Molecular Ecology (2007) **16**, 2755–2767 doi: 10.1111/j.1365-294X.2007.03315.x

Glacial *in situ* survival in the Western Alps and polytopic autopolyploidy in *Biscutella laevigata* L. (Brassicaceae)

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Abstract

Past climatic changes and especially the ice ages have had a great impact on both the distribution and the genetic composition of plant populations, but whether they promoted speciation is still controversial. The autopolyploid complex *Biscutella laevigata* is a classical example of polyploidy linked to glaciations and is an interesting model to explore migration and speciation driven by climate changes in a complex alpine landscape. Diploid taxa survived the last glacial maximum in several never-glaciated areas and autotetraploids are clearly dominant in the central parts of the Alps; however, previous range-wide studies failed to identify their diploid ancestor(s). This study highlights the phylogeographical relationships of maternal lineages in the Western Alps and investigates the polyploidy process using plastid DNA sequences (*trn*S-*trn*G and *trn*K-intron) combined with plastid DNA length polymorphism markers, which were transferable among Brassicaceae species. Twenty-one distinct plastid DNA haplotypes were distinguished in 67 populations densely sampled in the Western Alps and main lineages were identified by a median-joining network. The external Alps harboured high levels of genetic diversity, while the Central Alps contained only a subset of haplotypes due to postglacial recolonization. Several haplotypes were restricted to local peripheral refugia and evidence of *in situ* survival in central nunataks was detected by the presence of highly differentiated haplotypes swamped by frequent ones. As hierarchical genetic structure pointed to an independent evolution of the species in different biogeographical districts, and since tetraploids displayed haplotypes belonging to different lineages restricted to either the northern or the southern parts of the Alpine chain, polytopic autopolyploidy was also apparent in the Western Alps.

Keywords: Alps, autopolyploidy, homoplasy, microsatellite, nunataks, phylogeography, plastid DNA, post-glacial recolonization

Received 10 November 2006; revision accepted 5 February 2007

Introduction

Climatic oscillations that occurred during the Quaternary had several consequences on species' evolution (Hewitt 2004), although adaptation to new niches is not fully understood in this context (Davis & Shaw 2001). Most surviving species were forced to shift their range in order to track their ecological space, and several phylogeographical studies on lowland species have pointed to the importance of the last glacial maximum (LGM) in shaping intraspecific genetic diversity (Comes & Kadereit 1998; Taberlet *et al*. 1998; Hewitt 2004). In Europe, evidence from various studies have jointly attested that the Iberian and Italian peninsulas, as well as the Balkans have played a refugial role during the LGM and acted as major sources for many recolonizing species (*Tabula rasa* hypothesis; Hewitt 2000; Petit *et al*. 2002). Species that have responded to the LGM in such a way often harbour a high degree of genetic diversity in these refugial areas (i.e. regional hotspots of allelic richness; Widmer & Lexer 2001). Due to the recurrent founder effect affecting colonizing populations, it is expected that genetic diversity decreases when distance from the refugia increases, and this produces a long-lasting genetic signature that can be used to identify major recolonization routes (Hewitt & Ibrahim 2001). Nevertheless, this pattern

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can be obscured by secondary contact of vicariant lineages which results in admixing populations showing high heterozygosity and genetic diversity (melting spots; Petit *et al*. 2003). Whether climate-enforced range-shifts promote speciation through genetic differentiation in isolation or, in contrast, promote evolutionary stasis by increasing gene flow (Willis & Niklas 2004) is a central question of historical biogeography (Wiens & Donoghue 2004). Already, several studies have generally redefined the 'centre-periphery model' from showing mainly demographical stochastisity under equilibrium to a more dynamic view emphasizing the actual consequences of past climate-driven range-shifts. This change of perspective has helped to the further understanding of the fate of taxa undergoing climate change (Hampe & Petit 2005).

Alpine phylogeography

In phylogeography, the Alps have traditionally been seen as a major barrier blocking the advancement of lineages along their recolonization routes (e.g. Taberlet *et al*. 1998). However, due to its complex topography, the Alps are also a melting zone of lineages for several trans-alpine recolonizing species (Gugerli *et al*. 2001; Mátyás & Sperisen 2001). Several herbaceous alpine plants showed remarkable resistance to climate changes and this ability to survive the LGM in the Alps was much discussed in the last century (nunatak hypothesis; reviewed in Stehlik 2000). Phylogeographers have recently begun attempts to reconstruct the evolutionary history of species for which the Alps are the main habitat (Stehlik 2003), but are hampered by certain difficulties in their investigations such as scarce fossil evidence, chorological analyses giving ambiguous interpretations and cytogeographical evidence obscured by insufficient sampling. Hence, the nunatak hypothesis remains the focus of a long-standing debate. Recently, this debate has been revived aided by results obtained from the development and utilization of molecular markers in phylogeography. The presence of central nunataks, situated in the middle of the highly glaciated Alps, was recently attested by molecular phylogeography and several putative perialpine refugia were shown to favour the LGM survival of species (Schönswetter *et al*. 2005). Precise recolonization routes within the Alps remain obscure for most of the phylogeographical surveys because alpine topology mainly requires dense sampling and fast evolving markers in order to achieve sufficient resolution. In addition, *in situ* glacial survival is also supposed to result in ambiguous genetic signals caused by complex postglacial history (Hewitt 2004).

Polyploidy and glaciations

Polyploidy is a known, frequent process in plants and, as it is a major mechanism for abrupt speciation, it strongly affects their evolution (Levin 2002). The origin of polyploids, as well as their evolutionary advantages, are not yet fully understood (Ramsey & Schemske 1998; Comai 2005). However, natural young polyploids have often been interpreted as having periglacial origin (Favarger 1967), and thus offer an outstanding opportunity for investigations into the links between climatic events and speciation. Stebbins (1984) proposed the secondary contact hypothesis to explain the origin of periglacial polyploids postulating that LGM-climate-enforced range-shifts have promoted secondary contact between formerly allopatric taxa, and resultant hybrids have had to evolve through chromosome doubling to restore fertility. In the Alps, polyploidy studies that involved hybrids between fully differentiated species (i.e. allopolyploidy) were congruent with this hypothesis (Widmer & Baltisberger 1999). The secondary contact hypothesis can also explain the glacial origin of autopolyploid complexes, whereby the LGM had promoted the accumulation of genomic differences in refugial isolation and the subsequent chromosome doubling of the genomes of infraspecific hybrids. These polyploids are called segmental allopolyploids even though their meiotic behaviour resembles that of autopolyploids (i.e. with several multivalents).

Evolutionary advantages possessed by autopolyploids is still controversial (Ramsey & Schemske 2002; Comai 2005) where autopolyploids have largely been interpreted as heading towards evolutionary dead ends (but see Soltis & Rieseberg 1986). Frequent among alpine species, autopolyploids are often observed in young habitats (Favarger 1971). Some authors explained the success of autopolyploids after the LGM through purely historical reasons (Van Dijk & Bakx-Schotman 1997) while others invoked adaptive processes and attributed success to their inherent high genetic diversity (Soltis & Soltis 2000). Several autopolyploids were shown to have originated at multiple occasions from slightly different progenitors, leading to high levels of allelic diversity (Van Dijk & Bakx-Schotman 1997; Soltis & Soltis 1999). Furthermore, polysomic inheritance maintains high levels of heterozygosity and could strengthen the colonizing ability of these polyploids by buffering against recurrent founder effects, such as acting to reduce inbreeding depression (Ronfort 1999; Soltis & Soltis 2000). The hypotheses mentioned above are not mutually exclusive and should all be taken into account when trying to understand the advantage conferred by autopolyploidy in a natural setting.

The Mediterranean genus *Biscutella* (Brassicaceae) is composed of well-established clades (Olowokudejo 1986), two of which are of interest here. The first consists of an ancestral stock of annual species with a basic chromosome number of $n = 8$ and fragmented circum-Mediterranean distribution to which *B. didyma* belongs (Manton 1932). The second clade (subgenus *Biscutella*, series *laevigatae*) is

characterized by a basic chromosome number of $n = 9$ and represents diverse perennial species. *Biscutella laevigata* is the only species of the genus which range extends outside of the Mediterranean Basin to become a true (ad-) alpine taxon (Schönfelder 1968). It is found over most of the altitudinal gradient of the Alps in different open habitats. From early range-wide studies of Manton (1934, 1937), *B. laevigata* was thought to have colonized the Alps before the LGM in a diploid form ($2n = 2x = 18$) and then, pushed away by growing ice sheets, survived the Pleistocene vicissitudes in ice-free parts of the European continent. According to this *Tabula Rasa* scenario, this species recolonized the Alps as an autotetraploid ($2n = 4x = 36$) from extra-alpine diploid populations when the climate became favourable after the Würm maximum. Although this broad evolution of the autopolyploid complex has recently been confirmed by a range-wide study using allozyme markers (Tremetsberger *et al*. 2002), the origin of the tetraploids is still unclear. Tetraploids of *B. laevigata* ssp. *laevigata* clearly form a genetically diverse but coherent group, while diploids are split in three distinct clades. Most of the Austrian lowland and alpine populations of the diploid *B. laevigata* ssp. *austriaca* form a genetically coherent group that apparently diverged early from the other taxa. Most of the other diploid populations are found in lowlands north of the Alps (Fig. 1) and form two distinct evolutionary groups, named the northeastern and northwestern groups by Tremetsberger *et al*. (2002). The Austrian and the northeastern groups are phylogenetically well defined but only distantly related to the tetraploids, while the western populations (mainly, *B. laevigata* ssp. *varia*) form a loose core group linking diploids and tetraploids. Tremetsberger *et al*. (2002) also pointed out that a diploid taxon recently described by Raffaelli & Baldoin (1997) in the south of the Alps (*B. prealpina*) is more closely related to tetraploids than any other diploid group. From the range-wide study of Tremetsberger *et al*. (2002), it remains questionable whether the alpine autotetraploids of *B. laevigata* have originated from the northwestern and/or southern diploid populations.

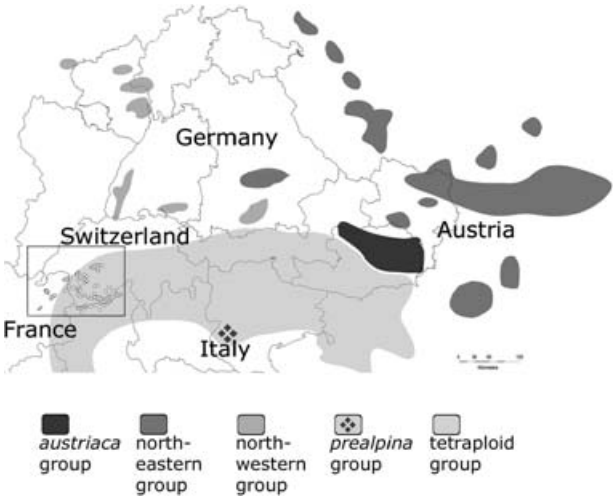

Fig. 1 Distribution of diploid and polyploid taxa in the *Biscutella laevigata* autopolyploid complex, following Tremetsberger *et al*. (2002). Black, dark-grey and medium-grey areas delimit, respectively, the extension of the diploid taxa: *B. laevigata* ssp. *austriaca*, the northeastern diploid and northwestern diploid (incl. *B. laevigata* ssp. *varia*) evolutionary groups. Alpine tetraploid *B. laevigata* ssp. *laevigata* is figured in light-grey and the location of the southern diploid *B. prealpina* is shown by small squares. The study area (named the Western Alps) is delimited by a thick rectangle.

As mentioned, probably due to complex alpine topography and history, previous range-wide studies have failed to clarify the evolutionary history of *B. laevigata*, hence a finer resolution obtained from dense sampling may result in deeper insights. The Western Alps investigated in this study provided an interesting framework representing several putative peripheral refugia and central nunataks. Furthermore, large internal valleys and high mountain chains contribute to make this area a suitable model to explore the evolutionary history of alpine species. As a frequent species that is representative of alpine meadows, *B. laevigata* is also a suitable model species to investigate the link between the ice ages and speciation through polyploidy, and the eco-genetic potential of a young autotetraploid species in its natural habitat. Adopting a dense sampling strategy and phylogeographical analysis of maternal lineages using highly variable plastid molecular markers, this study aimed to: (i) show that the species had diffuse LGM survival in multiple refugia in the Western Alps; (ii) identify the postglacial recolonization pathways within the Western Alps; and (iii) demonstrate that *B. laevigata* evolved by polytopic autopolyploidy.

Materials and methods

Sampling and DNA extraction

The study area (Western Alps) consists of four natural districts whose division is based on floristic data (Theurillat *et al*. 1993) that was further divided in putative glacial refugia on the one hand, and highly glaciated areas on the other hand (reviewed in Stehlik 2000). All the putative refugia and the four districts have been extensively surveyed by field trips and, even if *B. laevigata* was rare in the Vercors-Chablais-Chartreuse district, populations have been sampled in most putative refugia (14 in peripheral refugia and 7 in central nunatacks). Four individuals from 67 populations, respectively (a total of 268 individuals) were sampled in the field and immediately dried in silica gel. Location details are reported in supplementary material. Four individuals of *B. laevigata* ssp. *varia* (Mainz, Germany; leg. J.W. Kadereit) representing the northwestern evolutionary group of Tremetsberger *et al*. (2002), and three

individuals of *B. prealpina* (Recoaro, Italy; leg. M. Raffaelli) were included in the present investigation as extra alpine diploid outgroups in order to assess their putative parentage to the tetraploid *B. laevigata* ssp. *laevigata*. One Italian individual of the *B. laevigata* ssp. *lucida*, the only other tetraploid subspecies accepted (Raffaelli 1992) was also included. Finally, one individual of the distantly related species *B. didyma* (Crete, Greece; leg. F. Brüssow) was included as an outgroup of the *B. laevigata* complex.

Total DNA was extracted using the FastDNA kit (Q-Biogen) following the manufacturer's instructions, except that extracted DNA was washed twice. DNA quality and concentration were checked on agarose gels before further treatment.

Plastid DNA SSR markers

According to Petit *et al*. (2005a), long uninterrupted tracts of A/T can be suspected to present high mutation rates and thus represent good candidates to reveal infraspecific plastid polymorphism among maternal lineages. Such plastid chloroplast simple sequence repeat (cpSSR) DNA markers were developed by using the *Arabidopsis thaliana* and *Sinapis alba* sequences published in databanks. The *A. thaliana* sequence was manually screened to localize highly repeated mononucleotide motifs (i.e. more than 12 repeats) and homologous sequences were checked in *S. alba* to ensure that the putative cpSSR markers were conserved between these two model species. As the former two species are not closely related within the Brassicaceae (Koch *et al*. 2001), conserved poly A/T tracts found were surmised to be present also in *B. laevigata* and primers were designed in the conserved flanking regions. This procedure produced four putatively polymorphic cpSSR markers in *B. laevigata* (Table 1). The four markers, named Bras1 to Bras4, were PCR-amplified among different Brassicaceae species, indicating wide cross-species transferability within the family (*Capsella bursa-pastoris*, *Arabis hirsuta*, *Erophila verna*, *Brassica napus*, *Cardamine pentaphylla*, *Sinapis arvensis*, *Thlaspi arvensis*, *T. caerulescens*, *A. thaliana*; data not shown).

Table 1 Characteristics of the cpSSR markers developed in this study for *Biscutella laevigata* and transferable amongst the Brassicaceae. Size refers to the range of fragment length in *B. laevigata*

Code	Plastid location	Primers sequences (5′– 3′)	Size
Bras1	*trnE—trnT*	F: TCTTTTAAAGAAGTGATTGGTC	102–104
		R: TCTTAACAATGAGATGAGGC	
Bras2	*trnK*—intron	F: AAATTCGAATGGAAGCTCG	177–186
		R: GTATCAAGGGAGAATTCAGATAAC	
Bras3	*trnS—trnG*	F: AACCCTTCTCCACTTTATTC	210–239
		R: GTAAATAGAATTACTGAGAAAGTTC	
Bras4	*trnS—trnG*	F: GTCCACTCAGCCATCTCTCC	155–159
		R: TCGAACAAAGTAATCGGGAGTG	

Amplifications were performed in a 20 μL total reaction volume with 1× Q-biogen *Taq* buffer (containing 1.5 mM $MgCl_2$), 2.5 mM dNTPs, 10 μM of each primer, 1 U Q-Biogen *Taq* polymerase and about 10 ng of genomic DNA. Samples were amplified on a Biometra-T3 thermocycler with a denaturing step of 94 °C for 180 s, 36 cycles of 94 °C for 45 s, 53 °C for 45 s, 72 °C for 45 s and a final elongation cycle of 72 °C for 600 s. PCR-products were diluted 20 times and visualized using a ABI-PRISM 377 sequencer on 6% Long Ranger denaturing gels. Fragment sizes were estimated using the Genescan-350 ROX standard and scored using GENESCAN 3.1.2 (Applied Biosystems). This procedure was repeated on independent DNA extractions from 10 individuals to prove the reliability of the markers. Amplifications and scoring were repeated until all cpSSR loci were unambiguous for all individuals.

Sequencing

All different haplotypes from the multilocus cpSSR screening, as well as those from geographically distant individuals with identical haplotypes, were sequenced for a total of 53 individuals at two loci to better characterize the polymorphism and to detect putative homoplasy. The *trn*S-*trn*G region, including Bras3 and Bras4 (F: 5′-GATTCCTATCTAATGATCCAG-3′; R: 5′-GATCGGAAGATTAATCAAACC-3′), and the *trn*K–intron (Bras2; Table 1) were amplified following the procedure described above and purified using the FastPrep purification kit (Qiagen inc.). Products were directly sequenced using the Big Dye 3.1 Terminator cycle sequencing kit (Applied Biosystems) and analysed using the BIOEDIT freeware (Hall 1999). Sequences have been deposited in EMBL under accessions AM258996-AM259047 for *trn*S-*trn*G and AM259068-AM259117 for the *trn*K-intron.

Data analysis

The sequences were aligned using CLUSTALW software (Thompson *et al*. 1994). Indel and nucleotide polymorphisms were coded as present/absent and length variation at SSR loci were coded as multistate characters (alignment available from the authors upon request). Loci with a high mutation rate (SSR markers) were given a weight of 1, while indels and substitutions were given a weight of 2. Following Cassens *et al*. (2003), a median-joining network with a maximum parsimony postprocessing (Bandelt *et al*. 1999) was used to visualize phylogenetic relationships among the plastid haplotypes, because it allows the incorporation of alternative genealogies and generates 'median vectors' that represent unsampled or extinct haplotypes. The complete set of haplotype sequences was

used for the analysis and the network was computed using NETWORK 4.112 (www.fluxus-engineering.com). The tolerance parameter ε was set to zero, so that the branches connecting haplotypes represent the strict minimum number of mutations. Trials with relaxed ε were also explored but gave similar network topologies, suggesting a low amount of homoplasy in the dataset.

The statistical phylogeographical signal was estimated through the comparison of genetic structure with unordered alleles (G_{ST}) and ordered alleles (N_{ST}), as described in Pons & Petit (1996). N_{ST} takes the phylogenetic differentiation of haplotypes into account when estimating the genetic structure and thus uses the pairwise nucleotide differences among haplotypic sequences (Grivet & Petit 2002). Using DISTON 1.0 and PERMUT 1.0 software (http://www.pierroton.inra.fr/genetics/labo/Software), N_{ST} has been estimated and then compared to the G_{ST} and to zero by a 10 000 permutation test. Finally, using ARLEQUIN (Schneider *et al*. 2000) hierarchical G-statistics were computed by sorting the populations into: (i) putative refugial (21 populations, see supplementary material) vs. glaciated areas (46 populations); and (ii) natural districts (Theurillat *et al*. 1993), to evaluate the internal genetic structure in the dataset.

Patterns of isolation by distance were tested following Rousset (1997) by Mantel tests between a matrix of pairwise genetic differentiation among populations and a matrix of geographical distances among populations. In order to check for isolation by distance, pairwise G_{ST}, N_{ST}, $G_{ST}/(1 - G_{ST})$ and $N_{ST}/(1 - N_{ST})$ were investigated in the whole sample and in the refugial populations only. Tests were performed with 10 000 random permutations with SPAGEDI 1.1 (Hardy & Vekemans 2002).

Results

Molecular investigations

The four plastid cpSSR markers, Bras1 to Bras4, allowed the detection of 20 different haplotypes in the 268 individuals screened from the Western Alps (Table 2). The nine individuals included as outgroups showed six additional haplotypes. The four diploid individuals of subspecies *varia* were characterized by the same haplotype

Table 2 cpSSR profiles of the 21 haplotypes sampled in *Biscutella laevigata*. Capital letters represent the lineage of each haplotype in the Western Alps (Fig. 3) and VARIA, PRE 1–3, LUCIDA and DIDYMA show the six outgroups haplotypes (*B. laevigata* ssp. *varia*, *B. prealpina*, *B. laevigata* ssp. *lucida* and *B. didyma*, respectively). n stands for the number of individuals sequenced for each haplotype, and N represents the number of populations where corresponding haplotypes were found. The frequency of each haplotype in the Western Alps is presented below. *The haplotypes Ec1 and Ec2 had the same cpSSR profile but were shown to have slightly different DNA sequences (see text)

	Haplotypes								
	Aa	**Ab**	**Ba**	**Bb**	**Ca**	**Cb**	**Da**	**Db**	**Dc**
n	**1**	**1**	**1**	**1**	**1**	**2**	**9**	**1**	**1**
N	1	1	1	2	1	1	9	1	3
Frequency	0.01	0.01	0.00	0.01	0.01	0.01	0.12	0.01	0.03
Bras1	102	102	103	103	103	103	104	104	104
Bras2	177	177	179	180	184	184	186	185	185
Bras3	210	224	222	212	239	229	211	211	211
Bras4	158	157	156	155	156	156	157	158	157
	Ea	**Eb**	**Ec1**	**Ec2**	**Ed**	**Ee**	**Ef**	**Eg**	**Eh**
n	**1**	**15**	**1**	**2**	**1**	**1**	**1**	**1**	**1**
N	7	41	5*		1	2	1	1	1
Frequency	0.10	0.52	0.07		0.00	0.01	0.01	0.01	0.00
Bras1	103	103	103	103	103	104	103	103	103
Bras2	186	186	186	186	186	186	184	185	185
Bras3	212	212	212	212	226	212	212	212	212
Bras4	155	156	157	157	156	156	157	157	158
	Fa	**Fb**	**Fc**	**VARIA**	**PRE1**	**PRE2**	**PRE3**	**LUCIDA**	**DIDYMA**
n	**3**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
N	4	1	1	–	—	—	—	—	—
Frequency	0.02	0.00	0.00	–	—	—	—	—	—
Bras1	102	102	102	102	104	103	104	103	102
Bras2	178	178	178	179	187	183	180	186	179
Bras3	229	229	229	217	211	217	210	214	216
Bras4	159	158	157	157	156	154	156	155	137

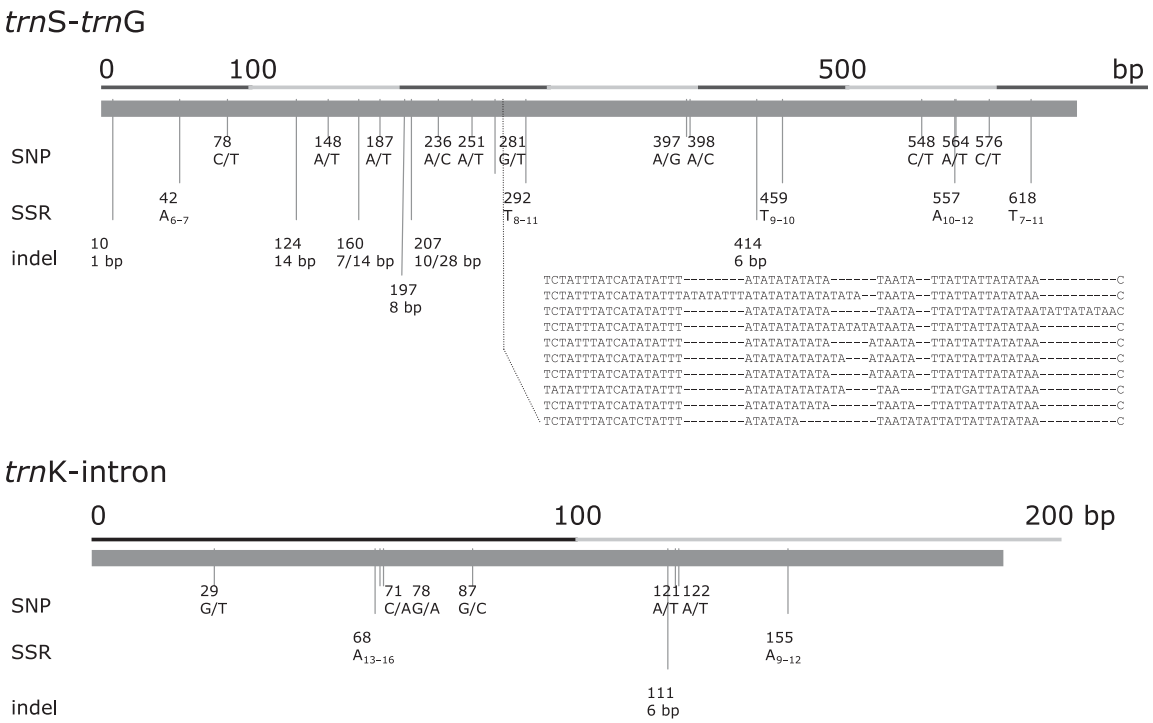

Fig. 2 Schematic representation of the plastid DNA regions sequenced in *Biscutella laevigata* and their multistate polymorphism. The thin horizontal line represents the scale in base pair and the broad line shows the DNA fragment. *trn*S-*trn*G and *trn*K-intron fragments show three types of polymorphism indicated at its position: SNPs (substitution, given by the base change); SSRs (microsatellites, with type and variation in repeat number); indels (insertions/deletions, indicated with their sizes). The alignment of a complex part is shown below *trn*S-*trn*G.

(VARIA), while the three *B. prealpina* diploids each revealed a new haplotype (PRE1 to PRE3). The sequencing of the different haplotypes at two loci was largely congruent with the multilocus cpSSR screening and allowed their phylogenetic divergence to be estimated. Together, the 660-bp *trn*S-*trn*G sequences and the 190-bp *trn*K-intron sequences from 52 individuals of the *B. laevigata* complex had 39 polymorphic sites (30 + 9, respectively; Fig. 2), among which 7 (5 + 2) were mononucleotide SSRs, 1 (1 + 0) was a dinucleotide SSR, 11 (10 + 1) were indels ranging from 1 to 28 bp, and 20 (14 + 6) were single nucleotide substitutions (Fig. 2).

Detailed molecular investigation revealed only two slight differences between the cpSSR screening and the sequencing: (i) Eb and Ee haplotypes presented different cpSSR profiles (nonsequenced Bras2 locus) though their sequences were identical; and (ii) although they presented identical multilocus cpSSR profiles, the haplotype Ec1 found in one population has a close but different *trn*S-*trn*G sequence from the Ec2 haplotype. This homoplasy can be explained by the fact that the Bras4 marker is composed of two closely linked SSR loci (Fig. 2). Thus, the Ec1 haplotype presents an 11 poly A (position 557) and a nine poly T (position 618), while the Ec2 haplotype is characterized by a 12 poly A and an eight poly T. These two haplotypes were thus considered identical for the statistical phylogeographical analysis. Altogether, the *B. laevigata* complex showed 21 haplotypes in the Western Alps and the molecular markers allowed the discrimination of 27 haplotypes in the whole sample including the outgroups. Only four haplotypes (Ea, Eb, Ec and Da) were frequently sampled (> 5%) and the haplotype Eb characterized 52.2% of the individuals. Seventeen other haplotypes were rare (< 5%), among which the haplotypes Ba, Eg, Fb, and Fc have been sampled in only one individual.

Molecular phylogeography

The haplotype network (Fig. 3) displayed a nonambiguous topology of the 27 haplotypes sampled. *Biscutella didyma* presented 47 characteristic mutations and was only loosely connected to the *B. laevigata* complex, but allowed a certain polarization of the relationships among haplotypes. In the complex it is worth noting that the diploid outgroups (*B. laevigata* ssp. *varia* and *B. prealpina*) were not in basal position. Nevertheless, *B. prealpina* presented larger haplotype diversity than *B. laevigata* ssp. *varia*. Haplotype PRE1 was closely related to the D haplotypes, and PRE2, PRE3 and the VARIA haplotypes were close to other Western Alps haplotypes. The haplotype of the tetraploid *B. laevigata* ssp. *lucida* was strongly connected to some Western Alps haplotypes (lineage E).

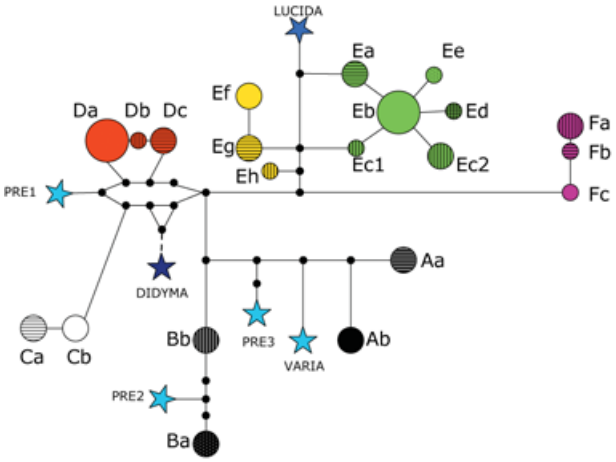

Fig. 3 Median-joining network with maximum parsimony of the plastid haplotype of *Biscutella laevigata* in the Western Alps. Outgroups are figured as blue stars (diploids: DIDYMA for *B. didyma*; VARIA for *B. laevigata* ssp. *varia*; PRE1, PRE2 and PRE3 for *B. prealpina*; tetraploids: LUCIDA for *B. laevigata* ssp. *lucida*). The 21 haplotypes sampled in the Western Alps are coloured according to the lineage (A to F) they belong to and dashed differently. The size of the pie chart is proportional to the frequency of each sampled haplotype (small, 1–3 individuals; medium, 4–20 individuals; large, > 20 individuals). Median vectors (i.e. unsampled or extinct haplotypes) are presented as small black circles.

The 21 haplotypes sampled in the Western Alps form six major groups of related haplotypes (lineages: A, B, C, D, E and F; Fig. 3) that were loosely connected by long branches (i.e. differentiated by several mutations). Multiple ploidy levels were observed in several lineages (Fig. 3). Lineages did not show similar geographical expansion (Fig. 4). Lineages B and C were restricted to putative peripheral refugia (STO, MOB and PAR, PAS), while lineages F (CHA, GRB, MOI, MOG, VID) and A (SIM and SAA) were mostly restricted to the area of central nunataks. Two lineages were widespread in the Western Alps (E and D). The first lineage was common in the northern part of the sampling area (Fig. 4) and presented a large diversity with nine different haplotypes. Haplotype Eb displayed a central position in the lineage E and was the most frequently sampled. Lineage D was common in the southern part of the Western Alps.

Statistical phylogeography

The coefficients of genetic differentiation among the 67 sampled populations of four individuals were significantly higher than zero: $G_{ST} = 0.840$ ($P < 0.001$) and $N_{ST} = 0.858$ ($P < 0.001$), but were not significantly different from each other ($P = 0.253$). The hierarchical analysis of molecular

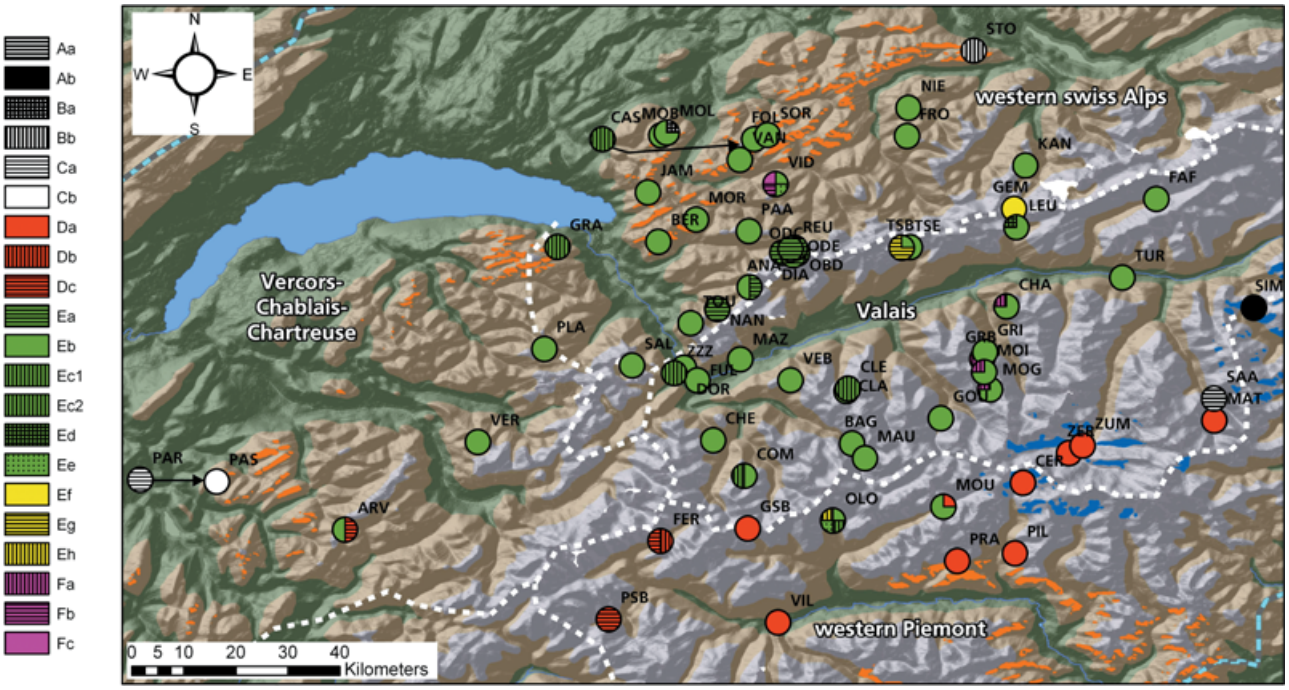

Fig. 4 Distribution of the haplotypes frequency in the 67 populations of *Biscutella laevigata* in the Western Alps. Dashed lines in blue represent the extension of the ice sheet during the last glacial maximum and white lines delimit the natural biogeographical districts according to Theurillat *et al*. (1993). The putative refugia are delimited following Stehlik (2000). Therein, the south-facing slopes have been coloured to represent the putative perialpine refugia in orange (between 1500 and 2500 m) and the central nunataks in blue (between 2000 and 3000 m). The haplotypes are coloured according to Fig. 3 and summarized in the left panel.

variance showed that the populations of refugial and glaciated areas were genetically slightly different (G_{ST} = 0.061, P = 0.024). On the contrary, the internal genetic structure was important when the populations were grouped according to natural districts (G_{ST} = 0.280, P < 0.001). None of the relationships tested between genetic and geographical distances were significant, which pointed to a lack of isolation by distance.

Discussion

Molecular investigation

The conjunct use of a multilocus cpSSR survey and DNA sequencing generated precise spatial signals from the investigation of numerous individuals with cpSSR markers and precise phylogenetic signals from the DNA sequences. Among the 27 haplotypes characterized in 277 individuals, the sequencing of 53 individuals revealed that multilocus cpSSR homoplasy resulted in only one case (population CAS, characterized by the haplotype Ec1) being wrongly assigned to a related haplotype (Ec2). The different cpSSR markers taken independently showed substantial homoplasy with similar length variants of independent origin (Table 2), but the multilocus cpSSR survey efficiently discriminated most of the haplotypes (20 out of 21). Contrary to the study of Navascués & Emerson (2005), which discouraged the practise of cpSSR for phylogenetic studies because of the potentially high level of homoplasy at the intraspecific level, this multilocus cpSSR survey did not lead to a biased interpretation. The cpSSR markers proved to be useful in identifying different haplotypes and revealed a very high level of polymorphism in a comparatively small area (150 × 150 km). Although microsatellites' length is rarely used in practice to generate phylogenetic signal, we found that cpSSR markers provided much more information compared to the commonly used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Stehlik 2003; Schönswetter *et al*. 2005). The cpSSR markers Bras1 to Bras4 are considered reliable and, as they were transferable amongst Brassicaceae, can potentially be used to explore the intraspecific diversity of maternal lineages. The *trn*S-*trn*G spacer also showed great potential for investigating intraspecific genealogies (Shaw *et al*. 2005) and has already been successfully exploited for phylogeographical studies in Brassicaceae (Alsos *et al*. 2005; Gaskin *et al*. 2005) and in tree species (Besnard *et al*. 2007).

Phylogenetic relationships among haplotypes and cytotypes

The *Biscutella laevigata* complex formed six connected groups of closely related haplotypes (called lineages; Fig. 3). The *B. didyma* outgroup, which belongs to another series of the genus *Biscutella* (Guinea & Heywood 1993), was phylogenetically loosely connected to the other sampled haplotypes and responsible for most of the reticulation in the network. *Biscutella laevigata* ssp. *lucida* is a widely accepted glabrous tetraploid subspecies (Raffaelli 1992; Raffaelli & Baldoin 1997), but its taxonomical status based on hairiness appears to be controversial. As Fig. 3 shows, the LUCIDA haplotype was more closely related to the northern hirsute tetraploids than to the southern glabrous ones (C. Parisod, unpublished data).

Although not in a strictly basal position, the extra alpine diploid outgroups of the *B. laevigata* complex were connected to the Western Alps' haplotypes. *Biscutella laevigata* ssp. *varia* showed only one haplotype in four sampled individuals. As already indicated by Tremetsberger *et al*. (2002), the lowland *varia* taxon seems to be genetically depauperate. *Biscutella prealpina* (Raffaelli & Baldoin 1997), on the other hand, presented three unrelated haplotypes out of three samples. Haplotype PRE1 was close to lineage D, while PRE2 and PRE3 were close to haplotype VARIA and connected to several Western Alps haplotypes (Fig. 3). Although the isozyme analysis of Tremetsberger *et al*. (2002) revealed a close relationship between the tetraploids to the extra alpine diploids of *B. prealpina* and the *varia* subspecies, it was not possible to precisely identify the maternal parentage of the Western Alps' samples within these taxa. The greater diversity of haplotypes found in the *prealpina* taxon compared to the *varia* subspecies suggests that the former was less affected by the LGM and also indicates that Italian populations are closely related to the source populations of alpine recolonisers. However, the extra alpine diploids were dispersed over the haplotype network (Fig. 3), indicating that the autotetraploids of *B. laevigata* appeared recurrently. Such a polyphyletic evolution through multiple events of polyploidy from genetically and morphologically differentiated diploid populations (Soltis & Soltis 1999) points to a dynamic genetic system in the *B. laevigata* complex and explains its taxonomic uncertainties (Guinea & Heywood 1993) thus requiring the use of a wide species concept in polyploid complexes (Mable 2003). Since the most common Western Alps tetraploids (lineage E) did not present a particularly close genetic relationship with the diploid taxa proposed as putative parents, the present results suggest that the *prealpina* taxon and the *varia* subspecies are only ancestral to the lineages that evolved into these autopolyploids.

Alpine phylogeography: diffuse LGM survival in multiple refugia

The network shows groups of related haplotypes (i.e. lineages; Fig. 3) connected together by long branches and such a topology is congruent with the allopatric refuge

model (Knowles & Maddison 2002). In a spatial context (Fig. 4), the molecular survey further showed that the frequently sampled lineages (D and E) were widespread over the highly glaciated areas of the Western Alps, while rare haplotypes (A, B, C and F) had a more restricted distribution and occurred exclusively in LGM refugia, suggesting that *B. laevigata* survived the LGM in several refugia of the Western Alps.

The LGM *in situ* survival was confirmed in several cases, as is presented in Fig. 4. Lineage B occurred in adjacent northern peripheral refugia (STO and MOL) and lineage C was restricted to the peripheral refugia in the south of the study area (PAR and PAS). Similarly, lineage A was restricted to populations (SAA and SIM) in the area of central nunataks. Results of this study strongly support the existence of survival within the Alps but, as was reported in previous alpine phylogeographical studies (Stehlik *et al*. 2001, 2002), most populations with particular haplotypes were located at the edge of the sampling area and therefore a postglacial migration cannot be strictly ruled out. In fact, this alternative hypothesis is in particular likely for lineage A because its range extends to the Simplon Pass area, which was envisaged as a major transalpine recolonization pathway (e.g. Mátyás & Sperisen 2001). On the contrary, lineage F had a scattered distribution within the study area with haplotypes Fb and Fc restricted to the VID population (together with haplotypes Eb and Ee) and they lie well within the limits of the northern peripheral Alps. Since this area had already been shown to be a narrow hybrid zone between diploids and autotetraploids of the *Anthoxanthum odouratum* L. complex (Felber-Girard *et al*. 1996), its role in LGM survival and as a secondary contact zone is further highlighted here by the high level of genetic diversity observed in *B. laevigata* (Fig. 4, with four haplotypes belonging to the unrelated lineages E and F). Furthermore, haplotype Fa was restricted to an isolated valley (CHA, MOG, MOI, GRB) in the immediate vicinity of the central nunataks area near Zermatt and, since individuals displaying this highly differentiated lineage F were surrounded by populations that contained the frequently sampled haplotypes Da and Eb, an explanation involving postglacial migration of this restricted haplotype is considered unlikely. Although early long-distance dispersal immigration from an nonsampled population cannot be strictly ruled out (Hewitt 2004), the pattern shown here suggests that the Central Alps nunatak provided a refugium for *B. laevigata* during the LGM and that haplotype Fa was postglacially swamped by recolonisers.

The presence of a population harbouring high genetic distinctiveness in the proximity of putative alpine refugia confirms the glacial *in situ* survival of *B. laevigata* in several alpine refugia and demonstrates a strong pattern of nunatak survival. The permanent *in situ* survival of plant populations in the Alps during the harsh LGM climatic conditions is intuitively hard to conceive, especially in the highly glaciated areas of the Central Alps. Nevertheless, LGM ice surface reconstructions (e.g. Kelly *et al*. 2004) indicated that several areas were emerging from the ice sheets in the Central Alps and, since snow does not accumulate on steep slopes, cliffs exposed to the south can be considered as microclimatically favourable habitats. Therefore, the peculiar ecological conditions prevailing in cliffs might have buffered the reaction of species against LGM mesoclimatic vicissitudes and favoured *in situ* survival in the Alps (Pawlowski 1970). *B. laevigata* is a likely candidate for LGM survival on nunataks as it is a typical species in siliceous as well as calcareous cliff-habitats (Delarze *et al*. 1998) and shows great tolerance against extreme temperature (i.e. continental ecology). Moreover, diaspores may also be considered as vehicles through time to explain patterns of genetic diversity in the glacial context. In fact, *B. laevigata* has already been shown to support high spatiotemporal ecological stochasticity because mature individuals are able to stay in the soil for years at a dormant stage before flowering again (Dannemann 2000).

This molecular phylogeography study performed with dense sampling reveals points of local refugia in the Central Alps and the northern external Alps. Most of these northern peripheral refugia on calcareous bedrocks were only anticipated in the calcifuge-species biased review of Schönswetter *et al*. (2005), and this area appears as a regional hotspot of genetic diversity in the present study. In the external Alps, neighbouring relict populations of *B. laevigata* were highly differentiated and are apparently much older than populations in the rest of the investigated area. In fact, the putative peripheral refugia appear to be an area with long-term persistence against climate change and it should be interesting to investigate other species to further determine its role as a rear edge (Hampe & Petit 2005) for alpine species.

Polytopic autopolyploidy and postglacial recolonization

With the exception of the central nunataks area that showed substantial genetic diversity, the northern peripheral Alps were the only part of the study area that harboured a high genetic diversity at the regional scale. This latter area presented six haplotypes within the common E lineage, while the Central Alps only presented a subset of haplotypes, with Eb dominating the previously glaciated areas (Fig. 4). A similar pattern was detected in the south of the study area, with several haplotypes of lineage D fading out in favour of Da within the previously glaciated areas. This implies that *B. laevigata* evolved through similar processes in the northern and southern parts of the Western Alps and suggests a postglacial recolonization from the external to the internal Alps. As most of the internal genetic structure (G_{ST} = 0.280) lies between the four natural districts

sampled following Theurillat *et al*. (1993), it seems that multiple polyploidy events resulted in independent autotetraploids of *B. laevigata* in the different biogeographical areas. This was also reflected in significantly differentiated morphological traits between the natural districts (C. Parisod, unpublished results). Furthermore, since the widespread tetraploid lineages D and E were genetically well-differentiated and apparently related to different diploid haplotypes, this pattern indicates that the species complex evolved through polytopic autopolyploidy. Therefore, it seems that different diploid populations evolved allopatrically into independent tetraploid lineages that recolonized the previously glaciated areas until the polyploid 'taxon' reached its nearly continuous range in the Western Alps. Therefore, some tetraploids might have evolved from refugial diploids in the external Alps before expanding over the previously glaciated areas of the Central Alps. Given that refugial populations harboured highly differentiated haplotypes, it may be interesting to strongly assess ploidy structure in order to test for the hypotheses of autopolyploidy out of refugia and/or evolution of *B. laevigata* through segmental allopolyploidy.

The genetic structure detected here with maternal plastid DNA markers was slightly higher than the average estimation found in angiosperms (Petit *et al*. 2005b). The global N_{ST} (0.858) was not different from the global G_{ST} (0.840), indicating that polymorphic populations were composed of both related and unrelated haplotypes. Furthermore, geographical distance failed to explain genetic differentiation among populations, which suggests that only a small part of the haplotype diversity expended rapidly from LGM refugia into the highly glaciated areas (Slatkin 1993). As the great expansion of some tetraploid haplotypes (e.g. Eb and Da) is easily discernable (Fig. 4), the phylogeographical pattern suggests that admixing and swamping of unrelated haplotypes is important in mixed populations. In fact, the two main tetraploid lineages of *B. laevigata* are restricted by the alpine landscape in either the north or the south of the main chain with the line of the main summit effectively acting as a major phylogeographical barrier that has only been permeable through high-altitude passes. For example, the presence of the southern Da haplotype in the northern area of Zermatt (ZER and ZUM) shows that high-altitude passes allowed a transalpine recolonization. The most accessible transalpine pass in this area (Theodule Pass, 3300 m) is at present too high to provide favourable habitat for the species, thus suggesting that migration probably occurred during the postglacial climatic optimum (Younger Atlantic, between 6000 and 4800 years ago; Burga 1988). Similarly, some populations (MOU and OLO) around other transalpine passes (Great St. Bernhard, 2475 m; Fenêtre Durand, 2812 m and Collon Pass, 3130 m) showed evidence of secondary contact between the two main tetraploid lineages in the south of the Central Alps (Fig. 4). Lineage F was mixed with common haplotypes Eb in MOG, MOI, GRB and VID and is probably swamped there by the widely recolonizing lineage E. Such a phylogeographical pattern highlights significant difficulties in statistically reconstructing the spatio-temporal dynamics of a frequent species. The complex postglacial history of *B. laevigata* reconstructed here with the help of dense sampling, resulted in a 'noisy' genetic signature, and this may explain the lack of significance in most of the statistical phylogeographical tests attempted here.

The complex Pleistocene history of the widespread *B. laevigata* raises interesting issues concerning the relationship between biological evolution and climate dynamics. Firstly, the northern peripheral Alps harbour high genetic diversity due to the long-term persistence of relict populations, while the highly glaciated Central Alps show substantial genetic diversity because of ongoing admixture between vicariant lineages. These different historical processes both lead to high genetic diversity across the studied species range. Furthermore, the main autotetraploid lineages of *B. laevigata* independently recolonized the space left by the retreat of glaciers over a wide range of habitats, and the populations growing in contrasting types of meadows over the whole altitudinal gradient are closely related. For example, populations sampled in alpine meadows as well as lowland, steppic meadows in both the Valais and the Aosta Valley harbour haplotypes that are valley-specific rather than habitat-specific. This indicates that tetraploids lineages of *B. laevigata* have great ecological amplitude and the pattern observed here contrasts with one expected if the wide ecological niche of polyploids was the consequence of multiple cytotypes adapted to different habitats (Favarger 1971) or due to the juxtaposition in the ecological or evolutionary landscape of independent, habitat-specific tetraploid lineages (Soltis & Soltis 2000). Unlike the study by Gasser (1986), which experimentally demonstrated local adaptation of *B. laevigata*, the evolutionary processes operating on the autopolyploid genomes within maternal lineages remains to be elucidated.

Finally, this study shows evidence of incipient speciation by polyploidy in relation to the ice ages in the Alps. The association between polyploidy and distribution, ecology or habitat has been addressed several times (e.g. Favarger 1971; Ehrendorfer 1980) and indicates that neopolyploids originate mainly under unstable conditions in areas of secondary contact (Stebbins 1984). Evolution by polyploidy linked to ice ages examined in arctic plants (Brochmann *et al*. 2004) confirm that highly glaciated areas do contain taxa with recently increased ploidy levels. The evolution of arctic polyploids seems to involve high levels of reticulation in areas drastically disturbed by recurrent past climatic changes. Allopolyploid taxa would have an advantage under these circumstances because of their ability to maintain genetic variation through fixed heterozygosity, in spite of

inbreeding and bottlenecks. It is tempting to fit this model on the evolution of the slightly diverged diploid species suggesting the presence of segmental allopolyploidy. The present molecular survey stresses the primary importance of the historical component in influencing the evolutionary ecology of the *B. laevigata* complex and provides some evidence to suggest the origin of the autotetraploid lineages after secondary contacts. However, this species has been shown to mostly form multivalents at meiosis (Manton 1937) and is characterized by tetrasomic inheritance (Tremetsberger *et al*. 2002), suggesting that this segregation pattern may be an advantage for autopolyploid-like species under changing climates.

Acknowledgements

We would like to thank L. Bavaud and S. M. Tam for English improvements, N. Galland and R. Holderegger as well as three anonymous reviewers for their helpful comments on the manuscript. We would like to thank O. Broennimann for help preparing the maps, F. Bancala for help in the field, D. Savova-Bianchi for assistance in the lab and the Société Académique Vaudoise for funding.

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Christian Parisod (INRA Versailles) is interested in polyploid evolution. This study is part of its PhD. He also works on landscape genetics and currently investigates the impact of transposable elements on allopolyploid genomes. Guillaume Besnard (UNIL) currently works on the phylogeography of the wild olive tree. He also studies the postglacial recolonisation of alpine plants and the evolution of adaptive traits (such as heavy metal tolerance and C4 photosynthesis).

Supplementary material

The following supplementary material is available for this article:

Supplementary material: Details of the sample localities and the haplotype frequencies. Pop. Code is the code used for each population. Alpine refugia summarises the information from the literature (Stehlik, 2000) and indicates whether populations were sampled in putative peripheral refugia (*) or in putative nunataks (**). Districts represent the natural biogeographical districts based on floristic data (Theurillat *et al.* 1993) with SOC for the 'Western Swiss Alps', POC for the 'Western Piemont', VCC for the 'Vercors-Chablais-Chartreuse' and VAL for the 'Valais' Alps.

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