.* 2002; Schönswetter *et al.* 2003, 2004, 2005; Pauls *et al.* 2006; Schmitt *et al.* 2006a;

Margraf *et al.* 2007; Borer *et al.* 2010, Alvarez *et al.* 2012). As a consequence, the southernmost parts of the Alps are also well known for their species diversity (Tribsch & Schönswetter 2003; Tribsch 2004; Schönswetter *et al.* 2005). In support of these assumptions, the *M. sylvaticum* populations from the Southern Alps showed higher genetic diversities compared to other populations, and we also found the highest number of private fragments in the southern Switzerland population from San Bernadino (CH-Sb).

4.5.2 Riss-Würm interglacial: up-hill shifts in all Riss refugia

The Riss-Würm or Eemian interglacial (130,000 – 116,000 years bp) was characterized by relatively stable climatic conditions and the temperature optimum of this interglacial period was even above today's mean temperatures in Europe (Zagwijn 1996; Aalbersberg & Litt 1998; Kukla *et al.* 2002; Kaspar *et al.* 2005). With the rising temperatures, *M. sylvaticum* must have shifted from the low altitudes occupied during the Riss glacial period to the now ice-free mountains colonising the entire Alpine Arch, but also surviving in the Pyrenees and Balkan mountains.

4.5.3 Würm glaciation: multiple refugia and extra-Mediterranean survival

When the temperature declined again with the onset of the Würm ice-age, *M. sylvaticum* also had to shift down-hill. In contrast to the Riss glaciation, which was more severe with stronger impacts on the survival of individuals in more northern areas (cf. Habel *et al.* 2011), some populations of more cold-tolerant species could survive the Würm ice age in more northern retreats (cf. Schmitt & Varga 2012), e.g. in some areas north of the Alps and in the lower areas in the proximity of the Tatra Mountains most probably in climatically buffered pockets (cf. Huck *et al.* 2012). These additional so-called extra-Mediterranean refugia often fostered further differentiation and thus increased the genetic complexity of the intraspecific genetic structures.

A similar scenario is also the most likely one for *M. sylvaticum*. The observed genetic differentiation among Alps, German upland and Tatra populations let us argue early Würm glacial expansion to these latter two regions, isolation in these regions with the glacial advance and subsequent genetic modifications. This scenario receives further support especially for the Tatra refugium by the high genetic diversity values in the Slovakian populations including three (SK-Ko) and four (SK-Sb) private fragments, which underline

the assumption of an extra-Mediterranean refugium in that particular region. Four private fragments in the Harz population (D-Ha) and higher than average *DW* values in Harz and Rothaargebirge let us, in analogy to the Tatra region, also argue for isolated Würm ice age refugia of *M. sylvaticum* in the central German uplands. Similar patterns supporting these two extra-Mediterranean centres of survival are also known for other mountain species (e.g. *Drusus discolor*, Pauls *et al.* 2006; *Polygonatum verticillatum*, Kramp *et al.* 2009; *Cicerbita alpina*, Michl *et al.* 2010; *Meum athamanticum*, Huck *et al.* 2012), and even trees like *Fagus sylvatica* (Magri *et al.* 2006; Magri 2008), the slug *Arion fuscus* (Pinceel *et al.* 2005) and the butterflies *Erebia medusa* (Schmitt & Seitz 2001) and *Parnassius mnemosyne* (Gratton *et al.* 2008), give strong genetic evidence for Würm glacial survival in the Tatra region, which consequently renders an important area of extra-Mediterranean survival (Schmitt & Varga 2012). Furthermore, as *M. sylvaticum* is a forest dwelling species, our data strongly support the previously existing hypothesis that even forests or forest-like habitats also should have survived at least the Würm glacial period in the German upland region north of the Alps and in the vicinity of the Tatras.

4.5.4 Postglacial: up-hill shifts and large northwards expansion

With the postglacial warming, populations of many mountain species simply shifted up-hill into the high-mountain systems as in the Pyrenees, Alps and Balkan mountains (cf. Schmitt 2009). This is also the most likely scenario for *M. sylvaticum*. However, the species' refugial populations north of the Alps are believed to have retracted to disjunct mountain peaks of the middle-high mountains of Central Europe, but did not retract to the Alps, a phenomenon also known for *Gentiana nivalis* (Alvarez *et al.* 2012), but also for some lineages of mountain butterflies (*Erebia sudetica*, Haubrich & Schmitt 2007; *E. epiphron*, Schmitt *et al.* 2006a). The time passed since this isolation on these mountains (about 10,000 years bp) was not sufficient for further differentiation among these *M. sylvaticum* populations.

Important postglacial range expansions are highly likely out of two of the Würm ice age retreats. The strong genetic cohesiveness between the eastern Balkan mountains all over the Romanian Carpathians strongly support postglacial expansion over major parts of the Carpathians from a more southern glacial refugium in the Balkan Peninsula maybe even extending as far north as the Southern Carpathians. A similar pattern was already documented for the mountain butterfly *Erebia euryale* (Schmitt & Haubrich 2008). Northern Europe was not, as in many other cases (e.g. Schönswetter *et al.* 2003; Muster

& Berendonk 2006) colonised from the same source as the Alps, but the populations sampled in the Tatras show the closest genetic coherence with the northern proveniences. Consequently, the refugium in the proximity of the Tatras is the most likely source, and the expansion to the North apparently has followed a pathway via the Baltic States and Finland to Scandinavia, and further west to Scotland and Iceland. This exclusive eastern pathway apparently is a common biogeographical feature in cold-adapted species like *Gentiana nivalis* (Alvarez *et al.* 2012) and the butterfly *Lycaena helle* (Habel *et al.* 2010).

The population of the Thuringian Forest grouped together with the population from the Pyrenees in all analyses performed. One possible explanation could be an intentional or unintentional anthropogenic seed transfer from the Pyrenees to this region. Their genetic patterns are quite similar and both populations share eleven alleles endemic for these populations and found in almost all of their individuals. Natural expansion from the Pyrenees to the Thuringian Forest seems to be highly unlikely due to the large distance between both areas, the relatively high weight of the seeds and the close geographic proximity of the Thuringian population to other genetic lineages.

5 Large Ringlet – *Erebia euryale*

5.1 Introduction – *Erebia euryale*

The extant-day distribution of many European species has been constantly shaped by Quaternary climatic fluctuations, more precisely by the oscillation between glacial and interglacial periods. Especially the ice ages, being considerably longer than the warm stages, had a strong impact (Hewitt 2000) and left deep imprints in the genetic structures of many species (Hewitt 1996, 2004; Avise 1998; Lascoux *et al.* 2004; Schmitt 2007). The extent of the continental ice sheets during the Pleistocene had a particularly important influence on the geographic distribution and genetic patterns of recent biota (Taberlet *et al.* 1998; Comes & Kadereit 2003; Hewitt 2004; Schmitt 2007). During glacial periods, Central Europe was mostly covered by tundra and cold steppe ecosystems, while most of Northern Europe and large parts of the European high mountain systems were covered by ice (Webb & Bartlein 1992; Dansgaard *et al.* 1993; Hewitt 2004; Schmitt 2007). As a result, almost all European species had rather different distribution patterns than today (Avise & Walker 1998; Hewitt 1999; Schmitt & Hewitt 2004a) and also performed remarkable range shifts after the end of the last ice age often resulting in the formation of secondary contact zones in recently colonised regions (Hewitt 2004).

The continuous development of genetic analysis methods have facilitated the unrevealing of the extant genetic structures and of many animal and plant species (e.g. Schmitt *et al.* 2005; Kramp *et al.* 2009; Schneeweiss & Schönswetter 2010; Huck *et al.* 2012; Sannikov & Petrova 2012), but also the Pleistocene changes in the European landscape (e.g. Hewitt 2004; Schmitt *et al.* 2006) were investigated in a considerable number of case studies to draw conclusions from recent patterns on the genetic fluctuations through space and time.

Initially, molecular biogeographic studies in Europe were mainly focussed on species expanding from the Mediterranean region, having their glacial survival centres in the classical Mediterranean refugia in the Iberian, Italian and the Balkan Peninsulas (de Lattin 1949), as evidenced by numerous genetic studies (reviews in Hewitt 2004; Schmitt 2007). However, recent genetic studies have increasingly focussed on the genetic structure of continental and (high) mountain species, supporting the existence of multiple glacial refugia within Europe beyond the above mentioned classical refugia. Indeed, the increasing number of genetic data reveals putative refugia for more cold-tolerant species

in Western, Eastern and Central Europe (Schmitt 2007; Schmitt & Varga 2012; Leppänen *et al.* 2013).

The molecular biogeography of cold-adapted arctic-alpine species is quite different from Mediterranean species and also varies remarkably from continental species (Schmitt 2009). Due to their extant distribution in the European high mountain systems and tundra belt in the North, which both have been covered by ice sheets during the glacial periods, many of the arctic-alpine species were shifting their distribution to the periglacial belt between the glaciated regions in the North and the South, but moving to higher elevations in the mountains and retreating northwards when the climate warmed again (de Lattin 1967; Müller 1980). This pattern has been supported by various genetic studies on plant and animal species (e.g. Schmitt & Hewitt 2004b; Schönswetter *et al.* 2006; Alvarez *et al.* 2012; García *et al.* 2012; Theissinger *et al.* 2013).

While important biogeographic questions on arctic-alpine species have already been answered and thus many ambiguities regarding distribution patterns of such species during glacial stages could be eliminated, only a few studies so far address species inhabiting coniferous mountain. Therefore, the large ringlet, *Erebia euryale*, is a good model to reveal the genetic history of such coniferous mountain forest elements, as this species is quite common in most areas of Southern and Central European coniferous mountain forests (Tolman *et al.* 1998; Kudrna 2002).

In former studies, the genetic structure of eleven *E. euryale* populations from the Pyrenees, Alps, Carpathians and Rila mountains (Schmitt & Haubrich 2008) and six populations from the Cantabrian Mountains in northern Spain (Vila *et al.* 2011) has already been analysed. These publications already give preliminary insights into the biogeographic history of *E. euryale* and its distribution shifts over time. Nevertheless, important questions still remain unresolved. Hence, we added 20 further populations, amongst others from the Massif Central, the Apennine Mountains and the Slovakian Tatra. The main goals of our study were:

(i) to verify and support or eventually falsify the hypotheses about genetic lineages and glacial refugia in the Cantabrian Mountains, Pyrenees, Alps, Carpathians and Rila, already established in previous studies on *E. euryale*;

(ii) to reveal the relationships of Apennines populations to the neighbouring genetic lineages in the Western and Eastern Alps and their fit into the overall picture;

(iii) to reveal the relevance of the Slovakian Tatra; has an independent glacial centre of survival for *E. euryale* existed in this region or is it representing a contact zone between different genetic lineages;

(iv) to picture the importance of the Massif Central region for *E. euryale* populations during glaciated and unglaciated periods;

(v) to reveal the biogeographic coherence between the Cantabrian Mountains, the Pyrenees and the Massif Central.

5.2 Study species – *Erebia euryale*

The large ringlet *Erebia euryale* (Esper 1805) (Lepidoptera, Nymphalidae, Satyrinae) is distributed over several European mountain systems, i.e. the Cantabrian Mountains, Pyrenees, Massif Central, Jura Mountains, Alps, mountains along the Czech-German border, Apennines, Carpathians, Balkans, but also Urals and Altai (Tolman *et al.* 1998; Kudrna 2002). The species' larval and imaginal habitats are grassy, flowery places in pine and spruce forest clearings and grassy slopes above the treeline (elevational range: about 750–2500 m asl.). Man-made meadows replacing these forests are similarly used. Larval host plants include Poaceae species (*Sesleria*, *Festuca*, *Poa* and *Calamagrostis*) as well as several species of the family Cyperaceae, i.e. *Carex flacca*, *Carex ferruginea*. The main flight period of the imagos starts in late June and ends mid-August. During bad weather conditions, the imagos survive in the shelter of coniferous trees (Sonderegger 2005) or *Juniperus* scrubs (Schmitt & Haubrich 2008). This taxon is classified Least Concern by the European Red List of Butterflies (van Swaay *et al.* 2010). Schmitt & Haubrich (2008) and Vila *et al.* (2011) reported four major genetic groups, mostly coinciding with the subspecific classification based on morphological characters: (i) Pyrenees and Cantabrian Mountains; (ii) western Alps representing *E. euryale adyte* (Hübner 1822); (iii) eastern Alps including the subspecies *E. euryale isarica* (Heyne 1895), but also *E. euryale ocellaris* (Staudinger 1861); (iv) southern Carpathians and Bulgarian mountains representing *E. euryale syrmia* (Fruhstorfer 1910).

5.3 Sampling – *Erebia euryale*

In total, 1298 individuals of *Erebia euryale* from 37 populations were sampled across major parts of its distribution in Central and Southern Europe (Northern Spain, Massif Central, Alps, Apennines, Tatras, Carpathians and Balkans; Table 5.1, Fig. 5.1). Individuals were frozen immediately after capture in liquid nitrogen and were stored accordingly until electrophoresis.

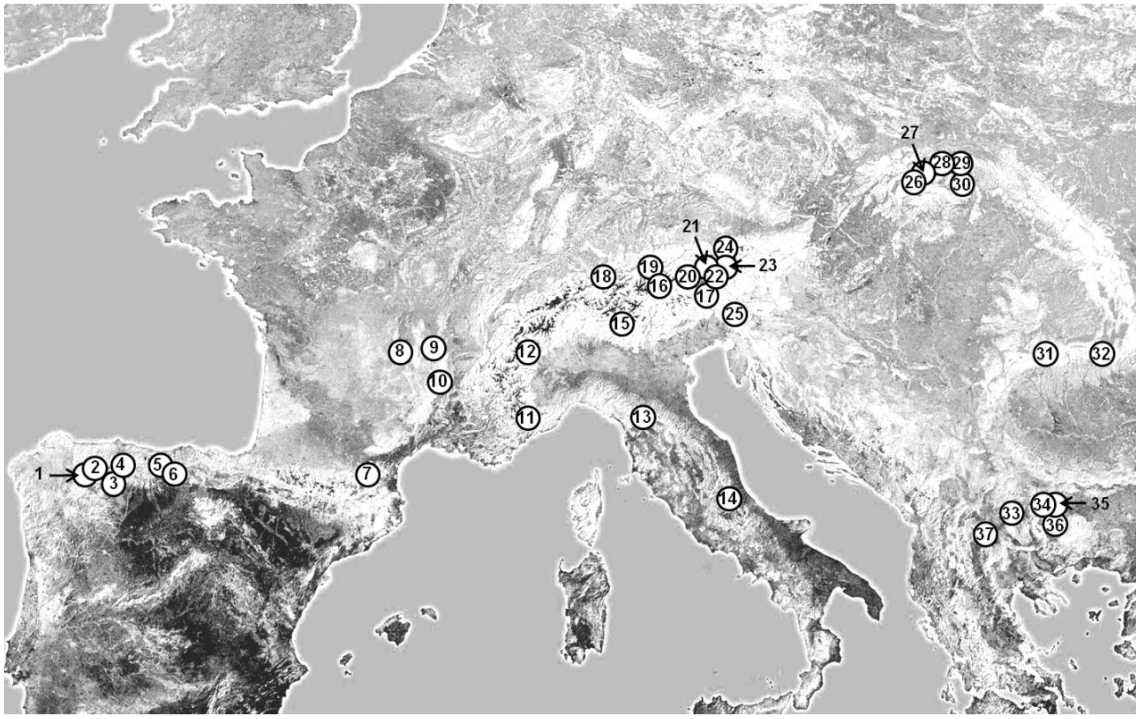

Fig. 5.1: Geographic distribution of the sample sites of the 37 populations of *Erebia euryale* analysed in this study. For further details see Table 5.1.

Table 5.1: Country code (CC), sampling locations, mountain ranges, sample sizes (*N*), altitude and coordinates of all *Erebia euryale* populations analysed in this study.

No.	CC	Location	Mountain range	Altitude	Coordinates	*N*
1	E	Leitariegos	Cantabrian Mountains	1600	N 42°59' W 06°24'	40
2	E	Somiedo	Cantabrian Mountains	1600	N 43°01' W 06°13'	35
3	E	Pajares	Cantabrian Mountains	1300	N 42°49' W 05°45'	36
4	E	San Isidro	Cantabrian Mountains	1500	N 43°04' W 05°25'	37
5	E	Pandetrave	Cantabrian Mountains	1200	N 43°06' W 04°32'	37
6	E	San Glorio	Cantabrian Mountains	1400	N 43°04' W 04°62'	33
7	F	La Glèbe	Pyrenees	1900	N 42°40' E 02°13'	40
8	F	Puy de Sancy	Massif Central	1800	N 45°32' E 02°49'	40
9	F	Col du Béal	Massif Central	1400	N 45°41' E 03°47'	40
10	F	Mount Mézenc	Massif Central	1400	N 44°54' E 04°12'	38
11	F	Val d'Isère	Alps	1900	N 45°27' E 06°59'	40
12	F	St Martin Vésubie	Alps	1400	N 44°04' E 07°15'	34
13	I	Cutigliano	Apennines	1400	N 44°06' E 10°45'	40
14	I	Prati di Tivo	Apennines	1500	N 42°30' E 13°33'	40
15	I	Passo di Croce Domini	Alps	1900	N 45°54' E 10°24'	39
16	I	Penser Joch	Alps	2000	N 46°48' E 26°16'	40
17	I	Plöckenpass	Alps	1400	N 46°36' E 12°57'	24
18	CH	Partnun	Alps	1800	N 46°59' E 09°51'	29
19	A	Sonnenstein	Alps	1900	N 47°08' E 11°22'	40
20	A	Kalkstein	Alps	1700	N 46°58' E 12°19'	36
21	A	Trauneralm	Alps	1500	N 47°07' E 12°48'	40
22	A	Schöneck	Alps	1800	N 47°03' E 12°48'	7
23	A	Obertauern	Alps	1800	N 47°15' E 13°34'	36
24	A	Ramsau am Dachstein	Alps	1300	N 47°25' E 13°39'	29
25	SLO	Šija	Julian Alps	1600	N 46°14' E 13°50'	11
26	SK	Kosodrevina	Tatra Mountains	1500	N 48°56' E 19°36'	40
27	SK	Demänovská Dolina	Tatra Mountains	1200	N 48°58' E 19°35'	24
28	SK	Rakuska Pol'ana	Belianske Tatry	1300	N 49°11' E 20°15'	40
29	SK	Pusté Pole	Tatra Mountains	750	N 49°12' E 20°54'	40
30	SK	Kojsovska holá	Tatra Mountains	1600	N 48°47' E 20°59'	40
31	RO	Groapa Seacă	Carpathians	1500	N 45°24' E 23°34'	40
32	RO	Sinaia	Carpathians	1500	N 45°21' E 25°31'	40
33	BG	Ruen	Osogovo	2100	N 42°09' E 22°31'	40
34	BG	Rila Monastry	Rila	2000	N 42°08' E 23°20'	39
35	BG	Granchar	Rila	2200	N 42°07' E 23°35'	14
36	BG	Bezbog	Pirin	2300	N 41°44' E 23°31'	40
37	MK	Solunska Glava	Jakupica	2000	N 41°42' E 21°26'	40

5.4 Results – *Erebia euryale*

All 15 loci analysed were polymorphic. The numbers of alleles per locus varied from three (PEP_{LGG}, FUM, GAPDH) to 13 (PGI) with an average of 5.64 (± 2.61). No general linkage disequilibrium was observed for any locus (all $p > 0.05$).

We calculated several population genetic parameters, based on allele frequencies. The mean number of alleles per locus (A) ranged from 1.53 to 2.93, with a mean of 1.99 (± 0.35) (see Fig. 5.2). Allelic Richness for seven individuals (A_{R7}) ranged from 1.19 to 1.72 with a mean of 1.51 (± 0.11) and for 30 individuals (A_{R30}) from 1.46 to 2.66 with a mean of 1.93 (± 0.31). The percentage of polymorphic loci with the most common allele not exceeding 95 % (P_{95}) ranged from 13 % to 67 % with a mean of 38 % (± 11); the total percentage of polymorphic loci (P_{tot}) ranged from 33 % to 67 %, mean 59 % (± 14). The mean expected heterozygosity (H_E) was 12.6 % (± 2.8) ranging from 4.2 % to 17.0 %, and the mean of the observed heterozygosity (H_O) was 11.7 % (± 3.0) varying from 4.0 % to 17.8 %. Details for all populations are shown in Table 5.2.

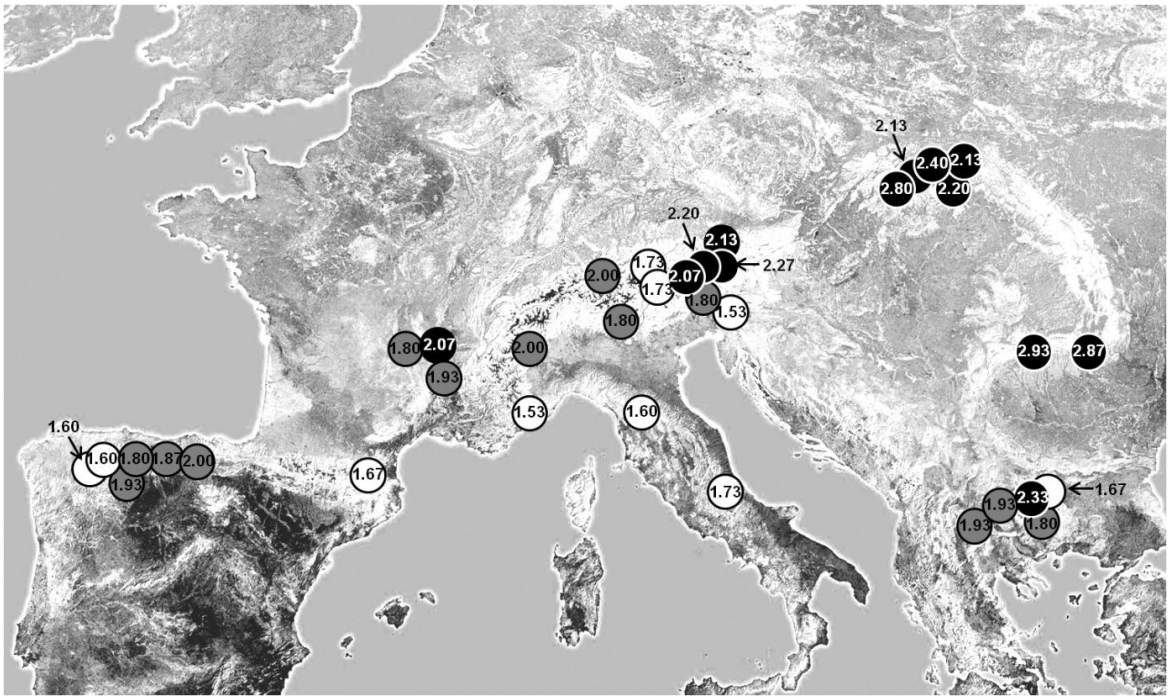

Fig. 5.2: Distribution of the mean number of alleles per locus (A) of *Erebia euryale*. Black circles: A > 2.0; grey circles: A ranging from 1.8 to 2.0; white circles: A < 1.8. For detailed information see Table 5.2. The population A-Schöneck was excluded due to insufficient sample size.

Table 5.2: Parameters of genetic diversity averaged over 15 allozyme loci of *Erebia euryale* analysed: country code (CC), number of individuals analysed (*N*), expected (H_E) and observed heterozygosity (H_O), total number of alleles (*A*), allelic richness for seven (A_{R7}) and 30 individuals (A_{R30}), total percentage of polymorphic loci (P_{tot}), percentage with the most common allele not exceeding 95 % (P_{95}), inbreeding coefficient (F_{IS}), deviations from Hardy–Weinberg equilibrium (HWE), number of private alleles (PA).

CC	Location	*N*	H_E [%]	H_O [%]	*A*	A_{R7}	A_{R30}	P_{tot} [%]	P_{95} [%]	F_{IS}	HWE	PA
E	Leitariegos	39.8	11.3	9.0	1.60	1.33	1.98	46.7	26.7	0.203	NS	-
E	Somiedo	34.6	11.9	10.7	1.60	1.40	1.89	46.7	40.0	0.097	NS	-
E	Pajares	36.0	15.8	16.1	1.93	1.53	1.76	60.0	33.3	-0.017	NS	-
E	San Isidro	37.0	12.3	12.2	1.80	1.48	1.58	53.3	46.7	0.016	NS	-
E	Pandetrave	36.8	15.9	17.8	1.87	1.57	1.53	46.7	40.0	-0.122	*	-
E	San Glorio	32.7	13.2	12.7	2.00	1.53	1.85	46.7	33.3	0.042	NS	1 (MDH2)
F	La Glèbe	39.9	14.7	12.4	1.67	1.48	1.65	46.7	40.0	0.160	*	-
F	Puy de Sancy	38.7	14.4	15.0	1.80	1.44	1.73	60.0	40.0	-0.056	*	-
F	Col du Béal	39.6	15.3	12.1	2.07	1.59	2.01	73.3	60.0	0.211	NS	-
F	Mount Mézenc	37.0	13.1	9.2	1.93	1.50	1.87	66.7	40.0	0.302	***	-
F	Val d'Isère	39.7	4.2	4.0	1.53	1.18	1.46	40.0	13.3	0.050	NS	1 (IDH2)
F	St Martin Vésubie	33.8	11.3	10.5	2.00	1.50	1.95	66.7	33.3	0.071	NS	-
I	Cutigliano	40.0	7.2	6.2	1.60	1.28	1.55	46.7	26.7	0.144	NS	-
I	Prati di Tivo	40.0	12.9	12.3	1.73	1.42	1.67	60.0	40.0	0.046	NS	-
I	Passo di Croce Domini	37.5	13.2	9.9	1.80	1.45	1.73	40.0	33.3	0.254	**	-
I	Penser Joch	39.1	11.4	8.6	1.73	1.40	1.67	46.7	26.7	0.247	***	-
I	Plöckenpass	22.9	15.2	14.2	1.80	1.57	-	46.7	40.0	0.163	***	-
CH	Partnun	28.1	12.4	11.9	2.00	1.51	-	60.0	33.3	0.040	NS	-
A	Sonnenstein	39.1	6.3	6.7	1.73	1.30	1.64	40.0	13.3	-0.061	NS	-
A	Kalkstein	35.7	12.8	13.5	2.07	1.55	2.02	73.3	40.0	-0.052	NS	1 (IDH1)
A	Trauneralm	39.9	12.8	11.7	2.20	1.53	2.08	80.0	33.3	0.090	NS	1 (PGI)
A	Schöneck	7.0	16.4	17.1	(1.60)	1.60	-	(53.3)	(53.3)	-0.039	NS	-
A	Obertauern	36.0	14.6	15.6	2.27	1.60	2.19	66.7	33.3	-0.067	NS	1 (IDH2)
A	Ramsau	28.3	15.9	13.5	2.13	1.66	-	60.0	53.3	0.147	***	-
SLO	Šija	11.0	10.3	9.1	1.53	1.43	-	33.3	26.7	0.115	NS	-
SK	Kosodrevina	39.8	12.8	10.7	2.80	1.71	2.60	73.3	46.7	0.163	***	1 (PGI)
SK	Demänovská Dolina	23.5	12.4	10.9	2.13	1.58	2.13	60.0	40.0	0.123	**	-
SK	Rakuska Pol'ana	38.5	10.5	10.9	2.40	1.56	2.25	66.7	40.0	-0.037	NS	-
SK	Pusté Pole	39.2	8.9	9.6	2.13	1.48	2.02	66.7	40.0	-0.074	NS	1 (6-PGDH)
SK	Kojsovska holá	38.7	12.2	12.8	2.20	1.55	2.11	66.7	53.3	-0.052	NS	-
RO	Groapa Seacă	40.0	14.8	14.8	2.93	1.71	2.66	86.7	40.0	-0.005	NS	3 (IDH1, IDH2, PK)
RO	Sinaia	40.0	14.7	14.0	2.87	1.70	2.61	86.7	46.7	0.046	**	2 (GAPDH, IDH1)
BG	Ruen	39.6	11.9	9.1	1.93	1.51	1.89	66.7	46.7	0.239	***	-
BG	Rila Monastry	38.9	17.0	15.9	2.33	1.72	2.26	73.3	66.7	0.061	NS	-
BG	Granchar	14.0	14.9	11.0	1.67	1.50	1.67	46.7	33.3	0.265	**	-
BG	Bezbog	39.9	12.3	10.2	1.80	1.45	1.76	73.3	26.7	0.165	***	-
MK	Solunska Glava	39.5	10.2	9.3	1.93	1.49	1.87	46.7	26.7	0.089	NS	-
	Mean	**34.6**	**12.6**	**11.7**	**1.99**	**1.51**	**1.93**	**58.9**	**37.6**	**0.080**		
	SD	**8.4**	**2.8**	**3.0**	**0.35**	**0.12**	**0.31**	**13.8**	**11.1**	**0.111**		

Values based on an insufficient number of individuals are given in parentheses and excluded from the calculation of means.
NS = $p > 0.05$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

A NJ phenogram based on Nei's genetic distances showed relatively unresolved results, scarcely supported by bootstrapping (Fig. 5.3). However, five clusters have been identified: (i) a cluster containing the populations from the Western Alps (ii) the populations from the Western Balkans and the Southern Carpathians, (iii) populations from Spain (Picos de Europa) and the Pyrenees, (iv) the Massif Central and (v) and an Eastern Alps group, also containing the populations from the northern and central Apennines. The populations from the Slovakian Tatra showed an intermediate position between Massif Central, Balkans, and Eastern Alps. The remaining populations could not unambiguously be assigned to a specific genetic group.

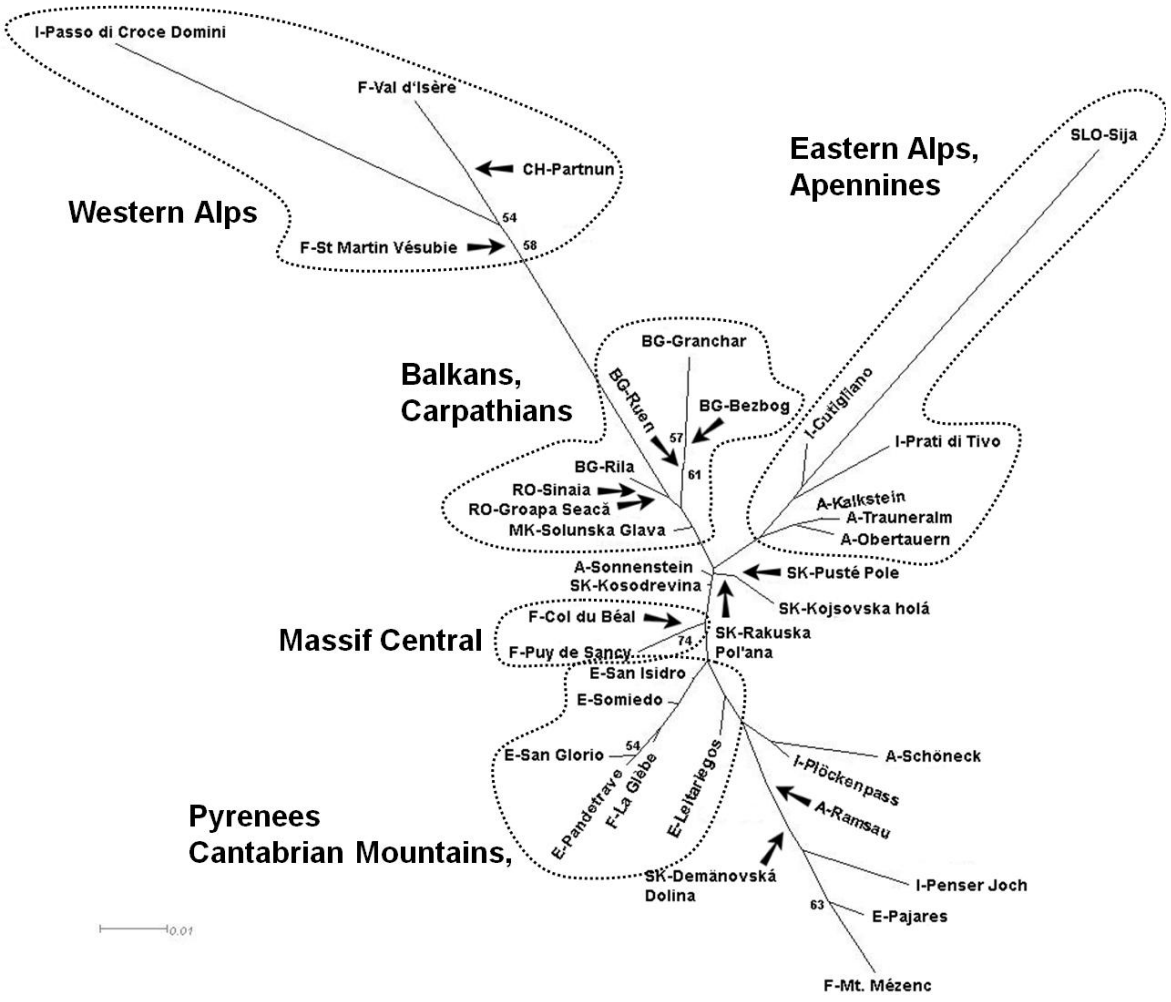

Fig. 5.3: Neighbour joining phenogram based on genetic distances (Nei 1972) of the 37 populations of *Erebia euryale*. Bootstrap values > 50% are given at the nodes. For more information see Table 5.1 and Fig. 5.1.

A better resolved genetic structure was revealed by the model-based Bayesian cluster analysis performed with STRUCTURE. The Evanno *et al.* (2005) method, implemented in the program STRUCTURE HARVESTER 0.6.93 (Earl & vonHoldt 2012), obtained the best resolution for $K = 5$. Although only five genetic lineages have been found, six genetic groups can be distinguished on that basis: (i) the Spanish populations from the Cantabrian Mountains and the population from the Pyrenees, (ii) the Massif Central, (iii) the Western Alps (two French populations, the Italian population from the Passo di Croce Domini and the Swiss population), (iv) the Eastern Alps (the remaining Italian populations, Austria and Slovenia) including the population from the Apennines, (v) the Balkans and the southern Carpathians and (vi) the Slovakian population from the Tatra mountains. The last group is mostly a mixture of different genetic lines present at the Balkan Peninsula and southern Carpathians as well as in the Eastern Alps (Fig. 5.4).

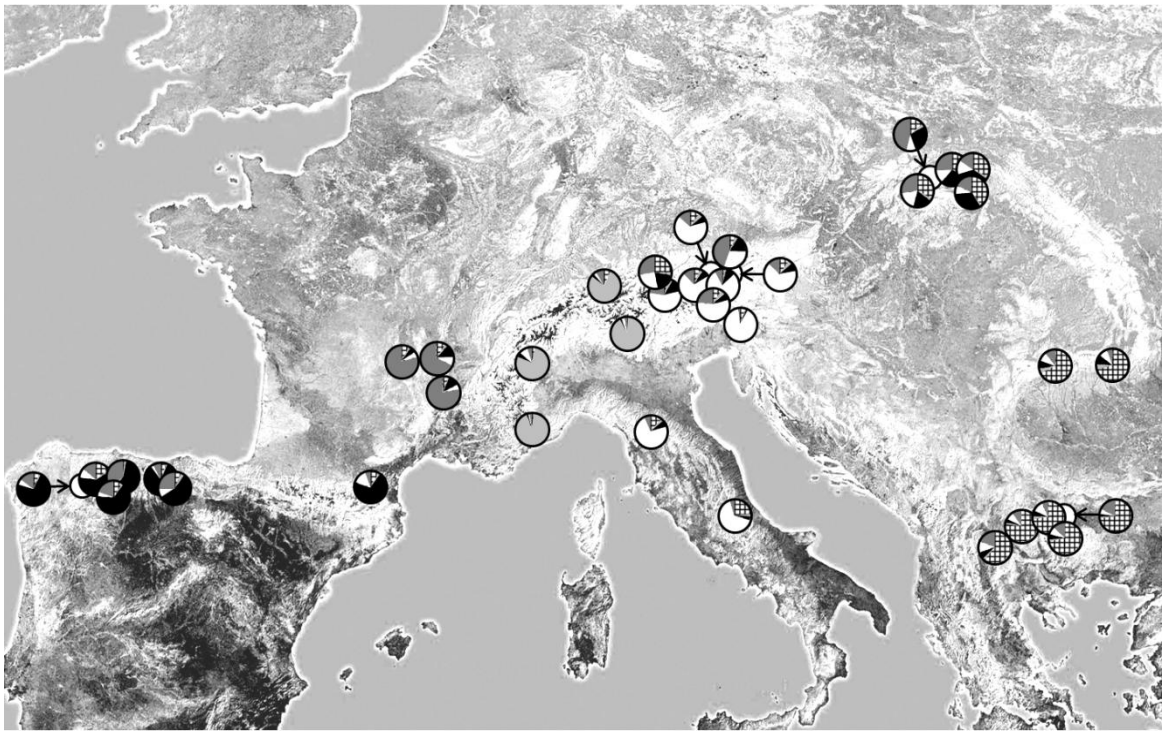

Fig. 5.4: Genetic clustering analyses of *Erebia euryale* genotypes. Different colours represent the proportional memberships of a population to the respective genetic clusters, averaged over individual membership proportions for $K = 5$ as calculated by the software STRUCTURE.

The unbiased genetic distances (Nei 1972) between all samples ranged from 0.002 to 0.250 with a mean of 0.055 (± 0.037). Regarding the pairwise mean genetic distances among the six STRUCTURE groups (five genetic lineages and the Tatra region as a potential zone of lineage intermixing), the Western Alps showed by far the highest values among all other groups; being most differentiated against the Cantabrian-Pyrenees group (0.107 ± 0.025), and least differentiated against the Tatra group (0.092 ± 0.039),

comparable to the genetic distance between the Tatra group and the populations from Southeastern Europe (Table 5.3).

Table 5.3: Mean genetic distances (Nei 1972) with their standard deviations among all pairs of the six genetic and geographic groups of *Erebia euryale*.

	Cantabria / Pyrenees	Massif Central	W Alps	E Alps	Tatra
Massif Central	0.034 ± 0.013				
W Alps	0.107 ± 0.025	0.105 ± 0.032			
E Alps	0.055 ± 0.026	0.055 ± 0.038	0.103 ± 0.040		
Tatra	0.029 ± 0.015	0.033 ± 0.026	0.092 ± 0.039	0.043 ± 0.027	
SE Europe	0.055 ± 0.018	0.052 ± 0.028	0.078 ± 0.042	0.057 ± 0.028	0.029 ± 0.017

Isolation by distance analysis based on Φ_{ST} values yielded a significant correlation ($p = 0.001$), but R^2 was 0.04 so that the explained variance was rather low (data not shown).

The genetic variance among all European populations was relatively high (locus-by-locus AMOVA results as a weighted average over 15 polymorphic loci; variance component = 0.309; $F_{ST} = 0.250$, $p < 0.001$). For means of comparison, the D_{est} value of 0.124 was also computed. The genetic variance within populations was moderate among individuals (variance component = 0.074, $F_{IS} = 0.079$, $p < 0.001$). However, the highest proportion of the genetic variance (i.e. 69.08%) was within the individuals (variance component = 0.856) (Table 5.3).

A hierarchical AMOVA testing the six STRUCTURE groups (five independent genetic lineages and the potential hybrid group found in the Slovakian Tatra Mountains) revealed 14.89% of the variation among groups, 11.99% among populations within groups and 73.12% within populations (Table 5.4).

Table 5.4: Locus-by-locus AMOVA (averaged over 15 loci) based on the total data set of the allozyme data for *Erebia euryale*. In the hierarchical AMOVA, populations were grouped according to the results given by the software STRUCTURE (five genetic lineages including the potential hybrid group in the Tatra).

Source of variation	SSD	CV	% total	*p*
Total (population level)				
Among populations	798.476	0.31031	**25.05**	<0.001
Within populations	2336.295	0.9286	**74.95**	
Total (individual level)				
Among populations	798.476	0.30922	**24.96**	<0.001
Among individuals within populations	1243.795	0.07389	**5.96**	<0.001
Within individuals	1092.5	0.8558	**69.08**	
Six groups (STRUCTURE)				
Among groups	453.313	0.18908	**14.89**	<0.001
Among populations within groups	345.163	0.15225	**11.99**	<0.001
Within populations	2336.295	0.9286	**73.12**	

SSD = sum of squares; CV = variance component estimates; % total = percentage of total variance; *p* = *p*-value.

Most of the pair-wise Φ_{ST} values were significant ($p < 0.05$). However, eight population pairs did not yield significant values: A-Kalkstein – A-Trauneralm, A-Schöneck – A-Trauneralm, BG-Bezbog – BG-Ruen, BG-Rila – RO-Groapa Seacă, BG-Rila – RO-Sinaia; RO-Groapa Seacă – RO-Sinaia, SK-Pusté Pole – SK-Demänovská Dolina and SK-Rakuska Pol'ana – SK-Demänovská Dolina. The maximum pair-wise Φ_{ST} reached 0.784 between the northern Apennines population I-Cutigliano and the population from Slovenia SLO-Šija ($p < 0.001$).

5.5 Discussion – *Erebia euryale*

Previous genetic studies on *Erebia euryale* already discussed the genetic structure from Northern Spanish populations (Vila *et al.* 2011), the Alpine region, the Pyrenees, the Southern Carpathians and partially from the Balkan region (Schmitt & Haubrich 2008), but based on a rather low number of populations analysed. This study therefore aims to test the previously formulated phylogeographic interpretations with an enlarged set of populations and to complete the scenario by revealing the origins of the populations in the Massif Central, the Apennines and the Northern Carpathians and their genetic relatedness to the populations already analysed in previous studies.

Our enlarged data set supported most of the results from these previous phylogeographic studies on *E. euryale*. (Schmitt & Haubrich 2008) found in their data the highest diversity values in the Southeastern European mountains. In our data set, we supplemented these populations by three populations from Bulgaria and one from Macedonia. Also in the enlarged data set, the Romanian populations had by far the highest values for example in the mean number of alleles per locus (Groapa Seacă: 2.93; Sinaia: 2.87) in combination with the highest number of private alleles (Groapa Seacă: 3; Sinaia: 2). The genetic differentiation among the Balkan and Carpathian populations based on the larger sample number was also as low as before (0.013 ± 0.008) supporting the postulated large continuous Würm ice age distribution all over a proposed extended forested area (or at least with forest-like structures) in Southeastern Europe (Schmitt & Haubrich 2008).

The Eastern Alpine populations are morphologically quite similar to the Southeastern European populations (Varga 1998), but showed remarkable genetic differentiation (Nei 1972) both in this study (0.057 ± 0.028) as well as in Schmitt & Haubrich (2008), now being better supported as we increased the number of populations from the Eastern Alps from four to nine, including one Slovenian population from the Julian Alps. Therefore, we now can strongly support the hypothesis of an important and spatially extended Würm glacial refugium at the unglaciated foothills of the Eastern and Southeastern Alps, which are also known as a survival area for several other species (e.g. Willis & van Andel 2004; Cheddadi *et al.* 2006; Mráz *et al.* 2007; Schmitt *et al.* 2010).

5.5.1 The Apennine Peninsula: unique refugium or postglacial expansion area?

The Apennine Peninsula is known as one of the three classic main glacial refugia for warm-adapted species in Southern Europe, besides Iberia and the Balkans (e.g. Taberlet *et al.* 1998; Hewitt 1999, 2000). However, glacial survival of more cold-tolerating species with mountain affinities were also reported, e.g. for *Picea abies* (Ravazzi *et al.* 2006; Vescovi *et al.* 2010) or the alpine leaf beetle *Oreina elongata* (Borer *et al.* 2010). On the contrary to these findings, our analyses indicated that the northern Apennines population (I-Cutigliano) and the central Apennines population (I-Prati di Tivo) do not represent a unique genetic lineage, but shared their heredity with the populations from the Eastern Alps (Fig. 5.4), a pattern supported by all analyses applied.

Furthermore, both Apennines populations showed low genetic diversity values (e.g. *A*: 1.60 in I-Cutigliano; 1.73 in I-Prati di Tivo) and no private alleles, which both argue against a glacial survival area in the Apennines region for *E. euryale*. Moreover, the mean number of alleles per locus was much higher in the Eastern Alps (e.g. A-Obertauern: 2.27; A-Traueneralm: 2.20). Decrease of genetic diversity from the Eastern Alps to the Apennines might indicate a postglacial expansion originating from a survival centre in the Eastern Alps southwards via the Tuscan Apennines to the central Apennines or even a continuous glacial distribution all over this region. However, the latter scenario is less likely due to unfavourable ecological conditions in the Po valley for *E. euryale* during glacial conditions (Schmitt 2007). The recent populations in the Apennines seem to have suffered from at least one bottleneck, either along the process of postglacial range expansion and / or *in situ* due to marginally suitable habitat conditions in the Apennines.

5.5.2 The Tatra region: glacial refugia or postglacial contact zone for different genetic lineages?

Regarding the results from STRUCTURE analysis, the populations from the Tatra Mountains represent a mixture of gene pools. These populations apparently combine the genetic information from the Eastern Alps and the Romanian Carpathians (Fig. 5.4). This intermediate position of the Tatra populations between Eastern Alps and Southern Carpathians was also supported by the results of the NJ analysis (Fig. 5.3).

The mean genetic diversity of the Tatra populations is remarkably high, as mirrored in the allele number per locus with all values being higher than 2.00 (e.g. 2.80 in the westernmost Slovakian population Kosodrevina) (Fig. 5.2, Table 5.2). High levels of genetic diversity in a particular area indicate either a contact zone between two genetic lineages during the postglacial recolonisation process (e.g. Bylebyl *et al.* 2008) or an important refuge area (Schmitt 2007). Especially the intermediate genetic position between the Eastern Alps and the Southeastern Europe lineage make a postglacial hybrid origin of the Tatra populations a likely scenario, in particular as Tatra Mountains are geographically located half-way between Eastern Alps and Southern Carpathians. This assumption is further supported e.g. by studies about arcto-alpine fungi which indicated the Tatra region as a contact zone between two postglacial immigrating lineages (Chlebicki 2002; Chlebicki & Suková 2004).

However, we still cannot exclude completely the other scenario of a centre of survival for *E. euryale* in the Tatra region during the last glaciation. This alternative hypothesis might be supported by the low genetic differentiation among all Slovakian populations (0.021 ± 0.019), which might evidence the existence of a large and continuous glacial survival centre. Some genetic studies on plant species already pointed out the Tatras as a glacial refugium, e.g. for *Polygonatum verticillatum* (Kramp *et al.* 2009), *Fagus sylvatica* (Magri *et al.* 2006) or *Pinus sylvestris* (Stewart & Lister 2001). *E. euryale* populates flowery places in pine and spruce forest clearings. The possible existence of some forests or forest-like structures in the Tatra region during the last glaciation therefore could be an indicator also for a glacial refugium for *E. euryale*, most likely at the foothills of the Tatra Mountains.

5.5.3 The Massif Central region as a genetically independent survival area

In various genetic studies, the Massif Central held different positions in terms of the distribution of genetic lineages and their relationships to the neighbouring high mountain ranges (Alps and Pyrenees) (Schmitt 2009). In our study, we found an endemic genetic lineage in the Massif Central for *E. euryale* (see results from the STRUCTURE analysis, Fig. 5.4). Similar results were obtained by Kerdelhué *et al.* (2006) for *Tomicus piniperda* or Kropf *et al.* (2012) for *Soldanella alpina*. However, other studies found genetic relationships to either the Pyrenees (e.g. Dixon *et al.* 2007 (*Androsace halleri*); Ronikier *et al.* 2008 (*Pulsatilla vernalis*)) or the Southwestern Alps (e.g. Kerdelhué *et al.* 2006 (*Thaumetopoea pityocampa*); Pauls *et al.* 2006 (*Drusus discolor*) or Kramp *et al.* 2009 (*Polygonatum verticillatum*)).

During the Last Glacial Maximum, the Massif Central region was marked by some smaller ice caps (Allen *et al.* 2008). Therefore, it should be expected that species today living in the Massif Central region at least suffered from range contraction and population decline, resulting in losses of genetic diversity. In contrast to this assumption, we observed genetic parameters with inter-mediate to high values (e.g. *A*: 1.80 – 2.07) for the Massif Central populations. High levels of genetic diversity were also found for some other species, for example *Lycaena helle* (Habel *et al.* 2010), *Meum athamanticum* (Huck *et al.* 2009) or *Saxifraga stellaris* (Kropf *et al.* 2008).

These results on genetic diversity and the independent genetic lineage are strong indicators for an important and strong glacial refugium of *E. euryale* in the Massif Central. This region was also postulated in other studies, e.g. for *Calluna vulgaris* (Rendell & Ennos 2002), *Drusus discolor* (Pauls *et al.* 2006) or *Bythinella* spp (Benke *et al.* 2009).

6 Moorland Clouded Yellow – *Colias palaeno*

6.1 Introduction – *Colias palaeno*

The biogeography of many animal and plant species has been intensively studied in Europe (reviews in Hewitt 2004; Schmitt 2007). Especially the group of the Mediterranean species is already well understood (review in Schmitt 2007) but also typical high mountain species (reviews in Schönswetter & Tribsch 2005; Schmitt 2009) and continental species (reviews in Stewart & Lister 2001; Stewart *et al.* 2010; Schmitt & Varga 2012) are relatively well studied. However, the phylogeographic knowledge on species associated with cold habitats, such as oligotrophic bogs, often performing a boreo-montane distribution, is still rather limited, and our knowledge is mostly restricted to ecological analyses (e.g. Pax 1916; Krogerus 1960; Coulson & Butterfield 1985; Urák & Samu 2008) and some studies about local and regional genetic structures (e.g. Stenøien & Såstad 1999; Bernard & Schmitt 2010; Buczkowska *et al.* 2012; Rasic & Keyghobadi 2012). Consequently, phylogeographic studies of the European bog fauna species are still scarce (Nève 1994; Habel *et al.* 2010; Rees *et al.* 2010; Bernard *et al.* 2011).

Nevertheless, bogs with their flora and fauna are of particular biogeographical interest because most of them were already established in the late Pleistocene or the early Holocene and have continuously existed without major alterations since then (Spitzer & Danks 2006). Therefore, peat bogs are highly unique but vulnerable habitats for many protected terrestrial and (semi-)aquatic animal and plant species (Barber 1993; Barkham 1993). Additionally, they play an important global role in the carbon cycle e.g. by exchanging CO_2 with the atmosphere, producing dissolved organic carbon or storage of carbon (Moore *et al.* 1998). However, bogs suffered from massive peat extractions during the last centuries (Stoneman & Brooks 1997; Howie 2002); more recently, many bogs were lost to agriculture, urbanization and forestry (Wheeler & Proctor 2000; Stephens *et al.* 2011). In Europe, this destruction has slowed down over the last few decades, but these habitats are still highly endangered e.g. by increasing air eutrophication causing additional degradation (Spitzer & Danks 2006).

However, it is not only bogs but also their inhabitants, which are highly endangered. They suffer both, from decreasing habitats and the subsequent loss of food and shelter as well as from climate change. Due to this vulnerable situation of bogs and other wetland habitats, they became the main focus of the international Ramsar Convention (www.ramsar.org). Therefore, it is highly important to study bog species in more detail for

a better understanding of their distribution dynamics in order to develop well adapted conservation management strategies.

Here, we study the population genetic structure of the endangered Moorland Clouded Yellow, *Colias palaeno* (L., 1761), a character species of well preserved and interconnected bog habitat complexes (Bolotov 2004; Spitzer & Danks 2006). Due to habitat loss for the larvae and imagos, *C. palaeno* is highly endangered in most parts of Europe (van Swaay & Warren 1999). We analysed the genetic constitution (allozymes and mtDNA) of 523 individuals from 21 populations scattered over most of the species' European distribution, from the Alps to northern Scandinavia. To also address the more regional genetic structures, we studied in more detail the populations in the Czech Republic where, until 1994, the species has lost more than 40 % of its populations existing prior to 1950. However, this loss of the populations was not equally distributed over the country (Pavličko 2002). For all these reasons, the establishment of a conservation genetic concept is necessary. Therefore, we analysed the genetic structures at the European level including the search for Evolutionary Significant Units (ESUs) and the genetic structures at a more regional scale. Thus, we intend to answer the following questions:

(i) Is *C. palaeno* differentiated into several genetic lineages (i.e. ESUs) within Europe? If so, how are these lineages geographically distributed?

(ii) Did the species survive the last ice age in a single large retreat or did multiple but geographically less extended refugia exist with independent postglacial expansions?

(iii) Is habitat availability reflected in the local and regional genetic make-up of populations and the degree of differentiation among them?

(iv) What conservation implications can be derived for *C. palaeno* from our results?

6.2 Study species – *Colias palaeno*

The Moorland Clouded Yellow *Colias palaeno* has a Holarctic distribution, reaching from the French Jura and the Alps in the West over parts of Central, Northern and Eastern Europe, Siberia to Korea, Japan and North America (Huemer 2004). At its southern distribution border, the species has a montane-subalpine distribution with a vertical range from about 900 – 2500 m asl., but mostly found from 1800 to 2200 m asl. (Huemer 2004), whereas it is a lowland species in Northern Europe (Henriksen & Kreutzer 1982).

C. palaeno is a characteristic species of bogs and comparable wetlands in the montane regions (Erhardt 1985), but can also be found in dryer sites with *Juniperus communis,* mostly in higher regions of the Central Alps. Moderately disturbed (i.e. eutrophic and / or drying) bog sites offer especially favourable conditions, if enough sun-exposed bog bilberry shrubs (*Vaccinium uliginosum*) are present and if, at the same time, the supply of nectar plants is sufficient for the imagos. In case of further evolution of such areas into heath or pioneer stand sites, due to further succession or draining (e.g., as a consequence of peat extraction), *C. palaeno* will rapidly disappear (Settele *et al.* 2009). Conservation actions can include e.g. in the restoration of former peat bogs. Extensive mowing at the edges of the marsh areas can have a positive effect as it increases the variety of nectar plants nearby the habitats of the larvae (Rüetschi & Scholl 1985; Ebert & Rennwald 1991).

The caterpillar is green and has a sulfur-yellow lateral line, which is only interrupted by white stigmas, but the young caterpillar turns brown before winter. The species is monophagous and larvae only feed on *V. uliginosum.* The eggs are laid singly on sun-exposed leafs of small and marginal shrubs (Lepidopterologen-Arbeitsgruppe 1994; Hermann 1998). The caterpillars hibernate at the food plant as their third instar larvae. After sufficient food intake in spring, the caterpillar transforms into a pupa, which is hanging on the pedicles of the host plant. The pupal stage lasts for one to three weeks. Depending on spring temperatures, the flight period starts at the beginning of June and lasts until the beginning of August. Adults may visit nectaring sites such as flower rich meadows, rarely straying too far from larval host plant sites (Ebert & Rennwald 1991).

6.3 Sampling – *Colias palaeno*

We sampled 523 individuals of *Colias palaeno* from 21 populations in Central and Northern Europe (Austria, Czech Republic, Estonia, Finland, Lithuania, Latvia, Poland and Sweden; Table 6.1, Fig. 6.1). In the Czech Republic, the sampling was quite detailed (12 sites, 258 individuals), representing a majority of larger population in three separate mountain ranges. In Baltic and Nordic countries, much less detailed sampling formed an approximate South-North transect.

Individuals were frozen immediately after capture in liquid nitrogen and were stored accordingly until electrophoresis and mtDNA analyses. For allozyme electrophoresis, all of the individuals were analysed, whereas only 213 individuals from 11 populations were used for mtDNA sequencing.

Table 6.1: Sampling locations (with abbreviations and coordinates), region, sample sizes (*N*), and altitude of all *Colias palaeno* populations analysed with allozyme electrophoresis. Populations marked with * are also used for mtDNA sequencing.

Pop ID	Location	Region	N_{allo}	N_{mt}	Longitude	Latitude	Altitude (m asl)
A*	Obergurgl	Alps	23	21	11°00' E	46°51' N	1900
CZ-Kr1*	Pernink	Krušné hory - Ore Mountains	26	19	12°47' E	50°22' N	880
CZ-Kr2	Přebuz	Krušné hory - Ore Mountains	15	-	12°36' E	50°22' N	890
CZ-Kr3	Boží dar	Krušné hory - Ore Mountains	19	-	12°54' E	50°24' N	1100
CZ-Sl*	Krásno	Slavkovský les - Kaiserwald	15	9	12°45' E	50°06' N	780
CZ-Su1	Borková	Šumava - Bohemian Forest	14	-	14°03' E	48°41' N	730
CZ-Su2	Jezerní slat'	Šumava - Bohemian Forest	11	-	13°34' E	49°02' N	1100
CZ-Su3	Kapličky	Šumava - Bohemian Forest	20	-	14°13' E	48°35' N	920
CZ-Su4	Mrtvý luh	Šumava - Bohemian Forest	25	-	13°53' E	48°51' N	740
CZ-Su5	Záhvozdí	Šumava - Bohemian Forest	29	-	13°56' E	48°50' N	740
CZ-Su6*	Pasecká slat'	Šumava - Bohemian Forest	34	24	13°39' E	49°02' N	940
CZ-Su7	Spálený luh	Šumava - Bohemian Forest	19	-	13°47' E	48°50' N	800
CZ-Su8	Stráženská slat'	Šumava - Bohemian Forest	31	-	13°44' E	48°53' N	820
EST*	Puhja	Lake Võrtsjärv	34	24	26°10' E	58°20' N	40
FIN1*	Svenskby	Gretarbyviken	22	19	23°17' E	60°07' N	40
FIN2*	Joensuu	Mustapyörre	39	22	29°55' E	62°37' N	100
FIN3	Oulu	Humalalampi	27	-	25°47' E	65°08' N	50
LT*	Baliskes	Marijampolė	42	21	23°36' E	54°47' N	80
LV*	Rubeni	Jūrmala	34	17	23°44' E	56°52' N	20
PL*	Lewsze	Białystok County	15	15	23°41' E	53°00' N	140
S*	Arvidsjaur	Norrland	29	22	19°55' E	66°01' N	200

N_{allo} = number of individuals used for allozyme electrophoresis
N_{mt} = number of individuals used for mtDNA sequencing

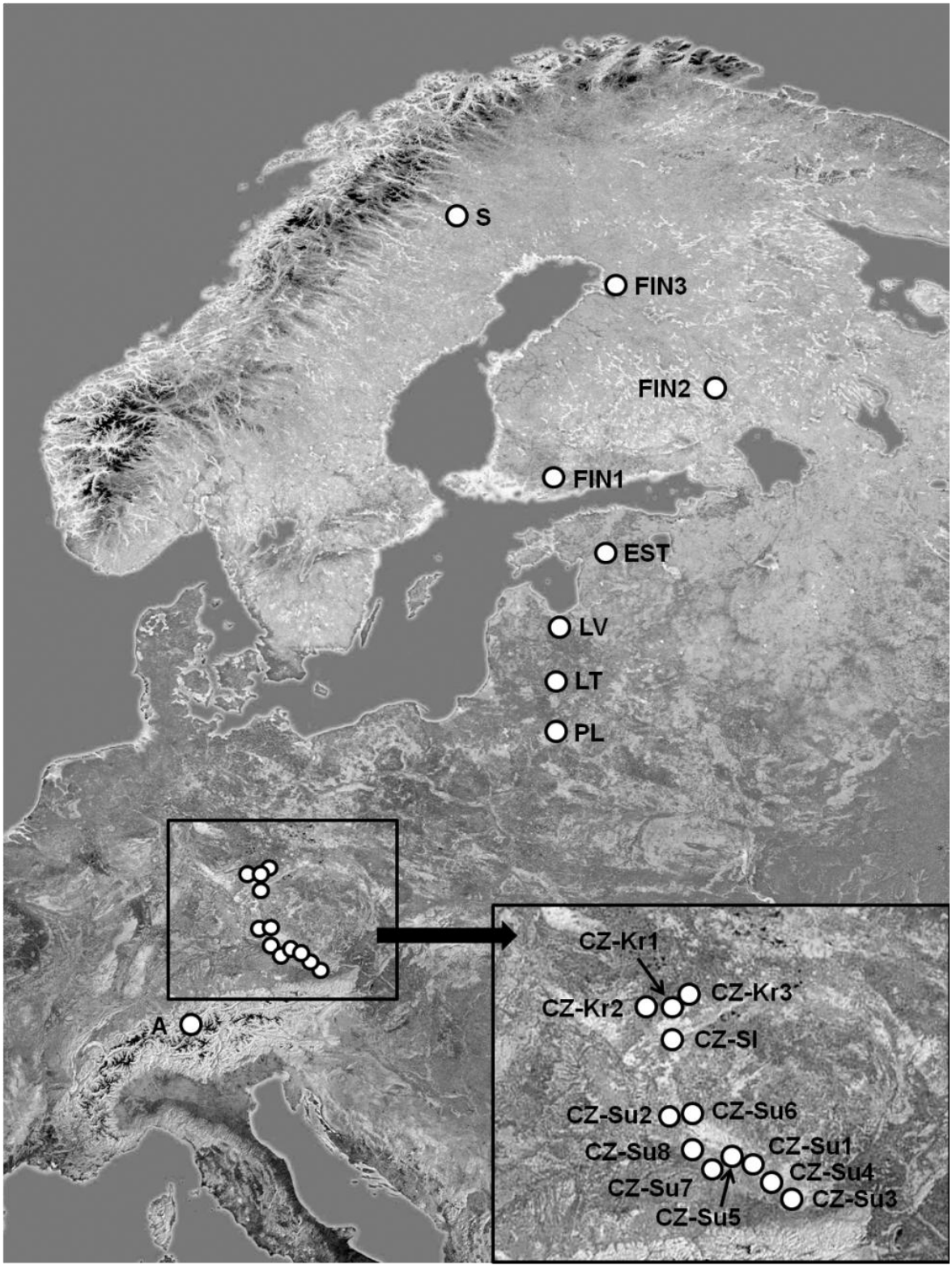

Fig. 6.1: Geographic distribution of the 21 studied populations of *Colias palaeno*. For abbreviations and further details see Table 6.1.

6.4 Results – *Colias palaeno*

6.4.1 Allozymes

18 of the 20 analysed loci were polymorphic. Only GAPDH and HBDH were monomorphic for all individuals. The numbers of alleles per polymorphic locus varied from two to 12 (PGI) with an average of 3.85 (± 2.04). No general linkage disequilibrium was observed for any locus (all $p > 0.05$).

We calculated several population genetic parameters, based on allele frequencies. The mean number of alleles per locus (A) ranged from 1.70 to 2.45, with a mean of 2.23 (± 0.16 SD). Allelic Richness for 11 individuals (A_{R11}) ranged from 1.56 to 1.78 with a mean of 1.65 (± 0.05 SD) and for 20 individuals (A_{R20}) from 1.92 to 2.34 with a mean of 2.13 (± 0.11 SD). The (P_{95}) ranged from 44 % to 70% with a mean of 54 % (± 6 SD); the total percentage of polymorphic loci (P_{tot}) ranged from 50 % to 80 %, mean 64 % (± 8 SD). The mean expected heterozygosity (H_E) was 19.3 % (± 2.2 SD) ranging from 16.3 % to 25.4 %, and the mean of the observed heterozygosity (H_O) was 15.9 % (± 1.9 SD) varying from 11.4 % to 19.5 %. Details for all populations are given in Table 6.2.

Table 6.2: Parameters of genetic diversity of allozymes averaged over loci for all 21 *Colias palaeno* populations analysed: number of individuals analysed (*N*), expected (H_E) and observed heterozygosity (H_O), total number of alleles (*A*), allelic richness (A_R) based on eleven and 20 individuals, total percentage of polymorphic loci (P_{tot}), percentage with the most common allele not exceeding 95% (P_{95}), inbreeding coefficient (F_{IS}), deviations from Hardy–Weinberg equilibrium (HWE) and number of private alleles (PA). For *A*, populations with less than 20 individuals analysed (given in parenthesis) are excluded from the calculation of mean due to insufficient sample size.

Samples	***N***	H_E **(%)**	H_O **(%)**	***A***	A_{R11}	A_{R20}	P_{tot} **(%)**	P_{95} **(%)**	F_{IS}	**HWE**	**PA**
A	23.0	21.0	15.7	1.95	1.69	1.94	55.0	50.0	0.244	***	0
CZ-Kr1	25.9	19.7	15.8	2.10	1.62	2.03	55.0	50.0	0.198	*	1 (PK)
CZ-Kr2	15.0	20.1	15.0	(1.70)	1.60	-	55.0	55.0	0.253	**	0
CZ-Kr3	18.9	21.3	15.4	(1.85)	1.66	-	60.0	60.0	0.280	***	0
CZ-Sl	15.0	19.0	17.0	(2.00)	1.63	-	65.0	55.0	0.098	NS	0
CZ-Su1	13.1	17.6	17.8	(1.70)	1.56	-	55.0	50.0	-0.003	NS	1 (MDH1)
CZ-Su2	11.0	19.1	15.9	(1.95)	1.63	-	65.0	50.0	0.189	**	0
CZ-Su3	19.5	22.7	17.8	2.10	1.72	2.10	65.0	65.0	0.217	***	1 (MDH2)
CZ-Su4	22.4	25.4	18.7	2.15	1.74	2.20	80.0	70.0	0.047	NS	0
CZ-Su5	29.0	19.8	16.4	2.00	1.62	1.92	70.0	55.0	0.173	**	1 (GOT1)
CZ-Su6	34.0	17.7	15.2	2.25	1.62	2.07	75.0	55.0	0.140	**	0
CZ-Su7	18.9	20.1	15.8	(1.95)	1.64	-	60.0	55.0	0.228	**	1 (G-6-PDH)
CZ-Su8	31.0	18.9	15.6	2.20	1.64	2.06	70.0	55.0	0.171	***	2 (MPI, PGI)
EST	34.0	16.3	13.5	2.30	1.61	2.12	60.0	45.0	0.170	***	1 (PK)
FIN1	22.0	17.0	11.4	2.25	1.61	2.22	65.0	50.0	0.309	***	0
FIN2	38.3	19.4	17.2	2.45	1.66	2.22	75.0	65.0	0.118	***	0
FIN3	26.9	22.1	19.5	2.45	1.78	2.34	60.0	55.0	0.118	**	0
LT	41.5	17.2	13.6	2.45	1.66	2.23	70.0	50.0	0.204	***	2 (IDH2, PGI)
LV	30.8	17.5	13.2	2.35	1.69	2.22	50.0	50.0	0.248	***	1 (PGI)
PL	15.0	16.7	17.4	(1.83)	1.61	-	61.1	44.0	-0.031	NS	1 (FUM)
S	28.9	17.5	15.3	2.25	1.63	2.12	70.0	55.0	0.123	**	1 (GOT2)
Mean	**24.5**	**19.3**	**15.9**	**2.23**	**1.65**	**2.13**	**63.9**	**54.3**	**0.166**		
SD	**8.4**	**2.2**	**1.9**	**0.16**	**0.05**	**0.11**	**7.7**	**6.3**	**0.086**		

NS = $p > 0.05$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

Little genetic structure was revealed by the model-based Bayesian cluster analysis performed with the software STRUCTURE. The Evanno *et al.* (2005) method obtained the best resolution for $K = 2$ (Fig. 6.2). Regarding the proportion of membership of each pre-defined population to a cluster, a clear East-West subdivision is apparent. Cluster one contains all populations from Austria and the Czech Republic and cluster two includes the more eastern and northern populations from the Baltic (EST, LT, LV) and from Fennoscandia (S, FIN1, 2, 3). Having a more detailed look to each of the two clusters, four subgroups can be distinguished: (i) more than 80 % of the individuals belong to cluster one (the Austrian population and the Slavkovský les population), (ii) 50 % to 80 %

of the individuals belong to cluster one (all other populations from the Czech Republic), (iii) more than 50 % of the individuals belong to cluster two (all Scandinavian and Baltic populations) and (iv) more than 80 % of the individuals belong to cluster two (the Polish population).

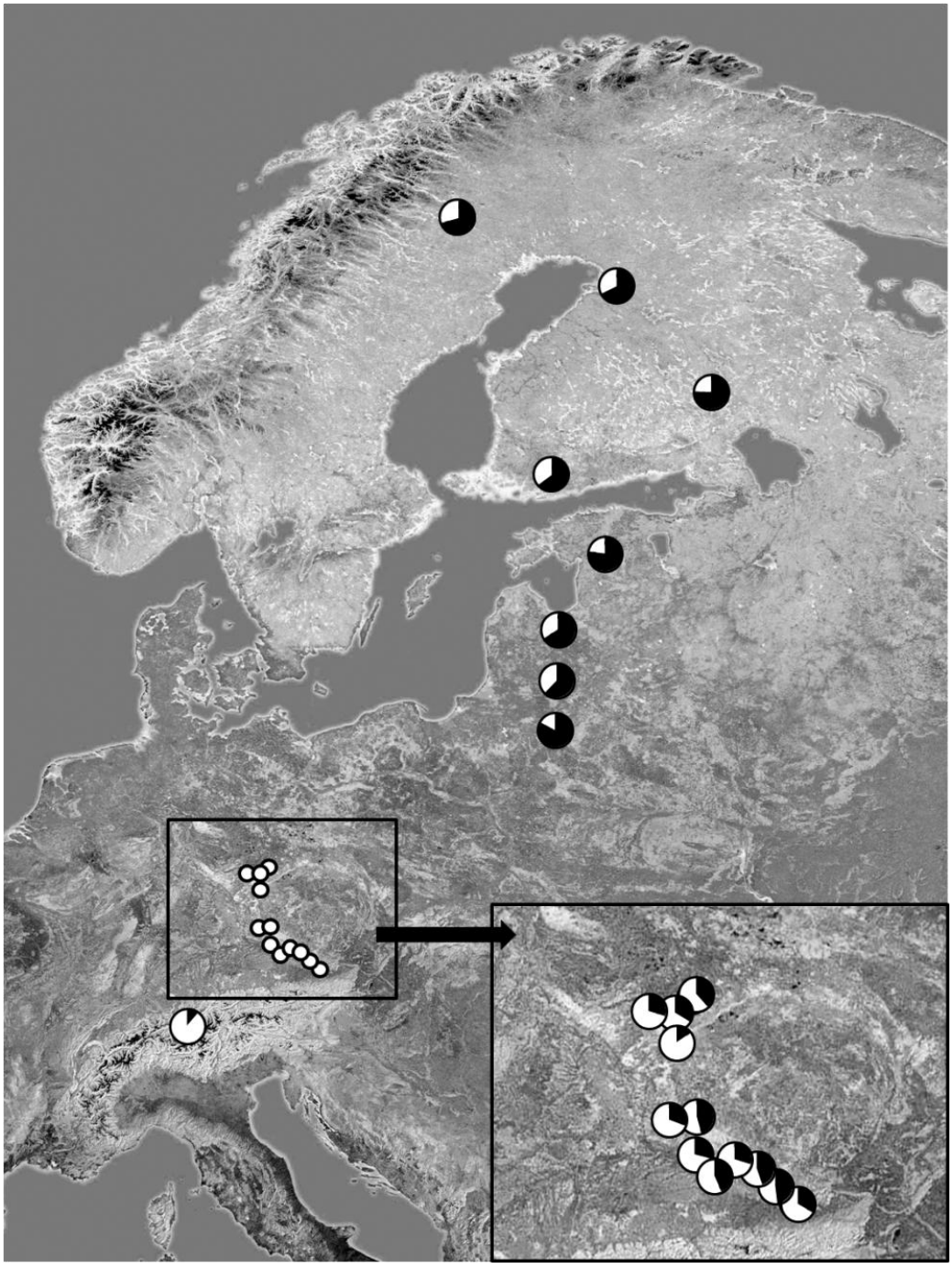

Fig. 6.2: Genetic clustering analyses of *Colias palaeno* genotypes as calculated by STRUCTURE. Black and white represent the proportional memberships of a population to the two respective genetic clusters, averaged over individual membership proportions for *K* = 2.

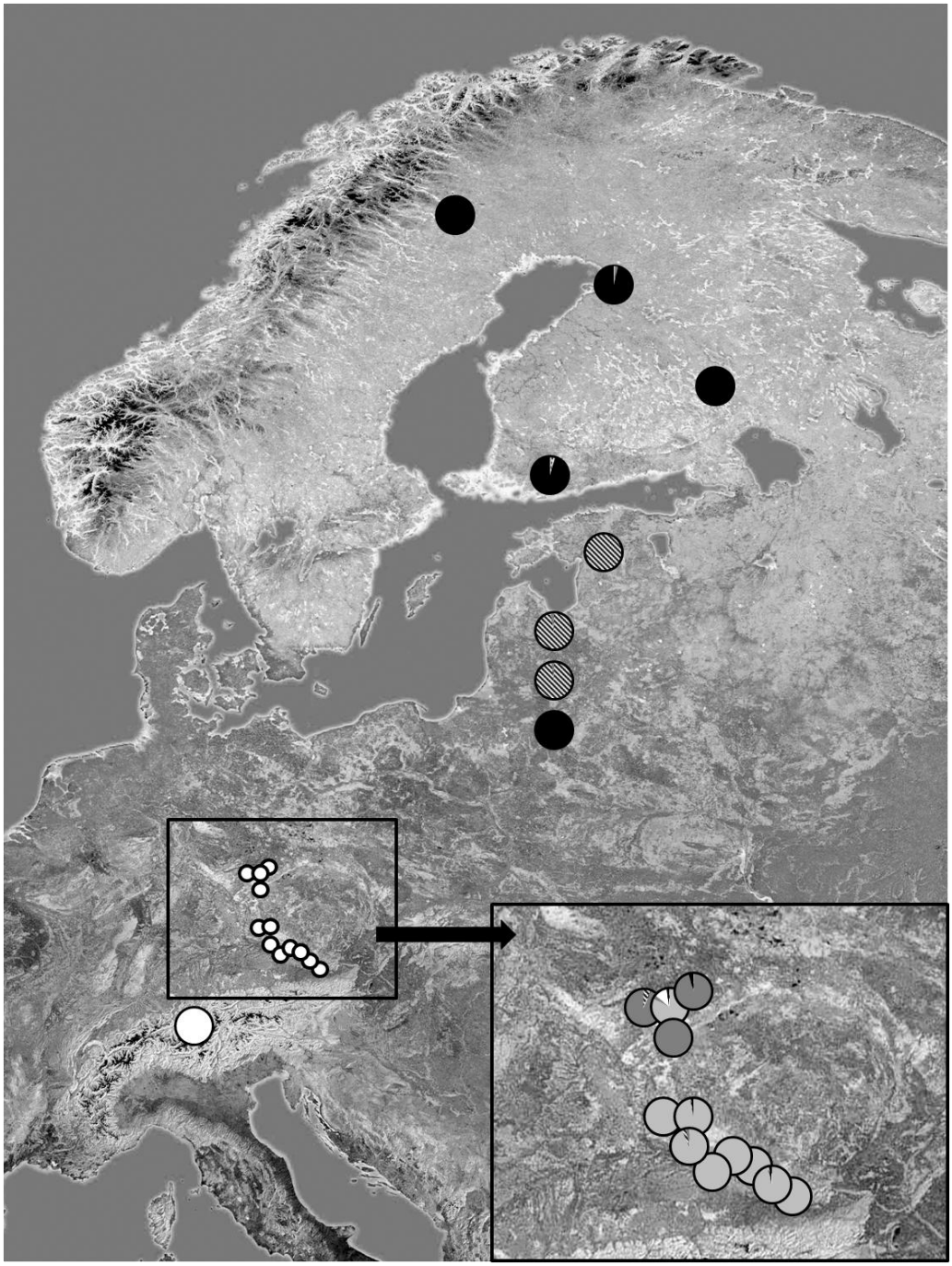

Fig. 6.3: Genetic clustering analyses of *Colias palaeno* genotypes as calculated by BAPS. Different colours represent the proportional memberships of a population to the respective genetic clusters, averaged over individual membership proportions for $K = 5$.

Bayesian clustering analysis using the software BAPS (Fig. 6.3) had a remarkable correlation with the results of the Neighbour Joining analysis (Fig. 6.4). Here, the highest posterior probabilities (0.9999) were obtained for $K = 5$. One cluster is composed of only one population, the population from the Austrian Alps (A). The second cluster included all populations from the Czech Republic except the population from Slavkovský les (CZ-Sl) and two populations from the Ore Mountains (CZ-Kr2,3), which built a third group. The

fourth group contained the Baltic populations (EST, LT, and LV). The populations from Poland (PL) and Fennoscandia (S, FIN1, 2, 3) formed the last group.

The genetic variance among all European populations was moderate (locus-by-locus AMOVA results as a weighted average over 18 polymorphic loci: variance component = 0.193; F_{ST} = 0.092, p < 0.001). The D_{est} value amounted 0.035. The genetic variance among individuals within populations was higher than among populations (variance component = 0.322, F_{IS} = 0.169, p < 0.001). However, the highest proportion of the genetic variance (i.e. 75.49 %) was within individuals (variance component = 1.586) (Table 6.3). A hierarchical AMOVA tested the two STRUCTURE groups and the five BAPS groups. For the two STRUCTURE groups, the variation among groups was 4.45 %, 7.42 % among populations within groups and 88.13 % within populations. The variation among the five BAPS groups was 7.42 % among groups, 4.14 % among populations within groups and 88.44 % within populations.

Table 6.3: Locus-by-locus AMOVA (averaged over 18 polymorphic loci) based on the total data set of the allozyme data for *Colias palaeno*. In the hierarchical AMOVA, populations were grouped according to the best obtained K identified by the software STRUCTURE (K = 2) and for K = 5 as calculated by the software BAPS.

Source of variation	SSD	CV	% total	*p*
Total (population level)				
Among populations	232.5	0.200	9.51	<0.001
Within populations	1907.0	1.901	90.49	
Total (individual level)				
Among populations	232.5	0.193	9.19	<0.001
Among individuals within populations	962.0	0.322	15.32	<0.001
Within individuals	737.5	1.586	75.49	
two groups (STRUCTURE)				
Among groups	53.1	0.084	4.45	<0.001
Among populations within groups	162.1	0.140	7.42	<0.001
Within populations	1699.5	1.668	88.13	
five groups (BAPS)				
Among groups	127.6	0.140	7.42	<0.001
Among populations within groups	87.6	0.078	4.14	<0.001
Within populations	1699.5	1.668	88.44	

SSD = sum of squares; CV = variance component estimates; % total = percentage of total variance; p = p-value.

Most of the pair-wise Φ_{ST} values were significant (p < 0.05). However, four population pairs did not yield significant values: CZ-Su5 – CZ-Su6, CZ-Su5 – CZ-Su8, CZ-Kr2 – CZ-Kr3 and FIN2 – FIN3. The maximum pair-wise Φ_{ST} reached 0.447 between the Slavkovský les population CZ-Sl and the population from Poland (p = 0.001). The unbiased genetic distances (Nei 1972) between all 21 populations ranged from 0.006 to

0.124 with a mean of 0.034 (± 0.023). Isolation by distance analysis yielded no significant correlation (R = 0.03; *p* = 0.087) (data not shown).

A NJ phenogram of the 21 populations (Fig. 6.4) based on Nei's genetic distances showed relatively unresolved results, scarcely supported by bootstrapping. Nevertheless, five geographic clusters, showing striking similarity with the BAPS analysis, can be identified: (i) the Austrian Alps (A) (ii) the Ore Mountains and the Slavkovský les, (iii) Šumava, (iv) the Baltic populations (EST, LV, LT) and (v) a northern group (S, FIN1, 2, 3), together with the population from Poland (PL).

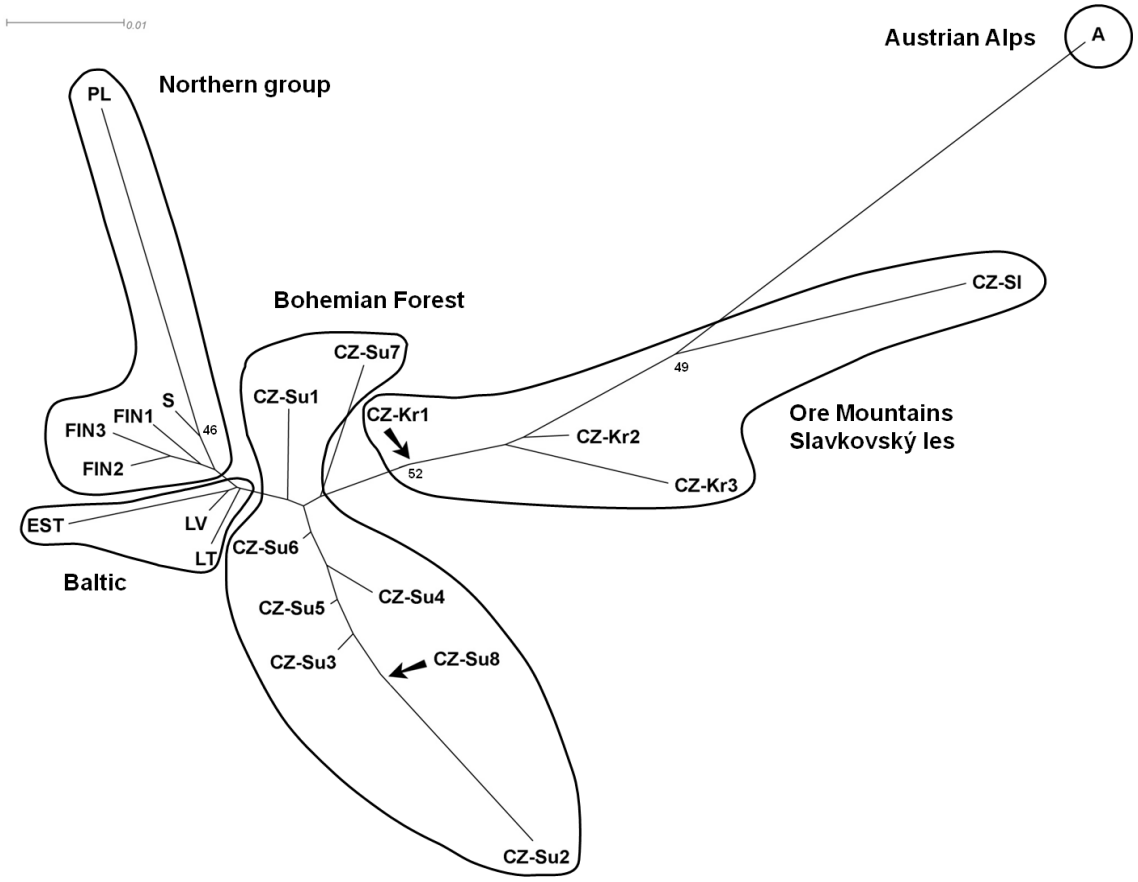

Fig. 6.4: Neighbour joining phenogram of the 523 individuals from 21 populations of Colias palaeno analysed. For abbreviations and more details see Table 6.1.

The population assignment test showed that most of the individuals of the populations have been assigned to their respective BAPS groups. The greatest probability showed group 1 (78.26 %), followed by group 2 (71.43 %) and group 3 (69.38 %). Group 4 and 5 showed a lower probability, but still, most of the individuals have been assigned to their own group (Table 6.4).

Table 6.4: Assignment test for the five genetic groups of *Colias* palaeno calculated by the software BAPS. All figures are given in %.

	group 1	**group 2**	**group 3**	**group 4**	**group 5**
group 1	78.26	2.04	2.87	0.93	0.00
group 2	13.04	71.43	7.18	7.48	11.28
group 3	8.70	14.29	69.38	20.56	16.54
group 4	0.00	2.04	6.70	51.40	12.03
group 5	0.00	10.20	13.88	19.63	60.15
total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**

group 1: A
group 2: CZ-Kr2, CZ-Kr3, CZ-Sl
group 3: CZ-Kr1, CZ-Su1-8
group 4: EST, LT, LV
group 5: PL, S, FIN1, 2, 3

6.4.2 Results mtDNA

For the COI analyses, 213 individuals from 11 locations were investigated. The alignment with 600 bp contained 58 (9.67 %) polymorphic sites of which 54 (9.00 %) were parsimony-informative. Haplotype diversity was low, ranging from 0.000 to 0.688, with haplotype numbers varying from one to three in each location. The population from Latvia (LV) contained with two the most private haplotypes of all populations analysed. Furthermore, the population from Estonia (EST) and the Slavkovský les population (CZ-Sl) each owned one private haplotype.

The average nucleotide diversity was also low, with values ranging from 0.0 % to 4.3 %. Overall mean haplotype and nucleotide diversities were 3.51 % (± 7.3 SD) and 1.2 % (± 0.7 SD), respectively (Table 6.5).

Table 6.5: Sampling locations, haplotypes per site and diversity values for the eleven *Colias palaeno* populations used for COI analyses. *N* = number of individuals; *n* = number of haplotypes; n_P = number of private haplotypes; *h* = haplotype diversity; Θ = nucleotide diversity; *PS* = number of polymorphic sites; *PD* = pairwise differences (TN model); SD = standard deviation.

Pop ID	Location	*N*	*n*	n_P	*h* ± SD	Θ ± SD	*PS*	*PD* ± SD
A	Obergurgl	21	3	0	0.552 ± 0.089	0.020 ± 0.010	48	11.74 ± 5.54
CZ-Kr1	Pernink	19	1	0	0.526 ± 0.040	0.043 ± 0.022	45	25.57 ± 11.74
CZ-Sl	Krásno	9	3	1	0.556 ± 0.165	0.006 ± 0.004	11	3.83 ± 2.13
CZ-Su6	Pasecká slat'	24	1	0	0.000 ± 0.000	0.000 ± 0.000	0	0.00 ± 0.00
EST	Puhja	24	2	1	0.370 ± 0.117	0.020 ± 0.011	52	12.14 ± 5.68
FIN1	Svenskby	19	2	0	0.433 ± 0.117	0.028 ± 0.015	45	17.10 ± 7.96
FIN2	Joensuu	22	3	0	0.688 ± 0.074	0.006 ± 0.003	48	19.69 ± 9.06
LT	Baliskes	21	1	0	0.000 ± 0.000	0.000 ± 0.000	0	0.00 ± 0.00
LV	Rubeni	17	3	2	0.640 ± 0.116	0.013 ± 0.007	53	7.72 ± 3.79
PL	Lewsze	15	1	0	0.000 ± 0.000	0.000 ± 0.000	0	0.00 ± 0.00
S	Arvidsjaur	22	2	0	0.091 ± 0.081	0.000 ± 0.000	1	0.09 ± 0.17
Total		213	11	4	0.351 ± 0.073	0.012 ± 0.007	58	11.56 ± 4.19

The haplotype network (Fig. 6.5) shows nine haplotypes arranged in four clades: Clade 1, the most abundant, consists of six different haplotypes (1, 2, 3, 4, 5 and 6) while all other clades consist of a single haplotype each (7, 8 and 9, respectively). Clades 1 and 4 were separated by 50 mutation steps, thus representing a considerable amount of divergence. Genetic distances among the other clades are much smaller. The difference between clade 1 and 2 amounted to 8 bp, while clade 1 and 3 are separated by 13 mutation steps.

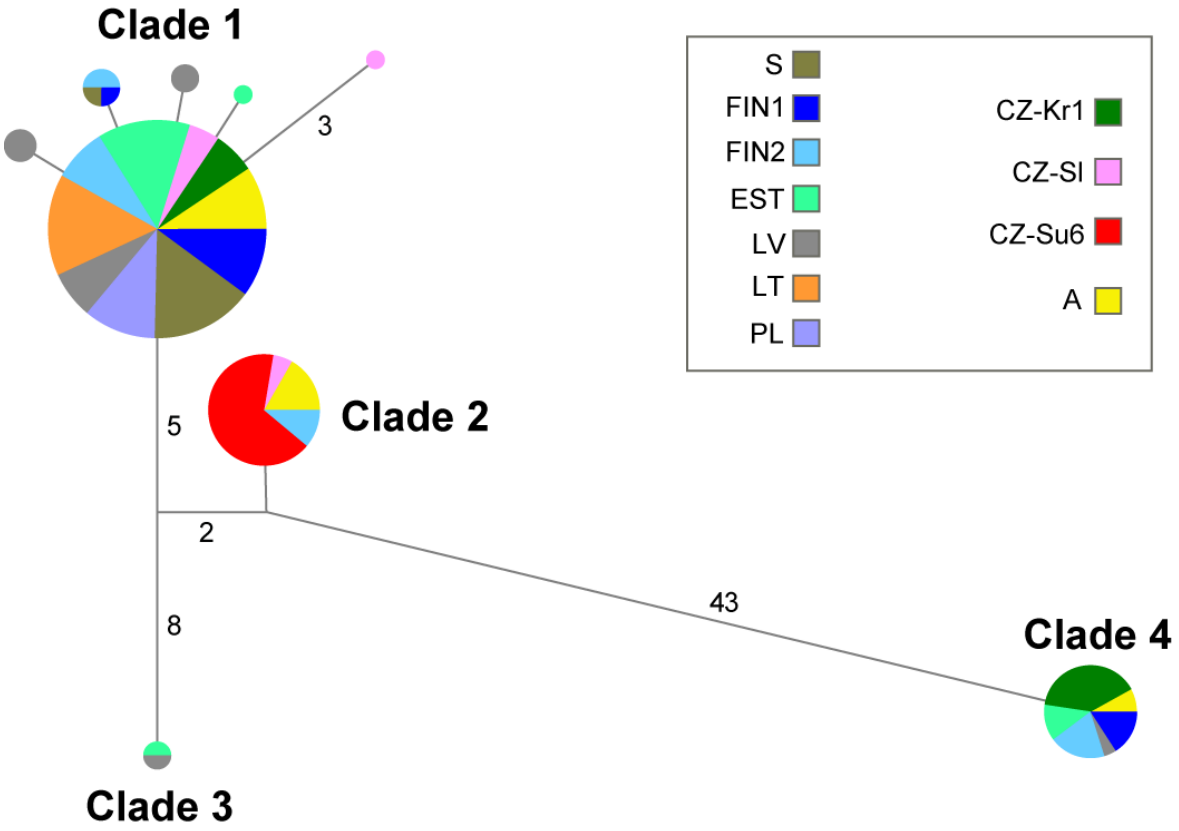

Fig. 6.5: Median joining haplotype network indicating the relationship amongst COI haplotypes of *Colias palaeno*. Each pie chart represents a different haplotype, made up of collection sites labelled by colour in which that haplotype occurs. Haplotypes connected by a line differ in sequence by one base pair unless otherwise indicated. Haplotype circle size denotes the number of sampled individuals.

The genetic distance, calculated with Kimura-2 Parameter for comparison purposes, ranged from 0.17 % to 0.67 % within clades and from 1.17 % to 9.17 % between clades. Clade 4 is the most differentiated clade from all other ones.

The four COI clades show different geographic distributions. Clade 1 was the only one found throughout the geographic range (except for Cz-Su6). Clade 2 was restricted to Austria, CZ-Su6 (the only 100 % clade 2 location) and Finland. Clade 3 was found only in a section of the Baltic range of the distribution (LV and EST). Clade 4 butterflies were found in Finland, Estonia, Latvia, Ore Mountains and Austria. Overall, there is no detectable trend in clade diversity. Although four sites (S, LT, PL and CZ-Su6) have only one clade present, no geographic replacement of clades is detectable (Fig. 6.6).

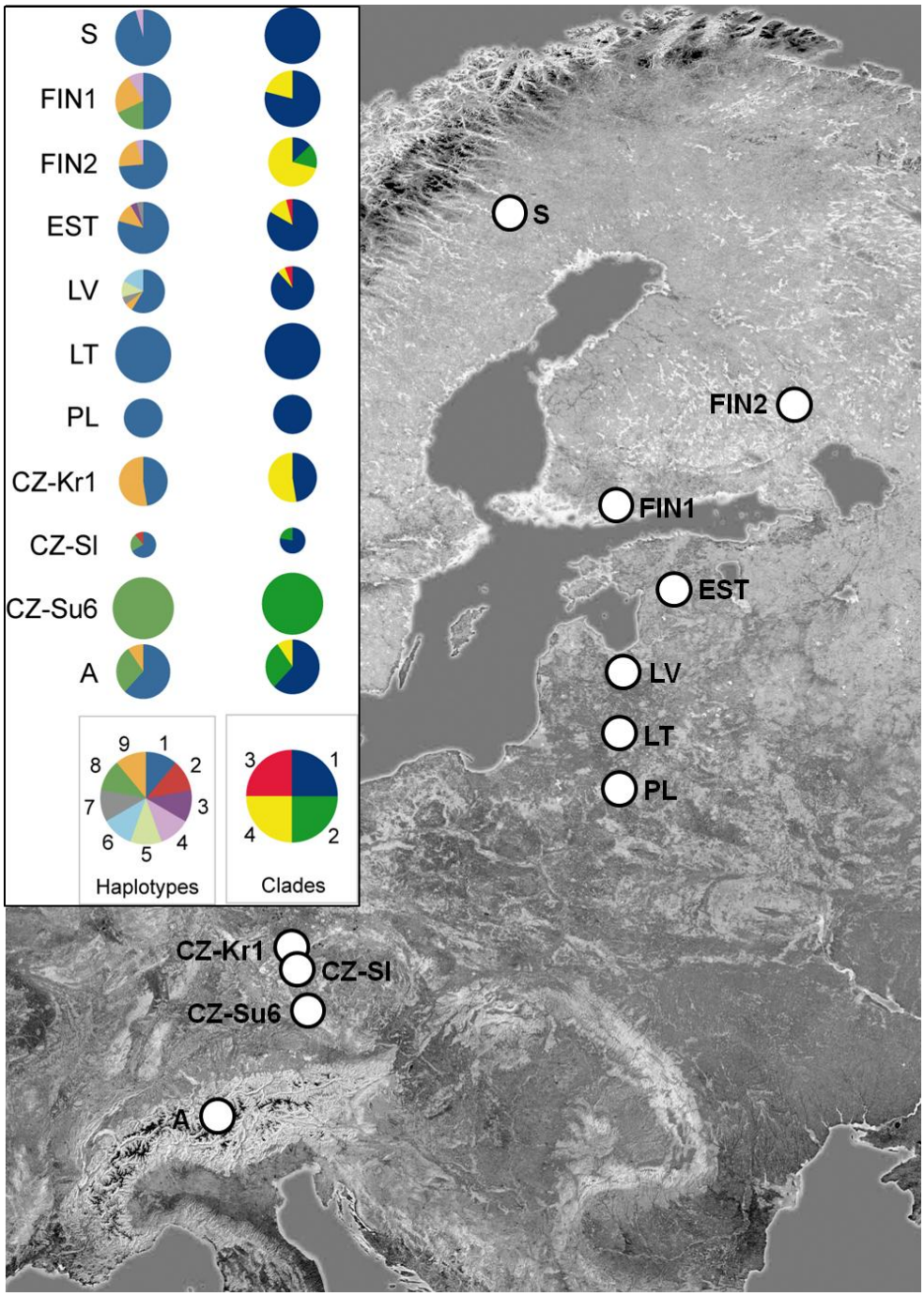

Fig. 6.6: Geographic distribution of the haplotypes found for the 11 *Colias palaeno* populations analysed. Colour circles represent mtDNA COI haplotype frequencies. Circle sizes are proportional to the sample size in each location. For abbreviations and more details see Table 6.5.

Based on COI NCBI BLASTn algorithm searches, the most closely related *C. palaeno* sequences belong to clades 1 and 2, the two most closely related clades in our dataset (Table 6.6). However, only clade 2 is 100 % *C. palaeno*, whereas clade 1 has also a high maximum identity of 99 % with other *Colias* species, namely the arctic species *C. tyche, C. hyperborea and C. hecla*. Clade 4 sequences all return as *C. alfacariensis*, while clade 3 is not possible to ascribe to a single species with certainty, as *C. phicomone*, *C. wiskotti*,

C. hecla or the nematode *Cooperia oncophora* have identical E-values, and high maximum identity and maximum scores.

Table 6.6: Network identified clades and haplotypes and corresponding top 4 species found by BLAST. Max score = highest alignment score for the aligned segments, calculated by summing the match rewards and mismatch and gap penalties for each segment; E-Value = the number of random sequences that can exist with the same score; Max identity = the percent similarity between the query and subject sequences over the length of the coverage area.

Clade	Haplo-types	Accession GenBank Number	GenBank Species	Max Score	E-Value	Max Identity
1	**1, 2, 3, 4, 5, 6**	GU828690	*Colias palaeno*	1109	0	100%
		FJ663430	*Colias tyche*	1099	0	99%
		EF457739	*Colias hyperborea*	1092	0	99%
		EU583872	*Colias hecla*	1082	0	99%
2	**7**	GU096759	*Colias palaeno*	1109	0	100%
		GU096746	*Colias palaeno*	1109	0	100%
		GU096743	*Colias palaeno*	1109	0	100%
		GU096745	*Colias palaeno*	1109	0	100%
3	**8**	HM393178	*Colias phicomone*	1075	0	99%
		EF457738	*Cooperia oncophora*	1075	0	99%
		EU583853	*Colias wiskotti*	1064	0	99%
		EU583869	*Colias hecla*	1059	0	99%
4	**9**	HQ004267	*Colias alfacariensis*	915	0	94%
		HQ004266	*Colias alfacariensis*	915	0	94%
		HQ004262	*Colias alfacariensis*	915	0	94%
		HQ004254	*Colias alfacariensis*	915	0	94%

A maximum likelihood (ML) analysis based on a fragment of the mt COI gene yielded the tree (-ln *L* = 2968.6) shown in Fig. 6.7. Most of the described species based on morphological criteria were recovered as either polyphyletic (e.g. *C. chrysotheme*) or paraphyletic (e.g. *C. alpherakii* with respect to *C. wiskotti*). The only species supported by high bootstrap values in the ML tree were *C. marcopolo*, *C. alfacariensis*, C*. nastes* and *C. myrmidone*.

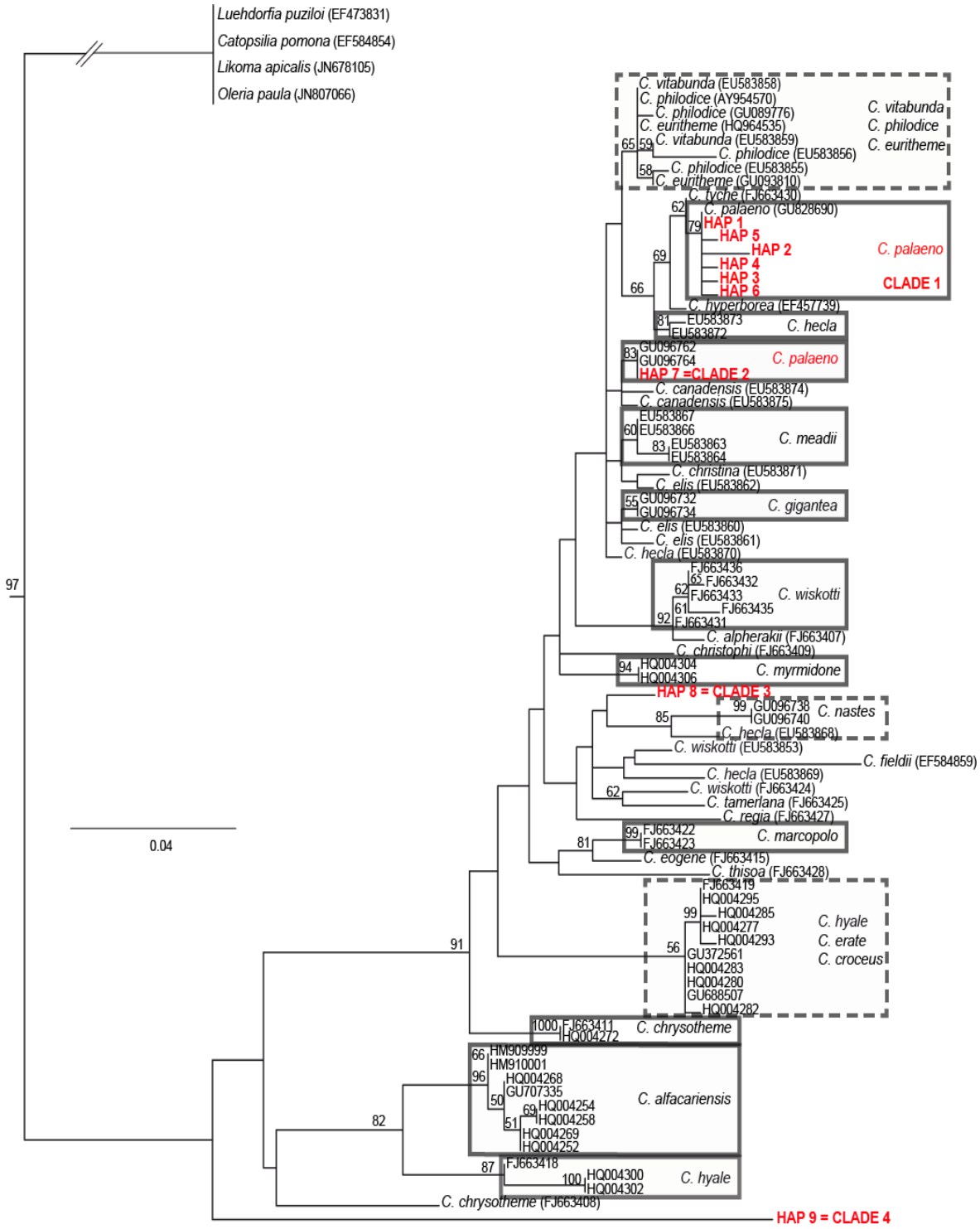

Fig. 6.7: The ML tree (GTR + I + Γ; I = 0.54, Γ = 0.68) for 83 sequences designated by their GenBank accession number, representing 28 putative species of *Colias* for mitochondrial partial COI. Bootstrap values > 50% are given. Putative *Colias palaeno* haplotypes sampled for this study are written in red. Boxes represent strongly supported clades; dashed boxes indicate hybridizing species.

6.5 Discussion – *Colias palaeno*

6.5.1 Biogeographic interpretation

The mean allozyme diversity of the analysed populations of *Colias palaeno* is lower than for other *Colias* species (e.g. Milovanov & Simchuk 2008), but is relatively high if compared with butterflies in general (e.g. Schmitt *et al.* 2005; Habel *et al.* 2010; Habel & Schmitt 2012). Furthermore, the genetic diversity is mostly equally distributed over our study area with no latitudinal gradients and no major regionally or locally impoverished populations. These findings strongly support constantly high population sizes and the general absence of major genetic bottlenecks in space and in time.

Genetic differentiation among populations was low, especially if considering mtDNA sequences of part of the COI gene. Most of the individuals share one common haplotype, which is found in all populations analysed apart for CZ-Su6. This haplotype has five satellites of low overall frequency with four of them only distinguished by one mutational step, a typical signal for a recent range expansion (Rogers & Harpending 1992). However, our GenBank search revealed relatively similar patterns of low within species differentiation for a number of other *Colias* species (*C. meadii*, *C. alfacariensis*, *C. wiskotti*), thus underlining a relatively shallow phylogeographic structure within species of this Pierid genus (Wheat & Watt 2008). The other three haplotypes are distinguished by eight to fifty mutational steps, but at least two of these three are most likely the result of hybridisation with other *Colias* species (e.g. with *C. nastes* or *C. hecla* in the case of clade 3, see below). Hybridisation between different *Colias* species has been frequently reported in the past (Hovanitz 1963; Wang & Porter 2004; Wheat & Watt 2008) and is also well supported by the high number of para- and polyphyletic *Colias* species in our COI phenogram (Fig. 6.7).

Thus, the mtDNA structure even might be compatible with postglacial range expansion from one Würm ice age refuge (cf. Paulo *et al.* 2001; Pinceel *et al.* 2005). In some contrast with this rather shallow mtDNA differentiation, allozymes reveal an F_{ST} of 0.09 and mean genetic distances (Nei 1972) among populations not compatible with postglacial range expansion and subsequent fragmentation. However, the overall differentiation in *C. palaeno* is lower than in most other mountain butterflies with population groups being separated for the entire Würm ice age or more (e.g. Schmitt *et al.* 2006b; Haubrich & Schmitt 2007; Schmitt & Haubrich 2008; Schmitt & Besold 2010; Vila *et al.* 2011). As a compromise between mtDNA and allozyme information, a continuous distribution over most of the Würm ice age with late Würmian disjunction (maybe during

the cryoxerotic Last Glacial maximum, LGM, some 20 ky ago) is the most likely scenario. All of our analyses support the strongest differentiation in allozyme frequencies between the northern proveniences (Fennoscandia, Baltic countries, Poland) and the Alps in the South, thus underlining the existence of two evolutionary significant units, but on a low level of differentiation and of rather recent origin. The Czech populations are either mostly similar to the northern ones (Šumava mountains) or intermediate.

This pattern is compatible with two retreats during the LGM, one north of the glaciers of the Alps and one south of the Fennoscandian glacier in eastern Central Europe. Survival of cold adapted species in retreats in the cold Steppic areas north of the Alps were postulated since long for species with arctic alpine distributions (e.g. Holdhaus 1954), however recent publications underline that in particular the more humid areas adjoining the Alps were of high relevance for the survival of mountain biota (reviewed in Schmitt 2009), even including the northern slopes of the Alps (e.g. Haubrich & Schmitt 2007; Huck *et al.* 2009; Hammouti *et al.* 2010; Michl *et al.* 2010).

The second Würm glacial core area in eastern Central Europe is supported in particular by the (recent) hybridisation with *C. tyche* or *C. hecla*, which both in Europe are currently restricted to the Arctic (Tshikolovets 2011). Therefore, introgression of haplogroup 3 into *C. palaeno* might have happened during glacial co-occurrence of these species in this area. However, the strong genetic differentiation of haplogroup 4 from all other so-far analysed *Colias* individuals convincingly supports its much more ancient process of introgression and thus a continuous hybridisation of *C. palaeno* with other *Colias* species.

The intermediate genetic position of the Czech populations in the Ore Mountains, Slavkovský les and, to a lesser extent, Šumava Mountains, might be the result of postglacial intermixing of the populations expanding from the two glacial core areas located to the southwest and the northeast of these regions. These regions between Bavaria and Bohemia are well known as important suture zones between biota postglacially expanding from different refuge areas (reviews in Hewitt 1999, 2000) with several well studied examples in other butterfly species (Schmitt & Müller 2007; Schmitt & Zimmermann 2012).

The colonisation of Fennoscandia after its postglacial deglaciation most probably follows the eastern pathways via the Baltic countries and Finland to Sweden and not by the western route via Denmark. The lack of genetic differentiation and the high genetic diversity of the Fennoscandian populations well support a phalanx-wise (cf. Ibrahim *et al.* 1996) colonisation by relatively high numbers of founder individuals (and thus without loss of genetic diversity, cf. Hewitt 1996) just using one single colonisation pathway. The

complete absence of the species in the northern German planes (Settele *et al.* 2005) and the mostly continuous distribution from northeastern Poland to Fennoscandia clearly favours this eastern corridor, which is also proofed for many other species (reviews in Taberlet *et al.* 1998; Hewitt 1999).

6.5.2 Conservation implication

The genetic diversity of *C. palaeno* at the allozyme level is astonishingly high for such a localised species. Such rare taxa in most cases show rather low levels of genetic diversity of their populations (e.g. Britten *et al.* 1994; Debinski 1994; Gadeberg & Boomsma 1997; Habel *et al.* 2009b). This genetic paucity apparently is one prerequisite for their survival in splendid isolation, while more genetically diverse species are more in need of gene flow among different populations to avoid the negative impact of inbreeding (Habel & Schmitt 2012). In this context, the high susceptibility of *Colias* species to inbreeding (Wang *et al.* 2009) might be related to the high genetic diversity of these taxa and might become a serious conservation problem for the often highly isolated and relatively small populations of Central Europe. Even without further habitat fragmentation and deterioration, many of these populations might become lost simply to genetic degradations, as also documented for most of the populations of the Hermit butterfly *Chazara briseis* in Czech Republic (Kadlec *et al.* 2010).

However, the loss of these populations in Central Europe might be less severe if simply focussing on the evolutionary potential of the species: Even a complete loss of the Central European populations would not eradiate unique genetic lineages if the populations in the Alps will be maintained, which also are not really endangered (Stettmer *et al.* 2007). The northern lineage also should be safeguarded by the more continuous and more extended populations in the Baltic countries and Fennoscandia (van Swaay & Warren 1999).

7 Conclusions

The results of the present work give further insight into the glacial floral and faunal history of mountain forest and peatland species by revealing the genetic structure and interpreting genetic diversity indices of two plant and butterfly species each and pointing out their postglacial (re)colonisation pathways into present habitats in Europe. Since these representatives of mountain forests and bogs are each characteristic accessory species, also the history of European mountain forests and bogs in general could be decrypted a bit further.

7.1 *Aposeris foetida*

For the calcicole woodland plant at least three important areas for glacial survival and postglacial expansion could be revealed:

(i) the foothills of the Northern Alps,
(ii) both flanks of the Dinaric Alps,
(iii) Central Balkans,

with further putative substructures in the first and the third group. High genetic differentiation within the Northern Alps group and the higher *DW* values support glacial survival at the northern foothills of the Alps at least during the last glacial period. This assumption is further supported by the relatively high number of private fragments within populations from the Northern Alps, compared to the considerably lower numbers in populations from the Southern Alps. Finally, all the average values of all investigated genetic diversity were significantly higher in the Northern than in the Southern Alps. A similar biogeographic structure was also found for *Polygonatum verticillatum* (Kramp *et al.* 2009). Some previous studies on alpine organisms postulate, that the majority of them had their glacial retreats mostly south of the glaciated Alps, or that northern retreats, if existing at all, were small and adding little to the postglacial recolonisation of these mountains (e.g. Tribsch *et al.* 2002; Schönswetter *et al.* 2003, 2004, 2005; Albach *et al.* 2006; Michl *et al.* 2010). However, plants like *A. foetida* or *P. verticillatum*, but also animals like the butterflies *Erebia epiphron* (Schmitt *et al.* 2006a) or *E. sudetica* (Haubrich & Schmitt 2007), strongly underline the great importance of more northern retreats for at least some alpine organisms. Furthermore, these glacial refugia of *A. foetida* provide strong evidence for the existence of forest ecosystems or at least habitats with some forest characteristics in the northern foothills of the Alps during the last glacial maximum.

As already evidenced over the last few years, forest ecosystems are highly likely to have persisted in the easternmost Alps and around the south-western Alps (Magri *et al.* 2006; Magri 2008; Schmitt & Haubrich 2008). However, our data show that these ecosystems were even more widespread than previously thought.

The second main centre of glacial persistence for *A. foetida* as well as an important expansion centre of postglacial recolonisation of the Southern Alps and the Tatra region is represented by the Dinaric Alps region, namely at both flanks of the mountain range. This assumption is well supported by high numbers of private fragments and high genetic diversity values found in the Croatian population studied. A similar pattern was also communicated by Magri *et al.* (2006) and Magri (2008), who assumed the Dinaric Alps as a refugial area for *Fagus sylvatica* or *Edraianthus serpyllifolius* (Surina *et al.* 2011), which also support a possible refugium of these species along the north-eastern Adriatic Sea.

Our data also support the great importance of the Balkans as expansion centre. This fact is well known for warm-adapted species (e.g. *Erinaceus concolor*, Seddon *et al.* 2001; *Maniola jurtina*, Habel *et al.* 2009a; *Melanargia galathea*, Habel *et al.* 2011b). The genetic structures of *A. foetida* support the idea that the Balkans also served as postglacial expansion centre for cold-adapted species. The results of the STRUCTURE analyses and the genetic diversity and uniqueness of the Balkan populations strongly support postglacial expansion from Würm glacial refugia at the Pannonian flank of the Dinaric Alps (see above) via the Slovenian mountain areas and throughout the southern calcareous Alps in western direction and to the calcareous parts of the Tatra Mountains in north-eastern direction.

7.2 *Melampyrum sylvaticum*

The results of *Melampyrum sylvaticum* indicate three main genetic groups:

(i) Pyrenees,
(ii) Southeastern Europe,
(iii) all other populations,

with subdivision of the third group at second level into three to four other groups (Alps, Central German uplands, Tatras, Scandinavia with Scottish highlands and Iceland, with the latter two groups not being separated unambiguously). These two levels of differentiation call for a more extended evolutionary time than Würm ice age processes and might therefore best be explained by subsequent evolutionary events during the last two full glacial-interglacial cycles.

All statistical analyses support the first separation into three genetic groups, which might be partially viewed as potential refugia during the Riss glaciation. As it is generally accepted that the rather extreme conditions during the Riss ice age enhanced the *tabula rasa* in Central Europe in relation to Würm ice age conditions (Habel *et al.* 2011b), one may also assume relatively southern Riss refugia for *M. sylvaticum*. In this context and reflecting the observed genetic structuring, it has to be assumed that areas in the vicinity of the Pyrenees and in the Balkan Peninsula served as refugial areas for *M. sylvaticum* during the Riss glacial.

The most likely Riss glacial refugium for the third genetic group should have been located south of the intensively glaciated Alps. Supporting this assumption, the Southern Alps populations of *M. sylvaticum* showed higher genetic diversities compared to other populations, and the highest number of private fragments was found in one of the southern Switzerland population.

With the rising temperatures of the Riss-Würm interglacial, *M. sylvaticum* shifted from the low altitudes occupied during the Riss glacial period to the now ice-free mountains colonising the entire Alpine region, with further centres of surviving in the Pyrenees and Balkan mountains.

At the beginning of Würm ice-age, *M. sylvaticum* shifted down-hill again. The genetic differentiation among Alps, German upland and Tatra populations indicate early Würm glacial expansion to the Northern Alpine and Tatra Mountains regions, followed by isolation and subsequent genetic modifications. This scenario receives further support

especially for the Tatra refugium by the high genetic diversity values in the Slovakian populations Furthermore, higher numbers of private fragments and higher than average *DW* values Harz and Rothaargebirge also suggest isolated Würm ice age refugia of *M. sylvaticum* in the central German uplands. As *M. sylvaticum* is a forest dwelling species, the previously existing hypothesis that even forests or forest-like habitats also should have survived at least the Würm glacial period in the German upland region north of the Alps and in the vicinity of the Tatras was substantiated.

With the postglacial warming, *M. sylvaticum* shifted up-hill into the high-mountain systems as in the Pyrenees, Alps and Balkan mountains. However, the species' refugial populations north of the Alps are believed to have retracted to disjunct mountain peaks of the middle-high mountains of Central Europe, but did not retract to the Alps. The time passed since this isolation on these mountains (about 10,000 years bp) was not sufficient for further differentiation among these *M. sylvaticum* populations.

Important postglacial range expansions are highly likely out of two of the Würm ice age retreats. The strong genetic cohesiveness between the eastern Balkan mountains all over the Romanian Carpathians strongly support postglacial expansion over major parts of the Carpathians from a more southern glacial refugium in the Balkan Peninsula maybe even extending as far north as the Southern Carpathians. Northern Europe was not most likely colonised from the refugium in the proximity of the Tatras and the expansion to the North apparently has followed a pathway via the Baltic States and Finland to Scandinavia, and further west to Scotland and Iceland.

In all analyses performed, the population of the Thuringian Forest grouped together with the population from the Pyrenees, which could be due to an anthropogenic seed transfer from the Pyrenees to this region. However, natural expansion from the Pyrenees to the Thuringian Forest seems to be highly unlikely due to the large distance between both areas, the relatively high weight of the seeds and the close geographic proximity of the Thuringian population to other genetic lineages.

7.3 *Erebia euryale*

Previous genetic studies on *Erebia euryale* by Schmitt & Haubrich (2008) and Vila *et al.* (2011), already discussed the genetic structure from Northern Spanish, Alpine, Pyrenean, Southern Carpathian and partially Balkan populations. This study presents an enlarged set of populations to test previously formulated phylogeographic interpretations and to complete the scenario by revealing the origins of the populations from Massif Central, the Apennines and the Northern Carpathians and their genetic relatedness to the populations already analysed in previous studies.

Most of the results from these previous phylogeographic studies on *E. euryale* have been supported by our data. Thus, with three additional populations we confirmed the highest diversity values in the Southeastern European mountains. The genetic differentiation among the Balkan and Carpathian populations based on the larger sample number was also as low as before, supporting the postulated large continuous Würm ice age distribution all over a proposed extended forested or forest-like area in Southeastern Europe.

The Eastern Alpine populations showed remarkable genetic differentiation, supporting the hypothesis of an important and spatially extended Würm glacial refugium at the unglaciated foothills of the Eastern and Southeastern Alps.

Although, the Apennine Peninsula is known as one of the three classic main glacial refugia for warm-adapted species in Southern Europe, all our analyses indicated that the Apennines populations analysed do not represent a unique genetic lineage, but shared their heredity with the populations from the Eastern Alps. Furthermore, both Apennines populations showed low genetic diversity values and no private alleles, which both argue against a glacial survival area in the Apennines region for *E. euryale*. The recent populations in the Apennines seem to have suffered from at least one bottleneck, either along the process of postglacial range expansion and / or *in situ* due to marginally suitable habitat conditions in the Apennines.

Regarding the results from STRUCTURE and NJ analyses, the populations from the Tatra Mountains represent a mixture of lineages combining the genetic information from the Eastern Alps and the Romanian Carpathians. The mean genetic diversity of the Tatra populations is remarkably high, which indicate either a contact zone between two gene pools during the postglacial recolonisation process or an important refuge area. Especially the intermediate genetic position between the Eastern Alps and the Southeastern Europe lineage make a postglacial hybrid origin of the Tatra populations a likely scenario.

However, the low genetic differentiation among all Slovakian populations might evidence the existence of a large and continuous glacial survival centre. *E. euryale* populates flowery places in pine and spruce forest clearings. The possible existence of some forests or forest-like structures in the Tatra region during the last glaciation therefore could be an indicator also for a glacial refugium for *E. euryale*, most likely at the foothills of the Tatra Mountains.

The results from the STRUCTURE analysis revealed for the Massif Central populations an endemic genetic lineage for *E. euryale.* Because of the glaciation of the Massif Central region during the Last Glacial Maximum, it should be expected that species today living in this area at least suffered from range contraction and population decline, resulting in losses of genetic diversity. In contrast to this assumption, we observed genetic parameters with inter-mediate to high values for the Massif Central populations. The higher genetic diversity and the independent genetic lineage are strong indicators for an important and strong glacial refugium of *E. euryale* in the Massif Central.

7.4 *Colias palaeno*

The comparatively low diversity in the allozymes of the analysed populations of *C. palaeno* is mostly equally distributed over the complete study area with no latitudinal gradients and no major regionally or locally impoverished populations. These findings strongly support constantly high population sizes and the general absence of major genetic bottlenecks.

Also, the genetic differentiation among populations considering mtDNA sequences of part of the COI gene was low. Most of the individuals share one common haplotype, which is found in all populations analysed apart for one population in the Šumava. This haplotype has five satellites of low overall frequency with four of them only distinguished by one mutational step with signalises recent range expansion (Rogers & Harpending 1992). This mirrors the pattern of low within species differentiation for a number of other *Colias* species according to a GenBank search. The other three haplotypes are distinguished by eight to fifty mutational steps, but at least two of these three are most likely the result of hybridisation with other *Colias* species (e.g. with *C. nastes* or *C. hecla*).

Thus, the mtDNA structure even might be compatible with postglacial range expansion from one Würm ice age refuge. In some contrast with this rather shallow mtDNA differentiation, allozymes reveal an F_{ST} of 0.09 and mean genetic distances (Nei 1972) among populations not compatible with postglacial range expansion and subsequent

fragmentation. As a compromise between mtDNA and allozyme information, a continuous distribution over most of the Würm ice age with late Würmian disjunction is the most likely scenario. All analyses support the strongest differentiation in allozyme frequencies between a northern group (Fennoscandia, Baltic countries, Poland) and the Alps, thus underlining the existence of two evolutionary significant units, but on a low level of differentiation and of rather recent origin. The Czech populations are more or less intermediate, with Šumava Mountains slightly similar to the northern populations. This pattern is compatible with two retreats during the LGM, one north of the glaciers of the Alps and one south of the Fennoscandian glacier in eastern Central Europe.

The second Würm glacial core area in eastern Central Europe is supported in particular by the (recent) hybridisation with *C. tyche* or *C. hecla*, which both in Europe are currently restricted to the Arctic (Tshikolovets 2011). Introgression might have happened during glacial co-occurrence of these species in this area and because of the strong genetic differentiation of one of the haplotypes from all other so-far analysed *C. palaeno* individuals supports a much more ancient process of introgression and thus a continuous hybridisation of *C. palaeno* with other *Colias* species.

The intermediate genetic position of the Czech populations might be the result of postglacial intermixing of the populations expanding from the two glacial core areas located to the southwest and the northeast of these regions.

The colonisation of Fennoscandia after its postglacial deglaciation most probably follows the eastern pathways via the Baltic countries and Finland to Sweden. The lack of genetic differentiation and the high genetic diversity of the Fennoscandian populations well support a phalanx-wise colonisation by relatively high numbers of founder individuals without loss of genetic diversity, just using one single colonisation pathway. The complete absence of the species in the northern German planes and the mostly continuous distribution from northeastern Poland to Fennoscandia clearly favours this eastern corridor.

With the results on *C. palaeno*, some conservation implications could be postulated. The genetic diversity at the allozyme level is astonishingly high for such a localised species. This genetic paucity apparently is one prerequisite for their survival in splendid isolation, while more genetically diverse species are more in need of gene flow among different populations to avoid the negative impact of inbreeding. In this context, the high susceptibility of *Colias* species to inbreeding might be related to the high genetic diversity of these taxa and might become a serious conservation problem for the often highly isolated and relatively small populations of Central Europe. Even without further habitat

fragmentation and deterioration, many of these populations might become lost simply to genetic degradations.

However, the loss of these populations in Central Europe might be less severe if simply focussing on the evolutionary potential of *C. palaeno*. Even a complete loss of the Central European populations would not eradiate unique genetic lineages if the populations in the Alps will be maintained. The northern lineage also should be safeguarded by the more continuous and more extended populations in the Baltic countries and Fennoscandia.

8 Summary

Comparing the results of the phylogeographies of the four species included in this thesis, some accordances have been found, even though certain patterns are only represented in one or two species. In all cases, the findings of the studied species strongly support the existence of forests or forest-like ecosystems beyond the classic forest refugia in the Mediterranean areas (Iberian, Apennine and Balkan peninsulas) during glacial times. However, evidence of glacial refugial areas in Southeastern Europe, especially the Balkans, have been found in this study as well. The analysed populations of *Aposeris foetida*, *Melampyrum sylvaticum* and *Erebia euryale* showed high genetic diversity values and mostly higher private fragments in this area, which is a strong indicator for centres of glacial survival during Würm and, regarding the results of *M. sylvaticum,* even during the Riss ice age.

Three of the analysed species (*A. foetida*, *M. sylvaticum* and *Colias palaeno*) supported a second main glacial refuge area located along the Northern Alps. Again, high genetic diversity values and the uniqueness of the populations living in this region today prove the importance of this area as a glacial centre of survival. Those results confirm several recently published studies on forest species and strongly indicate the persistence of forest-like structures or even forests during the ice ages along the foothills of the Northern Alps. Additionally, the persistence of *C. palaeno* in this area furthermore supports the existence of peatlands north of the Alps, at least during the last glacial.

The results of *M. sylvaticum* and *E. euryale* further indicate the vicinity of the Tatra Mountains as core areas for glacial survival. However, the genetic patterns found for *E. euryale* are ambiguous. Due to an intermediate position of two genetic lineages (originating in the Eastern Alps and Southeastern Europe), the Tatras could also reflect a postglacial mixture zone of those lineages. Moreover, the glacial and postglacial importance of this area for woodland species was accentuated, supporting other phylogeographic studies published.

Besides the congruities among the results of the study species, some unique patterns and therefore further potential glacial refugia have also been illuminated in this thesis. For instance, the calcicole species, *A. foetida,* most probably had further survival area at both sides of the Dinaric Alps, supported by high genetic diversity values and a high number of private fragments found in Croatian populations. Furthermore, the surroundings of the German Uplands and the margin of the Southern Alps provided suitable conditions for glacial survival for *M. sylvaticum*, while the Eastern and Southeastern Alpine region most

probably sheltered the Large Ringlet *E. euryale* during ice ages. Additionally, this butterfly species survived at least the glaciation along the foothills of the Massif Central, whose present populations showed a unique genetic lineage and their genetic diversity values have been measurably higher than in other populations for this species. Finally, a large and continuous Würm distribution is highly likely south of the Fennoscandian glaciers in Central Europe for *C. palaeno*, which might indicate extended peatland areas during Würm glacial.

With all the patterns found in this study, the understanding of glacial persistence of forest, respectively forest-like structures and peatlands during Würm or even Riss glacial in Europe could be advanced. The congruencies among the analysed woodland and bog species illustrate the importance and location of extra-Mediterranean refugia for European mountain forests and the glacial presence of Central European peatlands. Thus, already postulated theories could be supported and further pieces of the overall puzzle could be added. The varieties of the different survival centres once more clarified that further phylogeographic studies on mountain forest of different habitat requirements and especially peatland species have to be implemented to get a clearer picture of the glacial history of these habitats.

9 Zusammenfassung

Vergleicht man die Resultate der Phylogeographien der vier in dieser Studie untersuchten Arten, werden einige Übereinstimmungen deutlich. Dagegen wurden einige biogeographischen Strukturen nur für eine oder zwei Arten gefunden. In allen Fällen jedoch spiegeln die Ergebnisse, die für die jeweiligen untersuchten Arten gefunden wurden, das Vorkommen von Wäldern, bzw. waldähnlichen Ökosystemen zu Zeiten mitteleuropäischer Vergletscherung jenseits der klassischen Waldrefugien im mediterranen Raum (Iberische, Apenninen- und Balkan Halbinsel) wider. In dieser Studie wurden zusätzlich Hinweise gefunden, die auf eine Existenz von glazialen Rückzugsgebieten in Südosteuropa, speziell auf der Balkan Halbinsel, hinweisen. Die hier untersuchten Populationen von *Aposeris foetida*, *Melampyrum sylvaticum* und *Erebia euryale* zeigten hohe genetische Diversitätswerte und in den meisten Fällen eine hohe Anzahl von privaten Fragmenten in diesem Gebiet, was deutliche Anzeichen für ein glaziales Überdauerungszentrum während der Würm, und sogar der Riss Eiszeit sind, worauf die Ergebnisse von *M. sylvaticum* deuten.

Die phylogeographischen Strukturen von drei der hier analysierten Arten (*A. foetida*, *M. sylvaticum* und *Colias palaeno*) sprechen für die Lage eines zweiten Hauptrefugiums entlang der nördlichen Alpen. Die Bedeutung dieses Gebietes als glaziales Rückzugsgebiet wurde auch in diesem Fall durch hohe genetische Diversitätswerte und die genetische Eigenständigkeit der Populationen, die heute in dieser Region vorkommen, verdeutlicht. Diese Ergebnisse bestätigen einige bereits veröffentlichte Studien über Waldarten und weisen deutlich darauf hin, dass Wälder und waldähnliche Strukturen während der Eiszeiten im nördlichen Alpenvorland überdauern konnten. Zusätzlich wird durch die Persistenz von *C. palaeno* in diesem Gebiet deutlich, dass auch Moorlandschaften nördlich der Alpen während des letzten Glazials wahrscheinlich waren.

Die Resultate von *M. sylvaticum* und *E. euryale* deuten weiter darauf hin, dass die Umgebung der Tatra ebenfalls als Kerngebiet einer glazialen Überdauerung gedient hat, wobei die genetischen Strukturen, die in *E. euryale* gefunden worden sind nicht ganz eindeutig sind. Aufgrund der Mittelstellung der slowakischen Populationen zwischen zwei genetischen Linien, von der eine ihren Ursprung in den Ostalpen und die andere in Südosteuropa hat, könnte die Tatra ebenso eine postglaziale Kontaktzone dieser beider Linien darstellen. Nichtsdestotrotz wurde die Bedeutung dieses Gebietes für Waldarten im Glazial und Postglazial verdeutlicht und unterstützt somit einige Studien, die dieses ebenso bereits postulierten.

Neben den Übereinstimmungen innerhalb der Ergebnisse der untersuchten Arten, wurden in dieser Arbeit auch einige artenspezifische Muster gefunden, die auf weitere glaziale Refugien hindeuten. Die kalkholde Art *A. foetida* hatte beispielsweise ein Überdauerungsgebiet entlang beider Seiten der Dinarischen Alpen, was durch die hohen genetischen Diversitätswerte und die Anzahl der privaten Fragmente, die in den kroatischen Populationen gefunden worden sind, unterstützt wird. Die Umgebung der deutschen Mittelgebirge und der Rand der Südalpen stellten geeignete Bedingungen für ein glaziales Überleben für *M. sylvaticum* dar, während die Ost- und Südostalpen mit hoher Wahrscheinlichkeit dem Berg-Mohrenfalter *E. euryale* Schutz geboten haben. Diese Schmetterlingsart überlebte überdies mindestens die letzte Eiszeit entlang der Ausläufer des Zentralmassivs. Die derzeitigen Populationen in diesem Gebiet zeigten eine eigene genetische Linie und die genetischen Diversitätswerte waren deutlich höher als in den anderen untersuchten Populationen dieser Art. Letztendlich weisen die hier gefundenen genetischen Strukturen von *C. palaeno* auf ein großes und zusammenhängendes Würm-Refugium südlich des Fennoskandischen Gletschers in Zentraleuropa hin. Dieses Ergebnis könnte ein Hinweis auf ausgedehnte glaziale Moorlandschaften in diesem Gebiet sein.

Durch die Ergebnisse dieser Studie konnte deutlich zum weiteren Verständnis über das glaziale Vorkommen und Überdauern von Wäldern, bzw. waldähnlichen Strukturen in Europa während der Eiszeiten beigetragen werden. Die Übereinstimmungen in den phylogeographischen Strukturen der hier untersuchten Wald- und Moorarten veranschaulichen die Lage und Bedeutung extra-mediterraner Refugien für europäische Bergwaldarten und geben Hinweise auf das Vorkommen von zentraleuropäischen Moorlandschaften während des letzten Glazials. So konnten bereits aufgestellte Theorien unterstützt und ein weiteres Stück zu dem Puzzle hinzugefügt werden. Die Unterschiede in den Überdauerungszentren der einzelnen Arten machen jedoch deutlich, dass weitere phylogeographische Untersuchungen an Bergwald- und insbesondere Moorarten mit unterschiedlichen Habitatansprüchen durchgeführt werden müssen, um ein noch deutlicheres Bild der glazialen Geschichte dieser beiden Habitate bekommen zu können.

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