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Genetic structure and evolution of Alpine polyploid complexes: *Ranunculus kuepferi* (Ranunculaceae) as a case study

J. BURNIER,*[1] S. BUERKI,*[1] N. ARRIGO,* P. KÜPFER* and N. ALVAREZ†
**Laboratory of Evolutionary Botany, Institute of Biology, University of Neuchâtel, Rue Emile-Argand 11, CH-2009 Neuchâtel, Switzerland, †Laboratory of Evolutionary Entomology, Institute of Biology, University of Neuchâtel, Rue Emile-Argand 11, CH-2009 Neuchâtel, Switzerland*

Abstract

The alpine white-flowered buttercup, *Ranunculus kuepferi* Greuter & Burdet, is a polyploid complex with diploids endemic to the southwestern Alps and polyploids – which have been previously described as apomictic – widespread throughout European mountains. Due to the polymorphic status of both its ploidy level and its reproductive mode, *R. kuepferi* represents a key species for understanding the evolution of polyploid lineages in alpine habitats. To disentangle the phylogeography of this polyploid taxon, we used cpDNA sequences and AFLP (amplified fragment length polymorphism) markers in 33 populations of *R. kuepferi* representative of its ploidy level and distribution area. Polyploid individuals were shown to be the result of at least two polyploidization events that may have taken place in the southwestern Alps. From this region, one single main migration of tetraploids colonized the entire Alpine range, the Apennines and Corsica. Genetic recombination among tetraploids was also observed, revealing the facultative nature of the apomictic reproductive mode in *R. kuepferi* polyploids. Our study shows the contrasting role played by diploid lineages mostly restricted to persistent refugia and by tetraploids, whose dispersal abilities have permitted their range extension all over the previously glaciated Alpine area and throughout neighbouring mountain massifs.

Keywords: AFLP, apomixis, cpDNA network, phylogeography, polyploidy, Ranunculaceae

Introduction

Polyploidization describes the process that multiplies the number of chromosomes and genomic content within lineages (Otto 2003). It has occurred frequently in the evolutionary history of eukaryotes, and more particularly in plants (Adams & Wendel 2005). As a consequence, a large proportion of contemporary plant genomes are the result of past steps of genome doubling, often followed by massive silencing and elimination of duplicated genes (Levy & Feldman 2004; Paterson *et al.* 2004). Polyploidy also plays an important role in the evolution of extant species complexes (e.g. Koch *et al.* 2003; Albach 2007). This is particularly true in Palearctic species that were strongly influenced by ice ages. In these cases polyploidization is suggested to have been an important mechanism for generating new evolutionary lineages in areas that were glaciated during the Pleistocene [e.g. in Brassicaceae (Brochmann *et al.* 1992; Marhold & Lihovà 2006; Jordon-Thaden & Koch 2008), in Plantaginaceae (Albach 2007) and in Ranunculaceae (Hörandl *et al.* 2005)]. Several evidences also address the predominant role played by polyploids in the Quaternary evolutionary history of the Arctic flora (Abbott & Brochmann 2003; Popp *et al.* 2005; Brochmann & Brysting 2008). One possible reason for the persistence of polyploids is that the increase in ploidy level may be accompanied by adaptive advantages (Prentis *et al.* 2008): for instance, polyploids are able to compete

Correspondence: Philippe Küpfer, Fax: +4132 718 3001;
E-mail: philippe.kuepfer@unine.ch

[1] These authors have contributed equally and are considered joint first authors.

successfully with their diploid parents for available habitats (Lamuret 1988). A combination of genetic information from different ancestors can provide polyploids with a higher physiological and ecological flexibility than that of their diploid parents (Küpfer 1974; Bierzychudek 1985). Moreover, polyploidy might have direct causal links with switches in the reproduction mode of individuals, for instance through the evolution of apomixis (a type of asexual reproduction by seeds in which a somatic cell produces the embryo sac without either a meiotic stage or pollen fertilization). The formation of unreduced gametes is both a characteristic feature of apomicts and a necessary element in the production of polyploids in sexual individuals (Whitton *et al.* 2008). As a consequence, polyploidy might favor the occurrence and establishment of apomictic lineages (Roche *et al.* 2001).

Two major advantages of an apomictic mode of reproduction are (i) the ability to colonize new areas with single individuals (increased by the benefit of seed dispersal; Paun *et al*. 2006a, b) and (ii) the potential long-term persistence of the species despite unreliable pollinators or disturbed environments (Richards 2003). This latter advantage of an asexual mode of reproduction is notably highlighted in Palearctic species complexes composed of both diploids and polyploids. In these complexes, sexual diploid lineages tend to have a restricted distribution, while high latitude and altitude areas (previously covered by ice during the last glaciations) are often colonized by apomictic polyploids (Küpfer 1974; Bierzychudek 1985; Hörandl 2006). This phenomenon, referred to as 'geographical parthenogenesis' (Vandel 1928), might be caused by the invasion of newly opened habitats by self-compatible polyploids showing higher levels of heterozygosity due to genome doubling (Stebbins 1980, 1985). In areas that have experienced intense Quaternary climatic oscillations, it is widely accepted that causes underlying polyploidization processes may find their origin in the 'secondary contact model' as defined by Stebbins (1985). Accordingly, glacial advancement during the ice ages resulted in the fragmentation of diploid populations, which may have survived in nunataks or in peripheral refugia (Schönswetter *et al.* 2005). Consequently, with the retreat of the glaciers during interglacial periods, the divergent diploid lineages came into contact again in suture zones and produced polyploids by 'intra-specific' hybridization of diverging lineages.

Since the early 1990s, the evolutionary histories of several auto- and allo-polyploid arctic-alpine species have been disentangled by comparing plastid and nuclear molecular markers (e.g. Brochmann 1992; Koch *et al*. 1998; Franzke & Hurka 2000; Koch 2002; Marhold *et al*. 2002; Brochmann *et al*. 2004; Guggisberg *et al.* 2006). In parallel, recent considerations of paleoecological and paleoclimatic patterns in a phylogeographical framework have also advanced our understanding of the history of Alpine plant (e.g. Stehlik *et al.* 2001; Schönswetter *et al.* 2004, 2005; Paun *et al.* 2008). Although several studies have addressed the phylogeography of polyploid complexes with disjunct distributions (e.g. distributed both in the Arctic and in the Northern-Hemisphere mountain ranges), few molecular studies have focused on polyploid complexes strictly distributed throughout the 'European Alpine System' *sensu* Ozenda (2002; hereafter EAS, comprising the Alps, the Pyrenees, the Central Massif, the Carpathians, the Apennines, the Dinarides and the Northern Balkans). Interestingly, the cytotype patterns of several EAS polyploid complexes have been known for more than 30 years (Küpfer 1974). These patterns conform to the theoretical expectations of the geographical parthenogenesis hypothesis; diploids are mainly restricted to southern locations, whereas polyploids extend throughout the northern limits of the distribution areas.

Following Küpfer's (1974) work, through this and other studies, we aim to unravel the biogeographic history of these well-known polyploid complexes. This study focuses on an alpine white-flowered buttercup, *Ranunculus kuepferi* Greuter & Burdet (Greuter 1987), which exhibits a trend towards geographical parthenogenesis with diploids restricted to the southwestern Alps and tetraploids widely distributed across the Alpine range, the Apennines and Corsica. In this species complex, there also exist unbalanced cytotypes (tri- and pentaploids), which are encountered in a contact zone between diploid and tetraploid lineages in the southern Alps (Küpfer 1974). In this study, we aimed to:

1. investigate phylogenetic relationships between *R. kuepferi* and other taxa of Alpine white-flowered buttercups based on molecular markers;
2. clarify the distribution of cytotypes in *R. kuepferi* based on new chromosome counts;
3. further support the phylogenetic hypothesis with different clustering methods, using genome-wide screening (amplified fragment length polymorphism, AFLP);
4. develop hypotheses about the spatial and temporal historical biogeography of *R. kuepferi* throughout its distribution area;
5. propose hypotheses with respect to polyploidization and apomixis evolution in this species complex.

Finally, we will place our findings in the context of Quaternary evolution and dispersal of polyploid complexes in the EAS.

Materials and methods

Plant material

Based on karyological and morphological evidence, *Ranunculus kuepferi* was subdivided into two subspecies (Küpfer 1974; Huber 1985, 1988; Greuter 1987): (i) *R. kuepferi* subsp. *kuepferi*, the diploid cytotype (2n = 2x = 16) endemic to the southwestern Alps; and (ii) *R. kuepferi* subsp. *orientalis*, the polyploid cytotypes (including tri-, tetra- and pentaploids) widely distributed across the Alpine range (except in the southwestern margin of the Alps), the Apennines and Corsica. An overlap in the distribution of the two taxa occurs in the Southern Alps. These two subspecies can be distinguished by their flower morphology; *Ranunculus kuepferi* subsp. *kuepferi* has a regular corolla, <25 carpels and well-developed stamens, whereas *R. kuepferi* subsp. *orientalis* has an irregular corolla (due to the abortion of some petals), >60 carpels and few stamens (Küpfer 1974).

This study surveyed 33 populations (five in the eastern Alps, five in the central Alps, 17 in the southwestern Alps, two in the Apennines and four in Corsica; see File S1 in the Supporting Information) from which young leaves of 5–20 individuals were collected. In addition, one to five individuals were transplanted to the Botanical Garden of Neuchâtel to provide the root material needed for counting chromosomes. The sampling included 242 specimens of *R. kuepferi*, nine individuals of *Ranunculus platanifolius* L., four individuals of *Ranunculus aconitifolius* L. and four individuals of *Ranunculus seguieri* Vill. (see File S1 for more details). *Ranunculus platanifolius* is found throughout the Palearctic area, whereas *R. aconitifolius* is restricted to central and western Europe. Both species show a relatively wide ecological niche with an optimum in Megaphorbiae, and occur in montane and subalpine zones. In contrast, *R. seguieri* is distributed across southern and western European mountainous massifs and is confined to alkaline screes in alpine habitats. These species are considered the three most closely related species to *R. kuepferi* (Paun *et al.* 2005) and were used as outgroup samples. DNA extractions were performed using the QIAGEN DNeasy plant kit (QIAGEN), following the manufacturer's protocol.

Ploidy determination

Ploidy was determined in 91 living samples of *R. kuepferi* from 32 populations (only one population, CAY, was not analysed due to a lack of material) and within the complete set of outgroup samples (File S1). Root tips were pretreated in an alpha-bromonaphthalene water-saturated solution for 2 h and fixed in a solution of methanol–acetic acid (3 : 1) for several days. Finally, chromosomes were stained with acetocarmine and counted using a microscope.

DNA sequencing

Chloroplast [*rpL20-rps12* intergeneric spacer (IGS), *trnL* intron and *trnL-F* IGS] and nuclear [internal transcribed spacer (ITS) region: *ITS1*, *5.8S* and *ITS2*] markers were amplified and sequenced for a representative subset of *R. kuepferi* (107 individuals from 33 populations and from all cytotypes) and for the complete set of outgroup samples (File S1). Primers for the chloroplast regions are described in Shaw *et al.* (2005) for *rpL20-rps12* IGS region and in Taberlet *et al.* (1991) for *trnL* intron and *trnL-F* IGS regions. Primers for the ITS region are described in White *et al.* (1990). Amplification of selected regions was carried out in 25 μL of reaction mixture containing 5 μL of 5× PCR buffer, 1.5 μL of 25 mM $MgCl_2$, 0.5 μL of 10 mM dNTPs, 0.5 μL of 10 mM primers, 0.2 μL of GoTaq polymerase (5 u/μL) (Promega), and 14.5 μL of ddH_2O. PCRs were performed in a T3 thermocycler (Biometra). Initial template denaturation was programmed for 2 min at 95 °C, followed by 35 cycles at 95 °C for 45 s, 50 °C for 45 s, 72 °C for 1 min, plus a final extension of 10 min at 72 °C. Purification and sequencing were performed as a service provided by Macrogen, Inc.

Sequence alignment and phylogenetic reconstructions

ChromasPro version 1.34 (Technelysium Ltd., Helensvale, Qld, Australia) was used to assemble complementary strands and check software base-calling. The three chloroplast and ITS regions were initially aligned using Clustal X (Thompson *et al.* 1997) and subsequently manually adjusted using the similarity criterion (Morrison 2006). An incongruence-length difference test (ILD; Farris *et al.* 1995) between the chloroplast regions was performed as implemented in PAUP* version 4.0b10 (Swofford 2003) with 100 replicates. The ILD test was not significant ($P = 1.0$) and indicated that the three chloroplast data sets were congruent, thus allowing them to be combined for analysis in a total evidence approach (*sensu* Kluge 1989). Maximum parsimony analyses were performed on the combined chloroplast matrix and on the ITS matrix in PAUP* version 4.0b10 (Swofford 2003) by using a heuristic search strategy with a random addition sequence (10 replicates), TBR branch swapping, MULTREES, ALLSWAP, steepest descent in effect, MAXTREES = 1000 and GAPMODE = newstate. According to the phylogeny of the Ranunculaceae (Paun *et al.* 2005), *R. aconitifolius* was used as the most external outgroup to root the analyses.

A strict consensus tree was built on the basis of the most-parsimonious trees. To corroborate patterns resulting from classical phylogenetic analyses, we computed a cpDNA network, using the parsimony-splits network algorithm (Bandelt & Dress 1993), as implemented in SPLITSTREE version 4.6 (Huson & Bryant 2006).

AFLP analyses

Amplified fragment length polymorphism profiles were generated for all samples. Marker reproducibility was tested with nine replicated individuals randomly added in the PCR plates. The AFLP protocol was carried out following the procedure described by Vos *et al.* (1995) with minor modifications (Bonin *et al.* 2004; Gugerli *et al.* 2008) and using nonradioactive fluorescent dye-labelled primers (FAM-label; Applied Biosystems). Genomic DNA (~300 ng) was digested with the restriction enzymes *MseI* (New England Biolabs) and *EcoRI* (Promega), and further ligated (T4 DNA-Ligase; Promega) to double-stranded adapters in a programmable thermal controller (PTC-100; MJ Research Inc.) for 2 h at 37 °C. The ligation product was preamplified using primer pairs with a single selective nucleotide (i.e. *MseI*-C and *EcoRI*-A) and then selectively amplified using the two primer combinations *MseI*-CAC/*EcoRI*-ATC and *MseI*-CTG/*EcoRI*-ATG. Only the *MseI* primers were FAM-labelled. Selective amplification products were run by Macrogen, Inc., in a denaturing polyacrylamide gel with an internal size standard (ROX 400HD) on an automated DNA sequencer (ABI 377; Applied Biosystems). Fragments were further analysed using the ABI Prism GeneScan version 3.7 Analysis Software (Applied Biosystems). The presence or absence of each fragment (for the two primer combinations) was scored for each individual manually in a readable region ranging from 50 to 400 bp, using GENOGRAPHER version 1.6 (Benham *et al.* 1999). Scores of the primer combinations were further assembled into a binary data matrix.

Genetic diversity

Diversity patterns of *R. kuepferi* were investigated by considering the complete AFLP data set and using a 20 km × 20 km grid that covered the sampling area. For each intersection of the grid, samples located within a 40-km perimeter were used to compute the Nei's genetic diversity index (Nei 1972, 1987), an estimator of local genetic diversity that can be applied regardless of the ploidy level or the type of reproductive system in the studied organism (Nei 1987). This index was computed using the R CRAN package ADE-4 (Thioulouse *et al.* 1996; Chessel *et al.* 2004; R Development Core Team 2009). To provide unbiased estimates due to unequal sampling effort between the different sampling areas, computations were replicated 1000 times by re-sampling a constant number of samples (i.e. 10 samples) per grid point. Finally, genetic diversity measures were visualized in ARCGIS 9.1 (ESRI).

Genetic structure analyses

The genetic structure of the complete AFLP data set (242 *R. kuepferi* samples and the 17 outgroup samples) was investigated through three independent approaches. (i) A nonhierarchichal *K*-means clustering (Hartigan & Wong 1979) was performed using R CRAN (R Development Core Team 2009, script available on request). A total of 50 000 independent runs were carried out for each value of *K* clusters assumed. For each *K*-value, ranging from 2 to 19, only the first run yielding a positive value for the second derivative of the inter-cluster inertia (Kergoat & Alvarez 2008) was considered. Clusters within this best *K*-means run were characterized with an 'R'. (ii) A model-based Bayesian inference clustering was run using STRUCTURE version 2.2 (Pritchard *et al.* 2000; Falush *et al.* 2007). The analysis assumed an admixture model and independent allele frequencies between clusters. Five independent runs were carried out for each value of *K* ranging from 1 to 10, with parameters and model likelihood estimated over 1 000 000 Monte-Carlo Markov Chain generations (following a burn-in period of 200 000 generations). For each value of *K*, runs that obtained the highest maximum-likelihood values were taken into account for further analyses, and only the first run yielding a positive value for the second derivative of its likelihood was considered. Clusters within this best STRUCTURE run were characterized with an 'S'. The majority-rule criterion (>0.5 in the assignment probability) was applied to assign samples to a given cluster. (iii) *K*-means and STRUCTURE clustering results were independently displayed on a Principal Coordinate Analysis (PCoA) computed with the R CRAN package ADE-4 and using a Jaccard distance matrix based on the raw AFLP data. Finally, results of the two different clustering analyses were compared using a contingency table and further displayed on geographical maps using ARCGIS 9.1 (ESRI), by representing each population as a pie-chart addressing the number of samples assigned to each AFLP cluster.

Relationships between biogeographic clusters

Nei's genetic distances (Nei 1972) were computed pairwise between the *K*-means clusters using the R CRAN package ADE-4, and were displayed as a neighbour-joining tree using the package PHYLIP (Felsenstein 1993).

Results

Ploidy variation within Ranunculus kuepferi

Four different cytotypes were identified: diploid (2n = 2x = 16), triploid (2n = 3x = 24), tetraploid (2n = 4x = 32) and pentaploid (2n = 5x = 40). According to the cytotype distributions, four population compositions were identified: (i) seven populations were composed only of diploids [two of them were located on the westernmost border of the Alps in the Vercors massif (COB, NAT) and five were located in the southwestern Alpine area (VRN, CHA, MOU, COY, CMA)]; (ii) in one population (COM) only triploid specimens were observed; (iii) four populations, bordering the strictly diploid populations on their northern and eastern sides, were polymorphic regarding ploidy level (among them, the population CCH was composed of 2x and 3x; the population ALL was composed of 3x, 4x and 5x; the population TEN comprised 3x and 5x and the population AUT was composed of 3x and 4x); (iv) the 20 remaining populations were solely composed of the 4x cytotype. These populations were found in the central and oriental Alps (BRU, VAR, LAR, IZO, GSB, NAV, SIM, DEV, GLE, MUN, TON, HSL, GIA, SCO), the A-pennines (PIE, PRA) and Corsica (ERC, CAS, PUN, COS). The distribution of cytotypes is depicted in Fig. 1 (see also File S1 for more details), and as an illustration

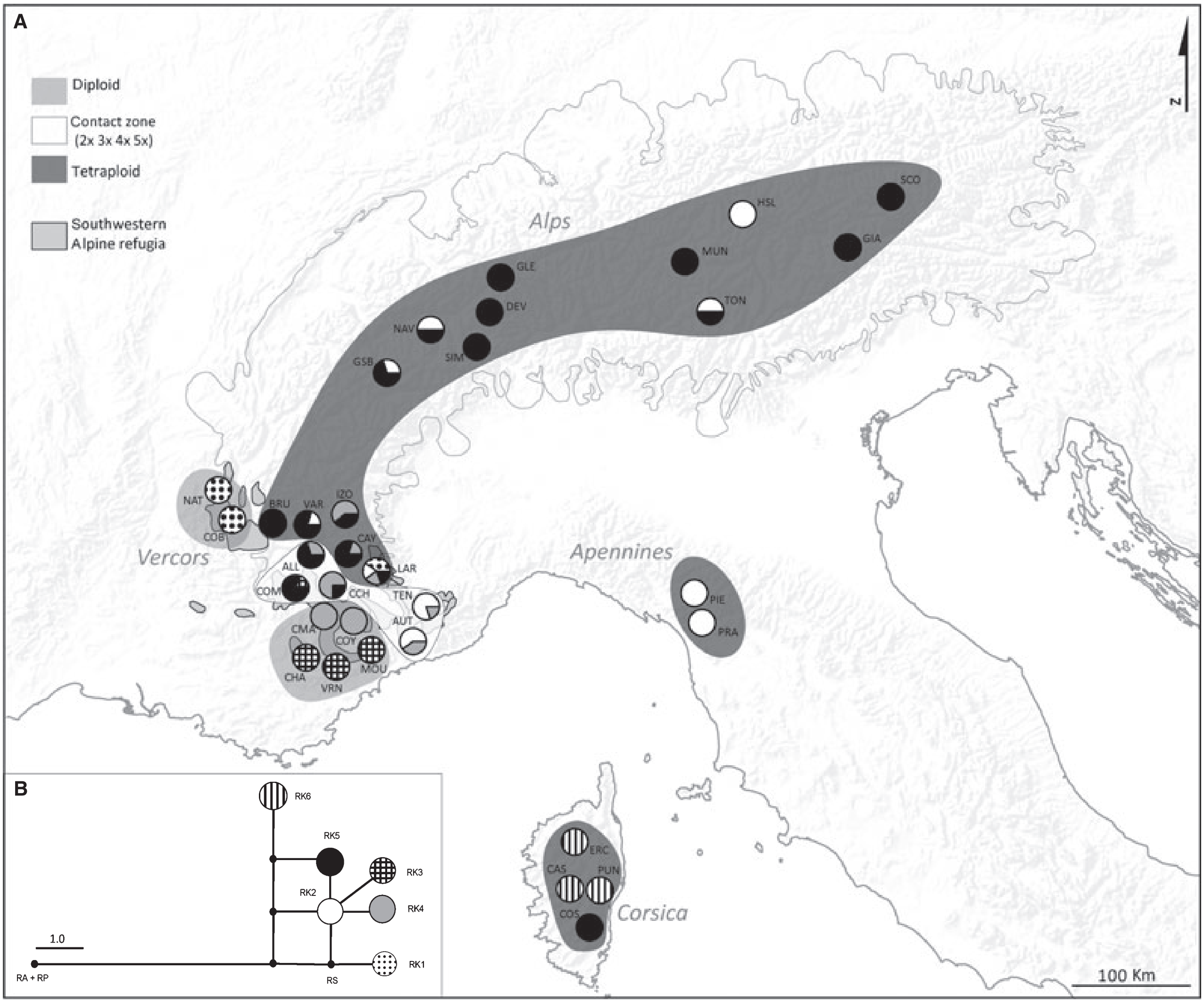

Fig. 1 (A) Geographical pattern of the six cpDNA haplotypes recovered in *Ranunculus kuepferi* (labelled from RK1 to RK6). Geographical distribution of cytotypes, glaciations limits and southwestern refugia are also indicated. (B) cpDNA parsimony-splits network of the four white-flowered buttercups species. For *R. kuepferi* haplotypes, symbols are as follows: dots – RK1, white – RK2, squares – RK3, grey – RK4, black – RK5, and vertical stripes – RK6.

of our chromosome counting technique, a picture of a mitotic metaphase in a 5x specimen is shown in File S2.

Phylogenetic reconstructions of cpDNA and ITS data sets

Alignment lengths of the chloroplast regions were 798 bp for *rpL20-rps12* IGS, 526 bp for *trnL* intron and 248 bp for *trnL-F* IGS. GenBank accession numbers for these three regions are provided in the File S1. The *rpL20-rps12* IGS included three substitutions and one indel, *trnL* intron contained two substitutions and three indels and *trnL-F* IGS included two substitutions (Table 1). Each of these 11 informative characters were 1 bp long and allowed the identification of six cpDNA haplotypes within *Ranunculus kuepferi* samples (i.e. RK1 to RK6) and two additional cpDNA haplotypes among the other sampled species (Table 1). The most external outgroup, *Ranunculus aconitifolius*, shared the same cpDNA haplotype with *Ranunculus platanifolius*, whereas *Ranunculus seguieri* had its own cpDNA haplotype. The total length of the single most parsimonious tree was 11 steps. As the most parsimonious phylogenetic tree (data not shown) had a high topological congruence with the cpDNA network, we analysed only the phylogenetic relationships expressed in the cpDNA network (Fig. 1B). Both phylogenetic analyses concurred in recognizing *R. aconitifolius* and *R. platanifolius* as one monophyletic unit, whereas *R. seguieri* was more closely related to the ingroup (Fig. 1B). The spatial distribution of the six *R. kuepferi* haplotypes is illustrated in Fig. 1A. As highlighted by the cpDNA network, RK1 is more closely related to *R. seguieri* than to any of the other cpDNA haplotypes within *R. kuepferi* (Fig. 1B). This cpDNA haplotype was found isolated in the Vercors, with one occurrence in the Southern Alps (LAR; Fig. 1A), whereas *R. seguieri* is widely distributed throughout the European mountainous massifs. Among *R. kuepferi*, RK6 was the most derived haplotype and was restricted to northern Corsica. The other cpDNA haplotypes were largely spread throughout the Alps, with RK2 and RK5 also occurring respectively in the Apennines and southern Corsica. Moreover, various cpDNA lineages were sympatric in several populations (e.g. COM, CAY, LAR, IZO, GSB, NAV, TON and VAR included between two and four different cpDNA haplotypes). Globally both phylogenetic and network analyses coincided in pointing out the polyphyletic status of the two subspecies defined within *R. kuepferi* (i.e. diploid and polyploids co-occurred in different cpDNA haplotypes; Fig. 1).

Alignment length of the ITS region was 672 bp (GenBank accession numbers: EU792758–EU792880). The ITS region included 19 informative characters and the maximum parsimony analysis yielded a most parsimonious tree with a length of 24 steps (data not shown). As for the cpDNA analysis, the most parsimonious tree had a high level of topological congruence with the network and to avoid redundancy only the latter will be discussed here (File S3). The ITS network analysis highlighted the monophyly of *R. seguieri*, the closely related status of *R. aconitifolius* and *R. platanifolius* (i.e. individuals were distributed in a monophyletic clade, but neither of the two taxa was monophyletic when considered individually) and the monophyly of *R. kuepferi*. In this ITS analysis, 12 steps separated *R. kuepferi* from *R. seguieri* and six steps separated *R. kuepferi* from the entity composed of *R. aconitifolius* and *R. platanifolius*. This contrasts with the cpDNA analysis in which *R. kuepferi* and *R. seguieri* were more closely related,

Table 1 Distribution of the eight cpDNA haplotypes sorted by chloroplastic markers and species: one for both *Ranunculus aconitifolius* and *R. platanifolius*, one for *R. seguieri* and six within *R. kuepferi*

	rpL20-rps12 IGS				*trnL* intron					*trnL-trnF* IGS	
Base pair	20	79	144	245	64	65	72	120	221	552	622
Site no.	1	2	3	4	5	6	7	8	9	10	11
RA+RP	`TTT`**A**`TTT`	`ATT`**C**`TTC`	`TTT`**C**`CAG`	`TAT`**-**`AAA`	`AAA`**A**`--G`	`AAA`**--**`GG`	`GCA-GTT`	`GCT`**G**`TTC`	`ATA`**G**`GAA`	`TTT`**T**`ATT`	`GTG`**A**`GAG`
RS	...**A**...	...**A**...	...**C**...	...**C**...	...**A**...	...**-**...	...**A**...	...**G**...	...**G**...	...**G**...	...**G**...
RK1	...**A**...	...**A**...	...**C**...	...**C**...	...**-**...	..-**-**...	...**A**...	...**G**...	...**G**...	...**G**...	...**G**...
RK2	...**A**...	...**A**...	...**C**...	...**C**...	...**A**...	...**-**...	...**A**...	...**A**...	...**G**...	...**G**...	...**G**...
RK3	...**A**...	...**A**...	...**C**...	...**C**...	...**A**A..	...**A**...	...**A**...	...**A**...	...**G**...	...**G**...	...**G**...
RK4	...**C**...	...**A**...	...**C**...	...**C**...	...**A**...	...**-**...	...**A**...	...**A**...	...**G**...	...**G**...	...**G**...
RK5	...**A**...	...**A**...	...**A**...	...**C**...	...**A**...	...**-**...	...**A**...	...**A**...	...**G**...	...**G**...	...**G**...
RK6	...**A**...	...**A**...	...**A**...	...**C**...	...**A**...	...**-**...	...**A**...	...**A**...	...**A**...	...**G**...	...**G**...

Only the 11 polymorphic sites (at base pairs 20, 79, 144, 245, 64, 65, 72, 120, 221, 552 and 622) are shown with variant characters in bold.

RA, *Ranunculus aconitifolius*; RK, *Ranunculus kuepferi*; RP, *Ranunculus platanifolius*; RS, *Ranunculus seguieri.*

while *R. aconitifolius* and *R. platanifolius* were more distant. However, the monomorphic status of individuals within *R. kuepferi* made this region worthless to address its within-species structure.

AFLP data set and patterns of genetic diversity

Ninety-four loci (i.e. 42 and 52 respectively for the two primer combinations *MseI*-CAC/*EcoRI*-ATC and *MseI*-CTG/*EcoRI*-ATG) were polymorphic and 100% reproducible in the replicates. When considering local genetic diversities, highest values were recovered in the Alpes Maritimes, in the contact zone between cytotypes (Fig. 2). In contrast, lower values were encountered in the central Alps, Apennines and northern Corsica. Diploid populations of the Vercors massif (COB and NAT) showed relatively high genetic diversities, considering the occurrence of only one single cytotype in this area.

AFLP-based spatial genetic structure: K-means, STRUCTURE, Jaccard-distances-based PCoA and among-clusters Nei distances

Evaluation of the inter-cluster inertia in the *K*-means method allowed selecting the best number of clusters. Accordingly, the best *K*-value was $K = 12$ (D''[inter cluster inertia] = $4 \times 10e\text{-}3$; File S4). The 12 *K*-means clusters, referred to as R1–R12, discriminated *R. kuepferi* (subdivided into eight clusters: R1–R8) from the outgroup species (*R. aconitifolius*, *R. platanifolius* and *R. seguieri* divided into four clusters: R9–R12). Within outgroups, only *R. seguieri* demonstrated its own cluster

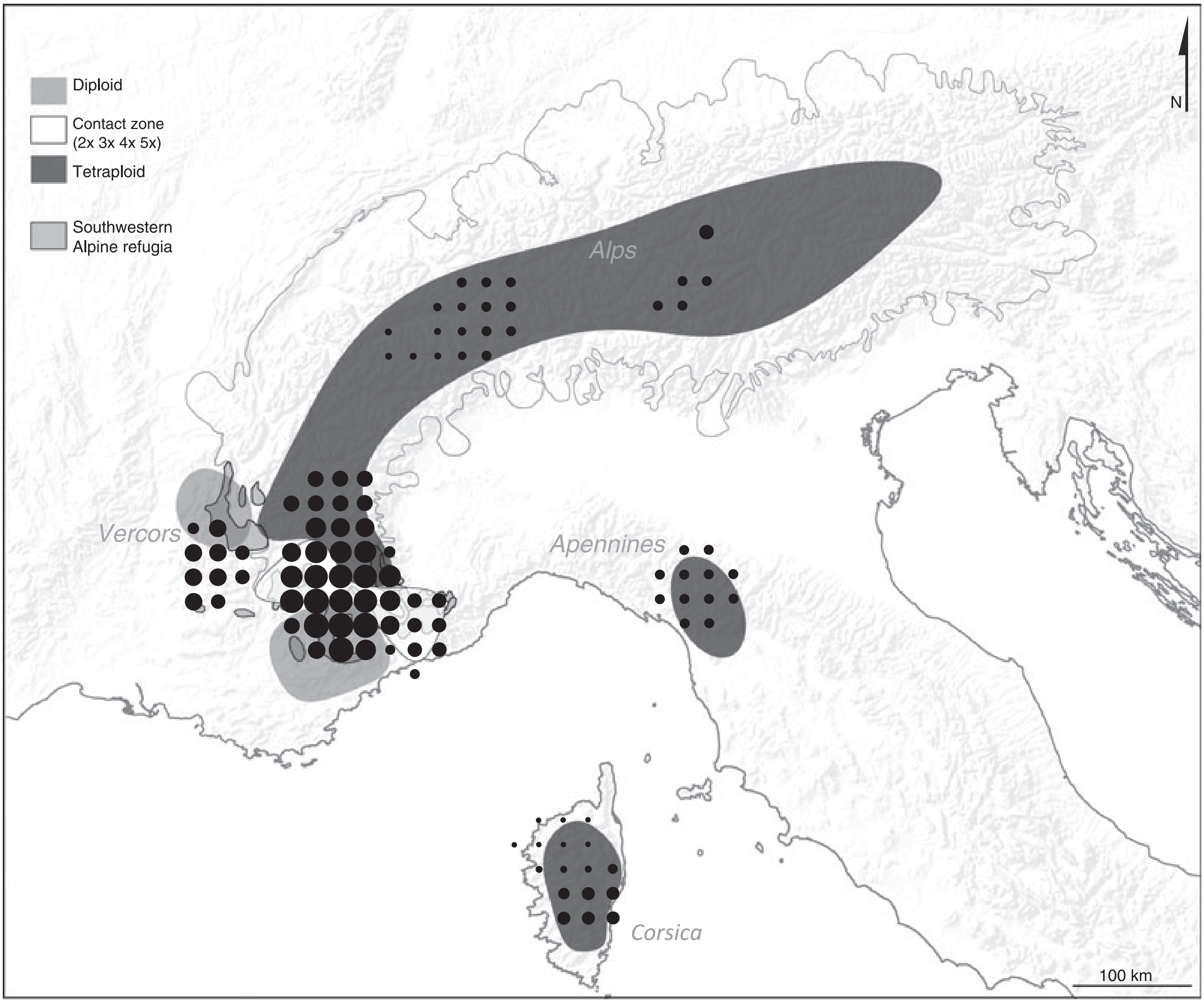

Fig. 2 Geographical distribution of the Nei's genetic diversities. Cytotypes distributions, glaciations limits and southwestern refugia are also indicated. See text for more details for the calculation of the genetic diversities.

(R9), whereas the two other species were mixed throughout the remaining clusters. Within *R. kuepferi*, the diploid populations from the Alpes Maritimes and those from the Vercors massif were respectively clustered in R1 and R2, whereas the remaining clusters were composed of polyploid populations. The STRUCTURE analysis reached the highest likelihood with $K = 8$ (ln[log] = −5852.7; $D'' = 2.1$; File S4). The eight STRUCTURE clusters, referred to as S1–S8, segregated the outgroup species from *R. kuepferi* and included all *R. platanifolius* and *R. aconitifolius* specimens, as well as one *R. seguieri* individual in one single cluster (S8). The other *R. seguieri* specimens were, however, not clearly assigned to any cluster. Similar to the results addressed by the *K*-means analysis, the *R. kuepferi* diploid populations from the Alpes Maritimes and those from the Vercors massif were respectively clustered in S1 and S2, whereas the remaining clusters were composed of polyploid populations. A contingency table was built to compare *K*-means and Structure clustering (Table 2): three clusters demonstrated a high level of congruence (>95%; R1–R3 and S1–S3), one group showed a slightly lover level of congruence (94.9%; R7 and S7) whereas the remaining clusters were less congruent (<75%, R4–R6, R8 and S4–S6; Table 2). Based on the comparison between *K*-means and STRUCTURE results, we centre our discussion on highly congruent entities, with minor emphasis on less-supported clusters. Moreover, we focus mainly on the *K*-means results (see Files S5 and S6 for more details on STRUCTURE analyses).

Table 2 Contingency table confronting the clustering results obtained through *K*-means and STRUCTURE analyses, with the best *K*-value obtained in each method (respectively 12 and 8 clusters).

	Structure								
K-means	S1	S2	S3	S4	S5	S6	S7	S8	*NA*
R1	**28**								
R2		**10**							
R3			**43**						*2*
R4			1	12					*14*
R5				2	22	5			*7*
R6					4	18			
R7							37*		*2*
R8				21	14				
R9								1	*3*
R10								4	
R11								3	
R12								6	

Shaded cells represent clusters comprising only outgroup specimens; bold values refer to clusters congruent at >95% between *K*-means and STRUCTURE analyses (i.e. R1–S1, R2–S2, R3–S3). The asterisk highlights the R7–S7 cluster, which is marginally non-congruent at the 95% threshold (i.e. 94.9%). NA, samples not attributed at a threshold of 0.5 in the STRUCTURE analysis (corresponding samples are in italics).

The monophyly of *R. kuepferi* and the segregation of clusters inferred by both structuring methods were confirmed by the PCoA (Fig. 3, Files S5). All specimens of

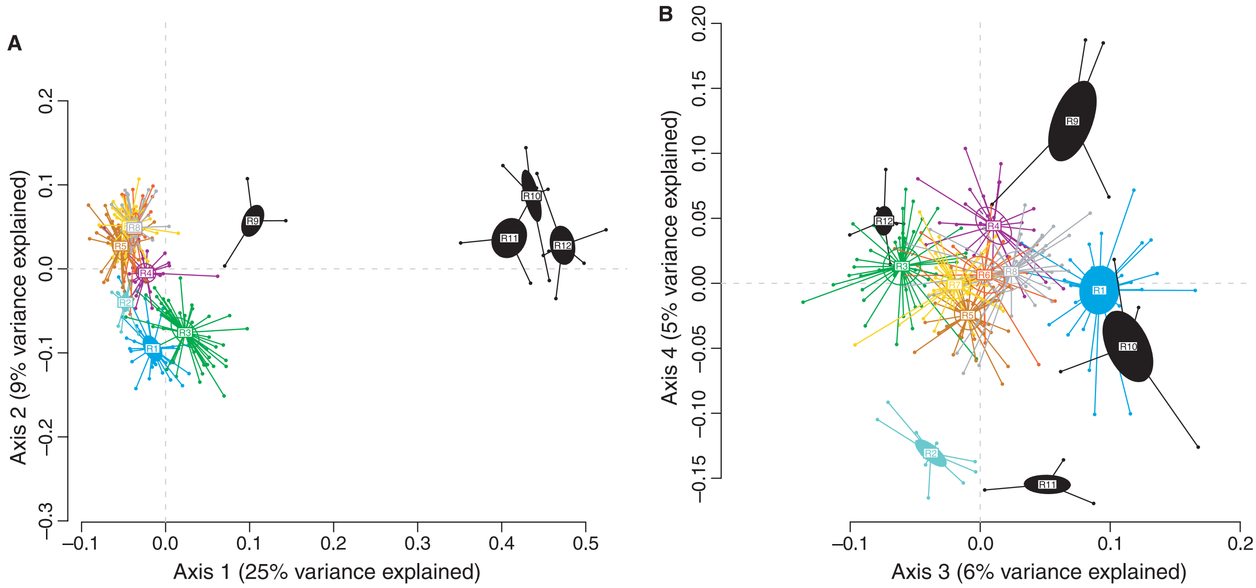

Fig. 3 Scatterplots of the PCoA analysis of the AFLP data set with *K*-means clusters. Symbols: full circles = diploids; empty circles = polyploids.

R. aconitifolius and *R. platanifolius* clustered together, with a transitional position of *R. seguieri* between the former and *R. kuepferi*. Within the ingroup, the two first axes of the PCoA (explaining 34% of the variance) recognized clusters R1 and R3 from the rest of the clusters, whereas cluster R2 is well discriminated by axes 3 and 4 (explaining 11% of the variance). *K*-means clustering results of the ingroup were geographically represented with information on southwestern alpine refugia and on the maximal extension of the Alpine ice sheet during the last glacial maximum (Fig. 4; see Files S6 for STRUCTURE results). The three well-supported clusters (R1–R3) are respectively distributed in the Vercors (diploid specimens included in R2), Alpes Maritimes (diploid specimens included in R1) and in the contact zone between cytotypes in the southern edge of the Alps (2x, 3x, 4x and 5x specimens are comprised in R3). The marginally non-congruent cluster R7, only composed of tetraploids, is much more spread out all over the central and eastern range of the Alps, with even an incursion into the Appenines. The remaining clusters are distributed throughout the Alps, Apennines and in Corsica (where *K*-means recognized a specific cluster R8 only shared with a few Alpine individuals from GSB, IZO, MUN and TON). These widespread clusters frequently occurred in sympatry and strictly included polyploid individuals.

The neighbour-joining tree based on the Nei's genetic distances between clusters was highly congruent with the PCoA and recognized clusters R1, R2 and R3 as

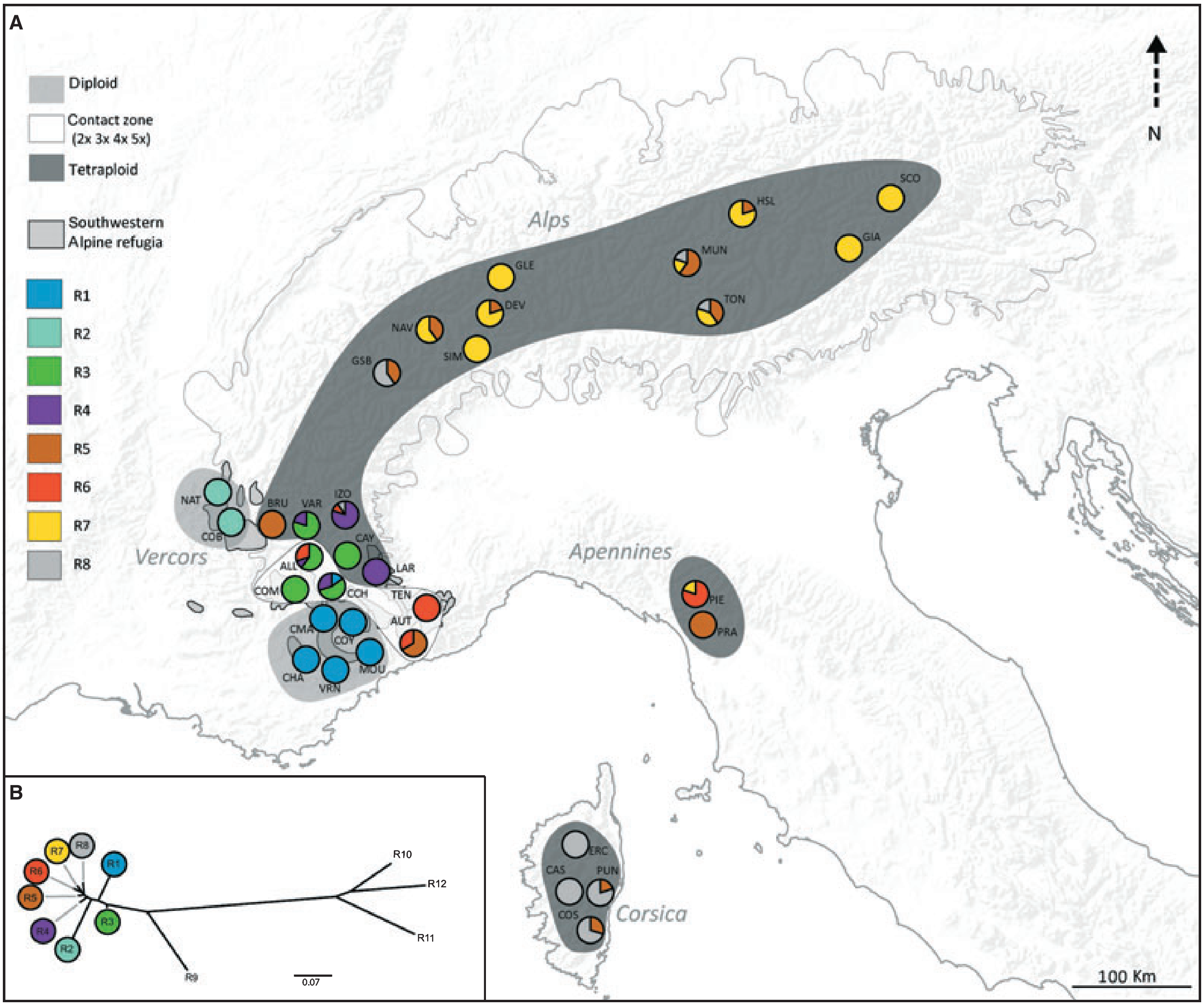

Fig. 4 (A) Geographical distribution of the eight *K*-means AFLP clusters within *Ranunculus kuepferi*. Cytotypes distribution as well as maximum extension of the Alpine ice sheet and putative southwestern refugia (according to Schönswetter *et al.* 2005) are also indicated. (B) Neighbour-joining tree based on the genetic distances between the 12 clusters defined by the *K*-means analysis.

discriminated entities, whereas the remaining clusters were much more closely related (Fig. 4B). This representation also illustrates the basal position of cluster R3 (composed of specimens ranging from diploids to pentaploids) compared with the other polyploid clusters (R4–R8).

Congruence between cpDNA haplotypes and AFLP structuring

Globally, AFLP and cpDNA data sets did not coincide in the distribution of clusters and haplotypes (Table 3). However, several lines of compatibility can be addressed. For instance, the diploid lineages were restricted to clusters R1 and R2, and corresponded respectively to RK1 and RK3–RK4 cpDNA haplotypes; nonetheless, no cpDNA haplotype was restricted to diploid specimens. Another example is found with clusters R6, R7 and R8, composed of a majority of specimens showing respectively RK2, RK5 and RK6 cpDNA haplotypes.

Table 3 Contingency table confronting cpDNA haplotypes and *K*-means clusters among *Ranunculus kuepferi*

	cpDNA haplotypes					
K-means	RK1	RK2	RK3	RK4	RK5	RK6
R1			6 (2x)	1 (NA) 5 (2x)		
R2	5 (2x)					
R3			1 (4x)	2 (NA) 1 (2x) 1 (3x)	5 (NA) 2 (3x) 6 (4x) 1 (5x)	
R4	2 (4x)	1 (NA) 1 (4x)		1 (NA) 2 (3x) 2 (4x)	2 (NA)	
R5		2 (3x) 5 (4x)			8 (4x)	1 (4x)
R6		4 (NA) 1 (3x) 2 (4x)		2 (4x)	2 (3x)	
R7		3 (4x)			12 (4x)	
R8		1 (4x)			6 (4x)	11 (4x)

Ploidy levels are indicated between parentheses.
NA, ploidy level unknown.

Discussion

Phylogenetic position of Ranunculus kuepferi *among closely related white-flowered buttercups*

With the aim of clarifying the phylogenetic position of *Ranunculus kuepferi* among the white-flowered buttercups, three closely related species were included in this study: *Ranunculus platanifolius*, *R. aconitifolius* (considered as the most external species in the maximum parsimony phylogenetic analyses) and *R. seguieri*. Although a considerable portion of the geographic range (throughout the Alpine range and for *R. platanifolius* even in Corsica) was sampled for the two first representatives, no cpDNA variation was encountered and both the AFLP-based clustering analyses and the ITS network grouped the two taxa together (Figs 1B and 3; File S3). These results provide additional evidence to how closely these taxa are related, as first proposed by Huber (1988), based on morphological and karyological (2n = 2x = 16) features. According to the cpDNA network, *R. seguieri* occupied a transitional position between the first two species and *R. kuepferi* (Fig. 1A). Unexpectedly, the *R. kuepferi* cpDNA haplotype RK1 is particularly closely related to *R. seguieri* (Fig. 1B), suggesting a potential polyphyletic status of *R. kuepferi* (Fig. 1B). Nevertheless, the status of *R. kuepferi* as a well-circumscribed entity is confirmed by the clustering analyses and by the ITS network (Fig. 3; File S3). Furthermore, the ITS network showed a well-supported distinction between *R. kuepferi* and *R. aconitifolius* plus *R. platanifolius* that are six steps distant, whereas *R. seguieri* is 12 steps distant. Such a high level of divergence between *R. kuepferi* and *R. seguieri* contrasts with the close relationship indicated by the cpDNA analysis.

Distribution of cytotypes

Chromosome counting confirmed the restriction of the diploid cytotype to the southwestern margin of the Alps as proposed by several authors (e.g. Küpfer 1974; Greuter 1987; Huber 1988), whereas the presumed apomictic tetraploid cytotype had a wider area of distribution than expected (with the exception of the southwestern margin of the Alps). A contact zone between diplo- and tetraploid cytotypes was confirmed by the presence of unbalanced cytotypes (tri- and pentaploids) (Fig. 2). Furthermore, the distribution of diploid populations and populations comprising multiple levels of ploidy coincided with southern alpine refugia, whereas a majority of tetraploid populations are encountered in the whole range of the Alps, previously covered by ice during the last glacial maximum (Schönswetter *et al.* 2005; Fig. 1). This pattern fits the predictions of the geographical parthenogenesis hypothesis (Vandel 1928; Hörandl 2006), in which the diploid sexual ancestors are confined to southern regions, while the polyploid relatives have larger distributional ranges and tend to colonize higher latitudes and altitudes (Bierzychudek 1985). In addition, it was shown that the polyploid (and presumably apomictic) plants had greater abilities to colonize previously glaciated areas than their sexual relatives (Bierzychudek 1985). In our case, the tetraploid cytotype colonized the Apennines and Corsica in addition to the whole range of the Alps, which suggests greater dispersal abilities relative to the diploid cytotype (Fig. 1). Genetic distances based on AFLP data supported the pattern highlighted above with higher diversities in the southwestern Alps (in diploid populations as well as in the contact zone) with a substantial decrease of diversity in the rest of the Alpine range (Fig. 2). In strictly tetraploid populations, the tendency towards a reduction of diversity might be due to bottleneck effects during the colonization of new areas (Fig. 2). In our opinion, this result clearly demonstrates a colonization edge syndrome in the easternmost distribution area. The results presented here attest the long history of *R. kuepferi* in the southwestern margin of the Alps, presumably due to the presence of refugia in this zone during the last glaciation (Schönswetter *et al.* 2005). Compared with the other diploids, populations from the Vercors massif (NAT and COB) show a lower genetic diversity and are the least distant from *R. seguieri* based on the cpDNA network (Fig. 1). These features are consistent with an ancient and long-lasting geographical isolation of this massif.

Phylogeography of R. kuepferi*: an intricate history revealed by plastid and nuclear evidence*

The cpDNA haplotypes associated with sexual diploids are restricted to the southwestern margin of the Alps and are divided into three geographical areas: Vercors (composed of the RK1 haplotype: NAT and COB), centre-south (composed of the RK4 haplotype: CMA, COY, CCH) and southernmost (composed of the RK3 haplotype: CHA, VRN, MOU). Populations from the Vercors massif are geographically separated by the Durance valley from the other diploids. As mentioned above, the cpDNA network provided evidence for an ancient segregation of RK1, compared with the other cpDNA haplotypes, particulary RK3 and RK4 (Fig. 1B). Interestingly, only one tetraploid specimen from LAR shares this cpDNA haplotype (Fig. 1A); this sample might be considered either as a relict of a first polyploidization event or as a recent recipient of long-distance gene flow. AFLP analyses also succeeded in discriminating the Vercors populations (R2), concurring in addressing the putative ancient history of *R. kuepferi* within this remote massif. Overall, AFLP data addressed the existence of three well-supported clusters (R1–R3), whereas the other clusters should be considered as one loosely defined entity (even if both *K*-means and Structure analyses recognized some subsplitting, with R7 being only marginally non-congruent at the 95% level; Table 2). Interestingly, the northern tetraploid populations of Corsica (ERC, CAS and PUN) possess a specific cpDNA haplotype (RK6), whereas the southern populations share their cpDNA haplotype (RK5) with northern Alpine populations of tetraploids (Fig. 1A). Based on the available data, we are unable to provide arguments to unambiguously explain the history of the northern Corsica cpDNA haplotype; however, *K*-means analysis also identified the cluster R8 as restricted to Corsica with only very few occurrences in the Alps (Fig. 4). Based on the Nei's neighbour-joining tree, both diploid clusters R1 and R2 could have been potential ancestors to tetraploid lineages (belonging to clusters R4-R8), whereas tetraploid specimens belonging to R3 exhibited a transitional position between diploid specimens of *R. kuepferi* and *R. seguieri* (R9; Fig. 4B). Two hypotheses might explain this pattern: (i) diploid ancestors of R3 were not sampled or extinct; or (ii) this tetraploid lineage results from hybridization between diploids of *R. kuepferi* and diploids of *R. seguieri*. Based on results presented here, we are not able to provide evidence in favour of one of these hypotheses. Nonetheless, there may have been at least two independent polyploidization events (one resulting in clusters R4–R8 and one being at the origin of cluster R3) followed by the colonization of the whole Alpine range by tetraploids that eventually reached the Apennines and southern Corsica (Fig. 4).

Inferences about apomixis in R. kuepferi

To fully understand the contemporary genetic structure of plants, both nuclear and plastid markers are needed (Eidesen *et al.* 2007). This is particularly true when studying species presenting sexual and apomictic modes of reproduction, such as what is presumed in *R. kuepferi*. The haploid cpDNA, usually nonrecombinant and maternally inherited provides relevant information about genetic structure mediated by seed dispersal, whereas AFLP markers provide insight about the genetic structure of the whole genomic DNA, intermixing evolutionary histories of seeds and pollen. Theoretically, in the context of strict apomixis, the patterns inferred by cpDNA and nuclear markers should be identical. In contrast, in the case of sexual reproduction, different patterns might be obtained; with deep phylogeographical histories revealed by cpDNA and shallower phylogeographical events revealed by nuclear markers (Eidesen *et al.* 2007). In our study, there were several discrepancies between cpDNA and AFLP patterns (Table 3). For instance, sexual diploid individuals segregated into three distinct lineages at the cpDNA level but were assigned to only two distinct clusters in the AFLP analysis (Figs 1 and 4 and Table 3). Among tetraploids, discrepancies are even present at the within-population level. In LAR, for example, the five individuals were assigned to the same AFLP cluster (R4), whereas they comprise four distinct cpDNA haplotypes (RK1, RK2, RK4 and RK5). This observation reflects possible gene flow between tetraploids. This type of pattern should not be observed in a strict apomictic mode of reproduction and therefore represents evidence suggesting sexual reproduction in polyploid lineages of *R. kuepferi*. The facultative apomictic reproduction in tetraploids is also supported by the presence of triploid and pentaploid individuals that are entirely restricted to the contact zone between diploids and tetraploids. Being limited to this sympatric area, these unbalanced cytotypes are likely to be the result of crossings between one diploid and one tetraploid parent. This hypothesis was previously argued by Huber (1985) and Vuille & Küpfer (1985), who suggested that the triploid individuals certainly resulted from the crossing of a diploid reduced gamete (n = 1x) and a tetraploid reduced gamete (n = 2x). In contrast, pentaploid individuals might result from the crossing of a diploid reduced gamete (n = 1x) with an unreduced tetraploid gamete (n = 4x), although one could also imagine that pentaploids might originate from the crossing of a triploid unreduced gamete (n = 3x) with a tetraploid reduced gamete (n = 2x). Nonetheless, it is evident that tetraploid gametes must be involved and must be fertile to produce triploid and/or pentaploid individuals. Experimental crosses performed more than 20 years ago tend to show that both scenarios are likely to have occurred in *R. kuepferi* (C. Vuille, unpublished data). Moreover, observation of aborted megaspores in pentaploid lineages (File S7) and combination of developed sexual and apomictic embryo sacs in tetraploids (File S8) provide additional lines of evidence supporting the possibility for polyploids to undergo sexual reproduction.

Evolutionary history of Alpine polyploid complexes

In this study, we demonstrate that the phylogeography of the *R. kuepferi* complex is in agreement with both a geographical parthenogenesis hypothesis (Hörandl 2006) and a colonization edge syndrome due to postglacial dispersals. However, the pattern addressed here contrasts with the major paradigms for postglacial recolonization pathways in Alpine plants, in which the genetic diversity of species across the Alpine range is shown to result from multiple range extensions from independent refugia (Schönswetter *et al.* 2005; Alvarez *et al.* 2009). Thus, our study highlights that in the case of polyploid complexes, postglacial range extension throughout the whole Alpine range might have resulted from the wide-scale dispersal of one single polyploid lineage that eventually spread out in other European mountain massifs. Higher dispersal abilities in such a lineage could have been driven by either its partially apomictic mode of reproduction – which ensures a higher probability of reproduction than in a strictly sexual reproduction – or by the wider ecological amplitude usually found in tetraploids. Conversely, clusters restricted to the Southern Alps can be considered as persistent refugia or in other words, as lineages that have not considerably increased their distribution range since the last glacial maximum. As a consequence, these lineages were not substantially involved in the colonization of the previously glaciated Alpine area. By contrasting the role played by multiple lineages restricted to persistent refugia (mostly composed of diploids) and that of one single tetraploid lineage mainly responsible for the species' postglacial recolonization, our study constitutes an innovative element, contributing to the understanding of the evolutionary history of Alpine polyploid complexes.

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References

Abbott RJ, Brochmann C (2003) History and evolution of the arctic flora: in the footsteps of Eric Hulten. *Molecular Ecology*, **12**, 299–313.

Adams KL, Wendel JF (2005) Polyploidy and genome evolution in plants. *Current Opinion in Plant Biology*, **8**, 135–141.

Albach DC (2007) Amplified fragments length polymorphisms and sequence data in the phylogenetic analysis of polyploids: multiple origins of *Veronica cymbalaria* (Plantaginaceae). *New Phytologist*, **176**, 481–498.

Alvarez N, Thiel-Egenter C, Tribsch A *et al.* (2009) History or ecology? Substrate type as a major driver of spatial genetic structure in Alpine plants. *Ecology Letters*, **12**, 632–640.

Bandelt H-J, Dress AWM (1993) A relational approach to split decomposition. In: *Information and Classification* (eds Opitz O, Lausen B, Klar R), pp. 123–131. Springer, Berlin Heidelberg, New York.

Benham J, Jeung JU, Jasieniuk M, Kanazin V, Blake T (1999) Genographer: a graphical tool for automated fluorescent AFLP and microsatellite analysis. *Journal of Agricultural Genomics*, **4**, 399.

Bierzychudek P (1985) Patterns in plant parthenogenesis. *Experientia*, **41**, 1255–1264.

Bonin A, Bellemain E, Bronken Eidesen P, Pompanon F, Brochmann C, Taberlet P (2004) How to track and assess genotyping errors in population genetic studies. *Molecular Ecology*, **13**, 3261–3273.

Brochmann C (1992) Polyploid evolution in arctic-alpine *Draba* (Brassicaceae). *Sommerfeltia Supplement*, **4**, 1–37.

Brochmann C, Brysting AK (2008) The Arctic – an evolutionary freezer? *Plant Ecology and Diversity*, **1**, 181–195.

Brochmann C, Soltis PS, Soltis DE (1992) Recurrent formation and polyphyly of Nordic polyploids in *Draba* (Brassicaceae). *American Journal of Botany*, **79**, 673–688.

Brochmann C, Brysting AK, Alsos IG *et al.* (2004) Polyploidy in arctic plants. *Biological Journal of the Linnean Society*, **82**, 521–536.

Chessel D, Dufour AB, Thioulouse J (2004) The ade4 package – I: One-table methods. *R news*, **4**, 5–10.

Eidesen PB, Alsos IG, Popp M, Stensrud Ø, Suda J, Brochmann C (2007) Nuclear vs. plastid data: complex Pleistocene history of a circumbolar key species. *Molecular Ecology*, **16**, 3902–3925.

Falush D, Stephens M, Pritchard JK (2007) Inferenceof population structure using multilocus genotype data: dominant markers and null alleles. *Molecular Ecology Notes*, **7**, 574–578.

Farris JS, Källersjö M, Kluge AG, Bult C (1995) Testing significance of incongruence. *Cladistics*, **10**, 315–319.

Felsenstein J (1993) *PHYLIP: Phylogeny Inference Package*, version 3.6b. Distributed by the author. University of Washinton, Seattle.

Franzke A, Hurka H (2000) Molecular systematics and biogeography of the *Cardamine* pratensis complex (Brassicaceae). *Plant Systematics and Evolution*, **224**, 213–234.

Greuter W (1987) *Ranunculus kuepferi* Greuter & Burdet, nom. nov. In: *Med-Checklist Notulae, XIV* (eds Greuter W, Raus T). *Willdenowia*, **16**, 439–452.

Gugerli F, Englisch T, Niklfeld H *et al.* (2008) Relationships among levels of biodiversity and the relevance of intraspecific diversity in conservation – a project synopsis. *Perspectives in Plant Ecology, Evolution and Systematics*, **10**, 259–281.

Guggisberg A, Mansion G, Kelso S, Conti E (2006) Evolution of biogeographic patterns, ploidy levels, and breeding systems in a diploid-polyploid species complex of *Primula*. *New Phytologist*, **171**, 617–632.

Hartigan JA, Wong MA (1979) A K-means clustering algorithm. *Applied Statistics*, **28**, 100–108.

Hörandl E (2006) The complex causality of geographical parthenogenesis. *New Phytologist*, **171**, 525–538.

Hörandl E, Paun O, Johansson JT *et al.* (2005) Phylogenetic relationships and evolutionary traits in *Ranunculus* s.l. (Ranunculaceae) inferred from ITS sequence analysis. *Molecular Phylogenetics and Evolution*, **36**, 305–327.

Huber W (1985) Neue Chromosomenzahlen bei *Ranunculus plantagineus* All. (Artengruppe des R. pyrenaeus L.). *Botanica Helvetica*, **95**, 19–24.

Huber W (1988) Natürliche Bastardierungen zwischen weissblühenden *Ranunculus*-Arten in den Alpen. *Veröffentlichungen des Geobotanischen Institutes der ETH, Stiftung Rübel Zürich*, **100**, 1–160.

Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution*, **23**, 254–267.

Jordon-Thaden I, Koch M (2008) Species richness and polyploidy patterns in the genus *Draba* (Brassicaceae): a first global perspective. *Plant Ecology and Diversity*, **1**, 255–263.

Kergoat GJ, Alvarez N (2008) Assessing the phylogenetic usefulness of a previously neglected morphological structure through elliptic Fourier analyses: a case study in *Bruchus* seed-beetles (Coleoptera: Chrysomelidae: Bruchinae). *Systematic Entomology*, **33**, 289–300.

Kluge AG (1989) A concern for evidence and a phylogenetic hypothesis of relationships among *Epicrates* (Boidae, Serpentes). *Systematic Zoology*, **38**, 7–25.

Koch M (2002) Genetic differentiation and speciation in prealpine *Cochlearia* (Brassicaceae): allohexaploid *Cochlearia bavarica* (Brassicaceae) compared to its diploid ancestor *Cochlearia pyrenaica* in Germany and Austria. *Plant Systematics and Evolution*, **232**, 35–49.

Koch M, Huthmann M, Hurka H (1998) Isozymes, speciation and evolution in the polyploid *Cochlearia* L. (Brassicaceae). *Botanica Acta*, **111**, 411–425.

Koch M, Al-Shehbaz IA, Mummenhoff K (2003) Molecular systematics, evolution, and population biology in the mustard Family (Brassicaceae). *Annals of the Missouri Botanical Garden*, **90**, 151–171.

Küpfer P (1974) Recherches sur les liens de parenté entre la flore orophile des Alpes et celle des Pyrénées. *Boissiera*, **23**, 1–322.

Lamuret R (1988) Adaptive strategies and ploidy levels. *Acta Oecologica*, **9**, 83–93.

Levy AA, Feldman M (2004) Genetic and epigenetic reprogramming of the wheat genome upon allopolyploidization. *Biological Journal of the Linnean Society*, **82**, 607–613.

Marhold K, Lihovà J (2006) Polyploidy, hybridization and reticulate evolution: lessons from the Brassicaceae. *Plant Systematics and Evolution*, **259**, 143–176.

Marhold K, Huthmann M, Hurka H (2002) Evolutionary history of the polyploid complex of *Cardamine amara* (Brassicaceae): isozyme evidence. *Plant Systematics and Evolution*, **233**, 15–28.

Morrison DA (2006) Multiple sequence alignment for phylogenetic purposes. *Australian Systematic Botany*, **19**, 479–539.

Nei M (1972) Genetic distance between populations. *American Naturalist*, **106**, 283–292.

Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Otto SP (2003) In polyploids, one plus one does not equal two. *Trends in Ecology and Evolution*, **18**, 431–433.

Ozenda P (2002) *Perspectives Pour une Géobiologie des Montagnes*. Presses Polytechniques et Universitaires *Romandes*, *Lausanne*.

Paterson A, Bowers JE, Chapman BA (2004) Ancient polyploidization predating divergence of the cereals, and its consequences for comparative genomics. *Proceedings of the National Academy of Sciences, USA*, **101**, 9903–9908.

Paun O, Lehnebach C, Johansson JT, Lockhart P, Hörandl E (2005) Phylogenetic relationships and biogeography of *Ranunculus* and allied genera (Ranunculacea) in the Mediterranean region and in the European Alpine System. *Taxon*, **54**, 911–930.

Paun O, Greuihuber J, Temsch EM, Hörandl E (2006a) Patterns, sources and ecological implications of clonal diversity in apomictic *Ranunculus carpatiola* (Ranunculus auricomus complex, Ranunculaceae). *Molecular Ecology*, **15**, 897–910.

Paun O, Stuessy TF, Hörandl E (2006b) The role of hybridization, polyploidization and glaciation for the origin and evolution of the apomictic *Ranunculus cassubicus* complex. *New Phytologist*, **171**, 223–236.

Paun O, Schönswetter P, Winkler M, Tribsch A, IntraBioDiv Consortium (2008) Evolutionary history of the calcareous alpine *Ranunculus alpestris* group (Ranunculaceae) in the European Alps and the Carpathians. *New Phytologist*, **171**, 223–236.

Popp M, Erixon P, Eggens F, Oxelman B (2005) Origin and evolution of a circumpolar polyploid species complex in *Silene* (Caryophyllaceae) inferred from low copy nuclear RNA polymerase introns, rDNA, and chloroplast DNA. *Systematic Botany*, **30**, 302–313.

Prentis PJ, Wilson JRU, Dormont EE, Richardson DM, Lowe A (2008) Adaptive evolution in invasive species. *Trends in Plants Science*, **13**, 288–294.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945–959.

R Development Core Team (2009) *A Language and Environment for Statistical Computing*. Available at http://www.R-project.org (last access 15 May 2009).

Richards AJ (2003) Apomixis in flowering plants: an overview. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, **358**, 1085–1093.

Roche D, Hanna WW, Ozias-Akins P (2001) Is supernumerary chromatin involved in gametophytic apomixis of polyploid plants? *Sexual Plant Reproduction*, **13**, 343–349.

Schönswetter P, Tribsch A, Stehlik I, Nikfeld H (2004) Glacial history of high alpine *Ranunculus glacialis* (Ranunculacea) in the European Alps in a comparative phylogeographical context. *Biological Journal of the Linnean Society*, **81**, 183–195.

Schönswetter P, Stehlik I, Holderegger R, Tribsch A (2005) Molecular evidence for glacial refugia of mountain plants in the European Alps. *Molecular Ecology*, **14**, 3547–3555.

Shaw J, Lickey Edgar B, Beck JT *et al.* (2005) The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany*, **92**, 142–166.

Stebbins GL (1980) Polyploidy in plants: unsolved problems and prospects. In: *Polyploidy, Biological Relevance* (ed. Lewis WH), pp. 495–520. Plenum Press, New York.

Stebbins GL (1985) Polyploidy, hybridization, and the invasion of new habitats. *Annals of the Missouri Botanical Garden*, **72**, 824–832.

Stehlik I, SChneller JJ, Bachmann K (2001) Resistance or emigration: response of the high- alpine plant *Eritrichium nanum* (L.) Gaudin to the ice age within the Central Alps. *Molecular Ecology*, **10**, 357–370.

Swofford DL (2003) *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods),* Version 4. Sinauer Associates, Sunderland, MA.

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of 3 non-coding regions of chloroplast DNA. *Plant Molecular Biology*, **17**, 1105–1109.

Thioulouse J, Chessel D, Dolédec S, Olivier JM (1996) ADE-4: a multivariate analysis and graphical display software. *Statistics and Computing*, **7**, 75–83.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **25**, 4876–4882.

Vandel A (1928) La parthénogénèse géographique: contribution à l'étude biologique et cytologique de la parthénogénèse naturelle. *Bulletin Biologique de France et Belgique*, **62**, 164–281.

Vos P, Hogers R, Bleeker M *et al.* (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**, 4407–4414.

Vuille C, Küpfer P (1985) Aposporie chez le *Ranunculus parnassifolius* L. I. Etude cytoembryologique. *Bulletin de la Société Neuchâteloise des Sciences Naturelles*, **108**, 123–134.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications* (eds Innis MA, Gelfand DH, Shinsky JJ, White TJ), pp. 315–322. Academic Press, San Diego, CA.

Whitton J, Sears CJ, Baack EJ, Otto SP (2008) The dynamic nature of apomixis in the angiosperms. *International Journal of Plant Sciences*, **169**, 169–182.

Julien Burnier completed this research as the main part of his Masters thesis at the University of Neuchâtel. He is interested in the phylogeography of alpine plants and in GIS, as a tool for testing hypotheses of spatial genetic structure. Sven Buerki is working on the evolutionary history of plants, at small and large spatio-temporal scales, with a focus on biogeographic and systematic questions. He is currently finishing his PhD at the University of Neuchâtel with a scope on the Sapindaceae family. Nils Arrigo investigates the fate of polyploid lineages and develops bioinformatic tools in different areas of research. He is currently a post-doctoral fellow at the University of Neuchâtel, characterizing gene flow between wheat and wild relatives. Philippe Küpfer has dedicated his career to the study of a large number of angiosperm families and has formulated numerous hypotheses regarding the evolution of alpine plants. He is currently honorary professor at the University of Neuchâtel. Nadir Alvarez investigates patterns and processes involved in the ancient and recent evolutionary histories of different groups in plants and insects. He is currently Junior Lecturer at the University of Neuchâtel.

Supporting information

Additional supporting information may be found in the online version of this article.

File S1 Population acronyms, species, locations, ploidy levels and number of individuals genotyped and sequenced (with the corresponding GenBank accession numbers) in the 42 investigated populations of white-flowered buttercups (i.e. 33 *Ranunculus kuepferi*, three *Ranunculus aconitifolius*, four *Ranunculus platanifolius* and two *Ranunculus seguieri*).

File S2 Mitotic metaphase of a pentaploid (2n = 5x = 40) specimen (ALL3) of *Ranunculus kuepferi*.

File S3 ITS parsimony-splits network of the four white-flowered buttercups species.

File S4 On the left, inertias and second derivates of the *K*-means analysis. On the right, likelihoods and second derivatives of the STRUCTURE analysis.

File S5 Scatterplots of the PCoA analysis of the AFLP data set with STRUCTURE clusters highlighted.

File S6 Geographical distribution of the seven STRUCTURE AFLP clusters within *Ranunculus kuepferi*. Cytotypes distributions, glaciation limits and south-western refugia are also indicated.

File S7 Ovule of a pentaploid (2n = 5x = 40) specimen of *Ranunculus kuepferi* from the Southern Alps. In this specimen, the archespore aborted before meiosis (thin arrow) and only an unreduced asexual embyro sac will be produced from a cell of the nucellus (2n = 5x = 40; thick arrow).

File S8 Ovule of a tetraploid (2n = 4x = 32) specimen of *Ranunculus kuepferi* from the Southern Alps. In this specimen, both asexual (thick arrow) and sexual (thin arrow) embyro sacs are produced synchronously. The thick arrow shows the aposphoric initial cell (2n = 4x = 32), whereas the thin arrow indicates the megaspore (n = 2x = 16).

Supplementary File #1

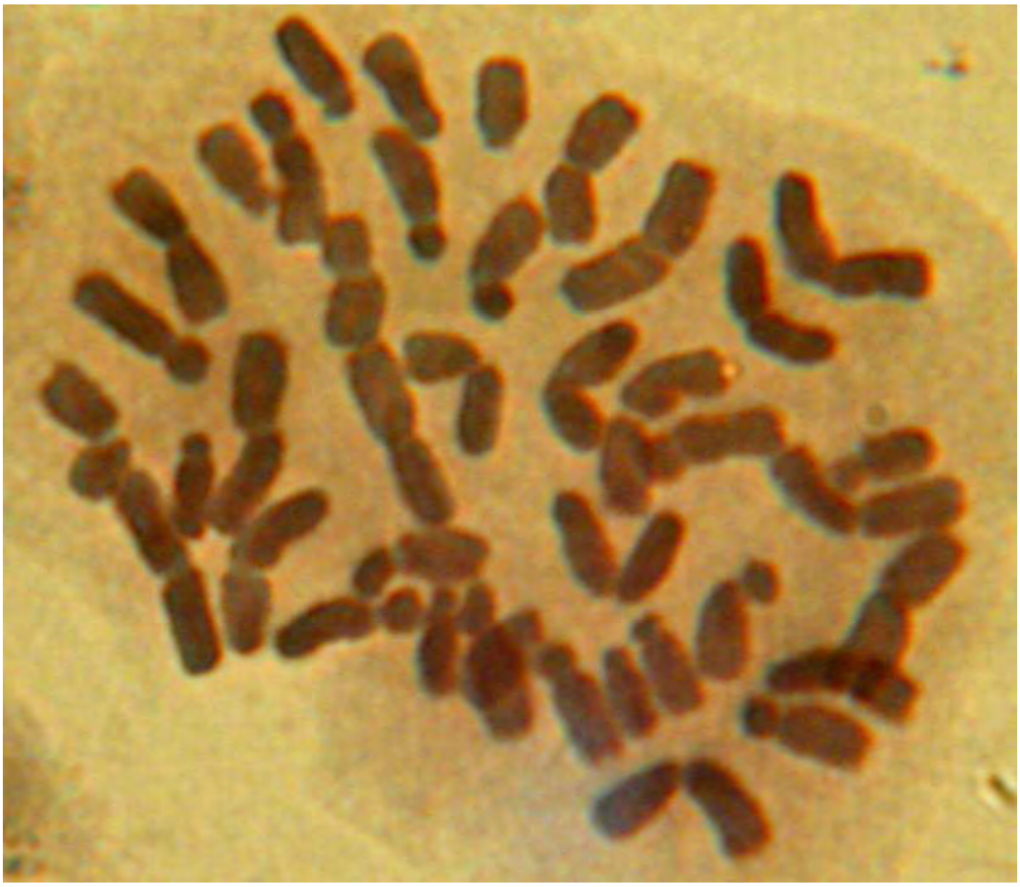
Population acronyms, species, locations, ploidy levels, and number of individuals genotyped and sequenced (with the corresponding Genbank accession numbers) in the 42 investigated populations of white-flowered buttercups (i.e., 33 *R. kuepferi*, three *R. aconitifolius*, four *R. platanifolius* and two *R. seguieri*).

Acronym	**Species**	**Country**	**Region**	**Location**	**Ploidy (2N)**	**Nb. individuals genotyped/ sequenced**	***rpL20-rps12* IGS**	***trnL* intron + *trnL-trnF* IGS**
ALL	*Ranunculus kuepferi*	France	Alpes de Haute Provence	Col d'Allos	3x, 4x, 5x	10 / 6	ALL1: *EU792634* ALL2: *EU792635* ALL3: *EU792636* ALL4: *EU792637* ALL5: *EU792638* ALL6: *EU792639*	ALL1: *EU792512* ALL2: *EU792513* ALL3: *EU792514* ALL4: *EU792515* ALL5: *EU792516* ALL6: *EU792517*
AUT	*Ranunculus kuepferi*	France	Alpes Maritimes	Plan Claval	3x, 4x	15 / 5	AUT1: *EU792642* AUT2: *EU792643* AUT3: *EU792644* AUT4: *EU792640* AUT5: *EU792641*	AUT1: *EU792520* AUT2: *EU792521* AUT3: *EU792522* AUT4: *EU792518* AUT5: *EU792519*
BRU	*Ranunculus kuepferi*	France	Hautes Alpes	Les Bruyères	4x	5 / 2	BRU1: *EU792647* BRU2: *EU792648*	BRU1: *EU792525* BRU2: *EU792526*
CAS	*Ranunculus kuepferi*	France	Corse du Sud	Cime de Castellaccion	4x	8 / 4	CAS1: *EU792673* CAS2: *EU792674* CAS3: *EU792675* CAS4: *EU792676*	CAS1: *EU792550* CAS2: *EU792551* CAS3: *EU792552* CAS4: *EU792553*
CAY	*Ranunculus kuepferi*	France	Alpes de Haute Provence	Col de la Cayolle	-	10 / 5	CAY1: *EU792653* CAY2: *EU792649* CAY3: *EU792650* CAY4: *EU792651* CAY5: *EU792652*	CAY1: *EU792531* CAY2: *EU792527* CAY3: *EU792528* CAY4: *EU792529* CAY5: *EU792529*
CCH	*Ranunculus kuepferi*	France	Alpes Maritimes	Col des Champs	2x, 3x	20 / 5	CCH1: *EU792655* CCH2: *EU792656* CCH3: *EU792657* CCH4: *EU792658* CCH5: *EU792654*	CCH1: - CCH2: *EU792533* CCH3: *EU792534* CCH4: *EU792535* CCH5: *EU792532*
CHA	*Ranunculus kuepferi*	France	Alpes de Haute Provence	Mourre de Chanier	2x	5 / 2	CHA1: *EU792690* CHA2: *EU792705*	CHA1: *EU792567* CHA2: *EU792582*
CMA	*Ranunculus kuepferi*	France	Alpes de Haute Provence	Montagne de Chamatte	2x	5 / 3	CMA1: *EU792659* CMA2: *EU792660* CMA3: *EU792661*	CMA1: *EU792536* CMA2: *EU792537* CMA3: *EU792538*
COB	*Ranunculus kuepferi*	France	Drome	Vallon de Combau	2x	5 / 3	COB1: *EU792662* COB2: *EU792725* COB3: *EU792726*	COB1: *EU792539* COB2: *EU792602* COB3: *EU792603*
COM	*Ranunculus kuepferi*	France	Alpes de Haute Provence	Les Combes	3x	10 / 5	COM1: *EU792664* COM2: *EU792663* COM3: *EU792665* COM4: *EU792666* COM5: *EU792667*	COM1: *EU792541* COM2: *EU792540* COM3: *EU792542* COM4: *EU792543* COM5: *EU792544*
COS	*Ranunculus kuepferi*	France	Corse du Sud	Plateau du Coscione	4x	10 / 6	COS1: *EU792670* COS2: *EU792671* COS3: *EU792672* COS4: *EU792694* COS5: *EU792756* COS6: *EU792757*	COS1: *EU792547* COS2: *EU792548* COS3: *EU792549* COS4: *EU792571* COS5: *EU792632* COS6: *EU792633*
COY	*Ranunculuskuepferi*	France	Alpes de Haute Provence	Petit-Coyer	2x	5 / 2	COY1: *EU792668* COY2: *EU792669*	COY1: *EU792545* COY2: *EU792546*

Acronym	**Species**	**Country**	**Region**	**Location**	**Ploidy (2N)**	**Nb. individuals genotyped/ sequenced**	***rpL20-rps12* IGS**	***trnL* intron + *trnL-trnF* IGS**
DEV	*Ranunculus kuepferi*	Italy	Piemonte	Alpe Devero	4x	10 / 2	DEV1: *EU792677* DEV2: *EU792678*	DEV1: *EU792554* DEV2: *EU792555*
ERC	*Ranunculus kuepferi*	France	Corse du Nord	Vallon de l'Erco	4x	10 / 5	ERC1: *EU792679* ERC2: *EU792680* ERC3: *EU792681* ERC4: *EU792682* ERC5: *EU792683*	ERC1: *EU792556* ERC2: *EU792557* ERC3: *EU792558* ERC4: *EU792559* ERC5: *EU792560*
GIA	*Ranunculus kuepferi*	Italy	Veneto	Passo di Giau	4x	5 / 2	GIA1: *EU792686* GIA2: *EU792687*	GIA1: *EU792563* GIA2: *EU792564*
GLE	*Ranunculus kuepferi*	Switzerland	Valais	Gletsch	4x	5 / 2	GLE1: *EU792688* GLE2: *EU792689*	GLE1: *EU792565* GLE2: *EU792566*
GSB	*Ranunculus kuepferi*	Italy	Val d'Aoste	Col du Gd St.Bernard	4x	5 / 3	GSB1: *EU792693* GSB2: *EU792691* GSB3: *EU792692*	GSB1: *EU792570* GSB2: *EU792568* GSB3: *EU792569*
HSL	*Ranunculus kuepferi*	Austria	Tirol	Hochsölden	4x	5 / 2	HSL1: *EU792695* HSL2: *EU792696*	HSL1: *EU792572* HSL2: *EU792573*
IZO	*Ranunculus kuepferi*	France	Hautes Alpes	Col d'Izoard	4x	10 / 3	IZO1: *EU792697* IZO2: *EU792698* IZO3: *EU792699*	IZO1: *EU792574* IZO2: *EU792575* IZO3: *EU792576*
LAR	*Ranunculus kuepferi*	Italy	Piemonte	Col de Larche	4x	10 / 5	LAR1: *EU792700* LAR2: *EU792701* LAR3: *EU792702* LAR4: *EU792704* LAR5: *EU792703*	LAR1: *EU792577* LAR2: *EU792578* LAR3: *EU792579* LAR4: *EU792581* LAR5:*EU792580*
MOU	*Ranunculus kuepferi*	France	Alpes Maritimes	Montagne de l'Estrope	2x	5 / 2	MOU1: *EU792685* MOU2: *EU792684*	MOU1: *EU792562* MOU2: *EU792561*
MUN	*Ranunculus kuepferi*	Switzerland	Grisons	Alp da Munt	4x	5 / 2	MUN1: *EU792706* MUN2: *EU792707*	MUN1: *EU792583* MUN2: *EU792584*
NAT	*Ranunculus kuepferi*	France	Drome	Col Naturel	2x	5 / 2	NAT1: *EU792708* NAT2: *EU792748*	NAT1: *EU792585* NAT2: *EU792624*
NAV	*Ranunculus kuepferi*	Switzerland	Valais	Nava	4x	5 / 2	NAV1: *EU792709* NAV2: *EU792710*	NAV1: *EU792586* NAV2: *EU792587*
PIE	*Ranunculus kuepferi*	Italy	Emilia Romagna	Monte Piella	4x	5 / 3	PIE1: *EU792711* PIE2: *EU792712* PIE3: *EU792713*	PIE1: *EU792588* PIE2: *EU792589* PIE3: *EU792590*
PRA	*Ranunculus kuepferi*	Italy	Emilia Romagna	Monte Prado	4x	5 / 3	PRA1: *EU792714* PRA2: *EU792715* PRA3: *EU792716*	PRA1: *EU792591* PRA2: *EU792592* PRA3: *EU792593*
PUN	*Ranunculus kuepferi*	France	Corse du Sud	Punta Bocca Dell'Oro	4x	5 / 4	PUN1: *EU792718* PUN2: *EU792645* PUN3: *EU792646* PUN4: *EU792717*	PUN1: *EU792595* PUN2: *EU792523* PUN3: *EU792524* PUN4: *EU792594*
SCO	*Ranunculus kuepferi*	Austria	Karntern	Höhe Tauern	4x	5 / 3	SCO1: *EU792737* SCO2: *EU792738* SCO3: *EU792736*	SCO1: *EU792613* SCO2: *EU792614* SCO3: *EU792612*
SIM	*Ranunculus kuepferi*	Switzerland	Valais	Col du Simplon	4x	5 / 2	SIM1: *EU792739* SIM2: *EU792740*	SIM1: *EU792615* SIM2: *EU792616*
TEN	*Ranunculus kuepferi*	Italy	Piemonte	Col de Tende	3x, 5x	9 / 5	TEN1: *EU792741* TEN2: *EU792742* TEN3: *EU792744* TEN4: *EU792745* TEN5: *EU792743*	TEN1: *EU792617* TEN2: *EU792618* TEN3: *EU792620* TEN4: *EU792621* TEN5: *EU792619*
TON	*Ranunculus kuepferi*	Italy	Lombardia	Paso del Tonale	4x	5 / 2	TON1: *EU792746* TON2: *EU792747*	TON1: *EU792622* TON2: *EU792623*
VAR	*Ranunculus kuepferi*	France	Hautes Alpes	Col de Vars	4x	10 / 5	VRA1: *EU792753* VRA2: *EU792749* VRA3: *EU792750* VRA4: *EU792751* VRA5: *EU792752*	VAR1: *EU792629* VAR2: *EU792625* VAR3: *EU792626* VAR4: *EU792627* VAR5: *EU792628*
VRN	*Ranunculus kuepferi*	France	Var	Varneige	2x	5 / 2	VRN1: *EU792754* VRN2: *EU792755*	VRN1: *EU792630* VRN2: *EU792631*

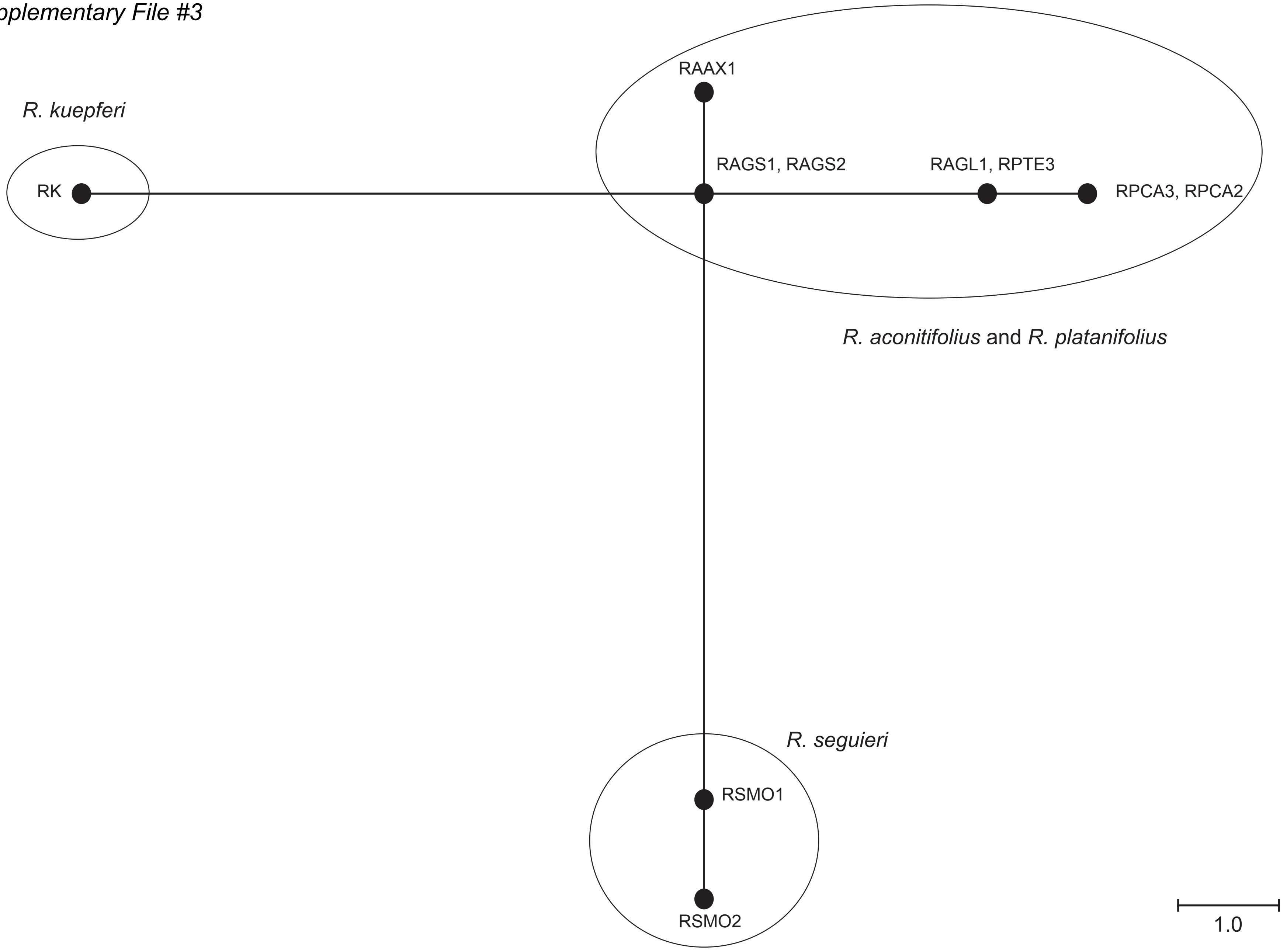
Acronym	**Species**	**Country**	**Region**	**Location**	**Ploidy (2N)**	**Nb. individuals genotyped/ sequenced**	***rpL20-rps12* IGS**	***trnL* intron + *trnL-trnF* IGS**
RAAX	*Ranunculus aconitifolius*	Switzerland	Bern	Axalpe	2x	1 / 1	RAAX: *EU792727*	RAAX: *EU792604*
RAGL	*Ranunculus aconitifolius*	Switzerland	Valais	Gletsch	2x	1 / 1	RAGL: *EU792719*	RAGL: *EU792596*
RAGS	*Ranunculus aconitifolius*	Italy	Val d'Aoste	Col du grand St.Bernard	2x	2 / 2	RAGS1: *EU792720* RAGS2: *EU792721*	RAGS1: *EU792597* RAGS2: *EU792598*
RPBO	*Ranunculus platanifolius*	Italy	Trentino-Alto Adige	Monte Bondone	2x	1 / 1	RPBO: *EU792728*	RPBO: *EU792605*
RPCA	*Ranunculus platanifolius*	France	Corse du Sud	Cime de Castellaccion	2x	4 / 3	RPCA1: *EU792729* RPCA2: *EU792730* RPCA3: *EU792731*	RPCA1: *EU792606* RPCA2: *EU792607* RPCA3: *EU792608*
RPMA	*Ranunculus platanifolius*	Italy	Lombardia	Passo de Maniva	2x	1 / 1	RPMA: *EU792722*	RPMA: *EU792599*
RPTE	*Ranunculus platanifolius*	Italy	Piemonte	Col de Tende	2x	3 / 3	RPTE1: *EU792723* RPTE2: *EU792724* RPTE3: *EU792732*	RPTE1: *EU792600* RPTE2: *EU792601* RPTE3: *EU792609*
RSCO	*Ranunculus seguieri*	Italy	Trentino-Alto Adige	Monte Cornet	2x	2 / 1	RSCO: *EU792734*	RSCO: -
RSMO	*Ranunculus seguieri*	France	Alpes Maritimes	Montagne de l'Estrope	2x	2 / 2	RSMO1: *EU792733* RSMO2: *EU792735*	RSMO1: *EU792610* RSMO2: *EU792611*

Supplementary File #2



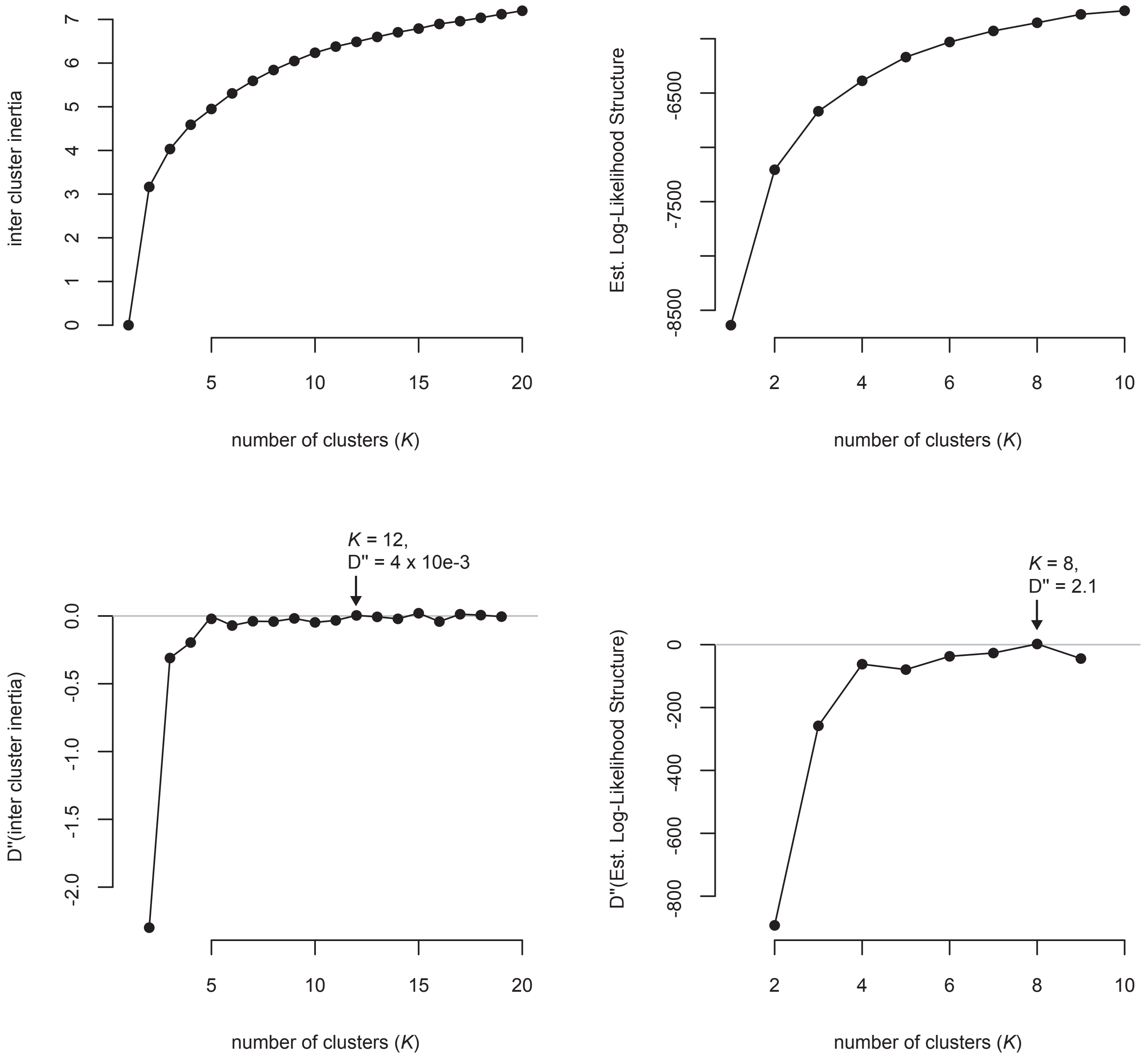
Mitotic metaphase of a pentaploid (2n=5x=40) specimen (ALL3) of *R. kuepferi*.
The picture was taken at a magnification of x1000. See manuscript for more details.

Supplementary File #3



ITS parsimony-splits network of the four white-flowered buttercups species. Specimen details are provided in supplementary file #1.

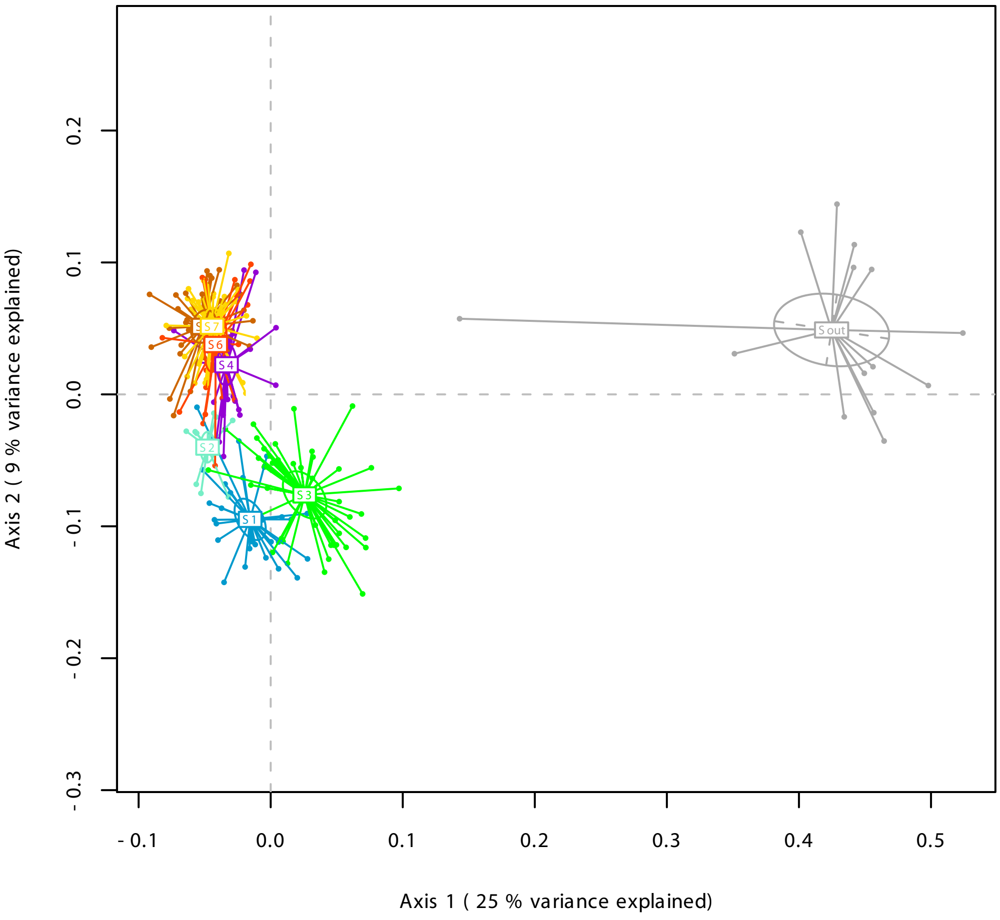
Supplementary File #4

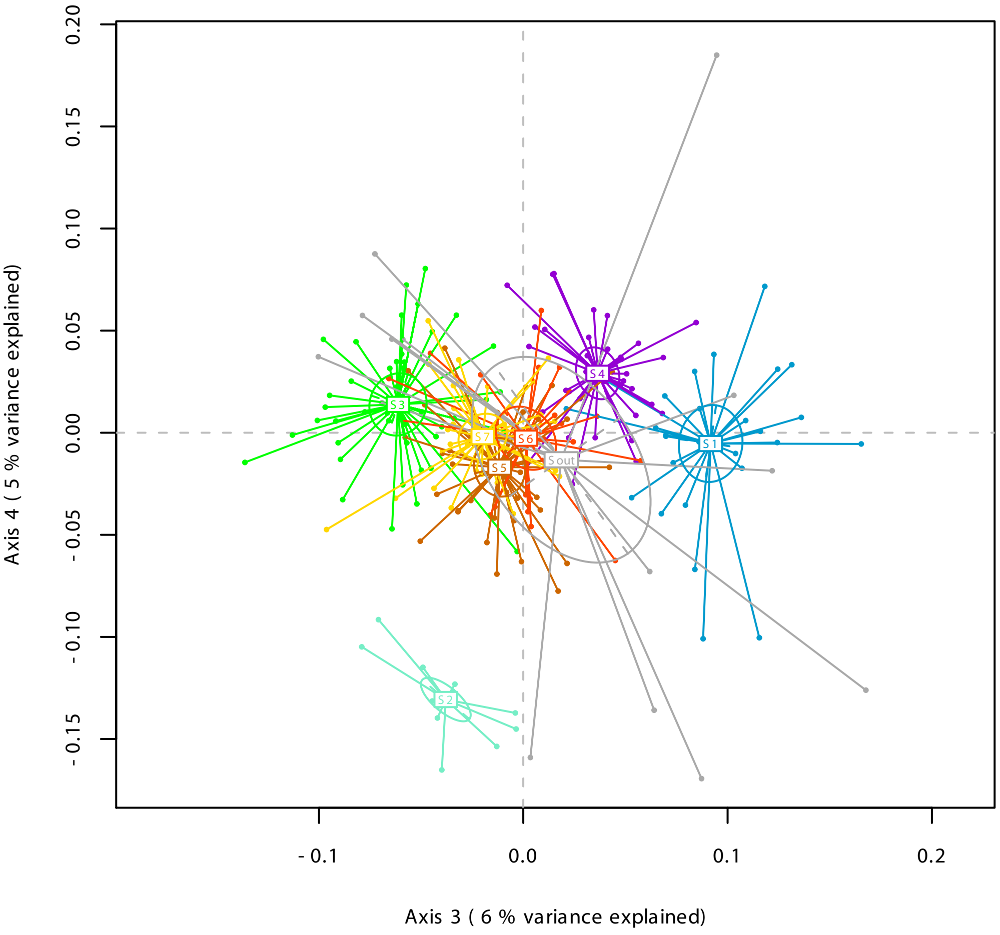


K = 12,
D'' = 4 x 10e-3

K = 8,
D'' = 2.1

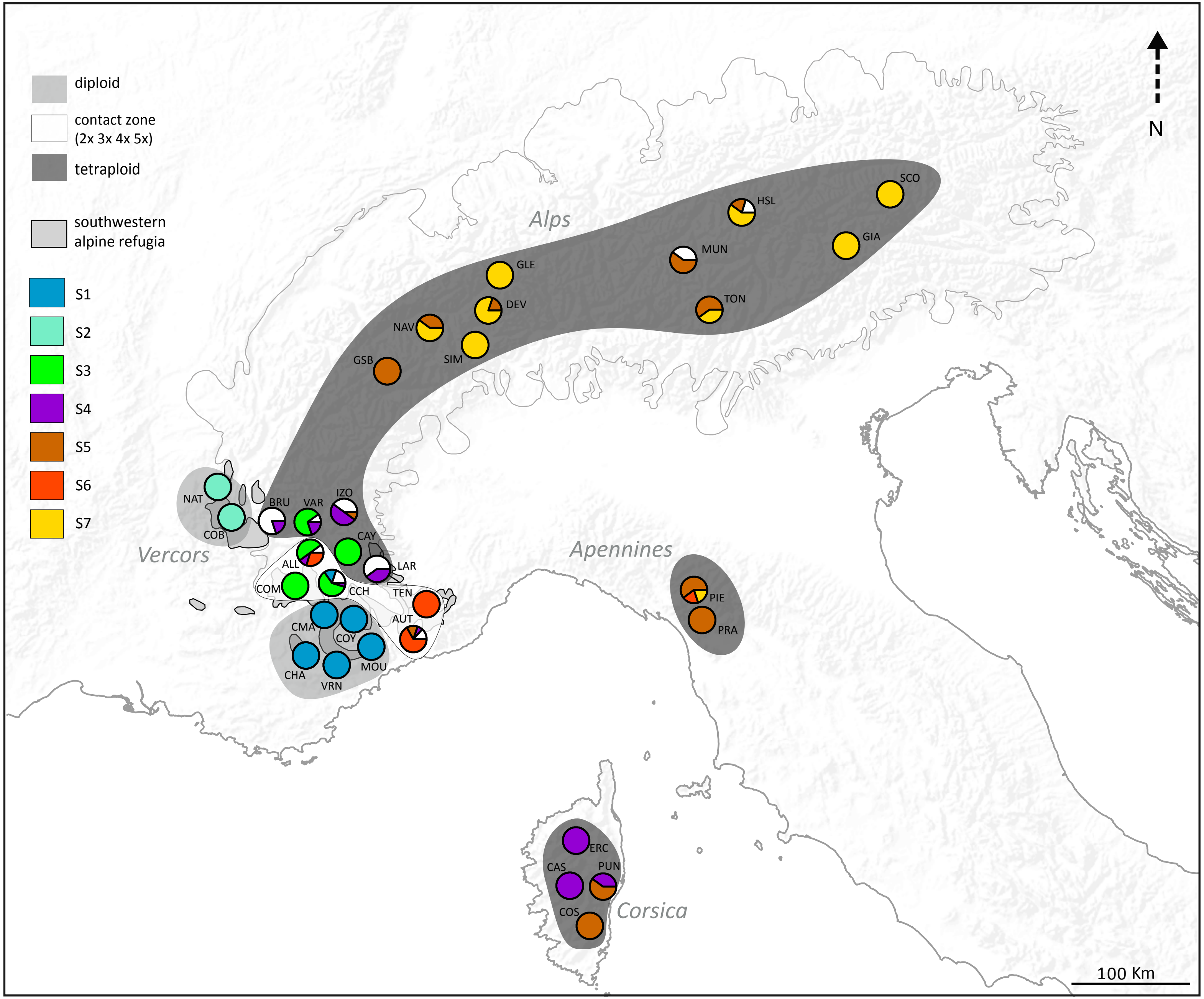
On the left, Inertias and second derivates of the K-means analysis. On the right, Likelihoods and second derivatives of the STRUCTURE analysis.





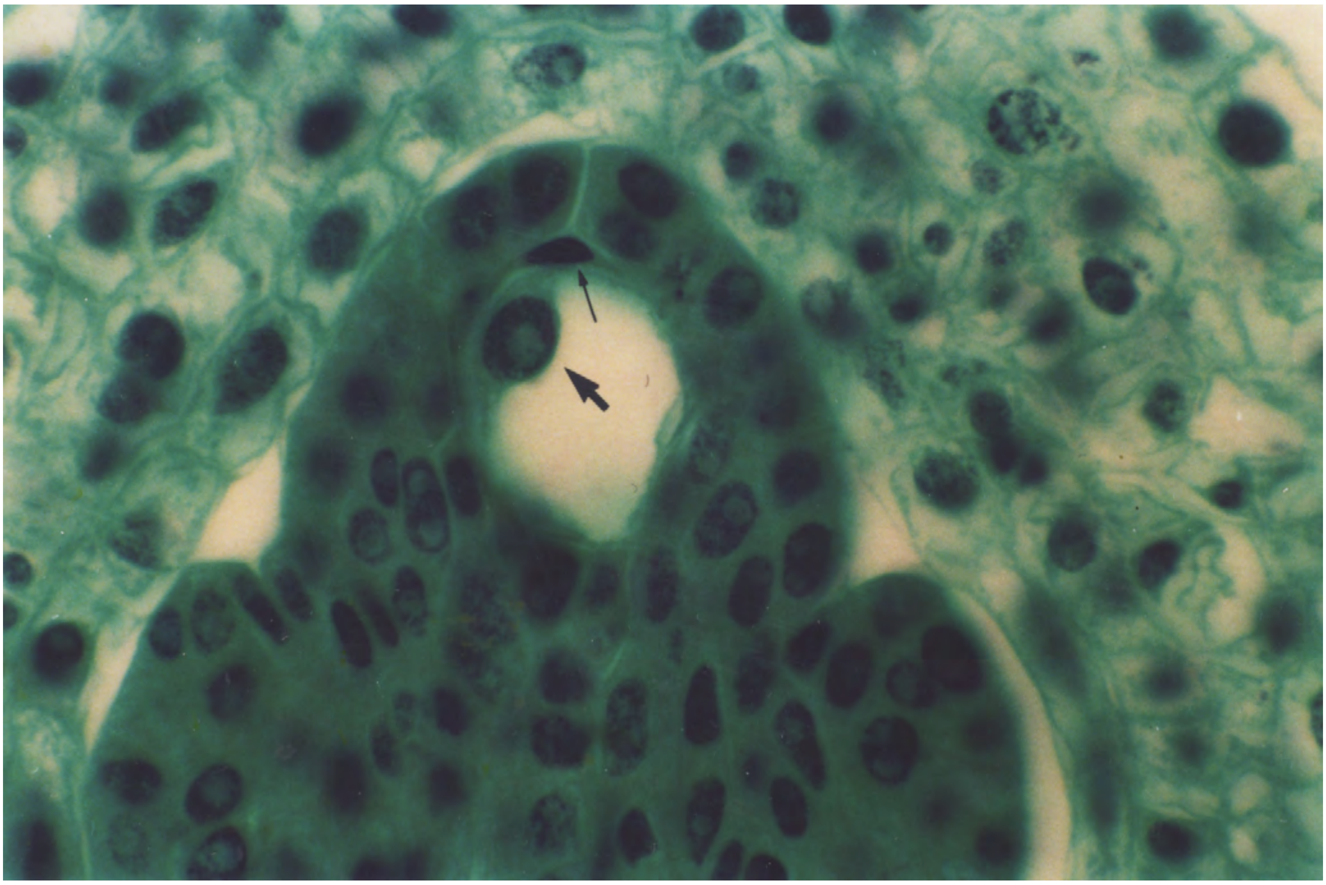
Scatterplots of the PCoA analysis of the AFLP dataset with STRUCTURE clusters highlighted.

Supplementary File #6



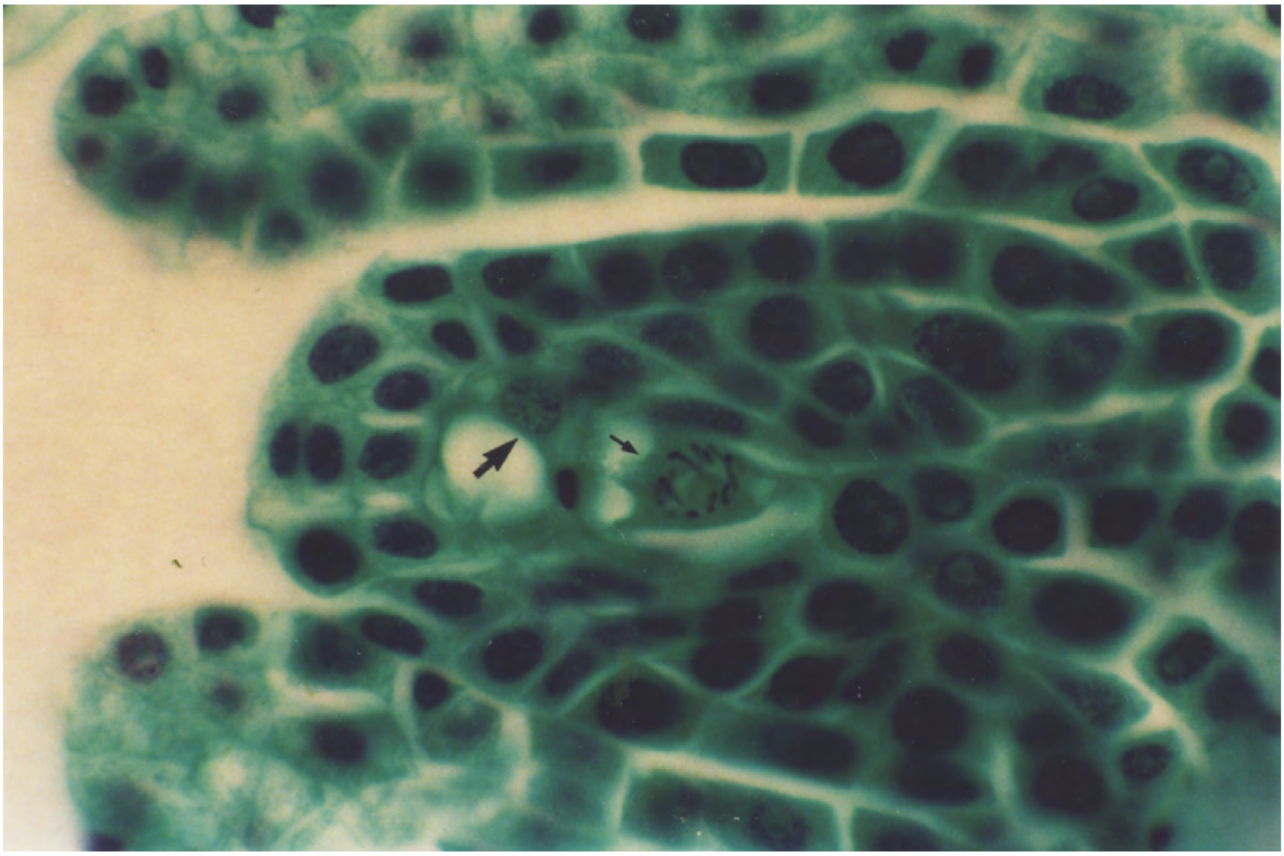
Geographical distribution of the seven STRUCTURE AFLP clusters within *R. kuepferi*. Cytotypes distributions, glaciations limits and south-western refugia are also indicated.

Supplementary File #7



Ovule of a pentaploid (2n=5x=40) specimen of *Ranunculus kuepferi* from the Southern Alps. In this specimen, the archespore aborted before meiosis (thin arrow) and only an unreduced asexual embryo sac will be produced from a cell of the nucellus (2n=5x=40; thick arrow). The picture was taken at a magnification of x100.

Supplementary File #8



Ovule of a tetraploid (2n=4x=32) specimen of *Ranunculus kuepferi* from the Southern Alps.
In this specimen, both asexual (thick arrow) and sexual (thin arrow) embryo sacs are produced synchronously. The thick arrow shows the aposporic initial cell (2n=4x=32), whereas the thin arrow indicates the megaspore (n=2x=16).
The picture was taken at a magnification of x100.