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Relationships among levels of biodiversity and the relevance of intraspecific diversity in conservation – a project synopsis

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Abstract

The importance of the conservation of all three fundamental levels of biodiversity (ecosystems, species and genes) has been widely acknowledged, but only in recent years it has become technically feasible to consider intraspecific diversity, i.e. the genetic component to biodiversity. In order to facilitate the assessment of biodiversity, considerable efforts have been made towards identifying surrogates because the efficient evaluation of regional biodiversity would help in designating important areas for nature conservation at larger spatial scales. However, we know little about the fundamental relationships among the three levels of biodiversity, which impedes the formulation of a general, widely applicable concept of biodiversity conservation through surrogates. Here, we present the set-up of an international, interdisciplinary project, IntraBioDiv (http://www.intrabiodiv.eu), which studied vascular plant biodiversity at a large scale, i.e. across the European Alps and the Carpathians. Our assessment comprises species richness (high-mountain flora), genetic variation (amplified fragment length polymorphisms, AFLPs) and environmental diversity (modelled potential habitat diversity). Our primary aims were to test for correlations between intra- and interspecific diversity and to identify possible environmental surrogates to describe biodiversity in the two study regions. To the best of our knowledge, IntraBioDiv represents the first multispecies study on intraspecific, molecular-genetic variation in relation with species and habitat diversity. Here, we outline the theoretical background, our sampling scheme, the technical approaches and the feasibility of a concentrated and standardized sampling effort. We further show exemplary results.

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Our three data sets will be made freely available and will provide a playground for further hypothesis testing in conservation, ecology or evolution open to the scientific community.

Keywords: Alps; Carpathians; Molecular-genetic diversity; Potential habitat diversity; Protected areas; Vascular plant species richness

Introduction

Biodiversity can be considered as a synonym of "variety of life" (Gaston, 1996c), which consists of various levels. These levels encompass ecosystems, species and their genes. Intraspecific genetic diversity is thus an integral part of biodiversity (Fiedler and Jain, 1992; Moritz, 2002). Genetic diversity defines the evolutionary potential of species and is consequently of prime importance for the long-term preservation of biodiversity in changing environments (Forest et al., 2007). However, intraspecific diversity is often neglected in conservation strategies because of difficulties not only of rating its significance, but also of merely quantifying it (Hughes et al., 1997; Till-Bottraud and Gaudeul, 2002).

The persistence of populations has been shown to be positively linked to genetic variability (Frankham and Ralls, 1998; Saccheri et al., 1998). Although Lande (1988) argued that demographic factors were more important than genetic ones in determining the short-term persistence of populations, it is now accepted that demographic and genetic processes often act synergistically (Allendorf and Luikart, 2007). Moreover, genetic variability may interact with demographic effects to foster the "extinction vortex" of small populations (Gilpin and Soulé, 1986).

Approaches for quantifying biodiversity

Species richness represents the most widely applied measure in biodiversity assessment (Gaston, 1996b) and conservation. Many attempts have been made to avoid the time-consuming and expensive direct assessment of all species distributions to facilitate conservation planning (Moreno et al., 2007). A popular approach in conservation is to rely on focal species (e.g. indicator, umbrella or flagship species; Simberloff, 1998) as surrogates for regional biota. However, recent investigations question this approach, which may not perform better than if any randomly selected species were studied (Caro and O'Doherty, 1999; Andelman and Fagan, 2000). In the same way, the use of higher-taxon sets (richness in genera, families or orders) as surrogates is often not satisfactory (Andersen, 1995; van Jaarsveld et al., 1998; but see Grelle, 2002; Prinzing et al., 2003). Alternatively, deducing overall species richness from a taxonomic subset of organisms, thought to be representative of other taxonomic groups, is widespread, but has yielded contradictory results (e.g. Sætersdal et al., 2003; Kati et al., 2004; Sauberer et al., 2004; Englisch et al., 2005).

As a consequence of the difficulties to assess species diversity using "taxonomical" surrogates, attempts using environmental data as surrogates for species diversity have been promising (e.g. Wessels et al., 1999; Wohlgemuth, 2002; Moser et al., 2005). Environmental surrogates, based on available data collected over large areas and extended time periods (e.g. climate records), would allow for an efficient evaluation of organismic diversity. However, using environmental parameters for this purpose remains controversial. For example, it has been shown that environmental diversity does not necessarily represent a good surrogate for vertebrate and vascular plant species diversity (Araújo and Humphries, 2001).

At the same time, there is very limited knowledge on the relationship between species richness (interspecific diversity) and genetic variation (intraspecific diversity). Currently, mainly theoretical considerations have been published (Vellend, 2005), while empirical data are mostly restricted to single-species studies (Vellend, 2004) or are limited in sample size or geographic and taxonomic representation (Vellend and Geber, 2005). Only recently, integrative studies have demonstrated that intraspecific diversity may have a positive effect on the associated species richness (Crutsinger et al., 2006; Whitham et al., 2006) and that genotypic diversity may enhance ecosystem resilience (Reusch et al., 2005).

But why should we expect that inter- and intraspecific diversity are positively correlated? Vellend and Geber (2005) argued that locality characteristics, e.g. area, geographical isolation or environmental heterogeneity, may affect both diversity levels in parallel, i.e. via similar neutral processes such as drift and immigration, making it plausible that intra- and interspecific diversity are positively correlated. Such a correlation was illustrated in single-species studies in island situations, but not for mainland situations (Vellend and Geber, 2005). Given the high degree of regional endemism and the rough topography, however, alpine habitats have often been considered as "islands in the sea of mountain ranges" (Riebesell, 1982), which let us expect that alpine plants

would likely display a positive relationship among the three diversity levels. Considerably more complex is the effect of adaptation and evolutionary history (see below) on intra- or interspecific biodiversity, and selective effects of species diversity on genetic diversity and vice versa are thought to impinge on the above parallel neutral processes (Vellend and Geber, 2005).

According to recent biogeographic and phylogeographic studies (e.g. Hewitt, 1996; Taberlet et al., 1998; Hewitt, 2000; Taberlet and Cheddadi, 2002; Tribsch, 2004; Schönswetter et al., 2005), both species richness and intraspecific diversity are often higher in areas that served as glacial refugia during Quaternary cold periods. In Europe, species richness is higher in southern regions than in northern regions because many species were able to survive glaciations in the South (Gaston, 1996a; Gaston et al., 1998). In the same way, for species with large distribution ranges, genetic diversity is usually higher in supposed refugia (Taberlet et al., 1998; Hewitt, 2000; Gugerli et al., 2001; Magri et al., 2006). In contrast, high levels of genetic diversity have also been found in suture zones outside of refugia, where species or evolutionary lineages originating from different refugia met after the last glaciation (Taberlet et al., 1998; Petit et al., 2003; Thiel-Egenter, 2007).

Designing a network of nature reserves

Strategies for preserving biodiversity in large protected areas such as national parks are vividly debated. The concept of complementarity has become an essential criterion thought to be important when developing networks of protected areas (Howard et al., 1998; Pimm and Lawton, 1998; Cabeza and Moilanen, 2001). The main aim of complementarity is to identify sets of protected areas that maximize the representation of regional biodiversity at minimum costs.

While there have been attempts to take into account alternative concepts of biodiversity, e.g. phylogenetic diversity of plants and animals, in designing networks of protected areas (Faith, 1992; Bowen, 1999; Moritz, 2002; Rodrigues and Gaston, 2002; Sechrest et al., 2002; Forest et al., 2007), consensus has emerged that directing conservation efforts at protecting species or ecosystems richness only does not suffice to allow for the preservation of the future evolutionary potential of species. At best, all three biodiversity levels should be adequately considered when setting priorities in biodiversity conservation and optimising the network of existing protected areas. At the same time, no empirical studies are available that evaluate the interrelations among the three biodiversity levels (Vellend, 2004; Lapointe and Rissler, 2005). This lack of knowledge is not surprising because the assessment of species distributions is already a challenge on its own, and because assessing intraspecific diversity is even more expensive and time-consuming. In contrast, environmental diversity can be easily assessed nowadays if digitalized geophysical maps and climate data are at hand.

IntraBioDiv – the flora of the Alps and of the Carpathians as a study system for investigating relationships among biodiversity levels

With the project IntraBioDiv (http://www.intrabiodiv.eu), an international research initiative was launched as a timely effort to fill the above gap in our knowledge on a fundamental aspect of biodiversity conservation. The primary goals of this project were (i) to test for relationships between interspecific (species) and intraspecific (genes) diversity on a large spatial scale, (ii) to describe potential habitat diversity and to identify efficiently assessible environmental proxies for plant biodiversity, by using a set of available environmental parameters and (iii) to elaborate guidelines for incorporating intraspecific, i.e. genetic, diversity in strategies for designing networks of nature reserves.

Such an endeavour required to integrate research teams with various expertise and wide geographic representation. At the same time, it was mandatory to limit the project to a geographically and ecologically well-delimited study system. Therefore, we chose the high-mountain vascular floras of the Alps and the Carpathians as study system.

Like most mountain ecosystems, the Alps and the Carpathians exhibit high species richness relative to their basal area (Väre et al., 2003), making them relevant for global biodiversity conservation (Körner, 2002). Furthermore, the alpine zone, i.e. the area above the timberline, is among the areas least disturbed by human activities in continental Europe, at least on a regional scale. The two mountain ranges also dispose of a dense network of meteorological stations. Potential habitats and their variability can thus be modelled by using climate records in combination with a digital elevation model (DEM) of high spatial resolution.

Plant distributions in the two mountain ranges studied are relatively well known (cf. Appendix B). However, the distribution data available, either included in regional distribution atlases or hosted in different databases, were not compatible across the entire study ranges. There were substantial differences in spatial reference systems used for data acquisition, in taxonomic classification and in the floristic coverage of the respective areas. Great effort was therefore required to combine, harmonize and complement the existing floristic databases.

Amplified fragment length polymorphisms (AFLPs; Vos et al., 1995) were chosen for describing intraspecific,

neutral genetic diversity. Recent overviews on AFLPs demonstrate their promises and pitfalls (Bonin et al., 2007a; Meudt and Clarke, 2007). We thus only briefly introduce their properties in relation to our project-specific requirements. Despite their limitations regarding marker interpretation, AFLPs have considerable advantages. They are universal, thus requiring no prior sequence information and little species-specific adjustment. Moreover, many loci can be screened simultaneously, characterizing the entire genome of an individual (genetic fingerprint). As these genetic markers are anonymous and dominant (Mueller and Wolfenbarger, 1999), only banding patterns (band presence/absence) are scored. AFLPs are highly reproducible if adequately selected and have proven informative for identifying phylogeographic structure and patterns of genetic diversity in vascular plants (Schönswetter et al., 2005).

Water and nutrient availability as well as temperature regime are among the key factors for a plant's ability to successfully grow and reproduce in a particular environment. Vapour pressure is highly correlated with leaf area index and, hence, serves as a surrogate for plant productivity, which itself is correlated with species richness (Hector et al., 1999). Species diversity may not only be a function of the annual means of climate variables but also depends on the latter's temporal variability. Therefore, standard deviations, in addition to means, of climatic variables may serve as surrogates for ecosystem diversity.

Can the principal conclusions taken from our study system, the vascular flora of the Alps and the Carpathians, be applied to other high-mountain ecosystems or to other organismic groups? The composition of any organismic assemblage results from several factors. (i) The geographic distribution of organisms has always been changing over time owing to natural environmental change, in parallel with the evolution or extinction of species. (ii) Organisms have survived past periods of climatic extremes in refugia. (iii) Colonization ability and genetic diversity differ among species. (iv) Endemism is not randomly distributed. (v) Perturbations due to natural (or anthropogenic) disturbance have lead to the destruction and fragmentation of habitats. In consequence, the same elementary processes likely caused similar general patterns of diversity. We thus suggest that the results of the IntraBioDiv project are indicative of correlations across all three biodiversity levels in a broad range of spatial, environmental and organismic settings (see approaches linking habitat and biodiversity in marine ecosystems; Ward et al., 1999; Thrush et al., 2001).

Specific research objectives of IntraBioDiv

The specific research objectives of the IntraBioDiv project were (i) to estimate the intraspecific (genetic) diversity of a large number of selected alpine vascular plant taxa over the entire Alps and Carpathians, (ii) to determine the geographic distribution of the alpine flora of the Alps and the Carpathians, based on available data and on new field surveys, (iii) to characterize environmental variation by generating maps of potential climatic habitat diversity across the two mountain ranges and (iv) to establish a database that combines the spatial distribution of intraspecific, interspecific and environmental diversity. For integration, we aimed at inter-relating the three data sets (intraspecific genetic diversity, species richness, environmental variation) to test for correlations among these diversity levels and to identify environmental surrogates for intra- and interspecific biodiversity. Given that the Alps comprise a contiguous high-mountain area and that a good coverage of environmental data is available, such surrogates will be established for the Alps. The models will then be tested for their predictability in the Carpathians, where mountain habitat is rather insular and climate data availability lower than for the Alps. If the spatial distributions of the parameters describing the three biodiversity levels were congruent, then it would be justified to use environmental data as biodiversity surrogates for finding the best strategy for designing networks of natural reserves to conserve biodiversity. However, should the spatial distribution of the three levels of biodiversity not be congruent, it will be essential to take into account measures of both intra- and interspecific diversity to optimize strategies for biological conservation.

The present article illustrates the specific approaches taken in IntraBioDiv to assess the three levels of alpine biodiversity at large scales. This information shall be valuable for future research involving research teams tackling fundamental multidisciplinary questions in ecology and evolution. We will specifically outline our sampling design, provide exemplary results, and discuss the prospects and limitations of the data sets obtained.

Methods

Study area

The sampling scheme was designed to assess the three levels of biodiversity in the entire ranges of the Alps and the Carpathians. We adopted the regular grid system used for the mapping of the Alpine and Carpathian flora, with cell sizes of 12′ latitude and 20′ longitude (ca 22.3 km × 25 km, ca 563 km^2; range: 529–596 km^2; Fig. 1A). Only cells with elevations >1000 m a.s.l. (353 and 208 grid cells in the Alps and the Carpathians, respectively) were taken into account. Mountain areas <1000 m a.s.l. with suitable habitats for high-mountain species were included if they were integrative

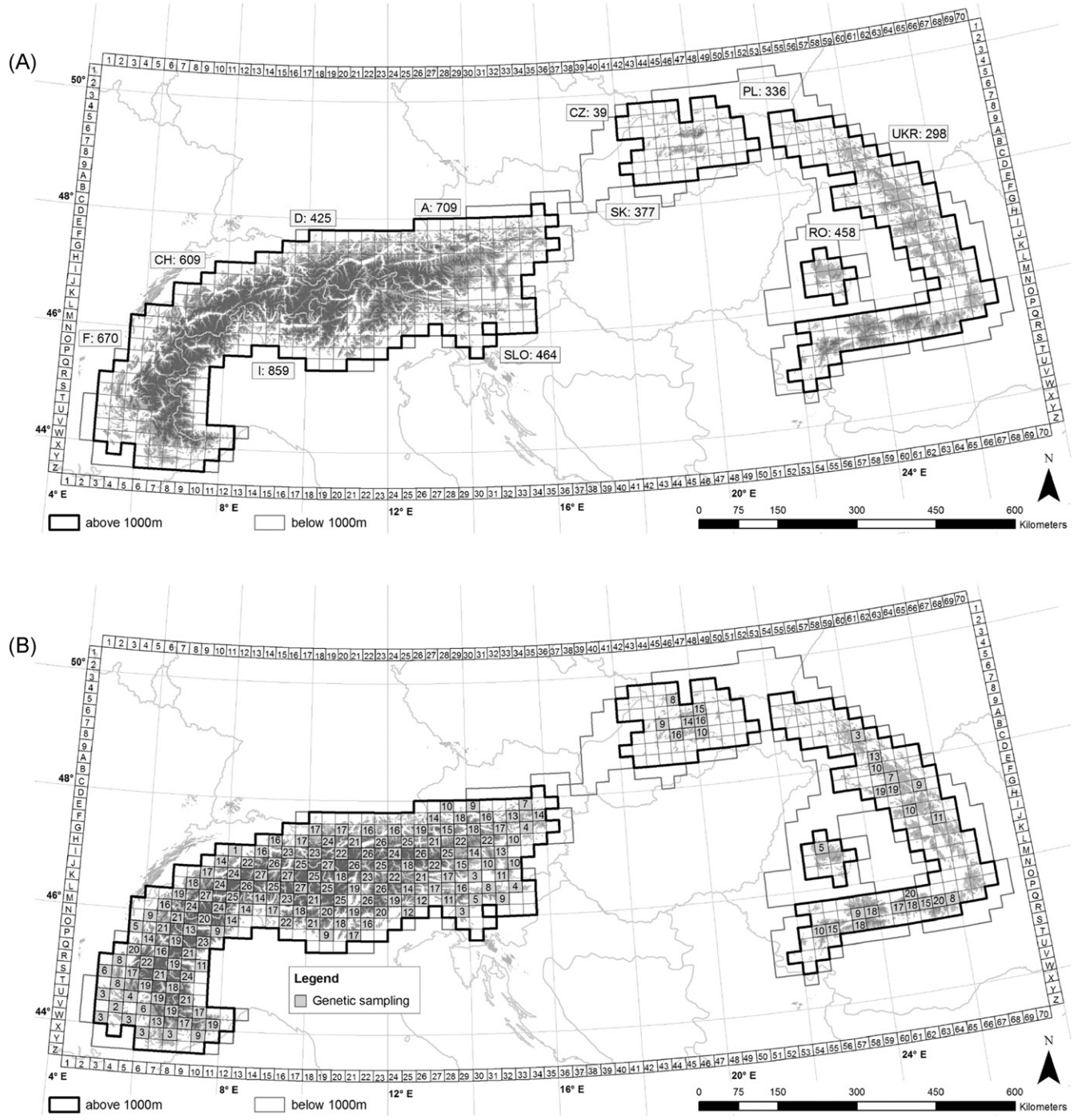

Fig. 1. Grid system adopted in the IntraBioDiv project for assessing ecosystem, species and genetic diversity of alpine vascular plants. Each grid cell represents 12′ latitude and 20′ longitude, i.e. approximately 563 km^2. (A) Grid system encompassing continuous mountainous areas in the Alps and the Carpathians. Numbers refer to the total alpine vascular plant species richness recorded within the respective countries (A, Austria; CH, Switzerland; CZ, Czech Republic; D, Germany; F, France; I, Italy; PL, Poland; RO, Romania; SK, Slovakia; SLO, Slovenia; UKR, Ukraine). (B) Grid cell selection (light grey) for sampling of genetically analysed species. The range was restricted to those cells comprising areas >1500 m a.s.l. Numbers reflect the total of species that were successfully genotyped within a given cell.

geomorphological parts of the Alps or the Carpathians, adding 35 (Alps) and 169 (Carpathians) cells. The total area summed up to 221,179 km^2 in the Alps and 209,718 km^2 in the Carpathians. For the genetic sampling, we only considered those cells that comprised areas >1500 m a.s.l. to account for a possible bias

owing to the particular situation of peripheral populations. Furthermore, we took genetic samples in only every second cell in the Alps (because of laboratory workload), while in the Carpathians, genetic sampling was adapted to the island character of alpine areas and included cells in all relevant Carpathian massifs (149 cells in the Alps, 30 cells in the Carpathians; Fig. 1B).

Genetic diversity

Two sets of 30 taxa were selected for genetic analyses in the Alps and the Carpathians, respectively. Of these, 14 taxa widespread in both the Alps and the Carpathians were analysed for both mountain ranges. The remaining taxa were either restricted to one of the two mountain ranges or else occurred in the Carpathians and in the eastern Alps. This resulted in a total of 45 species sampled for genetic analyses (Table 1). We applied the following criteria for taxon selection: (i) wide distribution in one or both study ranges, (ii) frequent occurrence, (iii) consistent ploidy level (as far as known), (iv) no taxonomic uncertainties and (v) easy identification in the field. We further aimed at a broad representation of families, biogeographical distribution types, life forms and other life history traits (reproductive mode, breeding system, dispersal type, etc.).

From an initial set of 60 widespread species collected in three Alpine areas (western, central, eastern Alps), preliminary laboratory tests identified those taxa that gave reliable AFLP patterns and were thus selected for the range-wide sampling in the Alps. No pre-sampling was carried out for the specific Carpathian taxa.

The vast fieldwork for the genetic sampling was shared among all project partners involved, so that each team was responsible for a particular number of cells. All specimens for genetic analyses were sampled within a single growing season (June–September 2004). Within each cell selected for genetic sampling, we considered one location per species, independent of its position within the cell. Specific locations were selected according to information from existing databases or based on geological maps. We sampled four individuals, numbered 1–4, per species along a horizontal transect, respecting 10 m distance between successive individuals to avoid sampling closely related individuals. A double sample of individual 1, labelled X, was sampled in each location for blind tests of AFLP pattern repeatability. We stored leaf tissue in scintillation tubes filled with silica gel, recorded the exact geographical position and elevation using GPS receivers and/or altimeters, and took one herbarium voucher in most locations. Herbarium specimens are deposited at KRAM, NE, WU and Z, while silica-dried material and DNA extracts are stored at the respective partner's laboratory (Table 1). Sampling effort per grid cell was set to 2 days, but revisiting of particular cells was sometimes required to increase the success rate of sampling.

To achieve consistency of AFLP scoring, all samples of a given species were analysed by a particular team (Table 1). Throughout the lab procedures, we adopted a strict sample arrangement on 96-well plates (Bonin et al., 2004), including two replicates among plates, two replicates within plates, and additional 5–10% blind duplicates (X samples). Samples were randomly arranged on the plates to avoid a systematic bias. Owing to workload restrictions, we genotyped three individuals per species per cell. DNA extractions were carried out with the DNeasy Plant kit (96-well plates or single columns; Qiagen, Hilden, Germany), using 10 or 15 mg of dried, ground leaf tissue. Each partner used lab-specific protocols and methods for AFLP and fragment length analysis (Appendix A). For fragment sizing and marker scoring after electrophoresis on an automated sequencer, GeneMapper 3.7 (Applied Biosystems) or Genographer 1.6.0 (http://hordeum.oscs.montana.edu/genographer) were used. The Applichem gels used for AFLP electrophoresis in one lab were manually scored for presence or absence of bands. We used the blind samples (X) to reliably estimate the reproducibility of AFLP banding patterns.

We assessed genetic diversity per cell based on Nei's (1973) average gene diversity, normalized within species to account for differences in mean diversity among species (Thiel-Egenter et al., in press). Cell-wise genetic diversity was calculated by averaging over all species, either in all cells or only in those in which at least ten species were genotyped. To evaluate the effect of the low local sample numbers on the regional patterns of genetic diversity in the Alps, we further developed two manually superimposed grid systems, i.e. 2×2 and 3×3 cells. Accordingly, we obtained average genetic diversity over two and 4–5 grid cells, respectively, with few, mostly marginal cells excluded owing to the irregular arrangement of the superimposed grids. The numbers of individuals per taxon accordingly increased to six and 12–15 per merged cells.

Floristic diversity

As a first step in the analysis of species diversity, a list of high-mountain taxa (HMT) for the Alps and the Carpathians was established. The fundamental criterion applied was that a given species had its optimum altitudinal distribution (Mirek, 1990) above timberline at least in one of the two study ranges (Englisch et al., unpubl. data). Only well-defined taxa were included in the list, whereas doubtful taxa and micro-species (e.g. within apomictic groups) were lumped to species aggregates for practical reasons. Information on total altitudinal range, on the optimum of range (i.e.

Table 1. List of plant taxa sampled for genetic analyses with the herbarium of voucher deposition, taxon occurrences in IntraBioDiv cells, sampling success (relative to mountain range(s) where a taxon was thoroughly sampled) and genotyping (sample sizes, success, number of polymorphic markers, responsible laboratory) with amplified fragment length polymorphisms (AFLPs)

Taxon	Family	Herbarium	Number of occurrences in IntraBioDiv grid cells				Sampling			Genotyping				
			Total	Sampling range										
				A	C	Total	Range[a]	Cells[b]	Success (%)	Locations[c]	Individuals	Success[d] (%)		AFLPs[e]
Partner[f]														
Androsace obtusifolia All.	Primulaceae	WU	174	80	7	87	A	46	57.5	45	131	94.9	134	UW
Arabis alpina L.	Brassicaceae	Z	403	149	27	176	A/C	155	88.1	148	442	95.1	150	UJF
Campanula alpina Jacq.	Campanulaceae	KRAM	93	22	23	45	C	19 (17)	82.6	36	108	100.0	155	IBPAS
Campanula barbata L.	Campanulaceae	NE	256	126	–	126	A	111	88.1	104	307	92.2	113	UNE
Campanula serrata (Kit.) Hendrych	Campanulaceae	KRAM	106	–	27	27	C	22	81.5	22	65	98.5	187	IBPAS
Carex firma Mygind	Cyperaceae	Z	229	103	4	107	A	83 (3)	80.6	79 (4)	223	86.4	58	UCSC
Carex sempervirens Vill.	Cyperaceae	Z	325	140	23	163	A/C	163	100.0	159	474	96.9	121	WSL
Cerastium uniflorum Clairv.	Caryophyllaceae	NE	160	77	2	79	A	48 (1)	62.3	45 (1)	133	90.5	89	UNE
Cirsium spinosissimum (L.) Scop.	Asteraceae	NE	241	121	–	121	A	112	92.6	110 (1)	325	96.7	95	UNE
Dryas octopetala L.	Rosaceae	Z	325	135	23	158	A/C	141	89.2	139 (1)	415	98.1	101	UJF
Festuca carpathica F. Dietr.	Poaceae	KRAM	36	–	17	17	C	9	52.9	9	27	100.0	103	IBPAS
Festuca supina Schur	Poaceae	KRAM	150	32	30	62	C	29	96.7	28 (1)	84	96.6	174	IBPAS
Festuca versicolor Tausch s.l.	Poaceae	KRAM	62	8	22	30	C	17 (5)	77.3	19	56	84.8	181	IBPAS
Gentiana acaulis L.[g]	Gentianaceae	NE	328	132	20	152	A	116	76.3	Not genotyped				UNE
Gentiana nivalis L.	Gentianaceae	NE	278	128	16	144	A/C	92	63.9	80 (2)	235	85.1	154	UNE
Geum montanum L.	Rosaceae	Z	353	137	27	164	A/C	141	86.0	141	420	99.3	93	WSL
Geum reptans L.	Rosaceae	Z	181	79	14	93	A/C	61	65.6	59	177	96.7	61	WSL
Gypsophila repens L.	Caryophyllaceae	Z	297	129	3	132	A	109	82.6	108	321	98.2	94	WSL
Hedysarum hedysaroides (L.) Schinz & Thell. s.l.[h]	Fabaceae	Z	249	108	18	126	A/C	90	71.4	87 (1)	251	93.0	122	UCSC
Hornungia alpina (L.) Appel s.l.[i]	Brassicaceae	WU	266	121	11	132	A	101	76.5	100	293	96.7	225	UW
Hypochaeris uniflora Vill.	Asteraceae	Z	300	104	28	132	A/C	90	68.2	86	257	95.2	94	UJF
Juncus trifidus L.	Juncaceae	Z	265	109	25	134	A/C	119	88.8	114 (2)	338	94.7	88	WSL
Leucanthemum waldsteinii (Sch. Bip.) Pouzar[g]	Asteraceae	KRAM	110	–	29	29	C	27	93.1	Not genotyped				IBPAS
Ligusticum mutellinoides (Cr.) Vill.	Apiaceae	Z	180	84	10	94	A	58 (6)	69.0	60 (3)	170	88.5	95	UCSC
Loiseleuria procumbens (L.) Desv.	Ericaceae	Z	253	116	17	133	A/C	105	78.9	103	309	98.1	121	UJF
Luzula alpinopilosa (Chaix) Breitst.	Juncaceae	NE	246	106	21	127	A/C	106	83.5	101 (2)	302	95.0	218	UNE
Peucedanum ostruthium (L.) W.D. Koch	Apiaceae	Z	283	138	2	140	A	124	89.9	117 (1)	350	94.1	113	UCSC
Phyteuma betonicifolium Vill. s.l.[j]	Campanulaceae	WU	270	124	–	124	A	112	90.3	104	305	90.8	158	UW
Phyteuma confusum A. Kern.	Campanulaceae	WU	47	11	12	23	C	23	100.0	15 (2)	44	63.8	152	UW
Phyteuma hemisphaericum L.	Campanulaceae	WU	210	104	–	104	A	78	75.0	76	225	96.2	234	UW

Table 1. (*continued*)

Taxon	Family	Herbarium	Number of occurrences in IntraBioDiv grid cells											
			Total	Sampling range			Sampling			Genotyping				
				A	C	Total	Range[a]	Cells[b]	Success (%)	Locations[c]	Individuals	Success[d] (%)		AFLPs[e]
Partner[f]														
Primula minima L.	Primulaceae	KRAM	126	42	21	63	C	55	87.3	54	162	98.2	169	IBPAS
Ranunculus alpestris L. s.l.[k]	Ranunculaceae	WU	239	106	12	118	A	91	77.1	86 (1)	255	93.4	434	UW
Ranunculus crenatus Waldst. & Kit.	Ranunculaceae	WU	18	1	12	13	C	9	75.0	9	27	100.0	97	UW
Ranunculus breyninus Cr.[g]	Ranunculaceae	KRAM	219	71	27	98	C	14 (10)	51.9	Not genotyped				IBPAS
Rhododendron ferrugineum L.	Ericaceae	Z	287	139	–	139	A	131	94.2	126	377	95.9	111	UJF
Rhododendron myrthifolium Schott & Kotschy	Ericaceae	KRAM	42	–	18	18	C	18	100.0	18	54	100.0	111	IBPAS
Salix reticulata L.[g]	Salicaceae	Z	270	126	16	142	A/C	107	75.4	Not genotyped				UJF
Saxifraga aizoides L.[g]	Saxifragaceae	Z	335	138	22	160	A/C	143	89.4	Not genotyped				WSL
Saxifraga stellaris L.[l]	Saxifragaceae	WU	281	126	17	143	A/C	119	83.2	113 (2)	319	89.4	190	UW
Saxifraga wahlenbergii Ball	Saxifragaceae	KRAM	12	–	5	5	C	4	80.0	4	12	100.0	127	IBPAS
Sempervivum montanum L. s.l.[m]	Crassulaceae	KRAM	246	100	19	119	(A)/C	11 (14)	57.9	22 (1)	66	88.0	107	IBPAS
Sesleria caerulea (L.) Ard.	Poaceae	Z	399	148	6	154	A	148	96.1	144 (1)	407	91.7	70	UCSC
Soldanella pusilla Baumg.[i]	Primulaceae	KRAM	187	85	12	97	(A)/C	8 (20)	66.7	28	84	100.0	90	IBPAS
Trifolium alpinum L.	Fabaceae	Z	157	73	–	73	A	66	90.4	64 (2)	188	94.9	95	UCSC
Veronica baumgartenii Roem. & Schult.	Plantaginaceae	KRAM	31	2	14	16	C	13	81.3	13	39	100.0	93	IBPAS
Total								3535 (76)		3063 (28)	9039		5377	
Mean (excl. species only partially sampled or not genotyped)									80.3			94.2	134.4	

Genotyping failed in five species, for which respective numbers are missing. A, Alps; C, Carpathians.

[a]Mountain range(s) for which a taxon was thoroughly sampled.

[b]Number of locations sampled in the targeted mountain range(s); number of additionally sampled locations in parentheses.

[c]Number of locations with AFLP data available/number of locations with only 1 individual genotyped and excluded from overall analyses.

[d]Relative to the number of individuals sampled for genotyping (i.e. 3/cell).

[e]Monomorphic markers excluded (monomorphic: marker present in all individuals or present/absent in all but one individual).

[f]University of Vienna (A); UJF, University Joseph Fourier, Grenoble (F); IBPAS, Institute of Botany of the Polish Academy of Sciences, Kraków (PL); UNE, University of Neuchâtel (CH); UCSC, Università Cattolica del Sacro Cuore, Piacenza (I); WSL, Swiss Federal Research Institute WSL, Birmensdorf (CH).

[g]No AFLPs available owing to technical difficulties or time constraints.

[h]Including samples of *H. h.* ssp. *exaltatum* (Kern) Chrtk.- Žert.

[i]Including samples of *H. a.* ssp. *brevicaulis* (Sternb. ex Spreng.) Appel and *H. a.* ssp. *austroalpina* (Trpin) Appel.

[j]Including samples of *P. persicifolium* Hoppe.

[k]Including samples of *R. bilobus* Bertol. and R. *traunfellneri* Hoppe.

[l]Including samples of *S. stellaris* ssp. *robusta* (Engl.) Murr and *S. stellaris* ssp. *prolifera* (Sternb.) Temesy.

[m]Including samples of *S. montanum* L. s.str., *S. carpathicum* Wettst. ex Pordan and *S. stiriacum* Wettst. ex Hayek.

vegetation belts) and on regional endemism was included in the list of HMT. Different groups of taxa restricted to particular biogeographic regions were distinguished (e.g. Pan-Carpathian, Pan-Alpine, Eastern Alpine, South-eastern Carpathian elements). However, only taxa having ranges completely confined to smaller biogeographic regions (i.e. Western Alps, Southern Alps, Eastern Alps, Western Carpathians, South-eastern Carpathians) or having at least 3/4 of their ranges within these regions were considered as endemic elements in subsequent analyses (Rabinowitz, 1981).

Data on the occurrence of HMT per cell were gathered across the entire grid, including additional information on the number of occurrences (frequency) within four sub-squares per cell. However, only the information per cell was used for mapping species distributions and analysing the relationship among the three biodiversity levels. Existing published (Appendix B) and unpublished floristic data were used to fill the IntraBioDiv project database. In the case of critical taxa (i.e. closely related taxa with sparsely distinct or only microscopic diagnostic characters or taxa which were not treated as distinct until recently), herbarium material was consulted to settle a uniform taxonomic treatment of particular taxa across the range and to avoid regional inconsistencies. Two consecutive seasons (2004 and 2005) were used for floristic gap filling in the field. The intensity of field work depended on the degree of existing floristic data in the respective parts of the study ranges, with the main gaps located in the Western Alps and the Romanian Carpathians.

Habitat diversity

Among the numerous habitat characteristics relevant for plants, climatic parameters proved to be the most suitable in the given context, and were selected for further analysis. In order to generate maps of climate variables for the Alps and the Carpathians, we used DAYMET (Thornton et al., 1997), a software environment that analyses daily records of climate variables in a spatial context and allows the interpolation of variables in a spatially explicit manner using climate station records and a 200-m DEM (Geosys Data Inc.). To generate predicted climate values for each pixel in a DEM, DAYMET applies a distance-weighted multiple regression in a circular moving window with the following characteristics (Thornton et al., 1997): (i) stations farther away from the target pixel have less weight than stations nearby; (ii) the independent predictors in the regression are latitude, longitude and elevation; (iii) the grid resolution to read elevation from a DEM into the regression is optimized to the processed climate variable (for precipitation, coarser grids of DEMs usually result in better predictions); (iv) the distance weighting follows a truncated Gaussian kernel, whose shape is optimized beforehand on a yearly basis in a cross-validation and is additionally adjusted for local variation in climate station density. In DAYMET, this regression-based approach is applied to daily maximum (TMAX) and minimum (TMIN) temperature as well as for precipitation (PRCP). Additional variables are either based on derivations from these precipitation and temperature variables or on global solar radiation (SRAD) and ambient water vapour pressure (VPA; Thornton et al., 1997; Thornton and Running, 1999; Thornton et al., 2000).

We used data from a set of climate stations available from the NOAA NCDC global historical surface climate data archive (www.ncdc.noaa.gov/oa/land.html). We supplemented these data with national data where available. In total, we used 977 and 435 stations for the Alps and the Carpathians, respectively. DAYMET was optimized regarding interpolation parameters on a yearly basis, and the total area was split into four tiles ($2 \times$ Alps and $2 \times$ Carpathians) for reasons of disk space and computing capacity. Per tile, each of the five basic climate variables was processed individually, year-by-year on a LINUX cluster of 64 nodes. The raw binary output from DAYMET was then processed in Interactive Data Language (IDL) for further derivations and aggregations. First, we derived additional climate layers on a daily basis: average temperature (TAVE), saturated vapour pressure (VPS), vapour pressure deficit (VPD), relative humidity (RELH), and potential evapotranspiration (ETPT) according to the empirical formula by Turc (1963, Table 2). Next, we summarized all daily climate variables into: (i) monthly and yearly summaries for each year and (ii) a long-term average of these monthly and yearly statistics (period 1980–1989). This time frame was selected because it provided the most complete and dense data coverage, thus avoiding spatial variation owing to varying data availability. Table 2 gives an overview of all 200-m gridded data available. These grids were the basis for analysing and explaining intra- and interspecific diversity patterns.

To express potential habitat diversity within grid cells from the site factors, we assembled their variety into defined classes of similar environments. To do so, we used spatial information on three highly important climatic variables that drive eco-physiological processes considered to constrain the distribution patterns of plants in space, namely (i) temperature in the form of degree-days, (ii) radiation in the form of potential direct radiation and (iii) precipitation based on annual rainfall totals. Radiation and temperature were classified linearly into 18 and nine classes, respectively. Precipitation was nonlinearly classified into nine classes because plant species diversity is not particularly sensitive to changes in high precipitation (i.e. water availability),

Table 2. Original climate layers derived for the project

Short	Name	Aggregation	Software
Direct output (daily)			
Tmin	Minimum temperature	Daily, monthly, yearly	DM, IDL
Tmax	Maximum temperature	Daily, monthly, yearly	DM, IDL
Prcp	Precipitation	Daily, monthly, yearly	DM, IDL
Srad	Global radiation	Monthly, yearly	ArcInfo
VPA	Ambient vapour pressure	Daily, monthly, yearly	DM, IDL
Derived output (daily)			
Tave	Average temperature	Monthly, yearly	ArcInfo
VPS	Saturated vapour pressure	Monthly, yearly	ArcInfo
VPD	Vapour pressure deficit	Monthly, yearly	ArcInfo
RelH	Relative humidity	Monthly, yearly	ArcInfo
ETpT	Potential evapo-transpiration (Turc)	Monthly, yearly	ArcInfo
Further derivatives			
RDay	Number of rain days	Monthly, yearly	IDL
RSiz	Rainfall per rain day	Monthly, yearly	IDL
DDeg	Degreedays (0 and 5.56 °C threshold)	Yearly	ArcInfo
Tmin.s	Standard deviation of Tmin	Monthly, yearly	IDL
Tmax.s	Standard deviation of Tmax	Monthly, yearly	IDL
Tave.s	Standard deviation of Tave	Monthly, yearly	IDL
Prcp.s	Standard deviation of Prcp	Monthly, yearly	IDL
RDay.s	Standard deviation of RDay	Monthly, yearly	IDL
RSiz.s	Standard deviation of RSiz	Monthly, yearly	IDL

The layers *DDeg*, *Srad* and yearly *Prcp* were used to generate the map of habitat diversity. The aggregation of daily data to months and years means that the variables are available as layers for each month and year, and additionally as long-term averages over the whole period. The following software packages were used to process the data: DM: Daymet (Thornton et al., 1997) was used to generate daily 200-m climate rasters from daily climate station data and a 200-m DEM; IDL: the Interactive Data Language (ITT Corp.) was used to statistically process the daily Daymet output into monthly and yearly summaries; ArcInfo (ESRI, Inc.) was used to re-calculate basic maps into new bioclimatic rasters.

while it is very sensitive to changes at the lower end of the water availability gradient (e.g. Wohlgemuth, 1998). We kept a higher number of temperature classes since elevation and its range were expected to be major drivers of diversity in alpine plants. Potential habitat diversity per grid cell was calculated as follows. (i) The three maps of temperature, radiation and precipitation were cut at 1000 and 1500 m a.s.l. (ii) The maps were classified according to the rules given above and (iii) aggregated in a compound map of class combinations (Fig. 2A). (iv) The number of class combinations per grid cell (2–14,137 pixels per cell above 1000 m a.s.l.) was determined in a GIS as an indicator of potential habitat diversity.

Exemplary results

Genetic diversity

In total, we sampled >14,000 individuals from 179 cells representing 3611 populations of the 45 high-mountain plant species. The number of cells sampled for single species ranged from four in *Saxifraga wahlenbergii*, restricted to the Carpathians, to 163 in *Carex sempervirens* (Fig. 3) from both mountain ranges (Table 1).

The sampling and genotyping success rates (Table 1) were determined against the number of cells in which a particular species was recorded in the IntraBioDiv floristic database. In several occasions, species were located in cells for which they were formerly unknown, thus complementing the floristic database. However, more often we were not able to find a population of a given species in a cell where its presence was documented, owing to time constraints. Averaged across species, we obtained samples from 80.3% of expected cells, varying from 100% in *Carex sempervirens*, *Phyteuma confusum* and *Rhododendron myrthifolium* to only 51.9% in *Ranunculus breyninus* (= *R. oreophilus*). Samples were excluded if they failed in DNA extraction or AFLP procedure or if they showed outlier AFLP patterns (i.e. misidentified individuals or PCR failure). The 28 locations (0.9%) where only one individual was successfully genotyped were omitted from overall analyses, while they were retained in species-specific genetic analyses. The resulting success rate in genotyping averaged 94.2% (63.8–100%; Table 1). We acquired genotypic data for up to 29 species

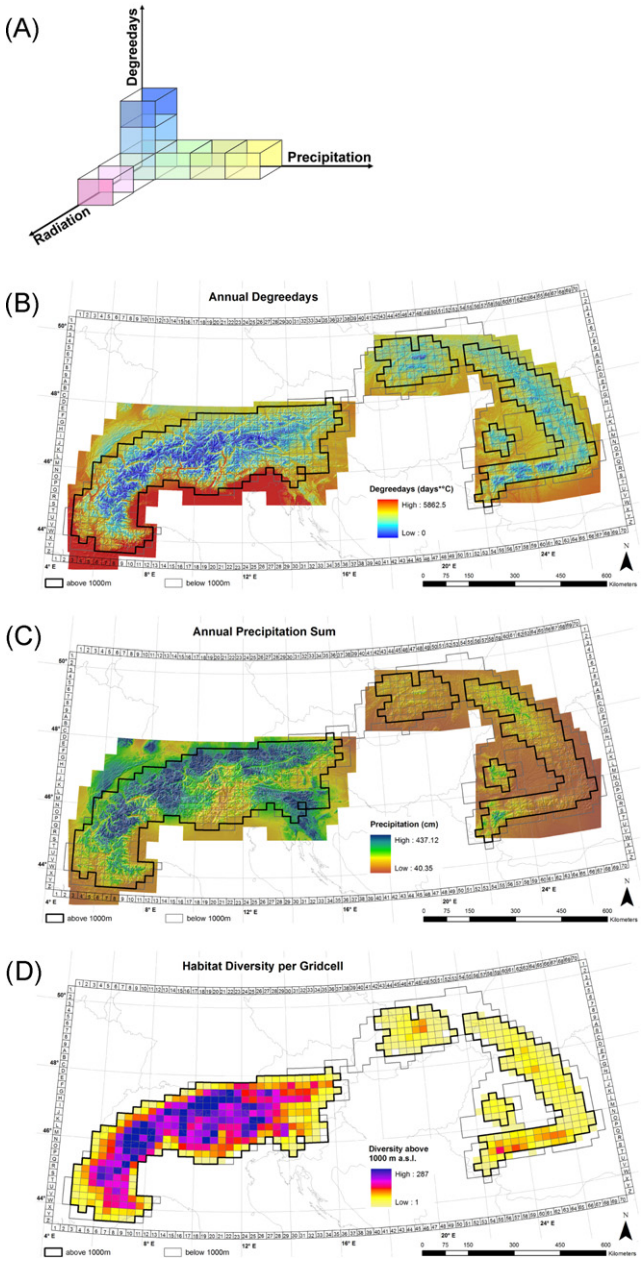

Fig. 2. (A) Illustration of the classification approach for potential habitat diversity mapping. Each colour represents a unique combination of environmental habitat variables. For each grid cell in the study range, we recorded the richness (number) of habitat types above the relevant threshold elevation of 1000 m a.s.l. by GIS overlay. Examples of modelled climate maps based on (B) long-term average degree days and (C) annual precipitation sum and (D) potential habitat diversity for the Alps and the Carpathians at 200-m spatial resolution.

per cell in the Alps and for up to 23 species per cell in the Carpathians (Fig. 1B).

We produced 58–434 polymorphic AFLP markers between 50 and 500 bp per species (mean: 134.4; Table 1; excluding species scored by laboratory UCSC where exact sizing was not feasible). Summed over all polymorphic AFLP markers, individuals, locations and species, but excluding replicates, approximately 1.1 million genetic data points were included in the overall analyses. Rates of AFLP reproducibility within species were above 95% for all species analysed. The AFLP data sets were subsequently used to calculate species-specific population genetic parameters (e.g. Mraz et al., 2007) and to infer genetic structures (Fig. 3).

Overall patterns of genetic diversity in the Alps showed a clear trend of high values along the northern margin and in the middle range of the Alps (Fig. 4A, B). After merging cells at the 2×2- and 3×3-cell level, the large-scale pattern was retained, but more diffuse owing to the disappearance of only local extremes (Fig. 4C–F). When considering only those cells or cell combinations comprising more than ten species (Fig. 4, right column), we observed less edge effects stemming from low sampling density as a consequence of low species occurrence (Fig. 1B).

Species diversity

Altogether, 176,978 records were collected in the floristic database for 1907 HMT in 674 grid cells. The list of taxa included 66 species groups (aggregates), 1468 species (including 349 apomictic species) and 373 subspecies. The number of taxa (Fig. 1A) and the proportion of restricted endemic taxa/species varied widely across countries or mountain ranges. The range size of taxa within the study area, measured as the number of grid cell occurrences, ranged from 1 to 543 (1.5–80.8%). Distribution maps were created for all HMT (IntraBioDiv floristic database; T. Englisch et al., unpubl. data; Fig. 5).

For single grid cells, HMT richness ranged from 11 to 503 in the Alps and from 2 to 335 in the Carpathians, evidently depending on the altitudinal range within cells. The total means of HMT richness per cell were 269.8 for the Alps and 75.9 for the Carpathians (apomictic species not included).

Habitat diversity

All resulting maps of environmental factors (Table 2) were exported to the ArcInfo GRID format. We illustrate these outputs with two examples, namely the long-term average degree days and the annual precipitation sum per cell (Fig. 2B, C). The available maps were used to derive measures of environmental heterogeneity within cells, a surrogate of potential habitat diversity (e.g. Kohn and Walsh, 1994; Fig. 2D).

The annual degree-day map revealed the well-known dependence on elevation, thus resolving the strong climatic (vertical) gradient in temperate mountain systems (Fig. 2B). The map also illustrates that the Carpathians

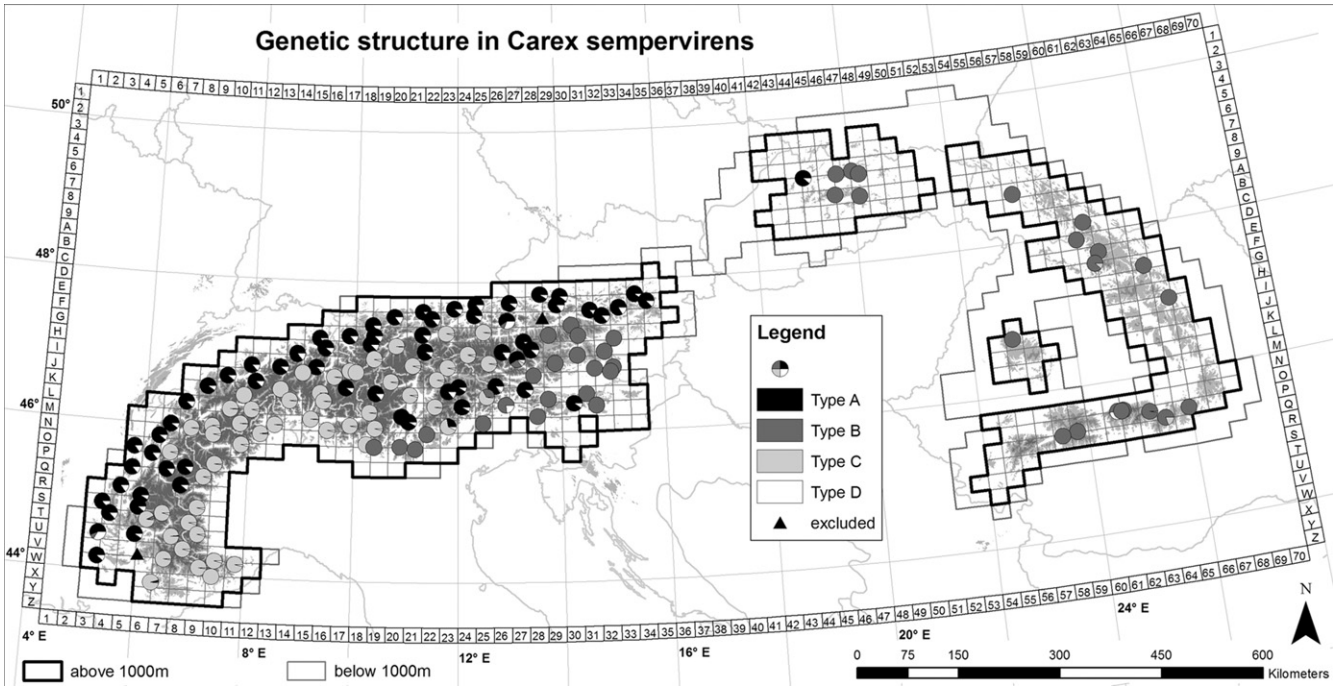

Fig. 3. Genetic structure based on amplified fragment length polymorphisms (AFLPs), exemplified by *Carex sempervirens* in the Alps and the Carpathians. The map displays all locations where plant samples were collected according to the standard grid system (Fig. 1B). Symbols distinguish the locations with successfully genotyped samples (pie chart at exact sampling location) and sampling locations dismissed from the data set owing to sample misidentification or unsuccessful genotyping (triangle). Genetic assignment to four clusters, relying on Bayesian inference (STRUCTURE; Pritchard et al., 2000) based on 121 polymorphic markers, is represented by the partitioning of pie charts.

are composed of several small mountain areas reaching above timberline. On the contrary, the Alps are a more coherent high-altitude ecosystem: only the margin of the Alps shares a similar degree of isolation as the Carpathians. The precipitation map demonstrated the strong humidity gradients in the Alps, while the Carpathians have much lower precipitation in general, and the within-system diversity is also much lower than in the Alps.

The patterns of potential habitat diversity per grid cell (Fig. 2D) reveal strong spatial gradients that generally follow species richness patterns. Grid cells in the centre of the Alps showed higher potential habitat diversity than did grid cells at the edge of the Alps. There were two areas of high habitat diversity in the south-western and in the central part of the Alps coinciding with known centres of floristic richness (Thiel-Egenter, 2007). The highest number of potential habitat richness totalled 287 classes (above 1000 m a.s.l. per grid cell), while the lowest number was one, originating from a cell barely reaching above 1000 m a.s.l.

Discussion

Biodiversity conservation is no longer a field of interest restricted to nature conservation enthusiasts or specialized researchers, but has become a relevant issue on the political and societal agenda. Knowing on the inter-relationships among the three levels of biodiversity, i.e. ecosystems, species and genes, is not only an academic issue, but would be useful for conserving biological diversity. If such inter-relationships exist, conservation research should then identify surrogates that can be assessed reasonably efficient.

To our knowledge, INTRABIODIV represents the first large-scale, multispecies empirical test of correlations among all three levels of biodiversity. The data are unprecedented in terms of range size, sampling density and species number. Maps of potential habitat diversity and of HMT distributions covered the full study ranges, while genetic analyses were restricted to a set of 45 widely distributed species and to only three individuals per species per cell. This, nevertheless, resulted in a consistent genetic data set representing $>80\%$ of the cells in which the respective species occurred (Table 1). Such an integrative study was only possible through the co-ordinated action of more than a dozen specialized research teams with complementary expertises and by building on large and long-term data collections (meteorological stations, floristic mapping).

Besides the specific aim of testing the correlations among the three levels of biodiversity, our data sets provide ample possibilities for further exploitation. These include predictive modelling (e.g. species range

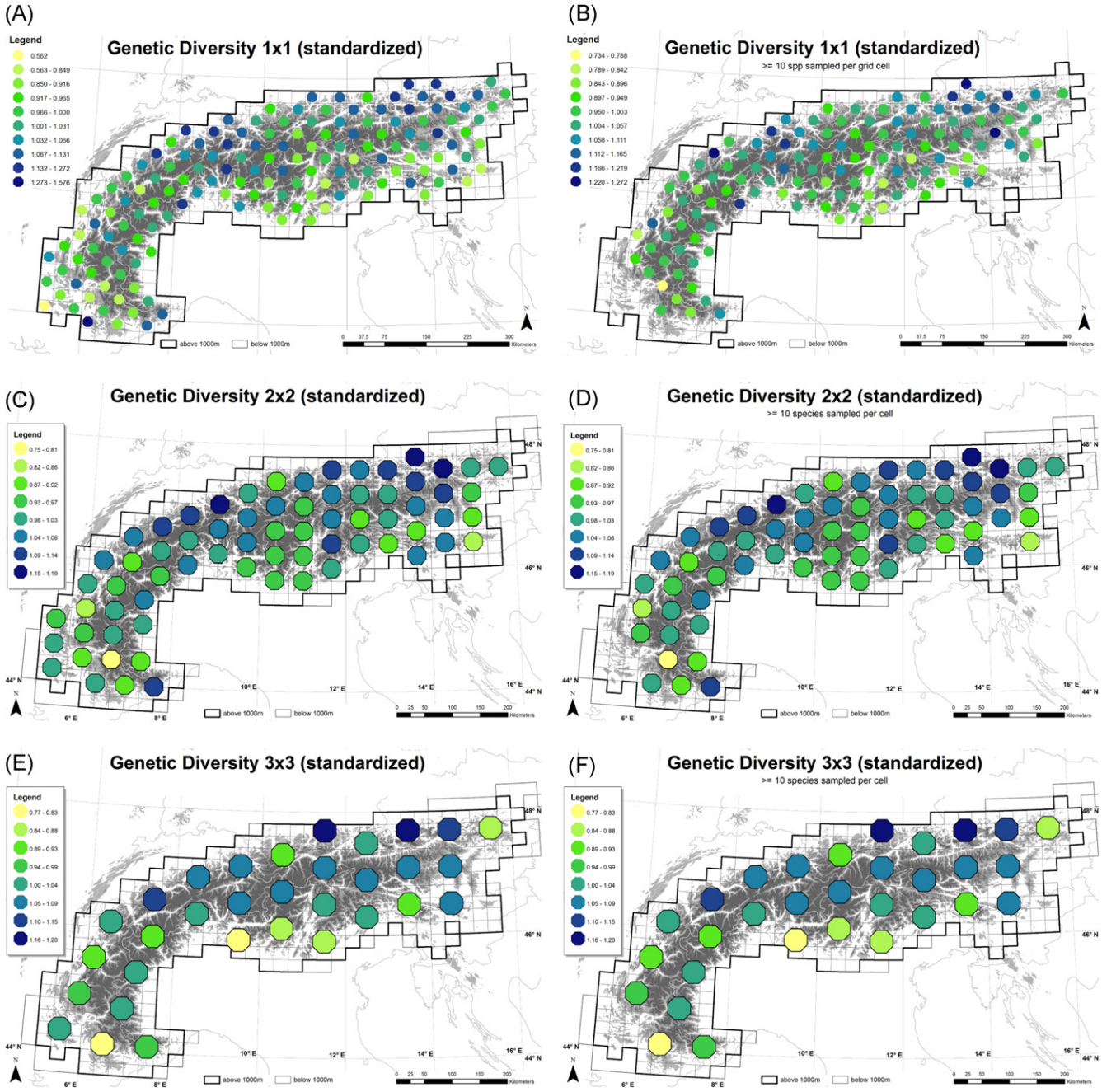

Fig. 4. Average genetic diversity estimated from molecular data of 27 high-mountain plant species sampled across the entire Alps (marked with "A" or "A/C" for sampling range in Table 1). Values were obtained for single cells (1×1; A, B), and for superimposed grids merging 2×2 (C, D) and 3×3 cells (E, F) of the basic grid, respectively. For each grid level, all cells (left column) or only those comprising data of at least ten species (right column) are considered.

shifts due to the on-going climate change), testing of floristic or population genetic hypothesis (Manel et al., 2007; Thiel-Egenter, 2007), describing biogeographical and phytogeographical patterns (Mraz et al., 2007; Thiel-Egenter, 2007; Ronikier et al., 2008; Paun et al., in press), or assessing the conservation status of high-mountain floras (Coldea et al., submitted).

The hitherto unprecedented contributions of the project, freely available for the scientific community, will be detailed distribution maps of the alpine flora of the Alps and the Carpathians, climatic maps as a basis for a multitude of future applications, and AFLP presence/absence matrices of 45 alpine taxa covering their Alpine and Carpathian distribution ranges. The latter offer a wealth of population genetic, phylogeographic and (infraspecific) taxonomic information, which can be used for validating theoretical models. However, owing to the coarse sampling applied for the

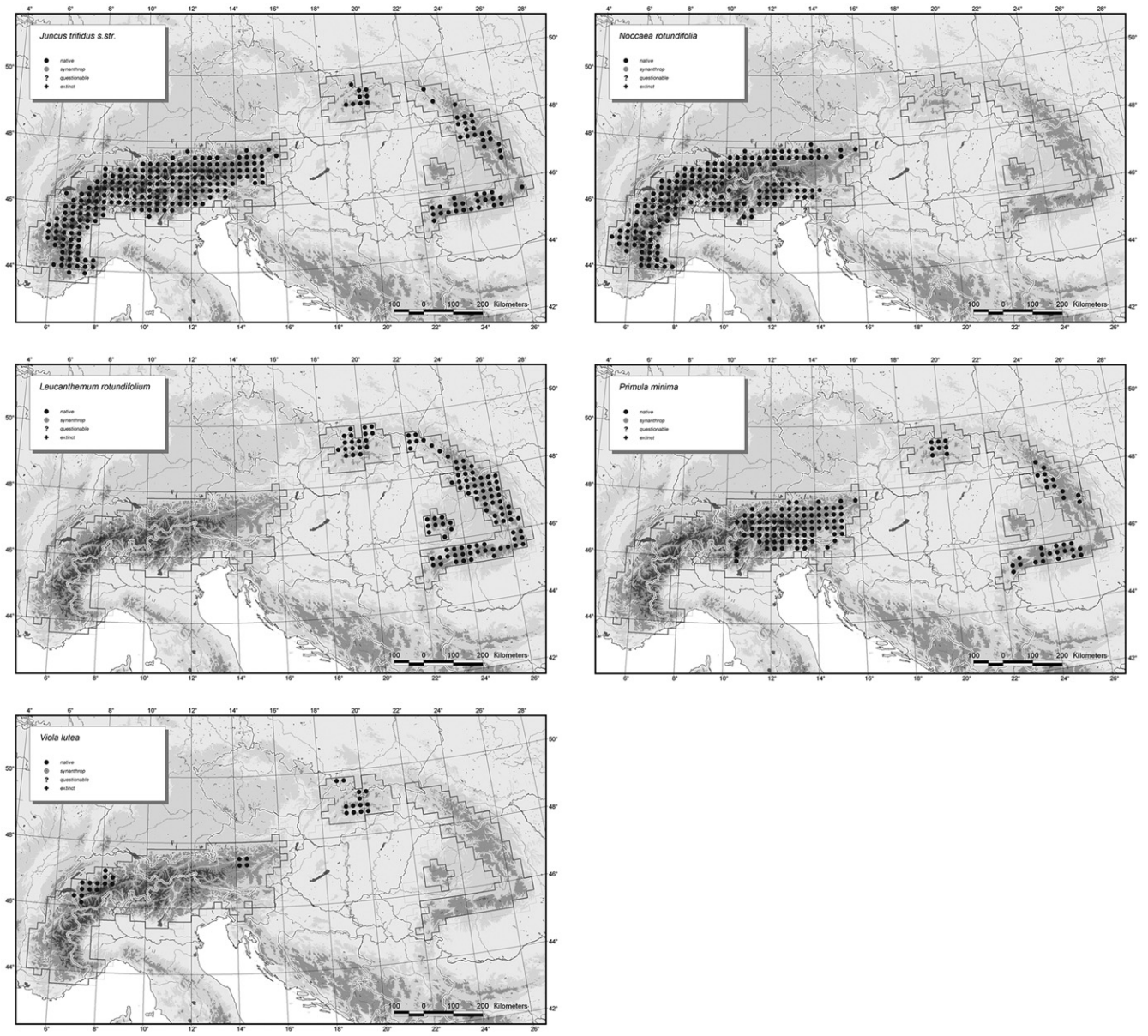

Fig. 5. Examples of characteristic distribution patterns of alpine plant taxa: (A) common to the Alps and the Carpathians (siliceous substrates, *Juncus trifidus*), (B) common to the Alps only (endemic; calcareous substrates, *Noccaea rotundifolia*), (C) common to the Carpathians only (*Leucanthemum rotundifolium*), (D) common to the Carpathians and the Eastern Alps (*Primula minima*), (E) disjunct occurrence with vicariant subspecies: one in the north-western Alps (*Viola lutea* ssp. *lutea*) and one in the eastern central Alps and in the Western Carpathians (*Viola lutea* ssp. *sudetica*).

genetic data, specific questions will require more intense sampling than the IntraBioDiv data can offer.

Prior doubts on only using three individuals per sampling location were dispelled by the clear genetic structures identified for the majority of species analysed (cf. Fig. 3). We take this as evidence that genetic structures can be reliably detected if low sample number per location is counterbalanced by a large number of sampling locations and by the use of many molecular markers. In accordance, our genetic data set could be relevant to infer long-term historical gene flow capacities of species over large distances (Alsos et al., 2007).

We used Nei's (1973) approach of average gene diversity to estimate genetic diversity, which Bonin et al. (2007a) proved most adequate for analysing AFLP genetic diversity. The consistent sampling intensity over the entire study range and across all selected species, in particular the constant sample size per location, ensured an adequate estimation of local genetic diversity. Calculating average genetic diversity on the

superimposed 2×2 and 3×3 grid systems further indicated that the large-scale pattern of diversity was not strongly biased owing to the low local sample number (Fig. 4, right column). This confirms our assumption that the coarse sampling strategy was adequate to represent the large-scale pattern of genetic diversity, while neglecting local phenomena.

Surprisingly, we found the highest average genetic diversity in the Alps along the northern margin, i.e. not in those southern-Alpine areas where glacial refugia are presumed (Stehlik, 2000; Schönswetter et al., 2005). Whether this pattern results from secondary contacts as opposed to diversity retained in glacial survival areas (Petit et al., 2003; Thiel-Egenter, 2007) remains to be investigated. However, we do not consider this as a sampling effect *per se*, since the pattern was retained when we reduced the data to those cells comprising $\geqslant$ ten species (Fig. 4, right column).

Limitations

Limitations in our data sets were mostly due to trade-offs related to limited labour and resource capacities. At the genetic level, we did not assess population size, which is often related to neutral genetic diversity (Allendorf and Luikart, 2007). But since we mainly included abundant species in our sampling, we believe that this effect is negligible at the spatial level of entire mountain ranges and that patterns of genetic diversity are rather related to other factors than current population sizes.

AFLP markers are anonymous, and no information is available on the contribution of organellar genomes to AFLP marker sets. Yet, it is generally assumed that the vast majority of the markers are of nuclear origin, which is supported by studies using AFLPs for parentage analysis (Gerber et al., 2000) or genome mapping (Herrmann et al., 2006). AFLPs are further prone to homoplasy, and some bands may even represent alleles of one locus (Wong et al., 2001). However, we consider such uncertainties as negligible given the large number of markers identified (Table 1). Moreover, the majority of the AFLP markers are likely to be neutral (Scotti-Saintagne et al., 2004). It may thus be argued that selective markers would show different patterns that are governed by habitat-driven processes in a similar way as species are affected (Vellend, 2005). There is no method established to date which would allow us to assess selective genetic variation on large spatial scales. However, new avenues are emerging, e.g. genome scans in relation to environmental factors (Bonin et al., 2006). Accordingly, our genetic data sets bear great potential for further analyses such as the detection of loci under selection, i.e. outlier loci (Bonin et al., 2007b; Holderegger et al., in press).

The floristic data set is constricted owing to some taxonomically difficult and unresolved groups. The analysis of complex taxa would require careful evaluation across their entire natural range (Landolt, 2006), demanding substantial additional taxonomical research. In the light of estimating species richness, however, we consider these restrictions in the floristic data as minor and the data set as homogenous and highly informative.

Restricting our analyses to HMT caused considerable difficulties when linking species richness patterns with potential habitat diversity. Habitat richness thus had to be restricted to areas above timberline per grid cell. However, altitudinal models on vegetation belts, combined with a DEM, allow one to estimate the area, e.g., above timberline per grid cell. Since information on altitudinal range is provided in the list of HMT, area-corrected habitat richess could be related to high-mountain species of certain range preferences.

At last, our results are restricted to a particular group of organisms, namely high-mountain vascular plants. Alpine mammals, birds, bryophytes, insects or any other group of organisms could well display divergent patterns, but this remains to be tested, and our project may serve as a reference for corresponding further studies.

Brief outlook

Despite the limitations mentioned above, we see great potential in our data sets for identifying consistently sampled large-scale patterns. The data give relevant information on the geographic distribution of species and habitats, and on the evolutionary history of species: data required for all-embracing conservation planning (Sechrest et al., 2002). A promise for future research will be the integrated, open-access database comprising data on intraspecific genetic polymorphism in multiple species, data on geographic distributions of more than 1900 HMT and maps of potential habitat diversity derived from several environmental parameters. These data sets will hopefully stimulate further research based on the results from the IntraBioDiv project.

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floristic data used for the project were based on – partly unpublished – materials that had previously been established in the frame of long-term national and regional floristic mapping and documentation schemes. Sincere thanks are due to the collaborators and those responsible for all these schemes for having made available subsets of those invaluable materials, as well as to the numerous voluntary botanists engaged in the respective field work. Without their enthusiasm, our project would not have been possible. We further acknowledge the substantial improvement of a former version owing to the constructive comments of three anonymous reviewers.

Appendix A

Molecular-genetic methods for amplified fragment length polymorphisms (AFLPs; Vos et al., 1995), and fragment length analysis

For abbreviations of the protocols refer to Table A1.

Std – *Standard protocol for DNA restriction*

Approx. 200 ng of genomic DNA were digested in a 20 μL reaction mix containing 2 μL 10 × Buffer 2 (New England Biolabs), 0.2 μL BSA (1 mg/mL), 5U *Eco*RI (New England Biolabs), 2U *Mse*I (New England Biolabs) and complemented with distilled water, placed at 37 °C for 2 h.

Std – *Standard protocol for ligation*

Twenty micro-litres of the digestion mix were mixed with 4 μL T4 DNA ligase buffer (Roche or Promega), 1U T4 DNA ligase (Roche or Promega), 1.44 μL of each 10 μM of *Eco*RI and *Mse*I adapters and distilled water in a total reaction volume of 40 μL. The mix was incubated at 37 °C for 2 h.

Std – *Standard protocol for pre-selective PCR*

In a total volume of 25 μL, we combined 3 μL of the ligation mix (diluted 1:10), 2.5 μL AmpliTaq PCR buffer or Buffer II (Applied Biosystems), 1.5 μL 25 mM $MgCl_2$, 2 μL 1 mM dNTPs (2.5 mM in IBPAS, Kraków), 0.5 μL of each 10 μM pre-selective primer, 0.5U AmpliTaq DNA polymerase (Applied Biosystems) and distilled water.

Thermocyclers were set to initiate amplification at 72 °C for 120 s, followed by 30 cycles with 94 °C for 30 s, 56 °C for 30 s, 72 °C for 120 s, completed by a 10-min final extension at 72 °C.

Std – *Standard protocol for selective PCR*

We used 5 μL of the pre-selective PCR mix (diluted 1:20), 2.5 μL AmpliTaq Gold PCR buffer or Buffer II (Applied Biosystems), 2.5 μL 25 mM $MgCl_2$, 2 μL 1 mM dNTPs, 0.5 μL 10 μM of each selective primer, 0.2 μL BSA (1 mg/mL), 1U AmpliTaq Gold DNA polymerase (Applied Biosystems) and distilled water in a 25 μL total reaction volume.

The profile for the thermal cycler was set to activate the hot-start DNA polymerase at 95 °C for 10 min, followed by 36 cycles with 94 °C for 30 s, a touch-down phase from 65 to 56 °C for 60 s over 13 cycles, and 56 °C for 60 s over the remaining 23 cycles, 72 °C for 60 s, completed by a final extension at 72° for 10 min.

Amplifications were run on the following thermocyclers: Geneamp 9600 Dual Block/2700/2720 (Applied Biosystems) at UJF, Geneamp 9700 (Applied Biosystems) at UCSC, UW and IBPAS, and PTC-100 (BioRad) at UNE and WSL (for partner lab abbreviations see Table A1).

A.1. Alternative protocols for digestion

(D-A) 200 ng genomic DNA was first restricted in a 25 μL total volume containing 2.5 μL 10 × *Taq*I buffer (New England Biolabs), 2.5 μL BSA (1 mg/mL), 5U *Taq*I (New England Biolabs) and distilled water, incubated at 65 °C for 2 h. A second restriction was performed adding 1.5 μL 10 × *Eco*RI buffer (New England Biolabs), 5U *Eco*RI (New England Biolabs) and distilled water in 40 μL in total. Restriction was performed at 37 °C for 2 h.

(D-B) RL buffer (5 ×) was prepared using 500 μL One-Phor-All Buffer PLUS (Amersham Biosciences), 25 μL 1 M DTT, 25 μL 10 mg/mL BSA and 450 μL water. The first restriction mix of 25 μL comprised 100–150 ng genomic DNA, 5 μL 5 × RL buffer, 5U *Taq*I (New England Biolabs) and distilled water, which was incubated at 65 °C for 1 h. A second digestion was performed by adding 3 μL 5 × RL buffer, 5U EcoRI (New England Biolabs) and water, incubating the 40-μL mix at 37 °C for 1 h.

(DL-C) A combined digestion/ligation was carried out in the following 12-μL reaction mix: 200 ng genomic DNA, 1.1 μL 10 × T4 DNA ligase buffer (Promega), 0.55 μL BSA (1 mg/mL), 5U *Eco*RI (Promega), 1U MseI (New England Biolabs), 1.2U T4 DNA ligase (Promega), 1 μL 5 μM *Eco*RI adaptor, 1 μL 50 μM *Mse*I adaptor, 1.1 μL 0.5 M NaCl and distilled water. The mix was incubated at 37 °C for 3 h.

A.2. Alternative protocol for ligation

(L-A) To the 40 μL digestion mix, the following ingredients were added to a total of 50 μL: 2 μL 5 × RL buffer (see above), 1U T4 DNA ligase (New England Biolabs), 1 μL 5 pM *Eco*RI adaptor, 1 μL 50 pM *Mse*I adaptor, 1 μL 10 mM ATP and distilled water. Incubation at 37 °C lasted for 3 h.

A.3. Alternative protocols for pre-selective PCR

(P-A) In 20 μL, we included 5 μL of the ligation mix (diluted 1:10), 2 μL Buffer II (Applied Biosystems),

Table A1. Overview of molecular lab protocols applied for each alpine plant species successfully analysed for amplified fragment length polymorphisms (AFLPs)

Partner laboratory	Species	Digestion	Ligation	Pre-selective PCR	Selective PCR	Selective bases		
UJF, Grenoble (F)	*Arabis alpina*	Std	Std	Std	Std	AAT/CTG	ACT/CTG	ATG/CAC
	Dryas octopetala	Std	Std	Std	Std	AAT/CAC	ATC/CAC	AGC/CAC
	Hypochaeris uniflora	Std	Std	Std	Std	ACA/CAC	ACC/CAG	AGG/CTG
	Loiseleuria procumbens	Std/D-A	Std	Std	Std	AAC/CTG	ACT/CTG	ATG/CTG
	Rhododendron ferrugineum	Std	Std	Std	Std	AAT/CAC	ATC/CAC	ATG/CTG
UCSC, Piacenza (I)	*Carex firma*	D-B	L-A	P-A	S-A	ACA/AAC	ACA/ACG	ACA/AGC
	Hedysarum hedysaroides	D-B	L-A	P-A	S-A	ACA/AAC	ACA/ACG	ACA/AGC
	Ligusticum mutellinoides[a]	D-B	L-A	P-A	S-A	AAT/CAC	ACA/CAC	AGC/CAC
	Peucedanum ostruthium	D-B	L-A	P-A	S-A	ACA/AAC	ACA/ACG	ACA/AGC
	Sesleria caerulea	D-B	L-A	P-A	S-A	ACA/AAC	ACA/ACG	ACA/AGC
	Trifolium alpinum	D-B	L-A	P-A	S-A	ACA/AAC	ACA/ACG	ACA/AGC
UNE, Neuchâtel (CH)	*Campanula barbata*	Std	Std	P-B	S-B	ACA/CTA	AGA/CAC	AGT/CTG
	Cerastium uniflorum	Std	Std	P-B	S-B	ATG/CTA	AGT/CTA	AGA/CTA
	Cirsium spinosissimum	Std	Std	P-B	S-B	ACT/CAC	ATC/CTG	ATG/CTG
	Gentiana nivalis	Std	Std	P-B	S-B	ACT/CAC	ATC/CAC	ATG/CTG
	Luzula alpinopilosa	Std	Std	P-B	S-B	ACC/CTG	AGA/CAC	AGA/CTA
UW, Wien (A)	*Androsace obtusifolia*	DL-C	(DL-C)	P-C	S-C	AAC/CA	ACA/CAT	AGG/CAA
	Hornungia alpina	DL-C	(DL-C)	P-C	S-C	AAC/CA	ACA/CA	ACG/CA
	Phyteuma betonicifolium	DL-C	(DL-C)	P-C	S-C	ACA/CAC	ACC/CAT	ATG/CTG
	Phyteuma confusum	DL-C	(DL-C)	P-C	S-C	AAG/CTC	ACC/CAG	ACT/CTA
	Phyteuma hemisphaericum	DL-C	(DL-C)	P-C	S-C	ACA/CAT	ACC/CAG	ACG/CA
	Ranunculus alpestris	DL-C	(DL-C)	P-D	S-D	AAG/CATA	ACA/CTGA	ACC/CAT
	Ranunculus crenatus	DL-C	(DL-C)	P-D	S-D	AAG/CATA	ACA/CTGA	ACC/CAT
	Saxifraga stellaris	DL-C	(DL-C)	P-C	S-C	AAC/CTT	AGG/CAA	ATC/CT
WSL, Birmensdorf (CH)	*Carex sempervirens*	Std	Std	Std	Std	ACA/CAC	ACA/CTG	ATG/CAG
	Geum montanum	Std	Std	Std	Std	ACA/CAC	ACA/CTG	ACC/CAT
	Geum reptans	Std	Std	Std	Std	ACA/CAC	ACA/CAT	ACC/CAT
	Gypsophila repens	Std	Std	Std	Std	ATG/CT	ACA/CT	ACT/CA
	Juncus trifidus	Std	Std	Std	Std	ATG/CT	ACT/CT	ACA/CA
IBPAS, Kraków (PL)	*Campanula alpina*	Std	Std	Std	Std	ACA/CAC	ACC/CAT	ACT/CAG
	Campanula serrata	Std	Std	Std	Std	AAG/CTG	ACC/CAG	AGA/CAC
	Festuca carpathica	Std	Std	Std	Std	ACA/CAAC	ACC/CAGA	ACT/CTGA
	Festuca supina	Std	Std	Std	Std	ACA/CAC	ACT/CAG	AGG/CAA
	Festuca versicolor	Std	Std	Std	Std	AAT/CAC	ACA/CAC	ACC/CAG
	Primula minima	Std	Std	Std	Std	ACC/CAT	ACT/CAG	AGA/CAC
	Rhododendron myrthifolium	Std	Std	Std	Std	AAT/CAC	ACC/CAT	ACT/CTA
	Saxifraga wahlenbergii	Std	Std	Std	Std	AAG/CTG	ACT/CAG	AGA/CAC
	Sempervivum montanum	Std	Std	Std	Std	ACA/CAC	ACC/CAG	AGA/CTG
	Soldanella pusilla	Std	Std	Std	Std	ACA/CAC	ACT/CTA	AGG/CAA
	Veronica baumgartenii	Std	Std	Std	Std	AAG/CTG ACC/CAT	ACT/CAG AGA/CAC	AGA/CTG ATG/CAA

"Std" refers to the standard protocols, letters indicate the alternative protocols as described in the text of Appendix A.

[a]Processed using *Eco*RI and *Mse*I (1U) enzymes (New England BioLabs) with a restriction temperature of 37 °C and an otherwise similar protocol to that used for *Eco*RI/*Taq*I enzymes.

1.2 μL 25 mM $MgCl_2$, 0.8 μL 5 mM dNTPs, 0.6 μL of each pre-selective primer (50 ng/μL), 0.4U AmpliTaq DNA polymerase (Applied Biosystems) and distilled water.

Thermocyclers ran 30 cycles with 94 °C for 30 s, 56 °C for 60 s, 72 °C for 60 s, followed by a 10-min final extension at 72 °C.

(P-B) The total volume of 20 μL contained 2 μL of the undiluted ligation mix, 2 μL PCR buffer (Promega), 1.6 μL 25 mM $MgCl_2$, 0.5 μL 10 mM dNTPs, 0.5 μL of each 10 μM pre-selective primer, 0.5U *Taq* DNA polymerase (Promega) and distilled water.

The thermal cycler started with 94 °C for 120 s, continuing with 28 cycles at 94 °C for 45 s, 56 °C for 45 s, 72 °C for 60 s and a final extension at 72 °C for 10 min.

(P-C) In 12.5 μL, the PCR mix was made up of 1.5 μL of the undiluted ligation mix, 1.25 μL PCR buffer (Applied Biosystems), 0.75 μL 25 mM $MgCl_2$, 1 μL 10 mM dNTPs, 0.5 μL 5 μM pre-selective primer each, 0.25U *Taq* DNA polymerase (Applied Biosystems), complemented with distilled water.

The thermal cycler program began at 72 °C for 120 s, continued with 30 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s and a final extension at 72 °C for 10 min.

(P-D) The 10 μL-mix contained 2 μL of the undiluted ligation mix, 1.14 μL 10 × RedTaq PCR buffer (Sigma), 0.22 μL 10 mM dNTPs, 0.29 μL 5 μM pre-selective primer each, 0.2U RedTaq DNA polymerase (Applied Biosystems) and distilled water.

The thermocycler was programmed to start at 72 °C for 120 s, followed by 20 cycles of 94 °C for 1 s, 56 °C for 30 s, 72 °C for 120 s and a final extension at 60 °C for 30 min.

A.4. Alternative protocols for selective PCR

(S-A) For the selective PCR mix of 20 μL, we used 5 μL of the pre-selective PCR mix (diluted 1:20), 2 μL Buffer II (Applied Biosystems), 1.2 μL 25 mM $MgCl_2$, 0.8 μL 5 mM dNTPs, 0.5 μL labelled forward primer, 0.6 μL reverse primer (50 ng/μL), 0.4U AmpliTaq DNA polymerase (Applied Biosystems) and distilled water.

The amplification profile consisted of 36 cycles with 94 °C for 30 s, a touch-down phase from 65 to 56 °C for 60 s over 13 cycles, and 56 °C for 60 s over the remaining 23 cycles, 72 °C for 60 s and a final extension at 72 °C for 10 min.

(S-B) The total mix of 20 μL was made up of 3 μL of the pre-selective PCR mix (diluted 1:20), 2 μL PCR buffer (Promega), 1.6 μL 25 mM $MgCl_2$, 0.5 μL 10 mM dNTPs, 0.8 μL of each 10 μM selective primer, 0.5U *Taq* DNA polymerase (Promega) and distilled water in a 25 μL total reaction volume.

The thermal profile was the same like the standard protocol, except that the initiation was at 94 °C for 120 s, the denaturing was only for 30 s and the final extension at 72 °C for 5 min.

(S-C) The total mix of 12.5 μL comprised 2.5 μL of the pre-selective PCR mix (diluted 1:10), 1.25 μL AmpliTaq Gold PCR buffer (Applied Biosystems), 1.25 μL 25 mM $MgCl_2$, 1 μL 10 mM dNTPs, 1 μL 1 μM *Eco*RI primer, 0.5 μL 5 μM *Mse*I primer, 0.1 μL BSA (1 mg/mL), 0.5U AmpliTaq Gold DNA polymerase (Applied Biosystems) and distilled water.

The thermocycler program was identical with the standard protocol.

(S-D) Within 10 μL, there were 2 μL of the pre-selective PCR mix (diluted 1:10), 1 μL RedTaq PCR buffer (Sigma), 0.22 μL 10 mM dNTPs, 0.54 μL of *Eco*RI and *Mse*I primers (1 and 5 μM, respectively), 0.2U RedTaq DNA polymerase (Sigma) and distilled water.

The thermal cycler was programmed with an initial cycle at 94 °C for 2 min, 65 °C for 30 s, and 72 °C for 2 min, continuing with 31 cycles of 94 °C for 1 s, touch-down annealing from 64 to 57 °C for 30 s over eight cycles and 23 cycles at 56 °C for 30 s, denaturing at 72 °C for 30 s and a final extension at 60 °C for 30 min.

A.5. Fragment length detection

We sized the amplified fragments on either automated capillary sequencers (ABI3100 or ABI3100-Avant, Applied Biosystems) using fluorescently labelled forward primers (mostly FAM or 6-FAM, alternatively VIC or NED) and internal size standard ROX500 (Applied Biosystems). UCSC used radioactive labelling and separated the fragments on acrylamide–bisacrylamide gels (29:1, Applichem). *Eco*RI primers were radioactively labelled with ^{33}P by an exchange reaction that transfers the radioactive phosphate in the gamma position of an ATP molecule to the dephosphorilated 5′ end of the primer by T4 polynucleotide kinase.

A mix containing 10 μL of gamma ^{33}P-ATP (10 μCi/μL), 5 μL of T4 buffer 10 × (250 mM Tris HCl pH 7.5, 100 mM $MgCl_2$, 50 mM DTT, 5 mM spermidine 3HCL-form), 1 μL of T4-kinase, 24 μL of H_2O were added to 10 μL of primer diluted at 50 ng/μL. The mix was incubated at 37 °C for 60 min and then heated to 70 °C for 10 min to inactivate the kinase.

Appendix B

References for vascular plant species distributions of the Alps and Carpathians

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