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# DISSERTATION

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## Geographical parthenogenesis – a case study on evolution, reproduction and genetic diversity of *Ranunculus kuepferi*


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# GEOGRAPHICAL PARTHENOGENESIS – A CASE STUDY ON EVOLUTION, REPRODUCTION AND GENETIC DIVERSITY OF *Ranunculus kuepferi*

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# ABSTRACT

This doctoral thesis aims at understanding geographical parthenogenesis, which means that sexual and asexual populations of the same species complex do not share the same distribution area. In general, asexuals occupy higher altitudes and latitudes and are more widespread. Geographical parthenogenesis combines different aspects that need to be considered for understanding the pattern: the reproduction system, poly-ploidisation events, genetic diversity, and colonization ability. The alpine species *Ranunculus kuepferi* is a model for studying geographical parthenogenesis, since polyploid, putative asexual populations are widespread throughout the Alps, while diploids sexual are confined to small refugial areas. I investigated different modes of reproduction and stability cytotypes via Flow Cytometry (FC) and Flow Cytometric Seed Screening (FCSS) in seeds. The origin of polyploidisation and genetic diversity of populations was analyzed with molecular methods, by using Amplified Fragment Length Polymorphism (AFLPs; dominant marker) and microsatellites (SSRs; codominant marker). These studies were conducted on 60 populations out of the whole distributional range of the species. All these markers helped to reveal the population genetic structure and the importance of modes of reproduction for the evolution of geographical parthenogenesis. The three chapters of the thesis treat the different aspects as follows:

Cytotype stability for each ploidy level was assessed over the distribution area with FC, and the mode of reproduction was determined via FCSS. This analysis revealed that diploids remain stable and fully sexual. Triploids in the contact zone are a product of backcrossing between diploids and tetraploids. Outside the contact zone, 30% of the seed display triploid embryos in tetraploid populations, but triploid adult plants occur only once in the whole tetraploid area. The majority of tetraploids maintain a stable ploidy level via gametophytic apomixis with either pseudogamous or autonomous endosperm formation. However, a few percent of seeds of tetraploids are formed in the sexual way.

Polyploidization events may give an explanation why apomixis originated in the species *Ranunculus kuepferi*. Evidence for an autopolyploid origin of the tetraploids was presented by Bayesian Analysis of Populations Structure (BAPS) analysis of SSRs, which showed that tetraploids originated from diploids without the contribution of another genome. Tetraploids obviously originated several time from diploids since they show almost no divergence

and only few new alleles in SSRs are present in tetraploids. AFLPs show in a Neighbor Joining analysis that diploids and tetraploids share the same gene pool. Therefore, geographical success of apomicts does not result from genomic novelty, which could be predicted in allopolyploids. Apospory seems to help to overcome the problems occurring in unbalanced meiosis caused by multivalent formation, which can be assumed from genotypes exhibiting multiple allelism in SSR loci. This uncommon combination of autopolyploidy and apospory stabilizes the reproductive system and hence the ploidy level.

Effects of breeding system and genetic diversity of populations were further studied by using AFLPs and SSRs. Tetraploids of *Ranunculus kuepferi* have a breakdown of the self-incompatibility system, allowing for pseudogamous selfing, whereas diploids remain self-incompatible. Therefore, apomicts can easily establish new populations, potentially with a single seed, and thus they have superior colonization ability. A BAPS analysis of AFLP data confirms populations-specific gene pools in apomicts which have probably resulted from multiple founder events. Diploids ought to have the advantage of higher genetic diversity via recombination, as our result show a genetic diversity typical for sexual outcrossers. However, tetraploids show the same level of diversity with respect to heterozygosity and $F_{ST}$ values, as well as they exhibit no clonality. Genetic diversity is maintained via facultative apomixis and allows maintenance of the widespread distribution pattern of tetraploids. Tetraploids profit from the best of both reproductive systems (better colonization ability and genetic diversity), and therefore they are more efficient than the diploids to spread over the major distribution area.

# ZUSAMMENFASSUNG

Diese Dissertation hat zum Ziel, das Phänomen der geographische Parthenogenese (GP) zu verstehen. GP bedeutet, dass sexuelle und asexuelle Populationen desselben Artkomplexes nicht die gleiche geographische Verbreitung aufweisen. Im allgemeinen besetzen asexuelle Populationen höhere Lagen und Breitengrade, und sind weiter verbreitet. Geographische Parthenogenese kombiniert verschiedene Aspekte, die für das Verständnis der Verbreiungsmuster betrachtet werden müssen: das Repoduktion-System, Polyploidisierung, genetische Diversität und die Fähigkeit zur Kolonisierung. Die alpine Art *Ranunculus kuepferi* ist als Modell zum Studium der geographischen Parthenogenese geeignet, da polyploide, asexuelle Populationen im gesamten Alpengebiet weit verbreitet sind, während die diploiden sexuellen Populationen auf glaziale Refugien beschränkt sind. Ich habe verschiedene Formen der Reproduktion und die Stabilität der Zytotypen mittels Flow Cytometry (FC) und Durchflusszytometrie an Samen (FCSS) untersucht. Die Herkunft der Polyploidisierung und die genetische Vielfalt der Populationen wurde mit molekular-biologischen Methoden, mit Amplified Fragment Length Polymorphism (AFLPs; dominante Marker) und Mikrosatelliten (SSR; kodominanten Marker) analysiert. Diese Studien wurden an 60 Populationen aus dem ganzen Verbreitungsgebiet dieser Art durchgeführt. All diese Marker haben dazu beigetragen, Aufschluss über die genetische Struktur und den Einfluss der Reproduktion auf die Entstehung von geographischer Parthenogenese zu erhalten. Die drei Kapitel der Arbeit behandeln die verschiedenen Aspekte wie folgt:

Die Stabilität der Zytotypen wurde mittels FC, der Fortpflanzungsmodus mittels FCSS bestimmt. Diese Analyse ergab, dass die diploiden Populationen stabil und voll sexuell bleiben. Triploide individuen kommen in der Kontaktzone vor und sind ein Produkt von Rückkreuzungen zwischen diploiden und tetraploiden Populationen. Außerhalb der Kontaktzone weisen in tetraploiden Populationen c. 30% der Samen triploide Embryos auf, aber triploide erwachsene Pflanzen treten nur einmal im ganzen tetraploiden Verbreitungsgebiet auf. Die Mehrheit der Tetraploiden behält eine stabile Ploidiestufe mittels gametophytischer Apomixis, wobei die Bildung des Endosperms entweder pseudogam oder autonom erfolgt. Einige wenige Prozent der Samen der tetraploiden Pflanzen sind auf sexuellem Weg entstanden.

Polyploidisierung kann eine Erklärung geben, warum Apomixis in der Art *Ranunculus kuepferi* entstanden ist. Die autopolyploide Herkunft der Tetraploiden wurde mittels Bayesian-Analyse der Populations-Struktur (BAPS) Analyse der SSRs nachgewiesen. Damit konnte gezeigt werden, dass die tetraploiden aus diploiden Populationen ohne den Beitrag eines anderen Genoms entstanden sind. Die tetraploiden entstanden mehrfach von diploiden Populationen, da sie fast keine genetischen Unterschiede und nur wenige neue SSRs-Allele zeigen. AFLPs bestätigen in einer Neighbor Joining Analyse, dass diploide und tetraploide Populationen den gleichen Gen-Pool aufweisen. Daher ist der geographische Erfolg der Apomikten nicht auf den Beitrag eines neuen Genoms zurückzuführen, wie es in Allopolyploiden anzunehmen ist. Apospory scheint die Probleme in der Meiose, die durch Multivalentbildung verursacht wird, zu überwinden, was vom Vorkommen von Genotypen mit multiple Allelen in den SSR Loci angenommen werden kann. Diese ungewöhnliche Kombination von Autopolyploidie und Aposporie stabilisiert das Reproduktionssystem und damit die Ploidiestufe.

Die Auswirkung von Reproduktionssystem und der genetischen Diversität der Populationen wurden durch die Verwendung von AFLPs und SSRs untersucht. Tetraploide Populationen von *Ranunculus kuepferi* haben die Selbst-Inkompati-bilität verloren, wodurch pseudogame Selbstbestäubung ermöglicht wird, während die diploiden selbst-inkompatibel bleiben. Daher können Apomikten leichter neue Populationen, potentiell sogar mit einem einzigen Samen gründen, und zeigen damit eine bessere Fähigkeit zur Kolonisierung. Eine BAPS Analyse der AFLP Daten bestätigt, dass die Apomikten populationsspezifische Gen-Pools aufweisen, die wahrscheinlich auf mehrfache Gründer-Effekte zurückzuführen sind. Diploide sexuelle Populationen hätten den Vorteil einer höheren genetischen Vielfalt durch Rekombination. Mein Ergebnis zeigt eine genetische Diversität, die typisch für sexuelle auskreuzende Arten ist. Die tetraploiden Apomikten weisen jedoch ein gleiches Ausmaß genetischer Diversität hinsichtlich Heterozygotie und der $F_{ST}$ Werte auf; außerdem zeigen sie keine Klonalität. Genetische Vielfalt wird über fakultative Apomixis beibehalten und ermöglicht die weite Verbreitung der Tetraploiden. Die tetraploiden Populationen profitieren von Vorteilen beider Reproduktions-Systeme (besser Kolonisierungsfähigkeit und genetische Vielfalt), und sind damit effizienter als die diploiden, ein großes Verbreitungsgebiet zu besiedeln.

# CO-AUTHORSHIP STATEMENT

The thesis was conducted in a research project designed by Univ.-Doz. Dr. Elvira Hörandl. I was responsible for sampling design and material collection, development of methodology, data collection and analysis, developing the specific research questions for the three chapters and preparation of the manuscripts.

Chapter one has been published in *Annals of Botany* in 2010, co-authored by Univ.-Doz. Dr. Elvira Hörandl (University of Vienna).

Chapter two has been submitted as a manuscript co-authored by Univ.-Doz. Dr. Elvira Hörandl (University of Vienna) and Mag. Jan Rodewald (University of Vienna) who did a part of the lab work. This chapter is submitted to *Molecular Ecology* for possible publication.

Chapter three will be submitted as a manuscript co-authored by Prof Dr. Johanna Wagner (University of Innsbruck), Dr. Ursula Ladinig (University of Innsbruck) and Univ.-Doz. Dr. Elvira Hörandl (University of Vienna). Prof. Dr. Wagner and Dr. Ladinig contributed data on breeding systems. This part will be submitted for publication to *Evolution*.

# INTRODUCTION

## GEOGRAPHICAL PARTHENOGENSIS: DEFINITION AND HYPOTHESES

Geographical parthenogenesis (GP) was first recognized by Vandel (1928), describing a pattern where sexual diploid and asexual polyploidy populations have different distribution areas. Later, the pattern was confirmed in many animals and plants (Bell, 1982; Bierzychudek, 1985; Asker and Jerling, 1992; Van Dijk, 2003; Haag and Ebert, 2004; Kearney, 2005; Hörandl, 2006). Asexual organisms tend to have a wider distribution range, occur at higher elevations and at higher latitudes, and colonize more frequently previously glaciated or otherwise devastated areas than their sexual relatives. For animals, geographical parthenogenesis is often referred to effects of polyploidy than to asexuality itself (Kearney, 2005; Lundmark and Saura, 2006; Kearney and Blacket, 2008). In plants, however, polyploidy is much more frequently tied to sexual reproduction than to apomixis (Bierzychudek, 1985; Asker and Jerling, 1992; Hörandl, 2006). However, in flowering plants sexual polyploidy is not correlated to large distribution areas (Stebbins and Dawe, 1987; Hörandl, 2006). Moreover, a GP pattern seems to be established in autopolyploid species as well, where the advantage of the genetic diversity brought by hybridization and heterozygosity cannot be the trigger of the establishment of GP (Bayer, 1991; Urbani et al., 2002; Thompson and Whitton, 2006; Cosendai and Hörandl, 2010).

Of the several non-exclusive hypotheses that may play a role to explain GP pattern based on reproduction system, ecology or genetic advantages, we can consider: 1) Bakers' law, which postulates an advantage to uniparental reproduction for colonization especially after long distance dispersal because of the capacity to found a new population with a single seed (Baker, 1965; Baker, 1967); 2) polyploidy; genome multiplication provides increased diversity and recombination (Comai, 2005) and 3) hybridization, where two species cross and combine their genomes and therefore have a specific vigorous hybrid genotype (Stebbins, 1959). Both polyploidy and hybridization assume advantages of genomic novelty and increased genetic diversity; 4) The Frozen niche variation model, which postulates a better use of ecological niches by broad arrays of clones that can use the resource space more efficiently than the sexual species (Vrijenhoek, 1979, 1984, 1994); 5) General purpose genotypes, which would rely on distributional success of

single, highly adaptive clones with specific vigorous genotypes that would have a fitness advantage (Baker, 1965; Lynch, 1984). Both rely on clonally inherited genotypes that would originate via asexual reproduction; 6) and Red Queen dynamics, which is based on differential response to biotic interactions and suggest that predators, pathogens or parasites would select for genetic diversity as created by sexual reproduction inducing rare host genotypes over clones. A reduced genetic diversity would tend to occur more frequently in areas with less biotic interactions (Van Valen, 1973; Hamilton, 1980; Vorburger, 2006; Neiman, 2009). However, genetic diversity and the response to selection can be altered by polyploidy (Levin and Levin, 2002) and by clonal diversity (e.g., Van Dijk 2003). But more recently, (Hörandl et al., 2008; 2009b) pointed out that in flowering plants, the reproduction system by itself, gametophytic apomixis, might play a rather important role in the establishment of the GP pattern.

Apomixis, the asexual way of seed production in flowering plants (Nogler, 1984a; Asker and Jerling, 1992; Hörandl et al., 2001; Koltunow and Grossniklaus, 2003; Hörandl, 2009b), provides a means to avoid an unbalanced meiosis or any other aspect which affects the fitness of the offspring. Two main pathways can be discriminated in the variety of forms (Koltunow and Grossniklaus, 2003; Tucker and Koltunow, 2009): embryos can develop directly out of somatic tissues of the ovule; this form of apomixis, called adventitious embryony, is usually not linked to polyploidy and therefore not of interest for our study. The second pathway, gametophytic apomixis, involves the formation of an unreduced embryo sac via two forms, apospory or diplospory. In apospory, the embryo sac develops from a somatic cell in the nucellus, whereas in diplospory the megaspore mother cell undergoes a restitutional meiosis to give an unreduced spore, which develops into an embryo sac. In both cases, the unreduced egg cell within the embryo sac develops without fertilization into an embryo. Therefore, both diplospory and apospory bypass segregation of chromosomes at meiosis and transmit the complete maternal chromosome set to the offspring.

The use of the pollen nuclei in apomixis plays a fundamental role for the seed formation, especially for the endosperm, in two different ways; autonomous endosperm formation provides an advantage over pseudogamous apomixis which still needs pollination for fertilization of polar nuclei. Thus, pseudogamous apomicts need a pollen donor and pollinators unless the plants can use self-pollen for endosperm fertilization (Dickinson et al., 2007; Hörandl, 2008). Sexuals, in contrast, are probably more efficient in habitats with regular pollinator frequencies and benefit from the advantage of genetic diversity. Since sexual species of apomictic complexes are usually self-sterile (Dickinson et al., 2007; Hörandl, 2010), their ability to found populations in

geographically distant areas is limited by the need of mating partners and pollinators. Depending on the use of pollen for endosperm formation (pseudogamy) or not (autonomous apomixis) pollination is needed or not (Nogler, 1984a; Asker and Jerling, 1992; Koltunow and Grossniklaus, 2003). Autonomous apomixis would easier establish new populations as pollination is not needed at all, whereas pseudogamous apomixis still needs pollen to fertilize the endosperm nuclei for seed production. If the pollen is not of sufficient quality, then the fitness of the seed might drop down. Most apomicts produce fertile, meiotically reduced pollen, and the genetic factors controlling apomixis can be inherited via the pollen (e.g., Asker and Jerling, 1992; Mogie, 1992). To benefit from uniparental reproduction, pseudogamous apomicts therefore must be hermaphroditic and self-compatible (SC) to allow the pollen tube to penetrate the style and to fertilize the endosperm (Hörandl, 2008; Hörandl, 2010). However, sexual selfers potentially do have the same advantage of uniparental reproduction (Baker, 1955). Baker's law postulates that uniparental reproduction allows for founding a new population after dispersal with a single individual, as there is no need of a mating partner; therefore the species should have a more efficient colonization ability (Baker, 1965; Baker, 1967). Baker's law is therefore only an advantage to apomixis if (1) the sexual species is self-incompatible (SI); (2) if the pseudogamous apomict is self-compatible (SC) and thus independent from cross-pollination, or has autonomous apomixis (Hörandl, 2010). Baker's law would provide an explanation for the broader distribution of asexuals in previously glaciated areas, because they might have faster colonized the open, devastated areas that have been left after the retreat of glaciers. However, the negative consequence of such a colonization event would be founder effects and genetic bottlenecks, because only a restricted number of genes would be transmitted to the next generation. The genetic diversity of the founder population would therefore be reduced (Hewitt, 2004). Sexual selfers are in this respect more affected as apomicts, as selfing leads to a rapid loss of heterozygosity, potentially causing inbreeding depression, while apomixis can maintain heterozygosity in their maternal offspring.

Another aspect to take into account is that partial apomixis can increase considerably the cytotype diversity within apomictic populations. Apomixis usually requires the coordination of embryo sac development without meiosis (apomeiosis) and the development of the egg cell without fertilization (parthenogenesis). Uncoupling of these processes leads to shifts in ploidy levels (Nogler, 1984a): meiosis plus parthenogenesis results in a dihaploid offspring (n+0), while fertilization of an apomeiotic egg cell results in an increase of ploidy level (2n+n, BIII hybrids). Dihaploids and BIII hybrids

are expected to undergo a decrease of fitness (Van Dijk and Vijverberg, 2005): continued reduction of ploidy levels results in the expression of previously masked recessive disadvantageous alleles, while continued increase of ploidy levels is limited by cellular constraints and functional disturbances of regulation mechanisms (Comai, 2005). A newly arisen, partially sexual cytotype may further suffer from minority cytotype disadvantages in the population (Levin, 1975), because it will mostly receive pollen of the wrong ploidy level. This may not only have negative effects on the fitness of the embryo, but also on endosperm formation. In the endosperm of flowering plants, a ratio of two maternal and one paternal copies of the genome are optimal for development, probably because of genomic imprinting; deviations from this ratio are sometimes tolerated, but often result in disturbances of seed formation (Spielman et al., 2003; Vinkenoog et al., 2003; Talent, 2009). Since the majority of apomictic plants are pseudogamous and need pollen for endosperm fertilization, interploidal crosses pose a problem because they cause endosperm imbalance and potentially seed abortion. For these reasons, stability of cytotypes is probably an important factor for fitness and the distributional success of apomictic lineages. To avoid the penalisation of meiotic disturbance, brought up by the polyploidization of the genome, apomixis might be a way for the species to retain fertility despite an irregular meiosis leading to formation of aneuploid embryos. Aneuploidy per se is in plants not necessarily the reason for reduced fitness in the embryo, but might be a cause for sterility in forthcoming generations. Moreover, the crucial 2 : 1 ratio between maternal and paternal genome contribution cannot be maintained, and therefore endosperm formation is disturbed and can lead to abortion of the seeds (Savidan, 2007; Talent, 2009). In the case of gametophytic apomixis, the embryo sac develops from an unreduced cell, thus avoiding aneuploid megaspores and potentially reduced fitness in the offspring (Koltunow and Grossniklaus, 2003).

In mixed populations, an apomictic pollen donor can fertilize a sexual plant, thereby transferring apomixis to the offspring of the sexual. Under a simple model of dominant, single locus control for apomixis, the offspring of the sexual will become apomictic. In turn, the pollen of the sexual does not fertilize an apomictic plant, because the egg cell develops parthenogenetically. This unidirectional hybridization would result in introgression of apomixis from the apomicts into the sexual populations, but not vice versa. Theoretically, sexuality should disappear from the population after a few generations (Mogie, 1992; Mogie et al., 2007). Among other factors, this process could contribute to geographical parthenogenesis by replacing sexuals by apomicts in sympatric areas. However, Mogie (1992)

already pointed out that the amount of actual introgression also depends on female fertility. Experimental studies in *R. auricomus* have shown that introgression is blocked if sexuals and apomicts differ in their ploidy levels, and that a significant higher fertility of sexuals prevents their replacement by apomictic cytotypes (Hörandl and Temsch, 2009). That is, stability of modes of reproduction and of cytotypes may be essential for maintenance of separated distribution areas. In fact, patterns of geographical parthenogenesis have so far not been reported for those taxa with a highly facultative, unstable apomixis (reviewed in Hörandl et al., 2008).

Asexual reproduction is in general frequently connected to polyploidy, and almost all apomictic plants are polyploid (Asker and Jerling, 1992; Grimanelli et al., 2001). The tight connection of polyploidy and apomixis has usually the consequence of a differentiation and reproductive isolation of cytotypes (Kao, 2007; Van Dijk, 2007; Mraz et al., 2008). Gametophytic apomixis is intimately linked to polyploidy in plants (Asker and Jerling, 1992), with a few exceptions of occasional apomixis in diploids (Bicknell, 1997; Kantama et al., 2007; Siena et al., 2008). Polyploidization, by increasing genome size or chromosome set (Hewitt, 2004; Comai, 2005), hybridization (Stebbins, 1959) and genome remodelling (Matzke et al., 1999; Otto, 2007a, 2007b) are some of the most important triggers of evolution as they cause major rearrangements of the genome. They increase heterozygosity and genetic diversity, and could enhance the adaptive potential to various ecological niches (e.g., Soltis et al., 2003; Soltis and Soltis, 2009). Heterosis, gene redundancy and potential sub-functionalization of duplicated genes are the main advantages of polyploidization (Comai, 2005). Changes in genome size and chromosome number, however, may disturb the pairing of homologous chromosomes at meiosis, the crucial step of reproduction of a sexual organism (Leitch and Leitch, 2008). Since sexual polyploids hardly ever show distributional patterns similar to geographical parthenogenesis, it is unlikely that polyploidy *alone* is a causal factor, but may have rather indirect effects on distribution patterns (Hörandl, 2006). Polyploidy is in general frequently connected to hybridization, resulting in allopolyploidy (Otto and Whitton, 2000). It is thought that hybridization and allopolyploidy play the major role in the GP pattern for animals (Kearney, 2005, 2006; Kearney and Blacket, 2008).

In allopolyploids, these meiotic problems are usually avoided, because doubling of the chromosome sets leads to a rebalance in the genome, promoting again bivalent formation during chromosome pairing at meiosis. In contrast, genome duplication within the same species, leading to autopolyploidy (Soltis and Rieseberg, 1986; Ramsey and Schemske, 1998), can induce drastic changes in the functionality of meiosis, because the pairing

of chromosomes will lead to formation of multivalents as each set of chromosomes has originated from the same species (Comai, 2005). Because of multivalent formation in autopolyploids, tetrasomic inheritance can be observed (Soltis and Rieseberg, 1986), and unbalanced chromosome segregation can lead to aneuploid and irregular chromosome numbers in the gametes.

Autopolyploid vs. allopolyploid mode of origin have different genetic consequences and are therefore of high relevance for understanding ecological and biogeographical success of polyploid cytotypes. Hybridization potentially creates genotypes with a higher vigor because of increased heterozygosity, and may enhance ecological flexibility by producing genotypic and phenotypic novelty (Kearney, 2005). This benefit is not expected in autopolyploids. In animals, GP has often been referred to genetic consequences of hybrid origin of the asexual species (Kearney, 2005, 2006) or to polyploidy (Lundmark, 2006; Lundmark and Saura, 2006). In plants, apomicts are almost exclusively polyploids, and the geographical pattern occurs mostly in apomictic allopolyploid complexes (reviews in Bierzychudek 1985; Hörandl, 2006; Hörandl et al., 2008). Some well-studied examples of sexual autopolyploid plants do not show significantly larger distribution ranges than the diploid cytotypes (Hörandl, 2006; Soltis et al., 2007). However, some case studies suggest that geographical parthenogenesis occurs in autopolyploid apomicts as well (Yahara, 1990; Bayer, 1991; Thompson and Whitton, 2006). The question arises whether genetic effects of polyploidization would explain the ecological and biogeographical success of asexual organisms, or whether apomixis helps to disperse polyploid cytotypes. Therefore, these modes of reproduction bypass potential meiotic disturbances and sterility that are especially expected in newly formed autopolyploids (Cifuentes et al., 2010). However, evidence for autopolyploid origins of apomicts is scarce (Thompson and Whitton, 2006; Hojsgaard et al., 2008). In fact, most apomicts have shown to be of hybrid origin (Asker and Jerling, 1992; Paun et al., 2006b; Palop-Esteban et al., 2007; Sharbel et al., 2009). Traditionally, apomixis has been seen as an evolutionary route to overcome hybrid sterility (Darlington, 1937).

## THE MODEL SYSTEM

The alpine species *Ranunculus kuepferi* Greut. & Burdet is an interesting model system for studying patterns and processes of geographical parthenogenesis in previously glaciated areas. Küpfer (1974) first recognized it as a separate species with diploid (2n = 16) and tetraploid (2n = 32) cytotypes, and rare (3x and 5x) cytotypes, which makes the species suitable for studying evolutionary origins of polyploid cytotypes. Diploid sexuals

occur only in the southwestern Alps, while tetraploid apomicts colonize the whole Alps, the northern Apennines, and Corsica (Cosendai and Hörandl 2010; see also Fig. 1). Triploids in the geographical contact zone represent probably backcrosses of diploids and tetraploids (Cosendai and Hörandl, 2010). Indications of facultative apospory in polyploids have been provided by Burnier et al. (2009). Later, tetraploids have been assessed as facultative apomictic throughout the range of the cytotype, while diploids are regular sexual (Cosendai and Hörandl, 2010). Küpfer (1974) found diploids only in the southwestern parts of the Alps that have remained ice-free during the last glacial maximum of Würm glaciation (c. 10,000 years ago). This area has long been known to be a glacial refugium for many plant species (Schönswetter et al., 2005). The tetraploids, in contrast, were observed in the previously glaciated central western Alps. Later, Huber (1988) refines the distribution and reports the tetraploid cytotype eastwards to Eastern Tyrol (Austria). He assessed the presence of triploids (2n = 24) in the sympatric area of the diploid and the tetraploid cytotype, suggesting a hybrid zone. Outside the Alps, tetraploid cytotypes have been detected in Corsica (Küpfer, 1974; Huber, 1988). The species has reduced pollen fertility (50-80 % aborted pollen grains), and reduced fertility (10-100% of achenes aborted) in the tetraploid cytotype, while the diploid cytotypes had good pollen (0-20% aborted) and achenes (0-10% aborted). Beside reduced pollen fertility only few other features distinguished diploids from the tetraploids, but the tetraploid cytotype of *R. kuepferi* has aborted petals, pollen and achenes (Huber 1988) similar to other, unrelated apomictic Ranunculi (Hörandl et al., 1997; Hörandl, 2008), which is probably due to developmental disturbances related to apomixis. Beside these features, no other morphological differences have been traced between the cytotypes (Huber, 1988). Information on other cytotypes and statistical evaluations of differences, however, were so far missing. Embryological comparative embryological studies by Vuille & Küpfer (1985) on the reproductive system of *R. parnassifolius*, *R. kuepferi* and some other Ranunculaceae assessed an embryo sac formation similar to the well-studied apomictic model system *R. auricomus* (Nogler, 1984a; Nogler, 1984b; Koltunow et al., 1995; Nogler, 1995). Here meiosis takes place, but the megaspore tetrad aborts during the later development. Instead, a somatic cell of the nucellus develops into an unreduced, 8-nucleate embryo sac of the *Polygonum* type. This apomeiotic (aposporous) embryo sac development is coupled to a parthenogenetic development of the egg cell. These two processes are facultative and can be uncoupled, resulting in shifts of ploidy levels in the offspring (Nogler, 1984a, b, 1995). However, the great majority of apomicts is pseudogamous, while autonomous apomixis occurs only at very low frequencies (c. 6% of the seed material; Cosendai and Hörandl, 2010).

In *R. kuepferi*, mode of reproduction and cytotype diversity within populations has not yet been assessed throughout the distributional range. Furthermore, the ploidy level and mode of reproduction of some geographically isolated outposts in the North Apennines (Mt. Cusna) and in the eastern central Alps (populations around Turracherhöhe) were so far unknown. These areas potentially could represent glacial refugia for sexual diploid populations. The easternmost populations (Figure 1, no. 59) are located very near to the last glacial maximum eastern refugia, a spot well known for its endemic flora (Schönswetter et al., 2005). Disjunct peripheral distributions of diploid species with related polyploid cytotypes in the centre of the Alps have been observed e.g. in the *R. auricomus* complex (Paun et al., 2006a; Hörandl, 2009a) and in sexual *Biscutella laevigata* (Parisod and Besnard, 2007). In North America, diploid sexual cytotypes of *Townsendia hookeri* have a strongly disjunct distribution in peripheral refugia of the Wisconsin glaciation, while polyploid apomicts occur in the central previously glaciated area (Thompson and Whitton, 2006). Alternatively, these outposts in *R. kuepferi* could have been founded after long distance dispersal by tetraploid apomicts. This scenario would support an idea of superior founder abilities of tetraploid apomicts according to Baker's law. Therefore, the study of SI systems is essential for understanding the actual capacity of uniparental reproduction in sexuals and apomicts. We further expect that multiple founder events would result in distinct local gene pools

The evolutionary origin and the mode of polyploidization of apomictic *R. kuepferi* was so far unkown. A phylogeographic study by Burnier et al. (2009) could not discriminate between auto- vs allopolyploid origin. The closest relatives of *R. kuepferi*, the sympatrical species *R. seguieri*, *R. aconitifolius* and *R. platanifolius*, have distinct morphological features that are by no means apparent in the tetraploid cytotype of *R. kuepferi* (Huber, 1988). It is therefore unlikely that any of the extant related species were involved in the origin of tetraploid *R. kuepferi*. However, theoretically also a more distantly related or extinct sexual species could have been involved in the parentage of an allopolyploid cytotype, as it has been demonstrated in *Limonium* by using microsatellite analysis (Palop-Esteban et al., 2007). In this case, the diverged gene pool of the "cryptic" second parent was still present in the polyploid taxon.

We further wanted to elucidate the genetic consequences of uniparental reproduction: multiple founder events by single or few individuals would result in distinct local gene pools, and in a loss of genetic diversity because of genetic bottlenecks. To test ecological hypothesis based on different genetic diversity of sexual and apomictic populations requires comparative

population genetic studies throughout the range of the species, but this was not available until now.

Testing of the main hypotheses:

Three main aspects for understanding GP were studied within the thesis: Stability, distribution and mode of reproduction of cytotypes via FCSS (Flow cytometric seed screening) and FC (flow cytometry) in first chapter; tests of auto vs allo-polyploid origin of apomicts with population genetic markers SSRs (Simple Sequence Repeat) and AFLPs (Amplified Fragment Length Polymorphism) data in the second chapter, and influence of genetic diversity and breeding systems over the pattern of GP using the same molecular markers and experimental approaches.

### CHAPTER ONE:

By analysis of ploidy levels and mode of reproduction via flow cytometry, we tried to answer the following questions: does this distribution pattern represent a geographic distribution of sexual polyploidy or geographical parthenogenesis? How constant is the ploidy level within populations over all the distribution range, and how can new cytotypes be formed? In the hybrid zone, is there an indication of introgression of apomixis into the sexual populations, and do apomicts have a potential to replace the sexual lineages? Are the areas outside or near the margin of the previous ice-shield refugias of diploid sexual populations or is a colonization scenario by tetraploid apomicts more likely?

### CHAPTER TWO:

Multilocus codominant data inform about multiple allelism and heterozygosity. Multilocus dominant data can reveal the composed structure of hybrid genomes in allopolyploids (e.g., Guo et al., 2006; Paun et al., 2006a) and are potentially informative about the overall genetic divergence and single vs. multiple origins of cytotypes. Since previous phylogenetic and morphological data did not provide any specific hypothesis for parentage of the tetraploid cytotype, we analyzed genomic structure of tetraploids to test for the presence of a cryptic hybrid genome. With this combined approach we tried to answer the following questions: (1) did the tetraploid apomictic cytotype originate via allopolyploidy or autopolyploidy? (2) Are polyploids of single or multiple origins? (3) Is the mode of polyploidization relevant for the biogeographical success of apomixis?

CHAPTER THREE:

However, GP has probably a complex causality (Hörandl 2006). We inferred autopolyploid origin of 4x apomicts by using microsatellite and AFLP analysis (Cosendai et al. subm.), which rules out hypotheses for GP based on hybrid origin. A rigorous testing of alternative hypothesis based on different genetic diversity of sexual and apomictic populations requires comparative population genetic studies throughout the range of the species. With all this information, we wanted to understand in this study different aspects: 1) Does population genetic structure support a hypothesis of multiple founder events, as expected after Baker's law? 2) Do breeding systems support a hypothesis of uniparental reproduction in apomicts? 3) Do apomicts show clonal diversity or widespread clones? 4) Is genetic diversity and heterozygosity different between sexual and apomictic populations?

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# CHAPTER 1 — CYTOTYPE STABILITY, FACULTATIVE APOMIXIS AND GEOGRAPHICAL PARTHENOGENESIS IN *RANUNCULUS KUEPFERI* (RANUNCULACEAE)

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Running title: Geographical parthenogenesis in *Ranunculus kuepferi*

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## ABSTRACT

BACKGROUND AND AIMS Asexual organisms are more widespread in previously glaciated areas than their sexual relatives (“geographical parthenogenesis"). In plants, this pattern is probably dependent on reproductive isolation and stability of cytotypes within their respective distribution areas. Both partial apomixis and introgressive hybridization potentially destabilize the spatial separation of sexual and apomictic populations. The wide distribution of apomicts may be further enhanced by uniparental reproduction which is advantageous for colonization. We study these factors in the alpine species Ranunculus kuepferi.

METHODS We assessed geographical distribution, diversity and mode of reproduction of cytotypes using flow cytometry (FC) and flow cytometric seed screening (FCSS) on samples from 59 natural populations of this species, and we compared seed set of cytotypes in the wild.

KEY RESULTS Diploid sexuals are confined to the southwestern parts of the Alps, while tetraploid apomicts dominate in previously glaciated and in geographically isolated areas despite a significantly lower fertility. Other cytotypes (3x, 5x and 6x) occur mainly in the sympatric zone, but without establishing populations. The tetraploids are predominantly apomictic, but also show a partial apomixis via an uncoupling of apomeiosis and parthenogenesis in the seed material. Both pseudogamy and autonomous endosperm formation are observed which may enhance uniparental reproduction.

CONCLUSIONS Diploids occupy a glacial relic area and resist introgression of apomixis probably because of a significantly higher seed set. Among the polyploids, only apomictic tetraploids form stable populations; the other cytotypes arising from partial apomixis fail to establish, probably because of minority cytotype disadvantages. Tetraploid apomicts colonize previously devastated and also distant areas via long distance dispersal, confirming Baker's Law of an advantage of uniparental reproduction. We conclude that stability of cytotypes and of modes of reproduction are important factors for establishing a pattern of geographical parthenogenesis.

Keywords: apomixis, flow cytometry, Geographical parthenogenesis, glaciations, polyploidy, Ranunculus kuepferi.

## INTRODUCTION

Vandel (1928) presented the term "geographical parthenogenesis" for the phenomenon that related sexual and asexual taxa have different distribution areas. Later, several authors discussed the causality of the pattern for animals and plants by addressing a number of new aspects related to the wider distributions of asexuals (Bell, 1982; Bierzychudek, 1985; Asker & Jerling, 1992; Law & Crespi, 2002; Van Dijk, 2003; Kearney, 2005, 2006; Hörandl, 2006). Plants reproducing via apomixis, ie. via asexually formed seed, tend to grow at higher altitudes and latitudes, and colonize more

frequently previously glaciated areas than their sexual relatives (Bierzychudek, 1985; Van Dijk, 2003; Hörandl *et al.*, 2008).

Asexual reproduction is in general frequently connected to polyploidy, and almost all apomictic plants are polyploid (Asker & Jerling 1992). The tight connection of polyploidy and apomixis usually leads to differentiation and reproductive isolation of cytotypes (Van Dijk, 2007; Kao, 2007, Mráz *et al.*, 2008). However, facultative apomixis can increase considerably the cytotype diversity within apomictic populations. Apomixis usually requires the coordination of embryo sac development without meiosis (apomeiosis) and the development of the egg cell without fertilization (parthenogenesis). The uncoupling of these processes leads to shifts in ploidy levels (Nogler, 1984a): meiosis plus parthenogenesis results in a dihaploid offspring (n+0), while fertilization of an apomeiotic egg cell results in an increase of ploidy level (2n+n, BIII hybrids). Dihaploids and BIII hybrids are expected to undergo a decrease of fitness (Van Dijk & Vijverberg, 2005): continued reduction of ploidy levels results in the expression of previously masked recessive disadvantageous alleles, while continued increase of ploidy levels is limited by cellular constraints and functional disturbances of regulation mechanisms (Comai, 2005). A newly arisen, partially sexual cytotype may further suffer from minority cytotype disadvantages in the population (Levin, 1975), because it will mostly receive pollen of the wrong ploidy level. This may not only have negative effects on the fitness of the embryo, but also on endosperm formation. In the endosperm of flowering plants, a ratio of two maternal and one paternal copies of the genome are optimal for development, probably because of genomic imprinting; deviations from this ratio are sometimes tolerated, but often result in disturbances of seed formation (Vinkenoog *et al.*, 2003, Spielmann *et al.*, 2003; Talent, 2009). Since the majority of apomictic plants are pseudogamous and need pollen for endosperm fertilization, interploidal crosses pose a problem because they cause endosperm imbalance and potentially seed abortion. For these reasons, stability of cytotypes is probably an important factor for fitness and the distributional success of apomictic lineages. In fact, patterns of geographical parthenogenesis have so far not been reported for those taxa with a highly facultative and unstable apomixis (reviewed in Hörandl *et al.*, 2008).

Most apomicts produce fertile, meiotically reduced pollen, and the genetic factors controlling apomixis can be inherited via the pollen (e.g., Asker & Jerling 1992, Mogie 1992). In mixed populations, an apomictic pollen donor can fertilize a sexual plant, thereby transferring apomixis to the offspring of the sexual. Under a simple model of dominant, single locus control for apomixis and an apomictic pollen donor heterozygous for the apomixis factor, a part of the offspring of the sexual will become apomictic. In turn, the

pollen of the sexual does not fertilize an apomictic plant, because the egg cell develops parthenogenetically. This unidirectional hybridization would result in introgression of apomixis from the apomicts into the sexual populations, but not vice versa. Theoretically, sexuality should disappear from the population after a few generations (Mogie 1992; Mogie *et al.*, 2007). Among other factors, this process could contribute to geographical parthenogenesis by replacing sexuals by apomicts in sympatric areas. However, Mogie (1992) already pointed out that the amount of actual introgression also depends on female fertility, and a significantly higher fertility of sexuals prevents their replacement by apomictic cytotypes (Hörandl & Temsch, 2009). The fertility of cytotypes is therefore an important factor for geographical parthenogenesis.

Other theories explain geographical parthenogenesis by superior colonizing abilities. The capacity to found a new population from a single individual or seed is a big advantage for colonization, especially after long distance dispersal (Baker's law; Baker, 1967; Hörandl, 2008, Hörandl & al., 2008). Here apomixis with autonomous endosperm formation provides an advantage over pseudogamy which still needs pollination, unless the plants can use self-pollen for endosperm fertilization (Dickinson *et al.*, 2007; Hörandl, 2008). Sexuals, in contrast, are probably more efficient in habitats with regular pollinator frequencies and benefit from the advantage of genetic diversity. Since sexual species of apomictic complexes are usually self-sterile (Dickinson et al., 2007; Hörandl in prep.), their ability to found populations in geographically distant areas is limited by the need of mating partners and pollinators.

Other theories explaining patterns of geographical parthenogenesis rather rely on genetic diversity of populations and a different response of sexual and apomictic populations to variable environments (e.g., host-parasite interactions, Van Valen, 1973; Vorburger, 2006; the benefit of general purpose genotypes, Lynch 1984; niche differentiation of clones, Vrijenhoek, 1984, 1994). However, genetic diversity and the response to selection can be altered by polyploidy (Levin, 2002) and by clonal diversity (e.g., Van Dijk 2003). Therefore, the assessment of distribution and stability of cytotypes and of modes of reproduction is indirectly of crucial importance for these models.

The alpine species *Ranunculus kuepferi* Greut. et Burdet is an interesting model system for studying patterns and processes of geographical parthenogenesis in previously glaciated areas. Küpfer (1974) first recognized it as a separate species with diploid (2n = 16) and tetraploid (2n = 32) cytotypes. He found diploids only in the southwestern parts of the Alps that

have remained ice-free during the last glacial maximum of Würm glaciation (c. 10,000 years ago). This area has long been known to be a glacial refugium for many plant species (Merxmüller, 1952, 1953, 1954; Schönswetter *et al.*, 2005). The tetraploids, in contrast, were observed in the previously glaciated central western Alps. Later, Huber (1988) and Burnier et al. (2009) refined the distribution and reported the tetraploid cytotype eastwards to Eastern Tyrol (Austria). They assessed the presence of triploids (2n = 24) in the sympatric area of the diploid and the tetraploid cytotype, suggesting a hybrid zone. Outside the Alps, tetraploid cytotypes have been detected in Corsica and in the Apennines (Küpfer, 1974; Huber, 1989; Burnier et al., 2009).

Küpfer (1974) already observed reduced pollen fertility (50-80 % aborted pollen grains), and reduced fertility (10-100% of achenes aborted) in the tetraploid cytotype, while the diploid cytotypes had good pollen (0-20% aborted) and achenes (0-10% aborted). Information on other cytotypes and statistical evaluations of differences, however, were so far missing. An embryological observation by Burnier et al. (2009) on a single tetraploid specimen suggested an embryo sac formation similar to the well-studied apomictic model system *R. auricomus* (Nogler, 1984, 1995). Here meiosis takes place, but the megaspore tetrad aborts during the later development. Instead, a somatic cell of the nucellus develops into an unreduced, 8-nucleate embryo sac of the *Polygonum* type. This apomeiotic (aposporous) embryo sac development is coupled to a parthenogenetic development of the egg cell. These two processes are facultative and can be uncoupled, resulting in shifts of ploidy levels in the offspring (Nogler, 1984a, b, 1995).

In *R. kuepferi*, mode of reproduction and cytotype diversity within populations has not yet been assessed on population samples throughout the distributional range. Furthermore, the ploidy level and mode of reproduction of some geographically isolated outposts in the North Apennines (Mt. Cusna) and in the eastern central Alps (populations around Turracherhöhe) were so far unknown. These areas potentially could represent glacial refugia for sexual diploid populations. The easternmost populations (Figure 1, no. 59) are located very near to the last glacial maximum eastern refugia, a spot well known for its endemic flora (Tribsch, 2004; Schönswetter *et al.*, 2005). Disjunct peripheral distributions of diploid species with related polyploid cytotypes in the centre of the Alps have been observed e.g. in the *R. auricomus* complex (Paun *et al.*, 2006, Hörandl, 2009) and in sexual *Biscutella laevigata* (Parisod & Bernard, 2007). In North America, diploid sexual cytotypes of *Townsendia hookeri* have a strongly disjunct distribution in peripheral refugia of the Wisconsin glaciation, while polyploid apomicts occur in the central previously glaciated area (Thompson and Whitton, 2006). Alternatively, these outposts in *R. kuepferi* could have been founded

after long distance dispersal by tetraploid apomicts. This scenario would support an idea of superior founder abilities of tetraploid apomicts according to Baker's law.

For *R. kuepferi*, a detailed study on cytotype diversity and mode of reproduction throughout the range of the species was so far missing. That is, it was so far uncertain whether the species shows a pattern of geographical polyploidy or geographical parthenogenesis. In the light of the reduced fertility of tetraploid cytotypes, their distributional success appears to be a paradox; advantages of apomixis may explain the observed pattern. Therefore, we want to study stability and modes of apomixis of the species throughout its distributional range. We further want to test stability of cytotypes and modes of reproduction within the respective areas. For this aspect, also possible introgression of apomixis into sexual species within the sympatric area, and the female fertility of cytotypes are of interest. Finally, we want to test whether geographically isolated outposts outside the iceshield of the Würm glaciation represent diploid sexual refugia or colonies of tetraploid apomicts. For these questions, flow cytometric methods are a powerful approach for studying cytotype diversity on large sample sizes. For the assessment of modes of reproduction and pathways of embryo formation, flow cytometric seed screening (FCSS) is a highly efficient method (Matzk *et al.*, 2000). We focus here on intrinsic features of apomixis as factors of geographical parthenogenesis; patterns of genetic diversity within and among populations, and hypotheses on origins of apomictic cytotypes will be presented in forthcoming papers.

The following specific questions were addressed in the study: Does this distribution pattern represent a geographic distribution of sexual polyploidy or geographical parthenogenesis? How constant is the ploidy level within populations over all the distribution range, and how can new cytotypes be formed? In the hybrid zone, is there an indication of introgression of apomixis into the sexual populations, and do apomicts have a potential to replace the sexual lineages? Are the areas outside or near the margin of the previous ice-shield refugias of diploid sexual populations or is a colonization scenario by tetraploid apomicts more likely?

# MATERIAL AND METHODS

## PLANT MATERIAL

The plants used in this research were collected in the wild during spring and summer 2004-2007; for details see Table 1 and Figure 1. About one third of the individuals were cultivated in the experimental fields of the botanical garden of the University of Vienna. The rest of the samples were dried in silica gel for further molecular analysis. Mature achenes were collected in the wild and dried with silica gel, or taken from the plants in the experimental garden directly after collection (which means that embryo sac formation and fertilization already has happened before on the natural site) and stored in the fridge at 4°C.

## PLOIDY LEVEL DETERMINATION

Previous chromosome counts of root tip squashes (Cosendai, 2005) were used to fix genome size measures to ploidy levels. Samples were measured via flow cytometry (FC) on fresh and silica dried material of leaves. The difference in genome size between dried and fresh material was never more than 10 % of variation, comparable to results obtained by Suda & Trávnícek (2006) for silica dried material. We can note that the genome size of the fresh material is in this case almost always slightly smaller than the silica gel material; some of the smallest DNA content values are due to old silica gel dried material (more than four years old) and look very degraded (yellow brownish material). These samples are indicated with an asterisk in Table 3 and may be unreliable for absolute genome size estimates, but nevertheless, allow for a reliable assessment of ploidy levels. Nuclei extraction was prepared following the procedure presented in Galbraith *et al.* (1983) and Dolezel *et al.* (2007a). Leaf material was chopped in Otto buffer I modified (with 1.26 g citric acid and 6 ml Triton® -X- 100 for 60 ml final volume) (Dolezel *et al.*, 2007b; Temsch *et al.*, 2008) with *Pisum sativum* (Ps) line "Ctirad" but mostly with *Zea mays* (Zm) line "CE-777" seedlings [4.38 pg, 2.73 pg DNA/2C, respectively (Dolezel *et al.* 1998)] as standard; then RNAse (3 mg/ml; Sigma) was added to the extract and incubated at 37 °C for half an hour in a water bath. Otto II buffer with propidium iodide (PI, VWR international; final concentration 50 µg/ml) (on the basis of Baranyi and Greilhuber, 1996; Temsch *et al.*, 2009) was added to the nuclei suspension and stored at 4°C for about one hour. Samples were analyzed on a CyFlow ML flow cytometer (Partec, Muenster, Germany) equipped with a green laser (100 mW, 532 nm, Cobolt Samba, Cobolt AB, Stockholm, Sweden). 5000 particles were measured per run; mostly three runs were conducted per

sample. Analyses of the runs and peak detection were made with the FloMax® software 2.0.0.1 (Partec, Muenster, Germany). The Cx-Value in the sense of Greilhuber *et al.*, 2005, was calculated based on a linear relation between the standard and the sample fluorescence intensity. The mean values are calculated on the basis of measure 25 samples per populations. Preparations were mostly repeated three times for statistical stability. Several measures were done by pooling the samples of several individuals. Appearance of a single peak indicated that the pooled samples were of the same genome size and consequently of the same ploidy level. The pg and Cx-values were calculated per population (Table 2).

Low differences in standard deviations and low coefficients of variation (mostly below 3%; Table 2) indicate a rather stable genome size within populations, falling clearly into distinct classes corresponding to cytotypes. A regression analysis confirmed that the increase of the genome size with ploidy levels is almost perfectly linear, so that ploidy levels are almost exactly the double or the 1.5 fold of the previous ploidy level (Figure 2). This analysis confirms the reliability of the measurements for the ploidy level assessments.

### FLOW CYTOMETRY SEED SCREENING (FCSS)

We base this part of the research on mature seeds and the mode of reproduction on the basis of *Polygonum* embryo sac type as found by Vuille & Küpfer (1985) in the species *Ranunculus parnassifolius* and in *Ranunculus auricomus* Nogler (1984b, 1995). Furthermore, some recent embryological observations (Burnier et al., 2009; J. Wagner, pers. comm.) confirm that *Ranunculus kuepferi* has an embryo sac of the *Polygonum* type. The principle here is to measure the quantity of the genome in the embryo and the endosperm, which appear in two different peaks in the histogram file. With this information, one can determine the ploidy level of the embryo and endosperm, and reconstruct the pathway of seed formation (Matzk *et al.*, 2000). We prepared achenes from 58 individuals according to the same protocol as for leaves. The achenes were softened in Otto I buffer for about 5 min on ice and then chopped following the same procedure as in FC with *Zea mays* (Zm) or *Pisum sativum* (Ps) standard. We use 4-7 achenes per sample, depending on the quality and the quantity of fruits produced by the plant. Figure 3 illustrates an example of the interpretation of an FCSS histogram. Beside the peaks of the standard (Zm), we observe three peaks for the seeds: the first peak (Rk) is the embryo corresponding to a triploid Cx value; the second peak is interpreted as the G2 of the embryo as it has the double amount of DNA of the embryo peak. The third peak has a ratio / pg value of 10 Cx, corresponding to an endosperm that has arisen from two polar nuclei (3x+3x) plus fertilization of two pollen nuclei (2x+2x). Based on these data,

we were able to determine the formation of seeds and the assumed mode of reproduction.

### FERTILITY OF CYTOTYPES

Female fecundity of cytotypes was assessed on 116 individuals from 21 populations, by using achenes collected in the wild or directly after transfer to the experimental garden. The number of well-developed and aborted achenes was assessed as in Hörandl (2008). Percentages of well developed achenes per collective fruit were calculated, pooled for cytotypes. After arcsin transformation of percentages, the significance of differences between cytotypes was tested via one-way ANOVA; for this test, the single sample from a hexaploid plant was pooled with the values of pentaploids. Since Levene's statistic revealed unequal variances among groups, Tanhame's test for pairwise multiple comparisons (not assuming equal variances) was used to test differences among cytotypes. We used SPSS for Windows vs. 12 for all calculations.

## RESULTS

### SPATIAL DISTRIBUTION AND FREQUENCIES OF CYTOTYPES

The geographical distribution of cytotypes largely confirms a spatial separation of the two most frequent ploidy levels, the diploids and tetraploids (Figure 1). The distribution of diploids is confined to the southwestern borders of the Alps outside the range of the last previous glacial maximum. Tetraploids are distributed all over the Alps in the previously glaciated area, including the population near the eastern margin of the former iceshield. Also the geographically isolated populations at Mt. Cusna and Corsica are tetraploid. A more diverse zone occurs at the overlapping distribution area of diploids and tetraploids in the southwestern Alps, where diploids, triploids, tetraploids, pentaploids and hexaploids are sympatric, and co-occur sometimes in the same population. Notably, triploids and hexaploids also do occur occasionally outside the hybrid zone, in Gran Paradiso (no. 33) and in the Bernina massif (no. 47). Only diploid and tetraploid cytotypes are predominant within their respective populations (100% frequencies; Figure 1). Triploid, pentaploid and hexaploid cytotypes are less stable, because they occur either only in mixed populations, or as low-frequency cytotypes within predominantly diploid or tetraploid

populations. Within mixed populations, frequencies of triploids are highest among all the other rare cytotypes.

### MODE OF REPRODUCTION

Flow cytometric seed screening revealed an unexpected high diversity of reproductive pathways (Table 3). FCSS confirms that diploids are sexual with a 2x embryo and a 3x endosperm. We had only one seed sample from a triploid mother plant (Allos, no. 23), which appeared to be apomictic and pseudogamous. Tetraploid mother plants showed a broader range of variation in the seed formation, indicating that the species has not a fixed apomixis. The majority of tetraploid plants, are apomeiotic and pseudogamous with the use of one or two pollen nuclei for fertilization of the endosperm (4x embryos and 10x to 12x endosperm); two cases of autonomous endosperm formation (8x) are also indicated. Three individuals from the population Col della Perla (no. 12) had a fully sexual reproduction with 4x embryos and 6x endosperms. One tetraploid plant from Gr. St. Bernard (no. 35) had seeds with an hexaploid embryo, and a highly ploid, 20x endosperm. Here fertilization of an unreduced 4x egg cell by 2x pollen, and an endopolyploid endosperm is likely. Surprisingly, several samples showed triploid embryos as in Figure 3. As they came from tetraploid parents, the embryo sac must have been formed via an unbalanced meiosis to produce triploid egg cells and polar nuclei. The egg cell developed partheno-genetically, while the endosperm was probably fertilized by one or two diploid sperm nuclei. Only this way the ratio of 3x : 8x or 3x : 10x can be explained. These seeds occur in a couple of populations with predominantly 4x adult plants outside the putative hybrid zone. Several tetraploid plants formed 3x or 4x embryos, but no endosperm peak was detected. Here either abortion or a rapid consumption of the endosperm by the growing embryo could have happened, as it is known from Asteraceae (Krahulcova and Suda, 2006). Pentaploid individuals from the population Col della Perla (no. 11) seem to form reduced embryo sacs and fertilized egg cells. From hexaploid plants, we had no seeds available for analysis.

### FERTILITY OF CYTOTYPES

The diploid cytotype has the highest fertility, with 30.8 to 97.1 % of achenes within collective fruits well-developed, while all the polyploids ranged from 0 to 60.9 % (Table 4, Figure 4). The tetraploid cytotype falls with its maximum within the range of the diploids, but the interquartile range remains below the minimum of the diploids (Figure 4). However, several samples (15 individuals) had no well-developed achenes at all, including the population from Mt. Cusna. This explains that also the mean values of tetraploids remain

low. ANOVA revealed a significant difference between percentages of well-developed achenes among the cytotypes (5x and 6x pooled; df among groups = 3; df within groups = 125; $F = 68.055$; $p < 0.001$). The pairwise multiple comparisons using Tanhame's post hoc tests revealed highly significant differences between the diploids and all polyploid cytotypes ($p < 0.001$ for all pairs). Between the 4x and the pooled 5x-6x cytotype, the difference is significant ($p = 0.017$), but there is neither a significant difference between 4x and 3x nor between 3x and 5-6x cytotypes ($p > 0.5$).

## DISCUSSION

### STABILITY OF TETRAPLOID CYTOTYPES ENHANCES GEOGRAPHICAL PARTHENOGENESIS

Our data confirm that the diploid sexual populations are confined to a relic area in the southwestern Alps outside the range of the ice cover of the last glacial maximum. On the whole distribution range in the previously glaciated area, tetraploids predominate; they are stable in the sense that only occasionally other cytotypes occur within the tetraploid populations. Since tetraploids are confirmed to be predominantly apomictic or at least facultatively apomictic, these results confirm geographical parthenogenesis in this species.

Within the tetraploid populations, only a few rare hexaploid and triploid individuals occur far away from the putative hybrid zone where they are common. It seems that new cytotypes arise occasionally from partial apomixis (ie., and uncoupling of apomeiosis and parthenogenesis), but they do have some difficulty to establish. They encounter probably few mating partners of the same ploidy level, resulting in meiotic disturbances and a lower fitness in the offspring. These factors may block the establishment of a critical number of individuals for survival of the new cytotype, as Levin (1975, 2002) described under the term minority cytotype disadvantage. The apomictic tetraploid cytotype, in contrast, avoids negative effects of meiotic disturbances and does not need a mating partner. Therefore, apomicts do not suffer from being a minority in the population (Hörandl, 2006), and can readily establish even aneuploid populations, as shown by e.g., Mraz *et al.* (2008) in *Pilosella*. For endosperm fertilization, apomictic *R. kuepferi* can either use self-pollen (Huber, 1988; E. Hörandl unpubl.), but also pollen of another cytotype in the population. The great variation of ploidy levels observed in the endosperm of well-developed seed suggests that seed formation in *R. kuepferi* is not highly sensitive against endosperm imbalance,

similar to some pseudogamous Rosaceae (Talent and Dickinson, 2007). Therefore, pollination by another cytotype would not confer a minority cytotype disadvantage for endosperm formation in this species. Autonomous endosperm formation, as also observed occasionally in *R. kuepferi*, is completely pollen-independent and may enhance uniparental reproduction. Nevertheless, strong endosperm imbalance could potentially contribute to the high amount of seed abortion in polyploid cytotypes.

A different pathway may lead to the formation of pentaploids in the contact zone: one seed sample from a 5x mother plant indicates a fully sexual pathway, probably via a meiotic reduction of the embryo sac (3x) and a fertilization by diploid pollen to form a 5x embryo and a 8x endosperm (Table 3). The origin of these pentaploids from other cytotypes, however, may involve also apomeiotic and parthenogenetic pathways.

Outside of the contact zone, no diploids occur which would explain the formation of triploids. But, triploid embryos seem to arise quite often in the seed of tetraploids populations (Table 3, Fig. 3), but almost never appear in the leaf material of adult plants. The reduction of ploidy level in the embryo can be only explained by a meiotically reduced embryo sac. We suppose that meiosis was disturbed in the mother plant, resulting in megaspores with unbalanced chromosome numbers. Molecular markers (microsatellites, AFLPs) do not indicate the contribution of another parental species to the genome of the tetraploid cytotype (Cosendai and Hörandl in prep.). Autopolyploid origin and multivalent formation during meiosis may cause such unbalanced chromosome numbers. The ratio obtained in the endosperm seems to confirm this. Effectively, with ratio 3x (embryo) : 8x; 9x; 10x (endosperm), the endosperm nuclei would be 6x, plus 2x, 3x and 4x derived from the pollen. Since the 3x embryo must have developed parthenogenetically, again either one or both pollen nuclei (2x +2x) could have been used for endosperm fertilization. In some of the samples with 3x embryos (populations no. 12, 41), an additional, but smaller 6x peak was observed, which represents the G2 peak of the growing embryo (e.g., Fig. 3). Moreover, disturbances of microsporogenesis and formation of unbalanced pollen (Izmailow, 1965, 1973, 1976; Jankun, 1965) can contribute to variation in the endosperm ploidy levels. Most important, these tetraploid plants show obviously only a partial apomixis by keeping a disturbed meiosis, but by developing the egg cell parthenogenetically. In the next generations, further reduction of ploidy levels would finally lead to haploid offspring, in which recessive deleterious mutations would be fully expressed. These processes may strongly reduce fitness of such lineages with only a partial apomixis (e.g. Van Dijk & Vijverberg 2005). Our data show only a single triploid adult plant in the whole area (Fig. 1; Table 2), but 17 triploid

embryos in the seeds from tetraploid adults. These plants come from 11 populations outside the area of diploid influence, suggesting a rather frequent phenomenon (Table 3). However, triploids that have been formed via this partial apomixis have probably difficulties to establish. In contrast, the fully apomictic pathway with a combination of apomeiosis and parthenogenesis maintains the tetraploid level and is obviously more successful for establishment of this cytotype, as seen in frequencies of adult plants. In two of the populations analyzed (Gr. St. Bernard and Simplon Pass), both 3x and 4x embryos occur in the seeds of tetraploids, but both populations had 100% adult tetraploid plants. In populations with both pathways, selection obviously favours individuals expressing full apomixis which stabilizes the ploidy level, over parthenogenesis alone which forms new cytotypes.

Hexaploids can be formed in a tetraploid population from a cross between an unreduced embryo and reduced pollen. The endosperm would be in this case 10x (8x+2x). We had only one seed sample with an 6x embryo, and an 20x endosperm. Here endopolyploidy could explain the double DNA content of the expected 10x. Endopolyploidy in the endosperm is known e.g. from *Zea mays* and other species (Kowles *et al.*, 1988; Barow & Meister, 2003; Barow, 2006). Such hexaploid $B_{III}$ hybrids may also give rise to triploids via meiosis and parthenogenetic development of the egg cell, but are obviously not stable.

These aspects would also explain why coexistence of cytotypes is rare in *R. kuepferi*, in contrast to what Kao (2007) showed for apomictic *Arnica cordifolia.* In *Arnica,* apomictic reproduction is predominant and probably stable enough to keep cytotypes reproductively isolated. Additionally, differences in phenology maintain a stable coexistence of cytotypes. Stable triploidy is also widespread in dandelions (*Taraxacum officinale* group; Van Dijk, 2003). In Asteraceae, apomixis may actually arise at the triploid level because of their mode of embryo sac development and endosperm formation (Talent, 2009). In *Ranunculus kuepferi*, our results rather suggest an origin of apomixis in tetraploid cytotypes, because of the existence of rare sexual tetraploids and a frequent uncoupling of apomeiosis and parthenogenesis on this ploidy level.

The variety of pathways indicates again the broad flexibility of modes of reproduction within this species. It appears to be able to express all possible solutions of embryo formation between fully sexual to complete apomixis. The coupling of apomeiosis and parthenogenetic development of the egg cell seems not yet fully established. Furthermore, *R. kuepferi* can undergo all kinds of endosperm development from the pseudogamous to the

autonomous type. These phenomena suggest a very young, postglacial or even recurrent evolutionary origin of apomixis. Further support for this hypothesis comes from linear correlations of genome size to ploidy levels (Figure 2), which infers that the frequently observed genome downsizing in polyploids has not yet occurred (see Leitch & Bennett, 2004).

### STABILITY OF THE DIPLOID SEXUAL POPULATIONS

Diploids maintain themselves via sexual reproduction and high seed set, but they can accept pollen from tetraploids in the sympatric zone to form triploid or pentaploid offspring. In the hybrid zone, triploids and pentaploids may be recurrently formed but still they are quite rare and do not form populations on their own. The relatively frequent triploids can arise from different pathways: a crossing between a haploid meiotic egg cell (x) and tetraploid meiotic pollen (2x) gives a triploid embryo, while the endosperm (2x) plus one pollen nucleus (2x) would be tetraploid; this 3x : 4x ratio was not actually observed in the wild seeds, but could account in our data for the major part of the triploids. The tetraploid pollen donor could be either sexual or apomictic. Alternatively, within a diploid population, occasionally unreduced pollen (2x) can be formed, and fertilize a haploid egg cell, resulting in the same embryo : endosperm ratio as above. The reciprocal cross would retrieve a 3x : 5x ratio. These processes can be a spontaneous step towards autopolyploidy via the triploid bridge (Ramsey and Schemske, 1998) and would not involve a cross between cytotypes. Our limited sampling of achenes in diploid populations may account for the lack of evidence on these pathways.

The hypothesis that apomixis can be transferred to sexual populations via the pollen of apomicts would suggest a replacement of sexual by apomictic cytotypes (Mogie, 1992; Mogie *et al.*, 2007). We have only little evidence for this pathway in our dataset, partly because of low seed set in triploids. A triploid plant from the population Allos (no. 23), that shows fully apomictic reproduction (Table 3), may have indeed originated from an apomictic pollen donor. In the seed material, the number of triploid embryos in the hybrid zone is very low (three), but these embryos have originated from a tetraploid mother plant. Therefore, introgression of apomixis into diploid sexuals cannot be proven directly with our seed dataset. Leaf material, however, shows that almost all triploid adult plants come from the hybrid zone, suggesting that crossings between diploid and tetraploid cytotypes probably do occur recurrently. This does not necessarily indicate an introgression process, because the tetraploid parents in this area could be also fully sexual, as shown in three individuals from population Col della Perla (population no. 12, Table 3). If triploids in the hybrid zones would be sexual or partly sexual,

than disturbances of meiosis, reduced fitness and minority cytotype disadvantages may strongly limit the establishment of triploid lineages. Triploid apomicts, in contrast, should be able to establish purely triploid populations, but such populations have not been observed. We conclude that triploids originated mainly from sexual events than from triploid apomictic parents.

Our results indicate that introgression of apomixis into sexual populations in the 2x-4x hybrid zone may occur, but frequencies need to be studied further. It remains questionable whether this process plays a major role for the observed pattern of geographical parthenogenesis. Moreover, the diploid sexual individuals of *R. kuepferi* do have a significantly higher fertility than all the polyploid cytotypes (Figure 4). In such cases, it is unlikely that apomixis can replace sexuality in mixed populations (see Mogie 1992; Hörandl and Temsch 2009). This is in accordance with results from *R. auricomus*, where findings of low crossability between different cytotypes, and a significantly higher fertility of sexuals rejected the introgression hypothesis (Hörandl & Temsch, 2009). Low frequencies of introgression were also found in population studies on *Taraxacum* (Brock, 2004).

### COLONIZING ABILITIES OF TETRAPLOIDS

The easternmost population at Turracherhöhe and the outposts at Corsica and Mt. Cusna are all tetraploid. For the population at Turracherhöhe, the FCSS data (Table 3) revealed fully apomictic reproduction. The samples from Mt. Cusna had completely aborted achenes, as typical for apomicts. For the populations at Corsica, we had no achenes, but Huber (1989) also reported seed and pollen abortion. Apomictic reproduction in both Corsica and Mt. Cusna population is further indicated by a clonal population genetic structure (Cosendai, 2005 and in prep.). These outposts have been most probably founded by efficiently colonizing tetraploid apomicts, probably via long distance dispersal. Corsica had no land bridge to the European continent since the Miocene (Loÿe *et al.*, 2004). The location of Mt. Cusna in the northern Apennines may lead to a hypothesis that the species could have migrated from the Alps towards Apennine, but there is no high mountain in Liguria to support this idea. As the diploid populations occupy a somewhat central position between Corsica and the major tetraploid area, it is likely that tetraploids originated in this area, but expanded their range rapidly via a centrifugal dispersal. Such distribution patterns of central diploid sexuals surrounded by expanding polyploid cytotypes apomicts are common in other cases of geographical parthenogenesis (*Antennaria*: Bayer, 1990; *Stevia*: Soejima *et al.*, 2001; *Paspalum*: Urbani, 2002; *Taraxacum* and *Chondrilla*: Van Dijk, 2003; *Ranunculus auricomus*: Hörandl, 2009). Long distance dispersal,

even between continents, is a frequent phenomenon in the genus *Ranunculus* (Emadzade *et al.* subm.). The achenes of *R. kuepferi* are small and have a cavity between the seed and the pericarp, aiding for wind dispersal (Müller-Schneider, 1986). Otherwise, birds or mammals may have been other important dispersal vectors. The ability of apomicts to found populations with a single diaspore may have had enhanced the success of rare dispersal events (Hörandl *et al.*, 2008). Since the apomicts do not have a fitness advantages with respect to female fecundity, it is likely that superior colonizing abilities, as postulated by Baker's Law (Baker, 1965, 1967), are a main factor for the observed centrifugal distribution. For the diploid sexual cytotype, self-sterility (Huber, 1988) and pollinator-dependence may strongly limit range expansions.

## CONCLUSION

The results on *R. kuepferi* overall confirm a high stability of the diploid and the tetraploid cytotype in their respective areas. Aneuploid cytotypes are probably recurrently formed, but fail to establish as long as remnants of the sexual pathway are maintained. The stability of the apomictic tetraploid cytotypes may consequently be important for establishing a large distribution area. In contrast, the significantly higher fertility of the diploids may stabilize the diploid cytotype in its relic area. Moreover, our data tend to prove the difficulty for a new cytotype to establish, as long as partial sexuality is maintained; minority cytotype effects sensu Levin (1975), Husband (2000) and Husband & al. (2008) seem to play an important role in this pattern for reducing the presence of different cytotypes in the population although the seeds are constantly newly produced.

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## TABLES

Table 1. Provenance of materials used in this study. Population number, the number corresponds to those in Figure 1. Country abbreviations: F, France; I, Italy; A, Austria; CH, Switzerland. Collectors: ACC, Anne-Caroline Cosendai; ACC-AC, Anne-Caroline Cosendai & André Cosendai; CS, Christoph Seger; EH, Elvira Hörandl; MH, Marc Hämmerli; PK, Philippe Küpfer; PS-GS, Peter Schönswetter & Gerald Schneeweiss. In locality, a roman number indicates repeated sampling on the same population in different years, arabic numbers are herbaria numbers of E. Hörandl (vouchers have been deposited in the herbarium of the University of Vienna, WU).

| Population number | Country | Province | Locality | Altitude | Coordinate North | Coordinate East | Collector | Date |
|---|---|---|---|---|---|---|---|---|
| 1 | F | Corse-du-Sud | Corsica | 1541 m | 42°01'46.4'' | 9°12'34.5'' | ACC | 28.05.2005 |
| 2 | I | Emilia-Romagna | Mt. Cusna | 1594 m | 44°18'06.7" | 10°22'26.6" | ACC-AC | 26.05.2006 |
| 3 | F | Var | La Chens_I | 1610 m | 43°44'59.3" | 6°39'25.5" | PK | 04.06.2004 |
| 4 | F | Var | La Chens_II | 1607 m | 43°44'58.5" | 6°39°29.1" | ACC-AC | 28.05.2006 |
| 5 | F | Alpes-Maritimes | Col de Tende | 1888 m | 44°09'03.0" | 7°33'56.3" | ACC | 09.06.2004 |
| 6 | I | Piemonte | Valle di Pesio_I_9589 | 1700 m | 44°11'43.68" | 7°39'33.85" | EH | 09.07.2007 |

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|---|---|---|---|---|---|---|---|---|
| 7 | I | Piemonte | Valle di Pesio_II_9592 | 1700 m | 44°11'43.68" | 7°39'33.85" | EH | 10.07.2007 |
| 8 | I | Piemonte | Passo del Duca_9525_/_9534 | 1700 m | 44°11'43.68" | 7°39'33.85" | EH | 14.07.2004 |
| 9 | I | Piemonte | Valle di Pesio_III_9593 | 1925 m | 44°11'43.68" | 7°39'33.85" | EH | 10.07.2007 |
| 10 | I | Piemonte | Vallone Cravina_9595 | 1960 m | 44°13'24.72" | 7°37'11.16" | EH | 12.07.2007 |
| 11 | I | Piemonte | Col della Perla_I_9596 | 2080 m | 44°9'11.05" | 7°37'21.14" | EH | 13.07.2007 |
| 12 | I | Piemonte | Col della Perla_II_9597 | 2200 m | 44°9'11.05" | 7°37'21.14" | EH | 14.07.2007 |
| 13 | F | Alpes-Maritimes | Notre Dame de la Fenestre | 1885 m | 44°05'45.3" | 7°21'34.2" | ACC | 11.06.2004 |
| 14 | F | Alpes-Maritimes | Col d'Isola | 2210 m | 44°11'43.7" | 7°09'19.7" | ACC | 11.06.2004 |
| 15 | I | Piemonte | Colle della Lombarde_I_9601 | 2260 m | 44°12'25.68" | 7°8'51.98" | EH | 15.07.2007 |
| 16 | I | Piemonte | Colle della Lombarde_II_9602 | 2477 m | 44°13'17.60" | 7°9'9.25" | EH | 16.07.2007 |
| 17 | F | Alpes-de-Haute-Provence | Vallette | 1820 m | 44°07.5' | 6°33.5' | PK | 04.06.2004 |

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|---|---|---|---|---|---|---|---|---|
| 18 | F | Alpes-de-Haute-Provence | Champs_I | 1925 m | 44°09'59.5" | 6°42'28.0" | ACC | 13.06.2004 |
| 19 | F | Alpes-Maritimes | Champs_II | 2080 m | 44°10'33.8" | 6°41'53.0" | ACC-AC | 29.05.2006 |
| 20 | F | Alpes-Maritimes | Cayolle_I | 2193 m | 44°15'13.6" | 6°44'52.1" | ACC | 13.06.2004 |
| 21 | F | Alpes-de-Haute-Provence | Cayolle_II | 2325 m | 44°15'34.4" | 6°44'41.3" | ACC-AC | 30.05.2006 |
| 22 | F | Alpes-de-Haute-Provence | Allos_I | 2247 m | 44°22'" | 6°37'" | PK | 04.06.2004 |
| 23 | F | Alpes-de-Haute-Provence | Allos_II | 2080 m | 44°18'03.4" | 6°35'06.0" | ACC-AC | 29.05.2006 |
| 24 | F | Hautes-Alpes | Vars | 2134 m | 44°32'21.3" | 6°42'05.6" | ACC | 14.06.2004 |
| 25 | F | Hautes-Alpes | Raboux | 1859 m | 44°38'38.4" | 5°58'43.1" | ACC | 15.06.2004 |
| 26 | F | Hautes-Alpes | Haute Queyras, Col de La Croix | 2000 m | 44°46'0'' | 7°01'" | MH | 22.05.2004 |
| 27 | F | Hautes-Alpes | Haute Queyras, Montette | 2000 m | 44°50'0'' | 6°55'0'' | MH | 23.05.2004 |

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|---|---|---|---|---|---|---|---|---|
| 28 | F | Drôme | Vercors_I | 1470 m | 44°54'07" | 5°28'039" | PK | 03.06.2004 |
| 29 | F | Drôme | Vercors_II | 1325 m | 44°50'26.1" | 5°25'23.1" | ACC-AC | 30.05.2006 |
| 30 | F | Hautes-Alpes | Col de Lautaret | 2060 m | 45°02'40.6" | 6°24'04.1" | ACC | 15.06.2004 |
| 31 | F | Savoie | Mt. Cenis | 2025 m | 45°13'55.4" | 6°53'53.6" | ACC-AC | 03.07.2007 |
| 32 | F | Savoie | Col d'Iseran | 2768 m | 45°25'09.2" | 7°01'52.9" | ACC-AC | 03.07.2007 |
| 33 | I | Valle d'Aoste | Gran Paradiso | 2079 m | 45°37'0.5" | 7°33'12.2" | ACC-AC | 04.07.2007 |
| 34 | F | Savoie | Petit St. Bernard | 2215 m | 45°40'40.4" | 6°52'55.2" | ACC-AC | 02.07.2007 |
| 35 | CH | Valais | Gr. St. Bernard | 2380 m | 45°52'09.9" | 7°09'33.3" | ACC-AC | 02.07.2007 |
| 36 | CH | Valais | Arpilles | 1830 m | 46°04.94' | 7°.78' | AC | 18.06.2006 |
| 37 | CH | Valais | Ovronnaz | 1840 m | 46°13.03' | 7°09.52' | AC | 18.06.2006 |
| 38 | I | Valle d'Aoste | Cervinia | 2200 m | 45°55'54.6" | 7°38'18.1" | ACC-AC | 04.07.2007 |
| 39 | CH | Valais | Jeizinen | 2020 m | 46°20'07" | 7°43'85" | ACC | 05.07.2004 |

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|---|---|---|---|---|---|---|---|---|
| 40 | CH | Valais | Lötschental | 1773 m | 46°26'05.5" | 7°51'48.0" | ACC | 29.06.2006 |
| 41 | CH | Valais | Simplon Pass | 2019 m | 46°15'03.2" | 8°01'48.2" | ACC | 29.06.2006 |
| 42 | CH | Valais | Furka Pass | 2162 m | 46°34'45.8" | 8°25'30.9" | ACC | 28.06.2006 |
| 43 | CH | Ticino | Lukmanier Pass | 1946 m | 46°33'49.2" | 8°47'54.7" | ACC | 28.06.2006 |
| 44 | CH | Graubunden | Rheinwald_9603 | 2100 m | 46°33'16.05" | 9°14'52.32" | EH | 18.07.2007 |
| 45 | CH | Graubunden | Julier Pass | 2277 m | 46°28'20.9" | 9°44'01.4" | ACC | 27.06.2006 |
| 46 | CH | Graubunden | Albula Pass | 2312 m | 46.58333° | 9.83333° | ACC | 26.06.2006 |
| 47 | CH | Graubunden | Bernina Pass | 2301 m | 46°24'47.3" | 010°01'17.3" | ACC | 27.06.2006 |
| 48 | A | Vorarlberg | Arlberg Pass | 2269 m | 47°08'49.1" | 10°14'55.3" | ACC | 25.06.2006 |
| 49 | CH | Graubunden | Umbrail Pass | 2463 m | 46°32'51.3" | 10°26'06.3" | ACC | 26.06.2006 |
| 50 | A | Tirol | Kaunertal | 2525 m | 46°52'21.8" | 10°42'37.5" | ACC | 25.06.2006 |
| 51 | I | Trento | Tonale Pass | 2400 m | 46°16'21.27" | 10°34'40.88" | EH | 14.07.2006 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 52 | A | Tirol | Timmelsjoch | 2105 m | 46°55'13.5" | 11°03'10.6" | ACC | 24.06.2006 |
| 53 | A | Tirol | Tuxer Alps | 2315 m | 47°07' | 11°34' | CS | 06.07.2006 |
| 54 | I | Trento | Rosengarten | 2500 m | 46°27'19.56" | 11°37'56.51" | EH | 11.07.2006 |
| 55 | I | Trento | Padon Pass | 2350 m | 46°27'47.80" | 11°53'42.88" | EH | 09.07.2006 |
| 56 | I | Veneto | Mt. Dürrenstein | 2400 m | 46°39'39.24" | 12°10'57.86" | EH | 18.07.2006 |
| 57 | A | Carinthia | Mt. Großglockner | 2220 m | 47°4'47.63" | 12°45'50.37" | EH | 12.08.2005 |
| 58 | A | Carinthia | Mt. Sadnig | 2200 m | 46°57'42" | 13°'35" | PS-GS | 07.07.2006 |
| 59 | A | Carinthia | Turracherhöhe | 2220 m | 46°55'20.47" | 13°52'44.35" | EH | 14.08.2005 |

Table 2. Summary of the population's genome size and ploidy level. s and f at the end of the population name indicate silica dried (s) or fresh (f) material, an asterisk* indicates old material (collected in 2004)

| Populations names | Mean values of population in pg | Standard deviation | Coefficient of variation in % | Number individuals | Percent of ploidy level in the population |
|---|---|---|---|---|---|
| **2x cytotypes** | | | | | |
| Vallone Cravina 9595_s | 4.433 | 0.040 | 0.911 | 17 | 100.0 |
| Lachens II_s | 4.144 | 0.069 | 1.675 | 22 | 100.0 |
| Colle della Lombarde I 9601_s | 4.459 | 0.091 | 2.037 | 24 | 100.0 |
| Colle della Lombarde II 9602_s | 4.405 | 0.027 | 0.602 | 11 | 100.0 |
| Valle di Pesio I 9589_s | 4.429 | 0.036 | 0.805 | 25 | 100.0 |
| Valle di Pesio II 9592_s | 4.462 | 0.042 | 0.946 | 4 | 100.0 |
| Valle di Pesio III 9593_s | 4.419 | 0.045 | 1.015 | 14 | 100.0 |
| Passo del Duca 9525_s * | 3.820 | 0.024 | 0.639 | 5 | 100.0 |
| Valette_s * | 3.941 | 0.164 | 4.149 | 24 | 79.2 |
| Vercors II_s | 4.058 | 0.050 | 1.220 | 18 | 100.0 |
| **Mean** | **4.257** | **0.244** | | | |
| **3x cytotypes** | | | | | |
| Allos I_f | 6.322 | 0.057 | 0.909 | 3 | 66.7 |

| | | | | | |
|---|---|---|---|---|---|
| Allos I_s | 6.487 | 0.058 | 0.893 | 12 | 91.7 |
| Allos II_s | 6.487 | 0.058 | 0.891 | 24 | 45.8 |
| Champs I_s | 6.431 | 0.015 | 0.228 | 1 | 100.0 |
| Champs II_f | 6.269 | 0.246 | 3.920 | 23 | 47.8 |
| Champs II_s | 6.450 | 0.040 | 0.624 | 7 | 28,60 |
| Gran Paradiso_s | 5.519 | | | 24 | 4,20 |
| Col della Perla I 9596_s | 6.599 | 0.079 | 1.195 | 23 | 47.8 |
| Col della Perla II 9597_s | 6.652 | 0.072 | 1.078 | 22 | 27,30 |
| Valette_s * | 5.964 | 0.318 | 5.325 | 24 | 20,80 |
| **Mean** | **6.318** | **0.340** | | | |

**4x populations**

| | | | | | |
|---|---|---|---|---|---|
| Arpilles_s | 8.372 | 0.223 | 2.666 | 19 | 100.0 |
| Albula_s | 8.704 | 0.095 | 1.087 | 25 | 100.0 |
| Allos I_s | 8.559 | 0.021 | 0.246 | 12 | 8,30 |
| Allos II_s | 8.597 | 0.128 | 1.485 | 24 | 25.0 |
| Allos I_f | 8.015 | 0.000 | 0.000 | 3 | 33.3 |
| Allos II_f | 8.341 | 0.025 | 0.299 | 2 | 50.0 |
| Bernina Pass_s | 8.707 | 0.226 | 2.599 | 24 | 95.8 |

| | | | | | |
|---|---|---|---|---|---|
| Cayolle II_f | 8.468 | 0.135 | 1.596 | 20 | 95.0 |
| Cayolle II_s | 8.543 | 0.142 | 1.662 | 9 | 88. 9 |
| Cervinia_s | 8.578 | 0.038 | 0.438 | 25 | 100.0 |
| Champs II_f | 8.419 | 0.082 | 0.973 | 23 | 52.2 |
| Champs II_s | 8.631 | 0.032 | 0.376 | 7 | 71.4 |
| Corsica_f | 8.196 | 0.277 | 3.385 | 2 | 100.0 |
| Mt. Cusna_f | 8.552 | 0.017 | 0.201 | 4 | 100.0 |
| Dürrenstein_s | 8.514 | 0.093 | 1.097 | 10 | 100.0 |
| Furka Pass_s | 8.524 | 0.071 | 0.831 | 25 | 100.0 |
| Gd Paradiso_s | 8.671 | 0.361 | 4.163 | 24 | 95.8 |
| Gr. St. Bernard_s | 8.716 | 0.091 | 1.048 | 24 | 100.0 |
| Col d'Iseran_s | 8.603 | 0.049 | 0.572 | 25 | 100.0 |
| Julier Pass_s | 8.251 | 0.051 | 0.621 | 25 | 100.0 |
| Kaunertal_s | 8.603 | 0.097 | 1.132 | 25 | 100.0 |
| Lötschental_s | 8.136 | 0.103 | 0.025 | 25 | 100.0 |
| Lukmanier_s | 8.158 | 0.059 | 0.720 | 25 | 100.0 |
| Mt. Cenis_s | 8.596 | 0.094 | 1.098 | 24 | 100.0 |
| Ovronnaz_s | 8.552 | 0.008 | 0.096 | 8 | 100.0 |
| Padon_s | 8.835 | 0.092 | 1.044 | 25 | 100.0 |
| Col della Perla I 9596_s | 8.658 | 0.125 | 1.438 | 23 | 34.8 |

| | | | | | |
|---|---|---|---|---|---|
| Col della Perla II 9597_s | 8.643 | 0.089 | 1.027 | 22 | 68.2 |
| Pt. St Bernard_s | 8.702 | 0.068 | 0.776 | 24 | 100.0 |
| Rheinwald 9603_s | 8.547 | 0.067 | 0.781 | 21 | 100.0 |
| Rosengarten_s | 8.584 | 0.067 | 0.780 | 20 | 100.0 |
| Mt. Sadnig_s | 8.749 | 0.067 | 0.771 | 15 | 100.0 |
| St. Anton_s | 8.834 | 0.094 | 1.068 | 25 | 100.0 |
| Simplon_s | 8.808 | 0.247 | 2.803 | 25 | 100.0 |
| Timmelsjoch_f | 8.314 | 0.077 | 0.931 | 1 | 100.0 |
| Timmelsjoch_s | 8.559 | 0.146 | 1.702 | 25 | 100.0 |
| Tonale Pass_f | 8.462 | 0.014 | 0.160 | 1 | 100.0 |
| Turracherhöhe_s | 8.617 | 0.065 | 0.753 | 26 | 100.0 |
| Tuxer Alps_s | 8.608 | 0.062 | 0.724 | 25 | 100.0 |
| Umbrail Pass_s | 8.731 | 0.295 | 3.375 | 25 | 100.0 |
| **Mean** | **8.552** | **0.184** | | | |

**5x populations**

| | | | | | |
|---|---|---|---|---|---|
| Allos II_f | 10.337 | 0.134 | 1.292 | 2 | 50.0 |
| Allos II_s | 10.649 | 0.163 | 1.533 | 24 | 29.2 |
| Col della Perla I 9596_s | 10.633 | 0.121 | 1.134 | 23 | 13,10 |

| | | | | | |
|---|---|---|---|---|---|
| Col della Perla II 9597_s | 10.755 | 0.052 | 0.484 | 22 | 4,50 |
| **Mean** | **10.540** | **0.175** | | | |

**6x populations**

| | | | | | |
|---|---|---|---|---|---|
| Bernina_s | 12.685 | 0.220 | 1.731 | 24 | 4,20 |
| Cayolle II_f | 12.671 | 0.114 | 0.900 | 20 | 5.0 |
| Cayolle II_s | 13.024 | 0.118 | 0.905 | 9 | 11,10 |
| Perla I 9596_s | 12.916 | 0.062 | 0.482 | 23 | 4,30 |
| **Mean** | **12.793** | **0.199** | | | |

| **Summary of ploidy level** | **Number of individuals** | **Percentage of cytotype** |
|---|---|---|
| 2x | 159 | 15.7 |
| 4x | 778 | 76.8 |
| 3x | 61 | 6.02 |
| 5x | 12 | 1.18 |
| 6x | 3 | 0.3 |
| Total | 1013 | |

Table 3. Summary of seed flow cytometric data with the observed ploidy levels in embryo and endosperm, and the inferred mode of reproduction. Data from the mother plant are taken from leaf measures. Pseudogamous I and II refer to a fertilization by one or two pollen nuclei, respectively (see also Hörandl *et al.*, 2008).

| | | | | | | **Mode of reproduction** | | | **Mother plant** |
|---|---|---|---|---|---|---|---|---|---|
| **population number** | **population name** | **Individual number.** | **embryo** | **second peak / G2 phase** | **endosperm** | **Origin of embryo sac** | **Egg cell** | **Endosperm development** | |
| 8 | Passo del Duca_9534 | 04 | 3x | | 8x | disturbed meiotic | parthenogenetic | pseudogamous I | N.A. |
| | | 08 | 3x | | 8x | disturbed meiotic | parthenogenetic | pseudogamous I | N.A. |
| 11 | Col della Perla_I_9596 | 13 | 5x | 10x | 8x | meiotic | fertilized | sexual | 5x |
| 12 | Col della Perla_II_9597 | 07 | 3x | 6x | 8x | disturbed meiotic | parthenogenetic | pseudogamous I | 4x |
| | | 01 | 4x | | 6x | meiotic | fertilized | sexual | 4x |
| | | 03 | 4x | 8x | 6x | meiotic | fertilized | sexual | 4x |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 03_new | 4x | 5x | 6x | meiotic | fertilized | sexual | 4x |
| | | 08 | 4x | | 10x | disturbed meiotic | parthenogenetic | pseudogamous I | 5x |
| | | 15 | 4x | | 10x | apomeiotic | parthenogenetic | pseudogamous I | 4x |
| | | 26 | 4x | | 8x | apomeiotic | parthenogenetic | autonomus | 4x |
| 13 | Notre Dame de la Fenestre | 05 | 3x | | 10x | disturbed meiotic | parthenogenetic | pseudogamous I | 4x |
| | | 02 | 4x | | 12x | apomeiotic | parthenogenetic | pseudogamous II | 4x |
| 15 | Colle della Lombarde_I_9601 | 01 | 2x | | 3x | meiotic | fertilized | sexual | 2x |
| 21 | Cayolle II | 09.1 | 4x | | 12x | apomeiotic | parthenogenetic | pseudogamous II | 4x |
| | | 09.2 | 4x | | 10x | apomeiotic | parthenogenetic | pseudogamous I | 4x |
| | | 15 | 4x | | 8x | apomeiotic | parthenogenetic | autonomous | 4x |
| 23 | Allos II | 09 | 3x | | 8x | apomeiotic | parthenogenetic | pseudogamous I | 3x |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 24 | Vars | 06 | 3x | N.A. | disturbed meiotic | parthenogenetic | aborted | 4x |
| | | 15 | 3x | N.A. | disturbed meiotic | parthenogenetic | aborted | 4x |
| | | 16 | 3x | N.A. | disturbed meiotic | parthenogenetic | aborted | 4x |
| 29 | Vercors_II | 05.1 | 2x | 3x | meiotic | fertilized | sexual | 2x |
| | | 05.2 | 2x | 3x | meiotic | fertilized | sexual | 2x |
| | | 06 | 2x | 3x | meiotic | fertilized | sexual | 2x |
| | | 07 | 2x | 3x | meiotic | fertilized | sexual | 2x |
| | | 19 | 2x | 3x | meiotic | fertilized | sexual | 2x |
| | | 23 | 2x | 3x | meiotic | fertilized | sexual | 2x |
| | | 05 | 2x | 3x | meiotic | fertilized | sexual | 2x |
| 31 | Mt. Cenis | 06 | 4x | N.A. | apomeiotic | parthenogenetic | aborted | 4x |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 32 | Col d'Iseran | 01 | 4x | | N.A. | apomeiotic | parthenogenetic | aborted | 4x |
| | | 02 | 4x | | N.A. | apomeiotic | parthenogenetic | aborted | 4x |
| 33 | Gr. St. Bernard | 11 | 3x | 5x | 8x | disturbed meiotic | parthenogenetic | pseudogamous I | 4x |
| | | 14 | 3x | | N.A. | disturbed meiotic | parthenogenetic | aborted | 4x |
| | | 15 | 3x | | 14x | disturbed meiotic | parthenogenetic | pseudogamous II | 4x |
| | | 11+12 | 4x | 6x | 12x | apomeiotic | parthenogenetic | pseudogamous II & sexual | 4x |
| | | 12 | 4x | | 10x | apomeiotic | parthenogenetic | pseudogamous I | 4x |
| | | 13_new | 4x | | 10x | apomeiotic | parthenogenetic | pseudogamous I | 4x |
| | | 21 | 4x | | 10x | apomeiotic | parthenogenetic | pseudogamous I | 4x |
| | | 21 | 4x | | 10x | apomeiotic | parthenogenetic | pseudogamous I | 4x |
| | | 26 | 4x | | N.A. | apomeiotic | parthenogenetic | aborted | 4x |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 13 | 6x | | 20x | apomeiotic | fertilized | pseudogamous II | 4x |
| 37 | Ovronnaz | 19 | 4x | | 12x | apomeiotic | parthenogenetic | pseudogamous II | 4x |
| 40 | Lötschental | 26 | 3x | | 8x | disturbed meiotic | parthenogenetic | pseudogamous I | 4x |
| 41 | Simplon Pass | 21 | 3x | | 10x | disturbed meiotic | parthenogenetic | pseudogamous I | 4x |
| | | 25 | 3x | 6x | 9x | disturbed meiotic | parthenogenetic | pseudogamous I | 4x |
| | | 08 | 4x | | 12x | apomeiotic | parthenogenetic | pseudogamous II | 4x |
| | | 18 | 4x | | N.A. | apomeiotic | parthenogenetic | aborted | 4x |
| | | 23 | 4x | | 12x | apomeiotic | parthenogenetic | pseudogamous II | 4x |
| 43 | Lukmanier Pass | 12 | 3x | | 8x | disturbed meiotic | parthenogenetic | pseudogamous I | 4x |
| | | 26 | 3x | | 12x | disturbed meiotic | parthenogenetic | pseudogamous II | 4x |
| 44 | Rheinwald | 03 | 3x | | 10x | disturbed meiotic | parthenogenetic | pseudogamous I | 4x |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 01 | 3x | 10x | disturbed meiotic | parthenogenetic | pseudogamous I | 4x |
| 47 | Bernina Pass | 22 | 3x | 8x | disturbed meiotic | parthenogenetic | pseudogamous I | 4x |
| 57 | Mt. Großglockner | 02 | 3x | 9x | disturbed meiotic | parthenogenetic | pseudogamous I | 4x |
| | | Herbar | 3x | 10x | disturbed meiotic | parthenogenetic | pseudogamous II | 4x |
| 58 | Mt. Sadnig | Herbar | 3x | 8x | disturbed meiotic | parthenogenetic | pseudogamous I | 4x |
| 59 | Turracherhöhe | 01 | 4x | 10x | apomeiotic | parthenogenetic | pseudogamous I | 4x |
| | | 02 | 4x | 10x | apomeiotic | parthenogenetic | pseudogamous I | 4x |
| | | 03 | 4x | 10x | apomeiotic | parthenogenetic | pseudogamous I | 4x |

Table 4. Descriptive statistics of the percentages of well-developed achenes per collective fruit. N = no. of collective fruits analyzed.

| Cytotypes | N | Mean | Minimum | Maximum | Std. Deviation |
|---|---|---|---|---|---|
| 2x | 21 | 67.79 | 30.77 | 97.14 | 17.96 |
| 3x | 9 | 11.73 | 1.25 | 33.33 | 9.96 |
| 4x | 93 | 17.96 | 0.00 | 60.87 | 15.51 |
| 5x | 5 | 7.83 | 0.00 | 15.56 | 6.24 |
| 6x | 1 | 3.17 | 3.17 | 3.17 | |

## FIGURES

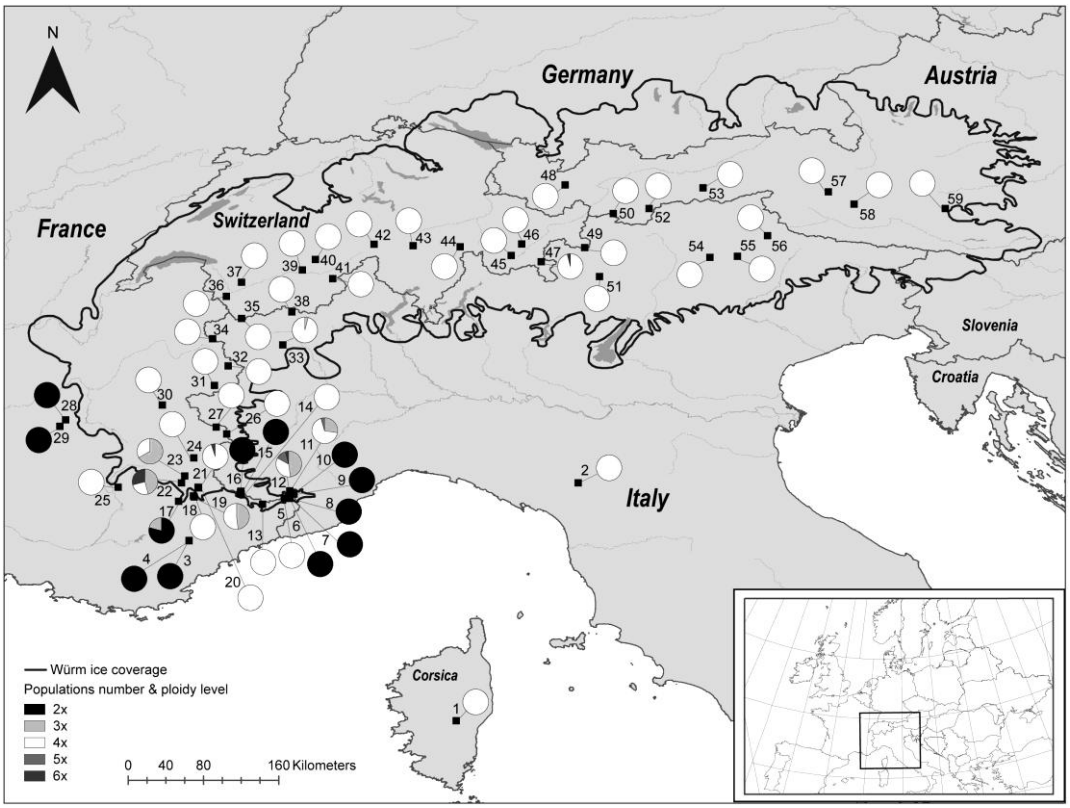


Figure 1. Map of the distribution of *Ranunculus kuepferi*. Black squares indicate the location, population numbers correspond to Table 1. Pie diagrams present the proportions of cytotypes for each population; black: 2x; light grey: 3x; white: 4x; middle grey: 5x; dark grey: 6x. The black line presents the extension of the last glacial maximum of the Würm glaciation.

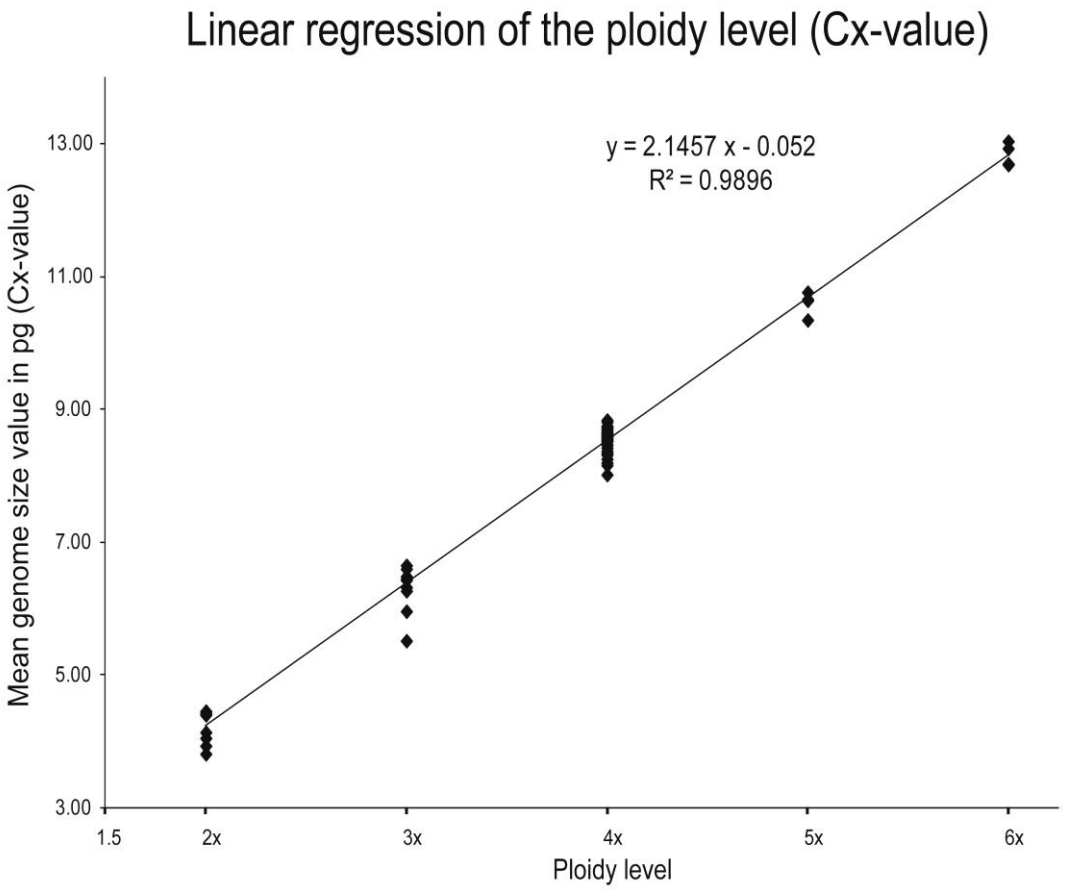


Figure 2. Linear regression of the relative ploidy level Cx-value and the genome size in pg based on leaf material; the slope of the regression is indicated.

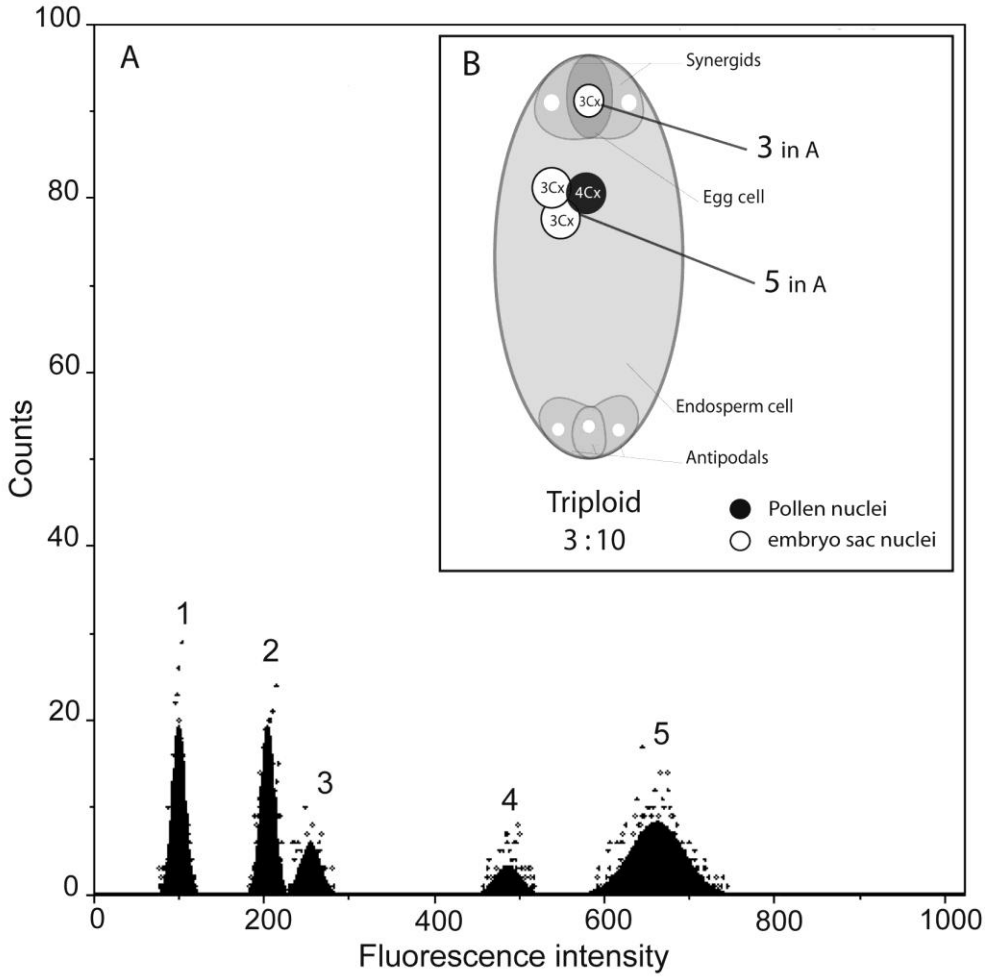


Figure 3. A, histogram of FCSS with five peaks: 1 and 2, standard *Zea mays* in the G1 and G2 phase, respectively; 3-5, *Ranunculus kuepferi* (Rk): 3: 3Cx, embryo G1 phase; 4: embryo G2 phase; 5: 10Cx endosperm (peak G1 phase). B; scheme of the respective embryo sac after fertilization, illustrating a triploid unfertilized embryo corresponding to peak 3, and the 10Cx endosperm corresponding to peak 5. Since the mother plant was tetraploid, the embryo sac must have developed via a disturbed meiosis. The 3x embryo developed without fertilization, the endosperm via pseudogamy.

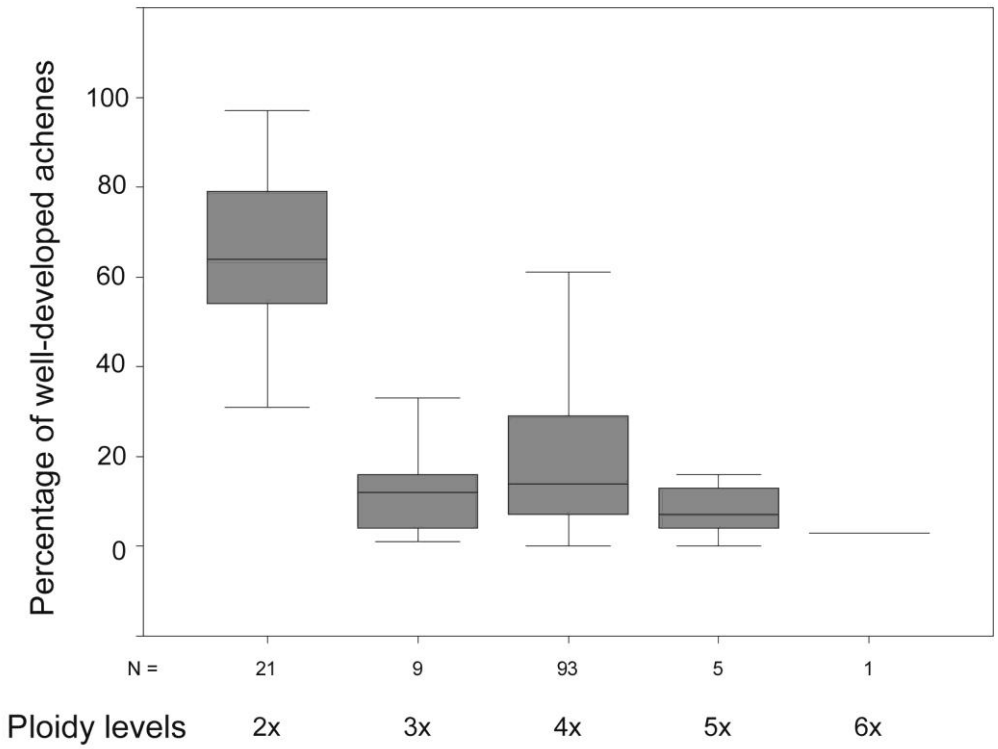


Figure 4. Boxplots of the variation of percentages of well-developed achenes per collective fruit for each cytotype. The box shows the 25$^{th}$ and 75$^{th}$ percentile range and the median value N = number of collective fruits.

# CHAPTER 2 — ORIGIN AND DISTRIBUTION OF AUTO-POLYPLOIDS VIA APOMIXIS IN THE ALPINE PLANT SPECIES *RANUNCULUS KUEPFERI* (RANUNCULACEAE)

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## ABSTRACT

The evolution of polyploids is strongly influenced by their mode of origin. Autopolyploidy is often hampered by disturbances of chromosome pairing and segregation at meiosis, and represents just a duplication of a diploid chromosome set. In contrast, allopolyploids exhibit fewer meiotic problems and potentially have selective advantages because of hybridity and genomic novelty. Apomixis, the asexual reproduction via seed in flowering plants, can overcome problems of meiotic reduction and potentially can enhance the establishment and geographical distribution of polyploid cytotypes. We elucidate the origin and genetic structure of apomictic polyploid cytotypes of the alpine species *Ranunculus kuepferi* by analyzing five microsatellite loci (SSRs) and amplified fragment length polymorphisms (AFLPs) on eight diploid and twelve polyploid (3x, 4x, and 5x) populations out of the range of the species. Polyploids possess a subset of SSR alleles of the diploids, with only two novel alleles in tetraploids. Multiple allelism appears in three SSR

loci in all polyploid cytotypes and is likely a result of facultative unbalanced meiotic events, as it is typical for autopolyploids. Admixture analysis of microsatellites revealed that the tetraploid apomicts derived their allelic diversity exclusively from the diploid populations without the contribution of another parental gene pool, which is confirmed by the absence of a second divergent parental genome in AFLP profiles. A Neighbor-joining tree of AFLPs revealed a low genetic divergence of cytotypes, and suggested at least three independent origins of tetraploid populations; triploid and pentaploids individuals represent probably backcrosses of diploid and tetraploid populations. Autopolyploidy is in *R. kuepferi* likely evolutionarily young and still has signatures of a facultative unbalanced meiosis. However, the shift to apomixis avoided consequences of meiotic problems and therefore helped for a rapid establishment of autotetraploid populations. The lack of hybridity in polyploid *R. kuepferi* suggests that autopolyploids can be successful via apomixis even without genomic novelty. The distributional success of polyploid cytotypes is probably more due to the effects of apomixis than to genetic consequences of polyploidy.

Keywords: AFLPs, Apospory, apomixis, geographical parthenogenesis, microsatellites, *Ranunculus kuepferi*, polyploidy.

## INTRODUCTION

Polyploidization, i.e. the multiplication of the chromosome set (Comai 2005), hybridization (Stebbins 1959) and genome remodelling (Matzke *et al.* 1999; Otto 2007a, b) are some of the most important triggers of evolution as they cause major rearrangements of the genome. Heterosis, gene redundancy and potential sub-functionalization of duplicated genes are the main advantages of polyploidization (Comai, 2005). Changes in genome size and chromosome number, however, may disturb the pairing of homologous chromosomes at meiosis, the crucial step of reproduction of a sexual organism (Leitch & Leitch 2008; Cifuentes *et al.* 2010). In allopolyploids, these meiotic problems are usually avoided, because doubling of the chromosome sets leads to a rebalance in the genome, promoting again bivalent formation during chromosome pairing at meiosis. In contrast, genome duplication within the same species, leading to autopolyploidy (Ramsey & Schemske 1998; Soltis & Rieseberg 1986), can induce drastic changes in the functionality of meiosis, because the pairing of chromosomes will lead to formation of multivalents as each set of chromosomes has originated from the same species (Comai

2005). Because of multivalent formation in autopolyploids, tetrasomic inheritance can be observed (Soltis & Rieseberg 1986), and unbalanced chromosome segregation can lead to aneuploid and irregular chromosome numbers in the gametes.

Apomixis, the asexual way of seed production in flowering plants (Asker & Jerling 1992; Koltunow & Grossniklaus 2003; Nogler 1984a), provides a means to avoid an unbalanced meiosis. Two main pathways can be discriminated in the variety of forms (Koltunow & Grossniklaus 2003; Tucker & Koltunow 2009): embryos can develop directly out of somatic tissues of the ovule; this form of apomixis, called adventitious embryony, is usually not linked to polyploidy and therefore not of interest for our study. The second pathway, gametophytic apomixis, involves the formation of an unreduced embryo sac via two forms, apospory or diplospory. In apospory, the embryo sac develops from a somatic cell in the nucellus, whereas in diplospory the megaspore mother cell undergoes a restitutional meiosis to give an unreduced spore, which develops into an embryo sac. In both cases, the unreduced egg cell within the embryo sac develops without fertilization into an embryo. Therefore, both diplospory and apospory bypass segregation of chromosomes at meiosis and transmit the complete maternal chromosome set to the offspring. Gametophytic apomixis is intimately linked to polyploidy in plants (Asker & Jerling 1992), with a few exceptions of occasional apomixis in diploids (Bicknell 1997; Kantama *et al.* 2007; Siena *et al.* 2008).

To avoid the penalisation of meiotic disturbance, brought up by the polyploidization of the genome, apomixis might be a way for the species to retain fertility despite an irregular meiosis leading to formation of aneuploid embryos. Aneuploidy per se is in plants not necessarily the reason for reduced fitness in the embryo, but might be a cause for sterility in forthcoming generations. Moreover, the crucial 2 : 1 ratio between maternal and paternal genome contribution cannot be maintained, and therefore endosperm formation is disturbed and can lead to abortion of the seeds (Savidan 2007; Talent 2009). In the case of gametophytic apomixis, the embryo sac develops from an unreduced cell, thus avoiding aneuploid megaspores and potentially reduced fitness in the offspring (Koltunow & Grossniklaus 2003). Therefore, these modes of reproduction bypass potential meiotic disturbances and sterility that are especially expected in newly formed autopolyploids (Cifuentes *et al.*, 2010). However, evidence for autopolyploid origins of apomicts is scarce (but see Hojsgaard *et al.* 2008; Thompson & Whitton, 2006). In fact, most apomicts have shown to be of hybrid origin (Asker & Jerling 1992; Palop-Esteban *et al.* 2007; Paun *et al.* 2006; Sharbel *et al.* 2009). Traditionally, apomixis has been seen as an evolutionary route to overcome hybrid sterility (Darlington 1937).

Autopolyploid vs. allopolyploid mode of origin have different genetic consequences and are therefore of high relevance for understanding ecological and biogeographical success of polyploid cytotypes. Hybridization potentially creates genotypes with a higher vigor because of increased heterozygosity, and may enhance ecological flexibility by producing genotypic and phenotypic novelty (Kearney, 2005). This benefit is not expected in autopolyploids. Asexual organisms in general tend to be more widespread than their sexual relatives, a phenomenon described as "geographical parthenogenesis" (Hörandl 2006; Kearney 2005; Vandel 1928). In animals, this pattern has often been referred to genetic consequences of hybrid origin of the asexual species (Kearney 2005, 2006) or to polyploidy (Lundmark 2006; Lundmark & Saura 2006). In plants, apomicts are almost exclusively polyploids, and the geographical pattern occurs mostly in apomictic allopolyploid complexes (reviews in Bierzychudek (1985; Hörandl 2006; Hörandl *et al.* 2008). Some well-studied examples of sexual autopolyploid plants do not show significantly larger distribution ranges than the diploid cytotypes (Hörandl, 2006; Soltis *et al.* 2007). However, some case studies suggest that geographical parthenogenesis occurs in autopolyploid apomicts as well (Bayer 1991; Thompson & Whitton 2006; Yahara 1990). The question arises whether genetic effects of polyploidization would explain the ecological and biogeographical success of asexual organisms, or whether apomixis helps to disperse polyploid cytotypes.

For observing these evolutionary pathways we use the alpine species *Ranunculus kuepferi* Greut. et Burd. (sensu Küpfer 1974). The presence of diploid and tetraploid populations, and rare (3x and 5x) cytotypes makes the species suitable for studying evolutionary origins of polyploid cytotypes. Tetraploids have been assessed as facultative apomictic throughout the range of the cytotype, while diploids are regular sexual (Cosendai & Hörandl 2010). Diploid sexuals occur only in the southwestern Alps, while tetraploid apomicts colonize the whole Alps, the northern Apennines, and Corsica (Cosendai & Hörandl 2010; see also Fig. 1). Triploids in the geographical contact zone represent probably backcrosses of diploids and tetraploids (Cosendai & Hörandl 2010). Therefore, the species is also a model system for studying the relevance of hybridity vs. genome duplication for geographical parthenogenesis. Indications of facultative apospory in polyploids have been provided by (Burnier *et al.* 2009). A comparative embryological study of *R. parnassifolius* and *R. kuepferi* (Vuille & Küpfer 1985) revealed an embryo sac formation similar to *R. auricomus*, a well-studied aposporous model system (Koltunow *et al.* 1995; Nogler 1984a, b, 1995). The tetraploid cytotype of *R. kuepferi* has aborted petals, pollen and achenes (Huber 1988) similar to other, unrelated apomictic Ranunculi (Hörandl 2008; Hörandl *et al.* 1997),

which is probably due to developmental disturbances related to apomixis. Beside these features, no other morphological differences have been traced between the cytotypes (Huber 1988). A phylogenetic study including related species within the genus (Hörandl *et al.* 2005; Paun *et al.* 2005) and based on dominant markers could not resolve the question of auto- and allopolyploid origin (Burnier *et al.* 2009). The closest relatives of *R. kuepferi*, the sympatrical species *R. seguieri*, *R. aconitifolius* and *R. platanifolius*, have distinct morphological features that are by no means apparent in the tetraploid cytotype of *R. kuepferi* (Huber, 1988). It is therefore unlikely that any of the extant related species were involved in the origin of tetraploid *R. kuepferi*. However, theoretically also a more distantly related or extinct sexual species could have been involved in the parentage of an allopolyploid cytotype, as it has been demonstrated in *Limonium* by using microsatellite analysis (Palop-Esteban *et al.* 2007). In this case, the diverged gene pool of the "cryptic" second parent was still present in the polyploid taxon.

For all these reasons, *Ranunculus kuepferi* appears to be an interesting model system for studying the evolution of polyploid apomictic cytotypes. Discriminating between auto- and allopolyploid origin of the tetraploid cytotype will help us to better understand the biogeographical success of apomixis. We use codominant (SSRs) and dominant AFLP markers to be able to discriminate between allo- and autopolyploidy. High sensitive co-dominant data are informative about multiple allelism and putative segregation patterns within the tetraploid cytotypes, fixed heterozygosity, and contributions of alleles from other parental species. Multilocus dominant data can reveal the composed structure of hybrid genomes in allopolyploids (e.g., Paun *et al*., 2006, Guo *et al.,* 2008) and are potentially informative about the overall genetic divergence and single vs. multiple origins of cytotypes. Since previous phylogenetic and morphological data do not provide any specific hypothesis for parentage of the tetraploid cytotype, we analyze genomic structure of tetraploids to test for the presence of a cryptic hybrid genome. With this combined approach we try to answer the following questions: (1) did the tetraploid apomictic cytotype originate via allopolyploidy or autopolyploidy? (2) Are polyploids of single or multiple origins? (3) Is the mode of polyploidization relevant for the biogeographical success of apomixis?

# MATERIAL AND METHODS

## PLANT MATERIAL

The plants used in this research were collected in the wild from 20 populations during spring and summer between 2004 and 2007; for details see Table 1 and Figure 1. A. By collecting between 10-25 individuals per population, we sampled at total of 372 individuals. Leaf material was dried in silica gel for molecular analysis. Determination of ploidy level of all individuals and the mode of reproduction of cytotypes has been presented in (Cosendai & Hörandl 2010).

## MOLECULAR METHODS

DNA was extracted following a slightly modified protocol based on the original CTAB-protocol (Doyle & Doyle 1990) using c. 0.5 g of silica gel dried leave tissue. After CTAB extraction, the DNA concentration of the extracts was measured photometrically with a Techne Specgene spectrophotometer (Techne, Cambridge, UK).

Microsatellite primers were developed by Savannah River Ecology Laboratory, University of Georgia, based on the protocol developed by Glenn & Schable (2005). PCR amplification was performed with a three primers system in a 12.5 µl volume (10 mM Tris HCl pH8.4, 50 mM KCl, 25.0 µg/ml BSA, 0.4 µM unlabelled primer, 0.08 µM tag labelled primer, 0.36 µM universal dye-labelled primer, 2.0-2.8 mM $MgCl_2$, 0.15 mM dNTPs, 0.5 Units JumpStart Taq DNA Polymerase (Sigma-Aldrich, St. Louis, MO, USA) and 20-40ng/µl DNA using an ABI thermal cycler). Primers were tested on a temperature gradient from 54-66°C with four touchdown programs based on Don *et al.* (1991) with a 10°C span of annealing temperature for the amplification. Then the PCR program starts with 5 cycles at 95°C for 30s, the highest annealing temperature 30s, and 72°C for 30s, then 21 cycles at 95°C for 30s, the highest annealing temperature with a decrease of 0.5°C per cycle for 30s and 72° for 30s, and last 15 cycles at 95°C for 30s, with the lowest temperature (start temperature -10°C) for 30s and 72°C for 30s. Final extension time at 72°C for 10 min. PCR products were purified using Sephadex G-50 Superfine (GE Healthcare Bio-Sciences, Uppsala, Sweden) according to the manufacturer's instructions. Cleaned products were run on a 3130xl Genetic Analyzer capillary sequencer (Applied Biosystems, Foster City, CA, USA) with GeneScan 500 ROX as the internal size standard (Applied Biosystems, Foster City, CA, USA). Within the plate one negative control was set. Raw data were sized and scored with GeneMarker 1.85 (SoftGenetics, State College, PA, USA) with parameter set at the size of selected

microsatellite as indicated in Table 2 with a threshold of 100 RFU. We analyzed 379 samples comprising 119 diploid, 15 triploid, 237 tetraploid and 4 pentaploid individuals.

AFLP fingerprint profiles were produced for 4–25 individuals per population, for a total of 377 individuals. A set of three primers combinations with four selective nucleotides was selected (fluorescent dyes in brackets): *Eco*RI-ACT / *Mse*I-CTCG (FAM), *Eco*RI-ACG / *Mse*I-CTCG (VIC), *Eco*RI-AGC / *Mse*I-CTGA (NED). Selective PCR products were purified using Sephadex G-50 Superfine (GE Healthcare Bio-Sciences, Uppsala, Sweden) according to the manufacturer's instructions. Cleaned products were run on a 3130xl Genetic Analyzer capillary sequencer (Applied Biosystems, Foster City, CA, USA) with GeneScan 500 ROX as the internal size standard (Applied Biosystems, Foster City, CA, USA). Each plate or run of sample was performed with one sample repeated over all plates, ca. 10 replicates within the plate and one negative control. Raw data were sized and scored with GeneMarker 1.85 (SoftGenetics, State College, PA, USA). Settings of parameters with GeneMarker for scoring used a size range of 120–510 bp, and a threshold set at 50 RFU (Relative Fluorescence Unit). The panel editor was used for filtering the irreproducible fragments, conserving only clear peak, for the 3 primers combinations. We did not score small fragments (50—120 bp) to avoid non-homologous fragments in this size class (Vekemans *et al.* 2002). Non-reproducible bands identified by comparisons among replicated individuals were excluded from the further analysis.

### ANALYSIS OF DATA

Microsatellite data were coded first as an allele matrix for five loci. We excluded the 6$^{th}$ locus (Rk 38) from the dataset as it presented up to two alleles additionally to the expected number in diploids and in tetraploids. We suspect that this pattern is due to a duplication or another specific evolution of the locus (e.g., Corral *et al.* 2009), and therefore we preferred to abandon the locus from this analysis. We genotyped all individuals for five loci to reveal multi-allelic patterns in polyploids. We tested the five remaining loci for linkage disequilibrium (LD; non-random segregation of loci) in the diploid samples with a Markov chain of 10000 steps in Arlequin 3.5.1.2 (Excoffier & Lischer 2010). Rk_11 and Rk_26 had a significant LD ($p < 0.005$) to each other and to the other loci, and were therefore excluded from calculations of observed heterozygosity. We refrained from progeny arrays or statistical segregation analysis because proportion of di- vs. multisomic inheritance would be likely distorted in the case of facultative apomixis. The allele matrix was computed with the program BAPS (Corander & Marttinen 2006), which provides a Bayesian inference analysis of population structure, using a

Bayesian probability algorithm to assign the different alleles to the optimal number of gene pools. To examine whether tetraploids have a gene pool of a hypothetical second parent different from the diploid gene pool, we designed a two-step analysis to test for allo- vs. autopolyploidy origin of tetraploids. First, we analyzed only the diploids sample for a simple clustering with 1000 iterations per sample to obtain the optimal partition of gene pools within the diploid cytotype, which revealed eight groups (alternative analyses with all five loci and with the three unlinked loci only revealed the same result, which infers that LD of two loci does not influence the grouping within the diploids). Second, we used these eight groups of the diploids and added the tetraploids to the partition as an unassigned ninth group to simulate another cryptic gene pool. This second admixture analysis filtered the sample of the unknown group through the setting of nine groups and was performed with 1000 iterations per sample. This analysis would assign the observed alleles of tetraploids either to the gene pools of the diploid cytotype or to another cryptic ancestral gene pool. Thus, we tested the hypothesis whether tetraploids have just duplicated the gene pool of the diploid cytotype, which is expected after autopolyploid origin, or whether they include a second parental genome, which would indicate allopolyploid origin. We proceed to the same two step analysis with the AFLPs data to get more confidence and reliability of the results, with a greater number of fragments (297) which should provide a more accurate response.

The AFLP dataset was analysed with FAMD 1.23 beta http:// www.famd.me.uk/famd.html (Schlüter & Harris 2006), using the Jaccard index for calculation of a similarity matrix and computing a Neighbour Joining tree. We performed a bootstrapping of the NJ tree with the options BS + RMD (BS randomly resample the loci; RMD replace missing data, 1000 repeats), and then proceeded with strict consensus and majority rule consensus trees. The strict consensus tree showed only a polytomy without any bootstrap support, but the majority rule consensus tree does present some bootstrap support that is reported on Figure 3. We performed a counting of specific fragments for each cytotype using FAMD as implemented in the program.

## RESULTS

### GENOTYPE AND ALLELE FREQUENCIES

In the microsatellite dataset, we obtained a total of 54 alleles in five loci (see supplementary Table for the summary of loci and the number of alleles per

locus). In the Rk_38 locus we obtained more alleles than expected according to ploidy level (up to four for diploids and up to six for tetraploids). In all the other loci, the maximum number of alleles corresponded to expectations after ploidy levels. Table 3 summarizes the genotype diversity sorted by ploidy level. The two linked loci Rk_11 and Rk_26 had a small number of alleles and therefore a low diversity of genotypes (two in diploids) compared to Rk_37, Rk_35 and Rk_27 (39 genotypes, 11 genotypes and 18 genotypes for the diploids). Based on the other loci Rk_37, Rk_35 and Rk_27, about half of the genotypes in diploids are heterozygous (mean observed heterozygosity = 52.66). Similar results were observed in other ploidy levels. Alleles were mostly shared among diploids and tetraploids, there are only two alleles found uniquely in tetraploids in the locus Rk_27. Genotypes with more than two alleles occur in all polyploid cytotypes, but only in tetraploids some genotypes with four different alleles are observed. This tendency of multiple allelism is most pronounced in Rk_37. The proportion of heterozygote genotypes (observed heterozygosity) differs between loci and between cytotypes (Table 3). Triploids show diallelic and triallelic genotypes, in the locus Rk_11 100% are triallelic. Tetraploids have also tetraallelic genotypes only in one locus Rk_37, but three other loci show diallelic and triallelic heterozygotes, while Rk_26 has only diallelic heterozygotes. Pentaploids show diallelic and triallelic heterozygotes, but for this cytotype the sampling is probably not representative.

The AFLP data set revealed a total of 297 fragments and revealed distinct multilocus genotypes for all individuals except for population 59 (Turracherhöhe), where two pairs of individuals each shared the same multilocus genotype. The counts of specific fragments within the ploidy levels revealed 13 private fragments for diploids and nine for tetraploids for a total of 292 polymorphic fragments, but no private fragments for triploids and pentaploids.

### GENETIC STRUCTURE

The admixture analysis of microsatellite data revealed eight main groups of diploids corresponding to the populations (Fig. 2A). Admixture occurs mainly within the four main disjunct geographical regions of the diploid area (Fig. 1, 2), but is remarkable low and restricted to a few individuals between these regions. The tetraploid populations present a strong admixture pattern with all of the allele groups of diploids. Most important, the 213 tetraploid samples did not form a group of their own. If the tetraploids would have a genome of another parental species, we would have seen an admixture of alleles from a ninth cluster/gene pool in the tetraploids (Fig. 2A). In contrast, all tetraploids share just the allele pool of the diploid cytotype of *R. kuepferi*.

The Bayesian analysis suggests that tetraploids are composed from the different allele pools of the diploids in contrast to the diploids without a genomic contribution of any other parental species. The admixture obtained with AFLPs data is more clear (Fig 2B), than the picture obtained with SSRs, diploids cluster in four groups as optimal partition, completely fitting to the geographical distribution and present very little admixture over 1000 replicates (observable in four diploids samples) and that even with 297 fragments analyzed. The admixture in tetraploids supports more than in SSR, that their origin is only from the diploids gene pool. There is no evidence of another cryptic gene pool that would be shown with a new color. Tetraploids present a mixture of the four diploids gene pool.

The NJ tree based on AFLP data shows only a weak structure mainly according to ploidy levels (Fig. 4). The diploid populations from Mt La Chens (3, 4), Vercors (28, 29) and the population group from Col de Tende comprising several populations (6, 7, 15, and 16), cluster quite apart from each other, with bootstrap support of 95%, 96% and <50%, respectively. Triploid backcross populations between 2x and 4x are nested within the diploid populations, like in the Col de Tende population (12), whereas Col des Champs (population 19) appears between tetraploids. Corsica (1) and Mt. Cusna (2), two geographically isolated populations, form strongly supported clusters (91% and 100% bootstrap support, respectively) and are nested within the backcross population of Champs (19). The cluster of the easternmost population (59 Turracherhöhe) had a bootstrap support of 98%, but the major cluster of tetraploids and its subcluster had no bootstrap support (violet branch in Fig. 4). Altogether AFLP data confirm the presence of a geographical structure in the diploids, and shows independent origins of three tetraploid populations (1, 2, 59), while the origin of the main tetraploid cluster remained unresolved.

## DISCUSSION

### ALLOPOLYPLOIDY VS AUTOPOLYPLOIDY

In the microsatellite data, the appearance of tetraallelic and triallelic genotypes in tetraploids indicates tetra- or trisomic inheritance, as it is typical for sexual autopolyploidy and has been often described in isozyme studies (Hörandl & Greilhuber 2002; Parisod *et al.* 2010; Rieseberg & Doyle 1989; Soltis & Rieseberg 1986). Presence of multiple allelism is commonly assumed as part of an autopolyploid pattern (Pupilli *et al.* 1997; Rieseberg & Doyle 1989; Stift *et al.* 2008). The multiallelic microsatellite pattern in extant

populations of apomicts is most probably not due to mutations, since only two private alleles have been observed in tetraploids. In contrast, we assume that autopolyploids were originally sexual, and have originated from crosses of various different diploid genotypes via unreduced gametes. This hypothesis is supported by findings of rare tetraploid sexual individuals in the Col de Tende region (no. 12 in Cosendai & Hörandl 2010). In the whole area, autopolyploids have kept multiple allelism after the shift to apomixis, and/or still exhibit residual sexuality. Frequent findings of 3x embryos in 4x mother plants, coupled to 8x to 10x endosperm ploidy levels (Cosendai & Hörandl 2010) indicates that still occasionally meiosis must occur, but with a disturbed segregation of chromosomes. Evidence of fixed heterozygosity, as often found in allopolyploid apomicts (Comai 2005; Comai *et al.* 2003; Nybom 2004) is not available from our data (Table 3). In the calculation of Ho (observed heterozygosity, Table 4) values of sexuals are comparable to means of values reported for mixed breeding systems based on microsatellite data (Nybom 2004). In two loci (Rk 11 and Rk 26) more than 90% of diploid individuals have heterozygote genotypes. In these two loci, segregation distortion, is likely which is a frequent phenomenon in microsatellite loci (Li *et al.* 2003; Ruiz & Asins 2003; Sargent *et al.* 2004). Linkage disequilibrium and/or non-neutrality, i.e., selection for heterozygous genotypes, may influence the observed allelic patterns on population level. However, our evaluation has a focus on evolutionary origins of cytotypes. A more detailed analysis of heterozygosity and breeding systems on population level will be presented elsewhere.

Results of both Bayesian analyses indicate a geographical differentiation within the diploid populations, more evident in AFLPs with only four groups (Fig. 2B), but almost no admixture among these geographical population groups, which may be explained by the putative non-neutrality in two loci (Rk 11 and Rk 26) or a very homogenous genome in diploids. Loss of genetic diversity due geographical isolation in refugial areas (Burnier *et al.*, 2009) may also influence the pattern in the sexual populations. However, the BAPS analysis with tetraploids strongly suggests that no other cryptic gene pool was involved in the origin of tetraploids than the diploid cytotype of *R. kuepferi*. In contrast, all eight gene pools of diploid *R. kuepferi* have contributed to the origin of autopolyploids, suggesting historical gene flow between diploid population groups in SSR data set. The gene of tetraploids populations is just a reshuffling of the four diploids gene groups in AFLPs. The genetic structure of the tetraploids exhibits always just a new mixture of alleles from the diploid source populations in both dataset. We cannot rule out that our microsatellite data are affected by null alleles, or by having not sampled loci that potentially could exhibit alleles from another species.

However, the absence of a second parental genome is confirmed by AFLP data, which provide with altogether 297 fragments a much broader representation of the genome and does not show a cryptic genome with this analysis as well. More over AFLP analysis revealed only 9 private fragments in the tetraploids (ca. 3% of total fragments), which is even lower than in diploids (ca. 4 % private fragments). Similar low frequencies of private fragments were found in the tetraploid cytotype of *Ranunculus cassubicifolius* (Paun et al. 2006), which is autopolyploid according to allozyme patterns (Hörandl & Greilhuber 2002). In contrast, allopolyploids exhibit usually balanced proportions of private fragments inherited from two divergent parental species (Guo et al. 2006; Paun et al. 2006). The AFLP data thus confirm the absence of a second, divergent parental genome in *R. kuepferi*.

Autopolyploid origin of tetraploids is further supported by the lack of morphological differences between the diploid and the tetraploid cytotype (Huber 1988). Aborted petals, pollen grains and fruits in tetraploids are likely due to developmental disturbances connected to apomixis (Asker & Jerling 1992; Hörandl, 2008). The closest relatives of *R. kuepferi* (*R. seguieri*, *R. platanifolius*, *R. aconitifolius* and *R. glacialis*) have all palmately divided leaves with multiple, more or less deep incisions, and also different fruit shapes. No such character, not even intermediacy, is apparent in the tetraploids that have the same lanceolate, entire leaves and the same fruit shape as the diploid cytotype. The scarcity of distinct macro-morphological characters compared to diploid progenitors is typical for autopolyploids (Soltis et al. 2007). In contrast, allopolyploidy often is connected to a pronounced morphological differentiation to the diploid parents (e.g., Soltis & Soltis 2009).

### FREQUENCIES OF ORIGIN AND GENETIC DIVERGENCE

The structure of the NJ tree based on AFLPs suggests independent origins of at least three of the tetraploid populations. For the main tetraploid cluster, however, with weak resolution of the backbone and the lack of bootstrap support at the basal branches, multiple vs. single origin remains an open question. The triploid cytotypes are nested within the diploids and are most likely backcrosses between diploids and tetraploids (Cosendai & Hörandl, 2010). Our results confirm a phylogeographic study that suggested several polyploidization events since the last glacial maximum within the species *Ranunculus kuepferi* (Burnier *et al.* 2009). Similar patterns of multiple origins were found in several other apomictic species, where polyploidization happened several time in their recent evolution, like in grasses (*Paspalum*, Siena *et al.* 2008), in ferns (Grusz *et al.* 2009), in hawthorns (*Crataegus*, Talent & Dickinson 2005) and in roses (*Rosa*, Koopman *et al.* 2008); Multiple

origins of autopolyploid cytotypes in *R. kuepferi* could not increase genetic diversity of the diploid gene pool, but may have enhanced a rearrangement of allelic combinations of the regional sexual gene pools within the polyploid cytotypes.

The little resolution and the short branches in the tree topology based on AFLP data (Fig. 3) suggest an overall lack of divergence between diploids and tetraploids. Polytomies at the basis of the tree confirm that the different cytotypes still share the same gene pool. In the SSR data, only two novel alleles appear in tetraploids compared to the diploids. This low divergence is striking because SSRs are selectively neutral and therefore a rapidly evolving marker (Schlötterer 2000). The lack of genetic divergence in *R. kuepferi* in all marker systems is probably due to a young evolutionarily origin. This hypothesis is further supported by the linear increase of genome sizes from diploids to tetraploids, and the lack of genome downsizing in polyploids (Cosendai & Hörandl 2010). This finding is consistent with phylogenetic evidence that all asexual flowering plants appear to be evolutionarily young (Whitton *et al.* 2008). The low genetic divergence between cytotypes and the geographical pattern further support the general hypothesis that apomictic cytotypes have originated predominantly during the Pleistocene, when climatic changes, glaciations and range fluctuations of species provided opportunities for origin and spread of apomixis (Hörandl 2009; Thompson & Whitton 2006; Van Dijk 2003).

### THE RELEVANCE OF APOMIXIS FOR THE BIOGEOGRAPHICAL SUCCESS OF AUTOPOLYPLOID CYTOTYPES

In sexual autopolyploids, chromosomes tend to multivalent formation at meiosis, which can lead to unbalanced segregation and aneuploid megaspores (e.g., Comai 2005, Cifuentes *et al.*, 2010). This mechanism likely explains multiple allelism in *R. kuepferi* in the microsatellite loci. Flow cytometric seed screening analysis of *R. kuepferi* (Cosendai & Hörandl 2010) has given indications for occurrence for a facultative, unbalanced meiosis. This study revealed that tetraploid adults produced triploid embryos in about one third of the seed samples. Because of the observed endosperm ploidy levels, these triploid embryos must have resulted from a triploid egg cell that has developed parthenogenetically. A triploid egg cell, however, can be only derived from a triploid embryo megaspore, which must have resulted from an unbalanced meiosis. These triploid lineages obviously failed to establish because triploid adults are almost absent from the natural populations. Obviously, the offspring arising from an unbalanced meiosis fails to establish in natural populations. In contrast, gametophytic apomixis, which appeared in c. two third of the seed samples, stabilizes the tetraploid

cytotype which predominates in the whole distribution area (Cosendai & Hörandl 2010). These findings, and our microsatellite data presented here, indicate that residual facultative meiosis is still present in autotetraploid *R. kuepferi*, but is selected against because it causes instability of cytotypes. In an evolutionary context, the consequence of sexual autopolyploidy could be a bottleneck until re-diploidization occurs, i.e. until bivalent formation becomes more and more regular (Comai 2005). The timeframe and selective mechanism for cytological diploidization likely takes many generations. During this timespan, the newly formed polyploid is hampered by reduced fertility (Cifuentes *et al.* 2010). In fact, tetraploid *R. kuepferi* has significantly higher percentages of aborted pollen grains (Huber 1988) and fruits (Cosendai & Hörandl 2010) than the diploid sexual cytotype. In contrast, the species can bypass meiotic segregation via apomixis, and the handicap of meiotic disturbance can be overcome rapidly. Apomixis would be a tool for the organism to escape from sterility, as already postulated by Darlington (1937). The lack of genetic divergence between the sexual and the apomictic cytotypes suggest very young and probably multiple evolutionary origin of autopolyploids, where cytological diploidization has probably not yet occurred. Therefore, apomixis may have aided an extremely rapid establishment of recurrently newly formed autotetraploid cytotypes.

The hypothesis that apomixis is an escape from sterility was traditionally applied to hybrids which often exhibit meiotic disturbances; however, our study suggests that apomixis evolves in autopolyploids via similar evolutionary mechanisms. The coupling of apospory to parthenogenesis, the development of the egg cell without fertilization, establishes a fully functional apomictic reproduction. Preliminary embryological studies on *R. kuepferi* suggest a predisposition to independent parthenogenetic development (J. Wagner, pers. comm.). From an evolutionary point of view, the capacity to turn to apomixis helps the organism to reproduce even if the reproduction is penalized by problems in meiosis. In the case of multiple, evolutionary young origins of autopolyploids, as in *R. kuepferi*, apomixis allows for preservation of many, genetically diverse lineages, that would be otherwise lost in a functionally biased sexual system.

The lack of a hybrid genome challenges traditional views on geographical parthenogenesis. We have evidence that also autopolyploidy allows for gaining large distribution areas in *R. kuepferi*, if coupled to apomixis. Most examples of geographical parthenogenesis have been described from allopolyploid systems (Hörandl 2006; Van Dijk 2003). However, the combination of autopolyploidy and apomixis established large distribution areas in other model systems as well (Bayer 1991; Thompson & Whitton 2006; Urbani *et al.* 2002; Yahara 1990). Our results do not support the

hypothesis that heterozygosity, hybrid vigor and genomic and phenotypic novelty would be crucial requirements for distributional success. In contrast, bypassing consequences of meiotic problems via apomixis is probably more important for establishment of newly formed polyploid cytotypes. Apomictic cytotypes further benefit from the advantages of uniparental reproduction in postglacial re-colonization scenarios (e.g., Van Dijk, 2003; Thompson & Whitton, 2006; Hörandl *et al.*, 2008; Cosendai & Hörandl, 2010). We conclude that facultative apomixis enhances the rapid spread and establishment of newly formed autotetraploid cytotypes.

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## TABLES

Table 1 Materials used in this study. Population numbers correspond to numbers in Figure 1; Country abbreviations: F; France, I; Italy, A; Austria, CH; Switzerland. Collectors: ACC; Anne-Caroline Cosendai, ACC-AC; Anne-Caroline Cosendai & André Cosendai, PK; Philippe Küpfer, EH; Elvira Hörandl, MH; Marc Hämmerli, CS; Christoph Seger, PS-GS; Peter Schönswetter & Gerald Schneeweiss. arabic numbers in localities indicate collection numbers in the herbarium of E. Hörandl. Voucher specimens have been deposited at the herbarium of the University of Vienna (WU).

| Population number | Country | Province | Locality | Ploidy level* | Altitude | Coordinate North | Coordinate East | Collector |
|---|---|---|---|---|---|---|---|---|
| 1 | F | Corse-du-Sud | Corsica | 4x | 1541 m | 0042°01'46.4'' | 009°12'34.5'' | ACC |
| 2 | I | Emilia-Romagna | Mt. Cusna | 4x | 1594 m | 0044°18'06.7" | 0010°22'26.6" | ACC-AC |
| 3 | F | Var | Mt. La Chens_I | 2x | 1610 m | 0043°44'59.3" | 006°39'25.5" | PK |
| 4 | F | Var | Mt. La Chens_II | 2x | 1607 m | 0043°44'58.5" | 006°39°29.1" | ACC-AC |
| 6 | I | Piemonte | Valle di Pesio_I_9589 | 2x | 1700 m | 0044°14'40'' | 007°37'42" | EH |
| 7 | I | Piemonte | Passo del Duca_9592 | 2x | 1700 m | 0044°11'43'' | 007°39'33" | EH |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 12 | I | Piemonte | Col della Perla_II_9597 | 3x, 4x, 5x | 2200 m | 0044°9'11.05" | 007°37'21.14" | EH |
| 15 | I | Piemonte | Colle della Lombarde_I_9601 | 2x | 2260 m | 0044°12'25.68" | 007°8'51.98" | EH |
| 16 | I | Piemonte | Colle della Lombarde_II_9602 | 2x | 2477 m | 0044°13'17.60" | 007°9'9.25" | EH |
| 19 | F | Alpes-Maritimes | Col des Champs_II | 3x, 4x | 2080 m | 0044°10'33.8" | 006°41'53.0" | ACC-AC |
| 28 | F | Drôme | Vercors_I | 2x | 1470 m | 0044°54'07" | 005°28'039" | PK |
| 9 | F | Drôme | Vercors_II | 2x | 1325 m | 0044°50'26.1" | 005°25'23.1" | ACC-AC |
| 33 | I | Valle d'Aoste | Gran Paradiso | 4x | 2079 m | 0045°37'0.5" | 007°33'12.2" | ACC-AC |
| 40 | CH | Valais | Lötschental | 4x | 1773 m | 0046°26'05.5" | 007°51'48.0" | ACC |
| 45 | CH | Graubunden | Julier Pass | 4x | 2277 m | 0046°28'20.9" | 009°44'01.4" | ACC |
| 48 | A | Vorarlberg | Arlberg Pass | 4x | 2269 m | 0047°08'49.1" | 0010°14'55.3" | ACC |
| 53 | A | Tyrol | Tuxer Alps | 4x | 2315 m | 0047°07' | 0011°34' | CS |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 55 | I | Trento | Padon Pass | 4x | 2350 m | 0046°27'47.80" | 0011°53'42.88" | EH |
| 58 | A | Carinthia | Mt. Sadnig | 4x | 2200 m | 0046°57'42" | 0013°00'35" | PS-GS |
| 59 | A | Carinthia | Turracherhöhe | 4x | 2220 m | 0046°55'20.47" | 0013°52'44.35" | EH |

* all data after Cosendai and Hörandl (2010).

Table 2 Summary of the primers used in this study, with the conservative sequence of flanking region, the base pair repeat, the recognition size, the total number of alleles found with the primer and GenBank accession number.

| Locus name | Forward primer sequence (5′– 3′) | Reverse primer sequence (5′– 3′) | Repeat | Size in bp | Total of alleles | GenBank accession no |
|---|---|---|---|---|---|---|
| Rk_11 | GTTTAAACCTTGCTGGTCCCGAG | CAGTCGGGCGTCATCACTTCAACGGAGAGGGGTGG | (CTT)^22 | 149-204 | 3 | XXX |
| Rk_26 | CAGTCGGGCGTCATCAGTTGGATTTCGCATGTTAGG | GTTTGGATTGAGGGAACAACGTCC | (GTTT)^4 | 205-234 | 2 | XXX |
| Rk_27 | CAGTCGGGCGTCATCACGAGTCACACAGATCCTGAAG | GTTTGGTCTTCGTTTGCCCATTC | (CT)^12 | 133-217 | 18 | XXX |
| Rk_35 | CAGTCGGGCGTCATCAATAAAAGGGTTCACTTCTTTCCAC | GTTTGAAATCATGGAGAGCGGTTTG | (CT)^6...(CT)^21 | 129-152 | 11 | XXX |
| Rk_37 | CAGTCGGGCGTCATCCCCATGACCCGAAC | GTTTGACACCGTATTCCGAGGC | (AG)^18 | 126-203 | 13 | XXX |
| Rk_38 | CAGTCGGGCGTCATCACACACACACCGTACACAG | GTTTCGAATCCCAACTAGCGGAC | (AC)^10...(AC)^13 | 139-156 | 7 | XXX |

Table 3 Summary of numbers and percentages of genotypes within the ploidy level for the microsatellite loci Rk 37, Rk 35, and Rk 27, and averages of observed heterozygosity (Ho) in the different ploidy levels. Heterozygote diallelic, triallelic and tetraallelic refer to the number of different alleles present in the genotypes of polyploids.

| | | **Rk37** | **%** | **Rk 35** | **%** | **Rk 27** | **%** | **Average observed heterozygosity** |
|---|---|---|---|---|---|---|---|---|
| 2x | Homozygote | 46 | **40.71** | 57 | **50.89** | 60 | **50.42** | |
| | Heterozygote diallelic | 67 | **59.29** | 55 | **49.11** | 59 | **49.58** | **52.66** |
| | Total | 113 | | 112 | | 119 | | |
| 3x | Homozygote | 6 | **40** | 8 | **53.33** | 6 | **40** | |
| | Heterozygote diallelic | 9 | **60** | 7 | **46.67** | 6 | **40** | |
| | Heterozygote triallelic | 0 | **0** | | **0** | 3 | **20** | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **All Heterozygots** | 9 | **60** | 7 | **46.67** | 9 | **60** | **55.56** |
| | Total | 15 | | 15 | | 15 | | |
| 4x | Homozygote | 82 | **42.27** | 94 | **40** | 115 | **53.99** | |
| | Heterozygote diallelic | 75 | **38.66** | 116 | **49.36** | 89 | **41.78** | |
| | Heterozygote triallelic | 27 | **13.92** | 25 | **10.64** | 9 | **4.23** | |
| | Heterozygote tetraallelic | 10 | **5.15** | | **0** | | **0** | |
| | **All Heterozygotes** | 112 | **57.73** | 141 | **60** | 98 | **46.01** | **54.58** |
| | Total | 194 | | 235 | | 213 | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| 5x | Homozygote | 1 | **25** | 1 | **25** | 1 | **25** | |
| | Heterozygote diallelic | 2 | **50** | 3 | **75** | 2 | **50** | |
| | Heterozygote triallelic | 1 | **25** | | **0** | 1 | **25** | |
| | **All Heterozygotes** | 3 | **75** | 3 | **75** | 3 | **75** | **75.00** |
| | Total | 4 | | 4 | | 4 | | |

## FIGURES

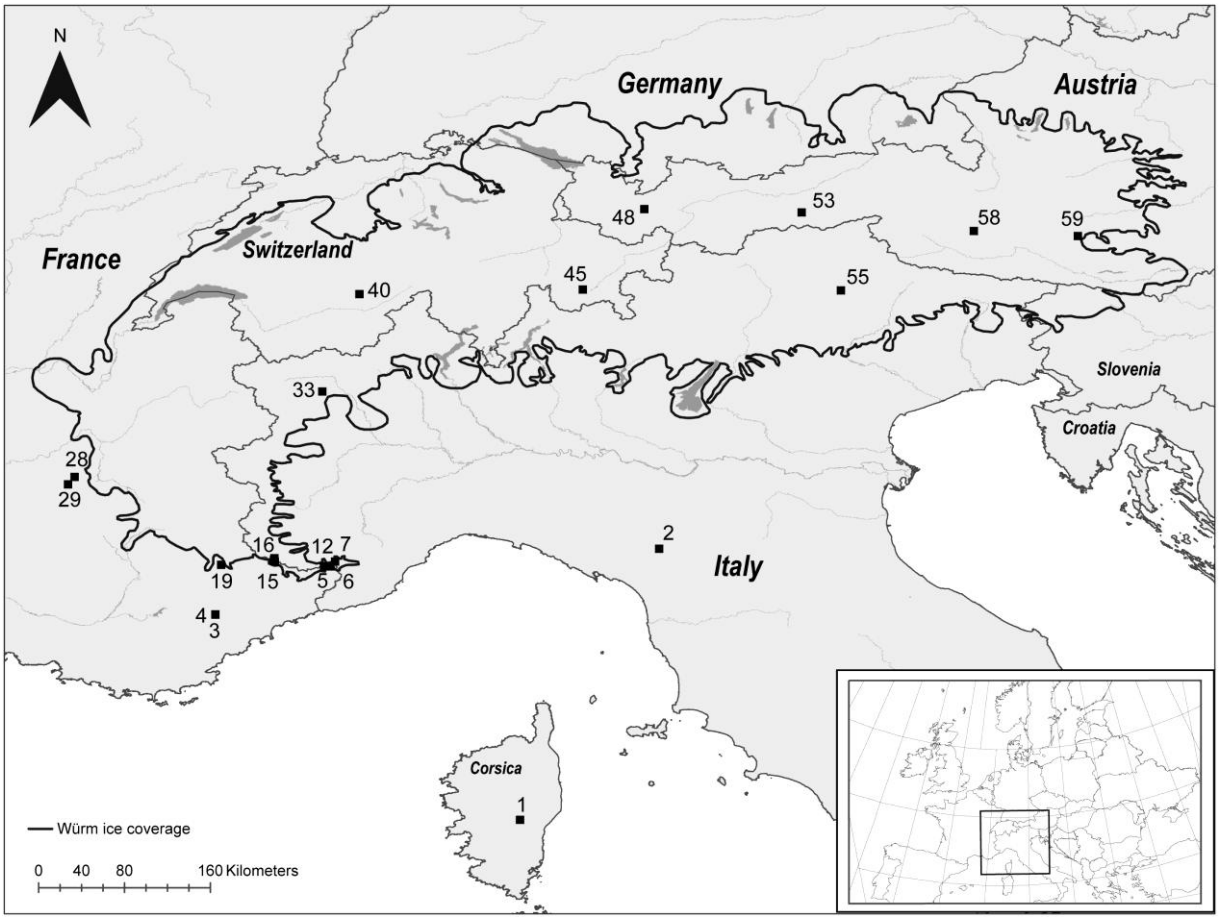


Figure 1 Map of the populations analysed. Dots and numbers indicate populations as listed in Table 1, the dark grey line represents the extension of the last glacial maximum of the Würm.

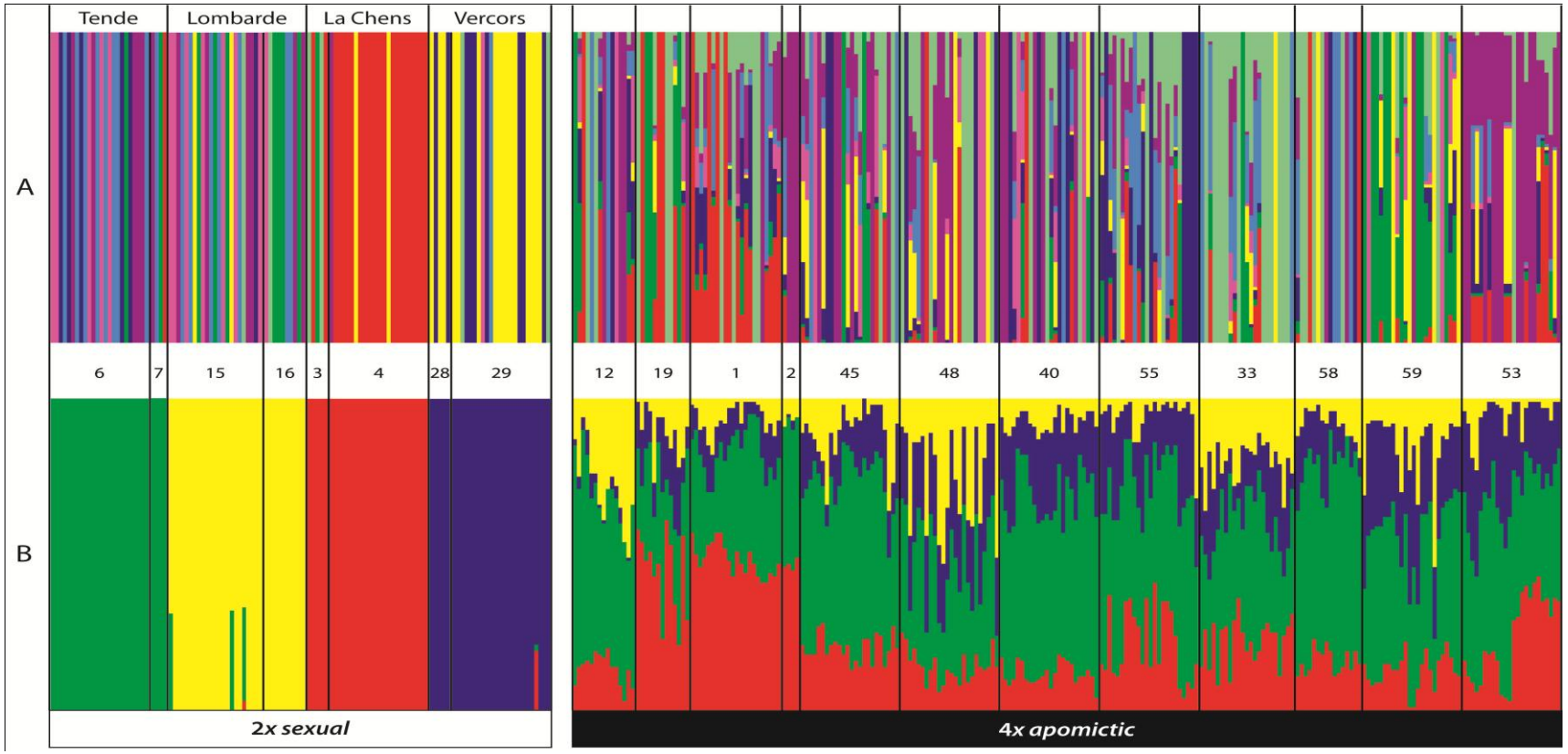


Figure 2 Admixture analysis of microsatellite data (A) and AFLP data (B). The numbers on the top correspond to the population number as in Table 1. Left A, eight main allele groups of the diploid sexuals, obtained with optimized settings. The main geographical regions are indicated at the top. Right A, admixture of the tetraploids to the diploid gene pool without indication of a ninth group of alleles. Left B, four main allele groups of the diploid sexuals, obtained with optimized settings. Right B, admixture of the tetraploids to the diploid gene pool without indication of a fifth group of alleles.

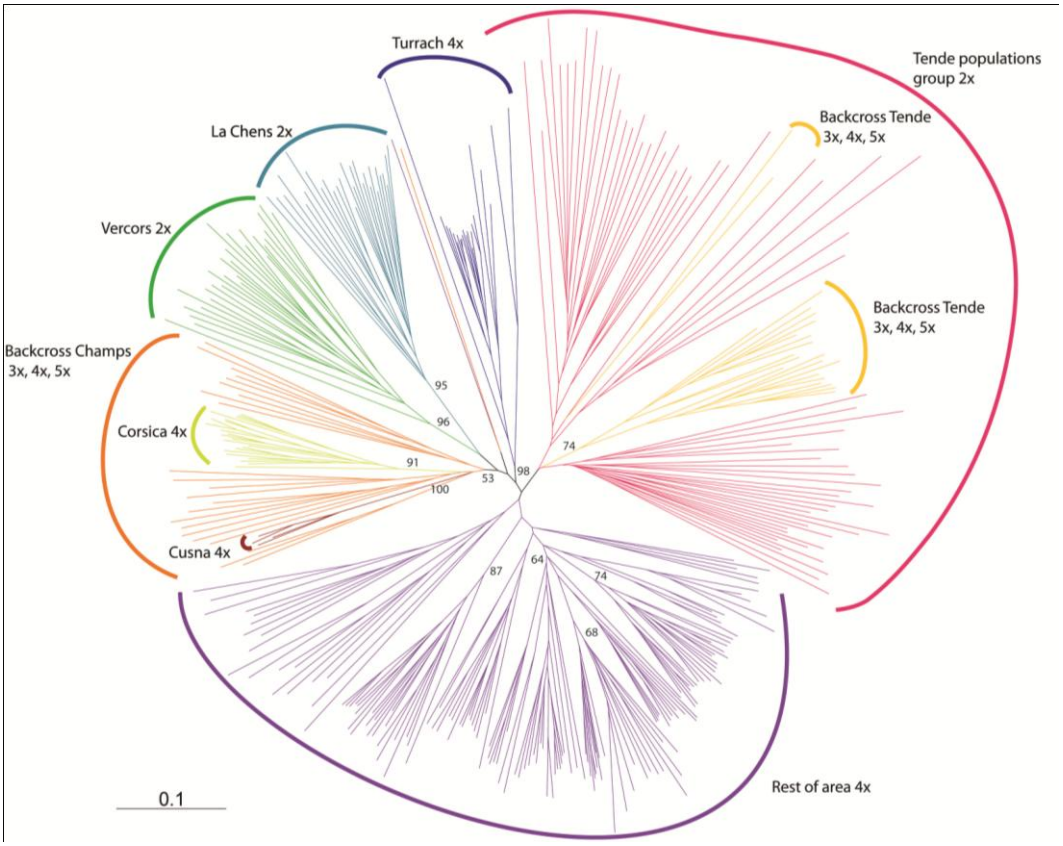


Figure 3 NJ tree based on the AFLPs dataset. Numbers on the branches indicate bootstrap support above 50. The diploid sexual populations and the main clusters of tetraploids are indicated by different colours.

Supplementary data. Summary of genotypes observed in the microsatellite loci Rk 37, Rk 35, Rk 11, Rk 26 and Rk 27 (see Table 2); Freq; indicate the frequency of the genotype within the ploidy level. 2x; diploids, 3x; triploids, 4x; tetraploids, 5x; pentaploids. Genotypes marked in grey are homozygote. Genotypes total; indicates the total number of different genotypes (in bold).

| | **Rk37** | **Freq.** | **Rk 35** | **Freq.** | **Rk 11** | **Freq.** | **Rk 26** | **Freq.** | **Rk 27** | **Freq.** |
|---|---|---|---|---|---|---|---|---|---|---|
| **2x** | 129/129 | 7 | 128/128 | 57 | 149/149 | 1 | 206/234 | 115 | 189/189 | 54 |
| | 129/136 | 1 | 128/131 | 7 | 149/191 | 118 | 234/234 | 4 | 189/191 | 1 |
| | 129/139 | 1 | 128/133 | 3 | | | | | 189/193 | 15 |
| | 129/163 | 7 | 128/135 | 1 | | | | | 189/195 | 3 |
| | 129/174 | 1 | 128/137 | 3 | | | | | 189/197 | 6 |
| | 129/196 | 2 | 128/140 | 1 | | | | | 189/199 | 1 |
| | 129/198 | 1 | 128/142 | 2 | | | | | 189/201 | 2 |
| | 129/200 | 10 | 128/144 | 4 | | | | | 189/203 | 2 |

| | | | | | |
|---|---|---|---|---|---|
| 129/202 | 1 | 128/147 | 9 | 189/205 | 4 |
| 129/205 | 4 | 128/149 | 8 | 189/207 | 9 |
| 133/174 | 1 | 128/152 | 17 | 189/209 | 3 |
| 136/136 | 4 | | | 189/211 | 6 |
| 136/163 | 1 | | | 189/213 | 2 |
| 136/205 | 1 | | | 189/217 | 3 |
| 139/139 | 1 | | | 191/191 | 1 |
| 139/160 | 1 | | | 193/193 | 4 |
| 160/167 | 1 | | | 193/211 | 2 |
| 160/174 | 1 | | | 197/197 | 1 |

| | |
|---|---|
| 160/202 | 2 |
| 163/163 | 13 |
| 163/167 | 3 |
| 163/198 | 1 |
| 163/200 | 1 |
| 163/205 | 3 |
| 167/167 | 1 |
| 167/174 | 1 |
| 167/198 | 1 |
| 167/200 | 2 |

| | |
|---|---|
| 167/202 | 3 |
| 174/174 | 3 |
| 174/202 | 2 |
| 174/205 | 3 |
| 196/205 | 3 |
| 198/198 | 1 |
| 198/200 | 1 |
| 200/200 | 3 |
| 202/202 | 4 |
| 202/205 | 7 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 205/205 | 9 | | | | | | | | |
| | **Genotypes total** | **39** | | **11** | | **2** | | **2** | | **18** |
| **3x** | 129/136 | 1 | 128/128 | 8 | 149/191/205 | 14 | 206/234 | 14 | 189/189 | 6 |
| | 129/163 | 1 | 128/135 | 2 | | | 234/234 | 1 | 189/195/211 | 1 |
| | 133/133 | 1 | 128/137 | 1 | | | | | 189/197/211 | 1 |
| | 133/174 | 1 | 128/152 | 3 | | | | | 189/205/207 | 1 |
| | 136/163 | 2 | 140/147 | 1 | | | | | 189/211 | 4 |
| | 160/174 | 1 | | | | | | | 189/221 | 2 |
| | 163/163 | 3 | | | | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 163/174 | 1 | | | | | | | |
| | 163/205 | 1 | | | | | | | |
| | 167/174 | 1 | | | | | | | |
| | 174/174 | 1 | | | | | | | |
| | 200/200 | 1 | | | | | | | |
| | **Genotypes total** | **12** | | **5** | | **1** | | **2** | **6** |
| **4x** | 129/129 | 5 | 128/128 | 94 | 149/149 | 1 | 206/234 | 197 | 185/189 | 1 |
| | 129/136/163/167 | 1 | 128/131 | 90 | 149/191 | 48 | 234/234 | 36 | 187/187 | 1 |
| | 129/136/163 | 1 | 128/131/152 | 23 | 149/191/205 | 160 | | | 187/189/207 | 1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 129/136/200/202 | 1 | 128/131/144 | 1 | 149/205 | 1 | 187/199 | 2 |
| 129/139/167 | 1 | 128/133 | 2 | 191/191 | 7 | 187/201 | 2 |
| 129/160/174/200 | 1 | 128/135 | 2 | 191/205 | 17 | 187/207 | 1 |
| 129/163/202 | 2 | 128/147 | 4 | | | 189/189 | 52 |
| 129/163/205 | 2 | 128/147/152 | 1 | | | 189/193/207 | 1 |
| 129/163 | 1 | 128/149 | 1 | | | 189/193 | 1 |
| 129/167/200 | 1 | 128/152 | 17 | | | 189/195 | 2 |
| 129/174/196 | 1 | | | | | 189/197/205 | 1 |
| 129/174 | 6 | | | | | 189/197/211 | 2 |
| 129/196 | 1 | | | | | 189/197 | 3 |

| | |
|---|---|
| 129/200 | 1 |
| 129/202 | 1 |
| 129/205 | 1 |
| 136/136 | 8 |
| 136/139/200 | 1 |
| 136/139/202 | 1 |
| 136/160 | 1 |
| 136/163/174/196 | 1 |
| 136/163/174/205 | 1 |
| 136/163/174 | 4 |
| 189/199/207 | 1 |
| 189/199 | 3 |
| 189/201 | 4 |
| 189/203/213 | 4 |
| 189/203 | 8 |
| 189/205 | 15 |
| 189/207 | 23 |
| 189/209 | 12 |
| 189/211 | 3 |
| 189/221 | 7 |

| | |
|---|---|
| 136/163/196/202 | 1 |
| 136/163/202 | 2 |
| 136/163 | 3 |
| 136/167/174/202 | 1 |
| 136/174 | 1 |
| 136/198 | 1 |
| 136/200/202 | 1 |
| 136/200/205 | 1 |
| 136/205 | 1 |
| 139/139 | 1 |
| 191/191 | 1 |
| 195/195 | 13 |
| 195/215 | 1 |
| 195/217 | 1 |
| 197/197 | 1 |
| 199/199 | 2 |
| 201/201 | 11 |
| 203/203 | 3 |
| 205/205 | 5 |
| 207/207 | 5 |

| | |
|---|---|
| 139/205 | 1 |
| 160/160 | 2 |
| 160/167 | 1 |
| 160/174/200/202 | 1 |
| 160/174 | 1 |
| 163/163 | 23 |
| 163/167/174 | 1 |
| 163/167 | 4 |
| 163/174/200/202 | 1 |
| 163/174/200/205 | 1 |
| 209/209 | 2 |
| 211/211 | 7 |
| 213/213 | 1 |
| 215/215 | 3 |
| 217/217 | 7 |

| | |
|---|---|
| 163/174/200 | 2 |
| 163/174/202 | 4 |
| 163/174/205 | 1 |
| 163/174 | 11 |
| 163/196 | 1 |
| 163/200 | 4 |
| 163/202 | 7 |
| 163/205 | 2 |
| 167/167 | 5 |
| 167/174/196 | 1 |

| | |
|---|---|
| 167/174 | 3 |
| 167/202 | 1 |
| 167/205 | 1 |
| 174/174 | 10 |
| 174/200 | 2 |
| 174/202 | 8 |
| 174/205 | 2 |
| 196/196 | 4 |
| 196/202 | 1 |
| 198/198 | 1 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 200/200 | 8 | | | | | | | | |
| | 200/202 | 3 | | | | | | | | |
| | 202/202 | 7 | | | | | | | | |
| | 202/205 | 4 | | | | | | | | |
| | 205/205 | 8 | | | | | | | | |
| | **Genotypes total** | **68** | | **10** | | **6** | | **2** | | **38** |
| **5x** | 129/163 | 1 | 128/128 | 1 | 149/191/205 | 4 | 206/234 | 4 | 189/189 | 1 |
| | 136/163 | 1 | 128/137 | 1 | | | | | 189/207 | 1 |
| | 136/163/174 | 1 | 128/149 | 1 | | | | | 189/211 | 1 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 174/174 | 1 | 128/140 | 1 | | | | | 187/189/199 | 1 |
| **Genotypes total** | **4** | | **4** | | **1** | | **1** | | **4** |

# CHAPTER 3 — MULTIPLE FOUNDER EVENTS AND MAINTENANCE OF GENETIC DIVERSITY CAUSE GEOGRAPHICAL PARTHENOGENESIS IN *RANUNCULUS KUEPFERI* (RANUNCULACEAE)

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Formated for *Evolution*

## ABSTRACT

Geographical parthenogenesis describes the phenomenon that asexual organisms have larger distribution areas, especially in previously glaciated areas. Several hypothesis have been discussed for the success of asexuals: uniparental reproduction may enhance rapid colonization (Baker's law), but applies to sexual selfing plants as well. Other models postulate advantages to asexuality because of clonal diversity, general purpose genotypes or reduced biotic interactions in colder climates. Here we analyze population genetic structure and breeding systems of the alpine species *Ranunculus kuepferi* as a model to understand the main factors for the different distribution success of sexual and asexual cytotypes on 59 populations out of the range of the species. Bayesian analysis of population structure based on Amplified Fragment Length Polymorphisms (AFLPs) revealed regional genetic clusters for sexuals, but unique gene pools for apomictic populations. Self-compatibility tests and analysis of pollen tube growth confirmed the ability for uniparental reproduction for pseudogamous apomicts, but not for diploid

sexuals because they exhibit stylar self-incompatibility. AFLP markers and five microsatellite loci suggest altogether low and mostly insignificant differences between sexuals and apomicts with respect to genotypic diversity, genetic diversity within and among populations, heterozygosity, and allelic diversity. Facultative recombination and autopolyploid origin maintain genetic diversity of apomictic populations. Our results suggest that the best explanation for geographical parthenogenesis is a combination of superior colonizing abilities of apomicts because of uniparental reproduction, and of the maintenance of genetic diversity which helps to overcome genetic bottlenecks after founder events.

Keywords: Bakers'law, founder events, geographical parthenogenesis, genetic diversity, self-compatibility.

## INTRODUCTION

Geographical parthenogenesis (GP) is a term coined initially by Vandel (1928), describing a pattern that sexual diploids and asexual polyploids have two different distribution areas. Later, the pattern was confirmed in many animals and plants (Bell 1982; Bierzychudek 1985; Asker and Jerling 1992; Van Dijk 2003; Haag and Ebert 2004; Kearney 2005; Hörandl 2006). Asexual organisms tend to have a wider distribution range, occur at higher elevations and at higher latitudes, and colonize more frequently previously glaciated or otherwise devastated areas than their sexual relatives. Of the several non-exclusive hypotheses that may play a role to explain GP pattern based on reproduction system, ecology or genetic advantages, we can consider: 1) Bakers' law, which assumes an advantage to uniparental reproduction for colonization (Baker 1967); 2) polyploidy (Comai 2005; Kearney 2005; Lundmark and Saura 2006) and 3) hybridization (Stebbins 1959; Kearney 2005), which both assume advantages of genomic novelty and increased genetic diversity; 4) Frozen niche variation, which postulates a better use of ecological niches by broad arrays of different clones (Vrijenhoek 1979, 1984); 5) General purpose genotypes, which would rely on distributional success of single, highly adaptive clones (Baker 1965), 6) and Red Queen hypothesis dynamics, which is based on differential response to biotic interactions (Hamilton 1980). More recently, Hörandl (2008; 2009) pointed out that in flowering plants, the reproduction system by itself, gametophytic apomixis, might play a rather important role in the establishment of the GP pattern.

Baker's law postulates that uniparental reproduction allows for founding a new population after dispersal with a single individual, as there is no need of a mating partner; therefore the species should have a more efficient colonization ability (Baker 1965; Baker 1967). Apomixis, the asexual mode of reproduction via seeds, potentially allows for uniparental reproduction, because the unreduced egg cell develops without fertilization into an embryo. Depending on the use of pollen for endosperm formation (pseudogamy) or not (autonomous apomixis) pollination is needed or not (Nogler 1984; Asker and Jerling 1992; Koltunow and Grossniklaus 2003). Autonomous apomixis would easier establish new populations as it is completely independent from pollination, whereas pseudogamous apomixis still needs pollen to fertilize the endosperm nuclei for seed production. To benefit from uniparental reproduction, pseudogamous apomicts therefore must be hermaphroditic and self-compatible (SC) to allow the pollen tube to penetrate the style and to fertilize the endosperm (Hörandl 2008; 2010). However, sexual selfers potentially do have the same advantage of uniparental reproduction (Baker 1955). Uniparental reproduction gives therefore only an advantage to apomixis if (1) the sexual species is self-incompatible (SI); (2) if the pseudogamous apomict is self-compatible (SC) and thus independent from cross-pollination, or has autonomous apomixis (Hörandl 2010). Baker's law would provide an explanation for the broader distribution of asexuals in previously glaciated areas, because they might have faster colonized the open, devastated areas that have been left after the retreat of glaciers. However, the negative consequence of such a colonization event would be founder effects and genetic bottlenecks, because only a restricted number of genes would be transmitted to the next generation. The genetic diversity of the founder population would therefore be reduced (e.g., Barrett and Pannell 1999; Hewitt 2004). Sexual selfers are in this respect more affected as apomicts, as selfing leads to a rapid loss of heterozygosity, potentially causing inbreeding depression, while apomixis can maintain heterozygosity in the maternal offspring.

Polyploidy, in the sense of genome multiplication (Comai 2005), has been assumed to be a major causal factor for geographical parthenogenesis; it originally was thought to be a part of the phenomenon (Vandel 1928). Polyploidy provides to the species the advantage of a higher genetic diversity and ecological flexibility (e.g., Soltis and Soltis 2009). Polyploidy is in general regarded as an activator of apomixis in flowering plants (Asker and Jerling 1992; Grimanelli et al. 2001). Polyploidy is in general frequently connected to hybridization, resulting in allopolyploidy (Otto and Whitton 2000). It has been thought that hybridization and allopolyploidy play the major role in the GP pattern for animals (Kearney 2005, 2006; Kearney and Blacket 2008).

However, in flowering plants sexual polyploidy is not correlated to large distribution areas (Stebbins and Dawe 1987; Hörandl 2006). Moreover, GP pattern seems to be established in autopolyploid species as well, where the advantage of the genetic diversity brought by hybridization and heterozygosity cannot be the trigger of the establishment of GP (Bayer 1991); Urbani et al. 2002; Thompson and Whitton 2006; Cosendai and Hörandl 2010).

Ecological hypotheses are in general based on a different population genetic structure in sexual and asexual organisms. The Frozen niche variation model and the General purpose genotype model rely both on clonally inherited genotypes that would originate via asexual reproduction. The wide distribution of asexual taxa could depend on two different strategies: the frozen niche variation model predicts that each clone is adapted to a particular ecological niche. A broad array of different clones would fill the resource space more efficiently than a sexual species (Vrijenhoek 1979, 1984), which could explain the GP pattern. On the other hand, a single clone could become widespread if this particular general purpose genotype would have a great phenotypic plasticity and could therefore adapt to various ecological niches (Lynch 1984; Vrijenhoek 1994; Vrijenhoek 2009). These alternatives hypothesis would explain the different success of asexual vs. sexual in colonization ability. The Red Queen hypothesis proposed the frequency dependence of selection by parasites and pathogens on clones over rare host genotypes (Glesener and Tilman 1978; Neiman 2009). Genotypic diversity would infer a general advantage to sexuality in the co-evolutionary arms-race. However, in colder climates with less biotic interactions, the advantage of high genetic diversity of sexuals disappears, and the quantitative advantage of clonal reproduction might easily establish broader distributions.

For testing these various hypotheses we study the alpine species *Ranunculus kuepferi* Greut. et Burd. sensu Küpfer (1974). The species has diploid, tetraploid, and rare 3x and 5x cytotypes; tetraploids have been assessed as facultative apomictic throughout the range of the cytotype, while diploids are regular sexual (Cosendai and Hörandl 2010). Diploid sexuals occur only in the southwestern Alps, while tetraploid apomicts colonize the whole Alps, the northern Apennines, and Corsica (Cosendai and Hörandl 2010; see also Fig. 1). The species exhibits thus a pattern of geographical parthenogenesis with apomicts colonizing previously glaciated areas, while sexuals are restricted in their distribution to ice-free marginal refugial areas. Triploids in the geographical contact zone represent probably backcrosses of diploids and tetraploids (Cosendai and Hörandl 2010). The tetraploid cytotype has partly aborted petals, pollen and achenes (Huber 1988) similar to other,

unrelated apomictic Ranunculi (Hörandl et al. 1997; Hörandl 2008), which is probably due to developmental disturbances related to apomixis. Seed set is in diploid sexuals populations significantly higher than in polyploid apomicts (Cosendai and Hörandl 2010). Previous phylogeographic studies revealed a colonization scenario out of the SW Alps (Burnier *et al.* 2009). However, the causality of the distributional success of asexual vs. sexual populations was not yet studied.

However, GP has probably a complex causality, and various factors may act in combination (Hörandl 2006). We inferred autopolyploid origin of 4x apomicts by using microsatellite and AFLP analysis, (Cosendai et al. subm.), which rules out hypotheses for GP based on hybrid origin. The success of *R. kuepferi* has been suggested to follow Baker's law (Cosendai and Hörandl 2010). However, the great majority of apomicts is pseudogamous, while autonomous apomixis occurs only at very low frequencies (c. 6% of the seed material; Cosendai and Hörandl 2010). Self-compatibility of sexuals was not yet studied. Therefore, the study of SI systems is essential for understanding the actual capacity of uniparental reproduction in sexuals and apomicts. We further want to elucidate the genetic consequences of uniparental reproduction: multiple founder events by single or few individuals would result in distinct local gene pools, and in a loss of genetic diversity because of genetic bottlenecks. A rigorous testing of ecological hypothesis based on different genetic diversity of sexual and apomictic populations requires comparative population genetic studies throughout the range of the species. With all this information, we want to understand in this study different aspects: 1) Does population genetic structure support a hypothesis of multiple founder events, as expected after Baker's law? 2) Do breeding systems support a hypothesis of uniparental reproduction in apomicts? 3) Do apomicts show clonal diversity or widespread clones? 4) Is genetic diversity and heterozygosity different between sexual and apomictic populations? 5) Is colonization hampered by genetic bottlenecks?

## MATERIAL AND METHODS

### PLANT MATERIAL

The plants used in this research were collected in the wild from 59 populations during spring and summer between 2004 and 2007; for details see Table 1 and Figure 1. By collecting between 10-25 individuals per population, comprising a total of 1009 individuals, the whole distribution area was covered. Leaf material was dried in silica gel for molecular analysis.

Determination of ploidy level of all individuals and the mode of reproduction of cytotypes has been presented in Cosendai and Hörandl (2010). A part of the living plant collection was transferred to the University of Innsbruck for analysis of self-compatibility and experimentation of further pollen growth.

### MOLECULAR METHODS

DNA was extracted following a slightly modified protocol based on the original CTAB-protocol (Doyle and Doyle 1990) using c. 0.5 g of silica gel dried leave tissue. After CTAB extraction, the DNA concentration of the extracts was measured photometrically with a Techne Specgene spectrophotometer (Techne, Cambridge, UK).

AFLP fingerprint profiles were produced for 4–25 individuals per population, for a total of 1009 individuals over 59 populations with 163 diploids, 45 triploids, 749 tetraploids, 13 pentaploids, 3 hexaploids and four individuals with unknown ploidy level.. A set of three primers combinations was selected (fluorescent dyes in brackets): *Eco*RI-ACT / *Mse*I-CTCG (FAM), *Eco*RI-ACG / *Mse*I-CTCG (VIC), *Eco*RI-AGC / *Mse*I-CTGA (NED). PCR reactions, program and sequencing procedure followed the same protocol as in Cosendai et al. subm. Sequencer results were sized and scored with GeneMarker 1.85 (SoftGenetics, State College, PA, USA) with the same setting as in Cosendai et al. subm.

Five Microsatellite primers were developed by Savannah River Ecology Laboratory, University of Georgia, based on the protocol developed by Glenn & Schable (2005). PCR amplifications were performed following protocols described in Cosendai et al. subm. Raw data were sized and scored with GeneMarker 1.85 (SoftGenetics, State College, PA, USA) with parameter set at the size of selected microsatellite with a threshold of 100 RFU. We analyzed a subset of 379 samples from 14 populations comprising 119 diploid, 15 triploid, 237 tetraploid and 4 pentaploid individuals. Primers and characterization of SSRs are provided in Table 2.

### BREEDING SYSTEMS

A part of the living samples was transferred to the Botanical Garden of the University of Innsbruck for analysis of breeding systems in 2x sexual and 4x apomictic cytotypes (the other cytotypes were not included because of the low number of individuals available and the low relevance of other ploidy levels for GP; see Cosendai and Hörandl, 2010). A total of 87 plants from 5 diploid and 13 tetraploid populations were bagged from bud to fruit stage. Whole plants were covered with a cellophane bag, which prevents both

animal and wind pollination (see also Hörandl 2008). During anthesis, three treatments were applied: 1) manual outcrossing: flowers that had been emasculated in the bud stage were pollinated with fresh allopollen from at least three different individuals when the stigma had become papillate; we did not try open pollination as pollinator spectrum in the experimental garden may be different to the natural condition in the alpine zone; 2) spontaneous selfing: no treatment, bagged only; and 3) manual selfing: self pollen was applied to the papillate stigmas). For all treatments, the percentage of well-developed achenes was calculated from the total of achenes as a measure for reproductive success for each collective fruit, following methods of Hörandl (2008). To test for stylar SI, flowers were hand-crossed (n = 7) or hand-selfed (n = 9) and fixed 3-5 d after pollination in FPA50 (50% ethanol, formalin, propionic acid; 90:5:5). In total, 105 carpels from tetraploid individuals and 37 carpels from sexual individuals were analyzed for pollen tube growth using the fluorescence standard method with aniline-blue (Linskens and Esser 1957) following Hedhly et al. (2003). Pistils were excised from the flowers, washed twice in dist. water (1h per wash), soaked in 8N NaOH-solution at 60°C for 15 min, rinsed twice more in dist. water and stained for at least 2 h with 0.1% aniline-blue in Sörensen phosphate buffer, pH 8. Pistils were gently squashed and examined under a fluorescence microscope (Olympus BH2, Tokyo, Japan; excitation filter 405-435 nm).Five categories of pollen tube growth were identified: 1) missing; 2) growth stop on the stigma surface; 3), growth stop within the stigma; 4) growth stop in the style and 5) pollen tubes reach the base of the style and enter the ovary.

### ANALYSIS OF MOLECULAR DATA

We performed a Bayesian analysis of population structure (BAPS) of AFLP data of all populations (Corander and Marttinen 2006; Corander et al. 2008) with 1000 iterations per sample. The analysis based on the Bayesian algorithm assigns samples to the corresponding gene pool, whereby the program sets the optimal number of groups (gene pools) for the dataset. To get insights into the origin of gene pool of the tetraploid populations, we further prepared a two step-analysis in BAPS using the AFLPs data set (Cosendai et al. subm.). First, we clustered the diploids alone, which revealed four geographical groups as the optimal partition. We used these four groups and set the tetraploids as an unknown fifth group gene pools to simulate a second, hypothetical parental gene pool, and performed the admixture with 1000 iterations per sample (supplement 1).

The AFLP dataset was further analyzed with FAMD 1.23 beta http://www.famd.me.uk/famd.html (Schlüter and Harris 2006), using the

Jaccard index for calculation of a similarity matrix and computing a Neighbor Joining tree (Fig2). We performed a bootstrapping of the NJ tree with the options BS + RMD (BS randomly resample the loci; RMD replace missing data, 1000 repeats), and then compute the majority rule consensus trees with SumTrees (Sukumaran and Holder 2009).

To test for the presence of clones, the total number of multilocus genotypes (G) was determined for each ploidy level, and proportion of distinguishable genotypes (PG) was calculated as the number of genotypes divided by sample size (Ellstrand and Roose, 1987). To calculate diversity within and among populations on the AFLPs data set, we calculated $F_{ST}$ values via AMOVA using Arlequin 3.5 (Excoffier and Lischer 2010). To test for correlations of genetic distances ($F_{ST}$ values) and geographic distances, we performed a Mantel test separately for the diploids and tetraploids. Geographical distances were calculated from coordinates with Hawthtools (Beyer 2004) in ESRI ArcGIS 9.3.1 (ESRI 2009). The Mantel test was performed with Addinsoft version 2010.4 for XLSTAT-Pro (Addinsoft 2010).

Microsatellite data were coded first as an allele matrix for five loci. In these loci, the maximum number of alleles fitted to the expections after the ploidy level of the respective individual. We tested the five remaining loci for linkage disequilibrium (LD; non-random segregation of loci) in the diploid samples with a Markov chain of 10000 steps in Arlequin 3.5.1.2 (Excoffier & Lischer 2010). Only Rk_11 and Rk_26 had a significant LD ($p < 0.005$) to each other and to the other loci. We therefore calculated observed heterozygosity Ho (number of heterozygotes/number of individuals per locus per population), and genetic diversity measures without these two loci. Genetic differentiation among populations was estimated by calculating $F_{ST}$ values following Wang (2002) based on an infinite allele model (IAM). We further calculated $G_{ST}$s as an equivalent to weighted averages of $F_{ST}$s for all alleles, based on equal weight of each populations (Pons and Petit 1996), and $R_{ST}$s based on allele size (Slatkin 1995; Rousset 1996), using a step-wise mutation model (SMM), both implemented in SPAGeDI 1.3 (Hardy and Vekemans 2002). All genetic measures were tested for significant differences between diploid and tetraploid populations by using 2-tailed t-tests.

Results of bagging experiments (percentages of well-devloped achenes per collective fruit) are shown as boxplots (Fig. 3). Data were arcsin transformed prior to testing for statistical differences among groups via one-way ANOVA. Since Levene's statistic revealed unequal variances among groups, Tanhame's test for pairwise multiple comparisons (not assuming equal variances) was used to test differences a) within each cytotype; b) between treatments within cytotypes and c) between cytotypes. The statistic evaluation of pollen

tube growth was accomplished by cross tables and Chi square test (Pearson). In all tests, the critical level of significance was $\alpha = 0.05$. All analyses were performed with SPSS for Windows vs. 12 (SPSS Inc., Chicago, IL, USA).

## RESULTS

### GEOGRAPHICAL POPULATION GENETIC STRUCTURE

We obtained 297 AFLP fragments for 1009 individuals. Our AFLP data analysed with BAPS including all populations revealed 28 groups as optimal partition (log marginal likelihood of optimal partition = - 91593.443) which are reflected by different colours in Fig. 1. The analysis revealed a different genetic structure of diploid and polyploid populations (Fig. 1). Most apomictic populations have each unique gene pools (nos. 1, 34, 38, 44, 47, 49, 51, 56 and 59), which is most pronounced in the Central and Eastern Alps (Fig. 1), with no or only admixture of single individuals from other populations. In the SW Alps, the tetraploid populations tend to share gene pools (e.g., nos. 5, 13, 22, 24, 25, 26, 27, 31, 32, 33, 34, 35, 41 and 47). In contrast, sexual populations share regional gene pools, like in Tende region (pop. 6, 7, 8, 9 and 10), Col de la Lombarde (15, 16), in the La Chens region (3 and 4) together with population of Valette (17) and in Vercors (28, 29). These four geographical groups were confirmed in the separate BAPS analysis of the diploid populations (log -11228.662), while the tetraploids retrieved their gene pools only from combinations of the diploid gene pools (supplement 1). The Neighbor joining tree (Fig. 2) confirmed these diploid population groups as separate clusters, but showed a lack of support for the basal branches and no geographical structure among the apomicts. Mostly only terminal clusters had bootstrap support. The Mantel test revealed a positive correlation between among-population diversity and geographic distance, which is lower in tetraploids ($y = 0.0003x + 0.2586$; $p < 0.0001$) than in diploids ($y = 0.002x + 0.1968$; $p < 0.0001$); supplement 2.

### BREEDING SYSTEM

Manual outcrossing revealed in diploid sexuals seed set in all flowers, with a broad range of reproductive success (13-92% of well-developed achenes per collective fruit). After spontaneous selfing, no seed set was observed except for one flower (13%), after manual selfing low amounts of seeds (0-3% with two outliers of 10% well-developed achenes); Fig. 3. In contrast, tetraploid apomicts had the highest seed set after manual selfing (0-52%), while spontaneous selfing and manual outcrossing revealed lower reproductive

success (0-29% and 0-25%, respectively), Fig. 3. Within cytotypes, only in diploid sexuals the differences in seed set between the three treatments (outcrossed, spontaneously and manually selfed) were highly significant, while in tetraploid apomicts, seed set did not differ between treatments (Table 6 a, b). The comparison between cytotypes revealed a significantly higher seed set between diploids and tetraploids after outcrossing, but no difference after spontaneous selfing. After manual selfing, tetraploid apomicts had a significantly higher seed set than diploid sexuals (Table 6c).

Seed set essentially reflected pollen tube growth (Fig. 4). In diploids, manual outcrossing led to full pollen tube growth in most carpels, whereas tube growth stopped within the stigma after selfing in most cases. Only in 17% of carpels tubes of self pollen reached the basis of the style. Differently in tetraploid apomicts: manual selfing led to full tube growth in about 35 % of the carpels, manual outcrossing only in 17 % of the carpels. In general, there was a high proportion of carpels without pollen germination or substantial tube growth at all in apomicts. Within cytotypes, pollen tube performance significantly differed between selfing and outcrossing (diploids: $p < 0.001$; tetraploids: $p = 0.004$, Chi square, Pearson). Differences in pollen tube growth between cytotypes and treatments were highly significant ($p < 0.001$) except for diploid selfing and tetraploid crossing (not significant).

## GENETIC DIVERSITY

Diploids, triploids and pentaploids had all individual multilocus AFLP genotypes (PG = 1.000), while in the tetraploids some individuals shared identical genotypes (Timmelsjoch, no. 52, one clone with three individuals, and in Turracherhöhe 59, two pairs with each two individuals) (PG = 0.995); Fig. 2. $F_{ST}$ values calculated for populations from AFLP data between all samples, between cytotypes, and diploids versus tetraploids and range from 0.332 and 0.385 for all groups (Table 3 A). $F_{ST}$ values of diploid sexuals differed from tetraploid apomicts only by 0.053, but this difference was significant ($p < 0.001$). In summary, gene pools had genetic diversity distributed more within populations (ca. 65%) than among populations (about 35%) in the dominant AFLP data. In SSRs (Table 3B), $F_{ST}$ values ranged between 0.169 and 0.130, with a difference of only 0.08 between 2x sexuals and 4x apomicts (not significant; $p < 0.846$). $G_{ST}$s had the highest values in triploid backcrossed populations (0.316), in tetraploids 0.182 and the lowest value in diploids (0.114). Differences between diploids and tetraploids were not significant (0.555). Genetic differentiation based on allele size ($R_{ST}$) was highest in diploids (0.125), intermediate in tetraploids (0.073) and lowest in triploids (-0.017). Differences between diploids and tetraploids were not significant ($p = 0.665$).

Levels of observed heterozygosity for three SSR loci differed between diploids and tetraploids and other ploidy levels with means of 53.37 % and 56.13%, respectively (Table 4) and were not significantly different (p = 0.665). A summary of allelic diversity is presented for all five SSR loci in Table 5. Diploids ranged from 2.60 to 4.00 and had a mean of 3.75 alleles per population, whereas tetraploids had a range of 2.40–5.60 and a mean of 4.1 alleles per populations; however, the number of alleles was not significantly different (P = 0.48), and values showed an overlap of ranges. Loci differed in their allelic diversity, as some loci presented over seven alleles (Rk 37), others only two (Rk 11 or Rk 26).

## DISCUSSION

### GEOGRAPHICAL POPULATION STRUCTURE OF APOMICTS AND SEXUALS

Our BAPS analysis of AFLP data (Fig. 1) shows that diploid sexuals tend to group together and share their gene pools within regions. However, no gene flow from other regions is observed except for the diploids population group in the Tende region (no. 6-10). In contrast, tetraploid asexual populations have mostly locally restricted gene pools, especially in the more isolated populations in the eastern Alps and in Corsica; share of gene pools between populations is either missing or remains restricted to single individuals (Fig. 1). The pattern in Fig 1. is probably due to several founder events by apomictic populations by single individuals. Share of gene pools with other populations could be due to multiple colonization or recombination within the population. Burnier (2009) already hypothesized the possibility of several founder events in the apomicts and described at least two centers of diversity. Our results suggest several founder events by apomicts in previously glaciated areas in the Alps, and in geographically isolated locations (Eastern Alps and Corsica). The second BAPS analysis suggests that gene pools of all apomictic populations are composed of a random re-distribution of the diploid source populations (supplementary data), which confirms previous results of autopolyploid origin of apomicts on a larger sample (Cosendai et al. subm.). This re-shuffling of the diploid source gene pool via facultative recombination may also explain the occurrence of more than one gene pool in apomictic populations. Genetic diversity is stronger correlated to the geographical distance in diploids than in the tetraploids, suggesting that isolation-by-distance is more pronounced in the sexual populations (supplement 2). The slight discrepancy of our results to Burnier et al. (2009) may be explained by different sampling strategies; the earlier

study did not cover the easternmost accessions and was not based on population samples.

A lack of geographical structure in the apomicts is also obvious in our Neighbor Joining analysis (Fig. 2), where only individuals of diploid geographical population groups cluster together. The lack of bootstrap support at basal branches confirms the low divergence among cytotypes, which is probably due to autopolyploid origin and a young evolutionary origin (Cosendai et al. subm.).

### BREEDING SYSTEM

Bagging and crossing experiences, and analysis of pollen germination and growth, confirm that diploids are largely self-incompatible. The data on pollen tube growth suggest that the S-I system is due to stigmatic or stylar S-I, and not to herkogamy. Diploids are therefore predominantly outcrossers, and do require mating partners for successful seed set. In tetraploids, the seed set is in general reduced, and the number of good achenes is lower than in diploids in every treatment; this result is in accordance with reproductive success observed in wild populations (Cosendai and Hörandl (2010) and is typical for pseudogamous apomicts (Hörandl, 2008, 2010). Disturbances of meiosis in neopolyploids, and developmental disturbances during embryogenesis may account for the low seed set. Moreover, since *R. kuepferi* is mostly pseudogamous, disturbances of pollen tube growth may limit endosperm fertilization and seed set. In the apomicts, the lack of significant differences in seed set after outcrossing, manual and spontaneous self-pollination suggests a breakdown of stylar self-incompatibility. The relative high percentage of pollen tubes growing to the basis of the style in manually selfed tetraploids (Fig. 4) confirms that pollen tube growth is not inhibited within the stigma and the style. The background of S-C in apomicts is not known and could have a genetic basis, probably following polyploidization (e.g. Richards 1997). Another possibility is the occurrence of mentor effects (i.e. damaged or foreign pollen could help to break the self-incompatibility system (Hörandl and Temsch 2009; Hörandl 2010). Since *R. kuepferi* has partly aborted pollen (Huber, 1988), SC could be also due to this form of pseudo-self-compatibility.

Altogether our results confirm the ability of uniparental reproduction in pseudogamous apomictic *R. kuepferi*, while sexuals are dependent on cross-pollination. The independence of apomicts from mating partners and pollinators might infer an important advantage in alpine climates. Unfavourable weather conditions in high altitudes can reduce pollinator activities (e.g., McCall & Primack, 1992), which may limit pollinations and

seed set for outcrossing plants (e.g., Munoz & Arroyo, 2006). Therefore, reproductive assurance, as it is provided by the combination of apomixis and self-compatibility, is advantageous in alpine habitats. Low frequencies of pollen-independent autonomous apomixis may contribute to reproductive success of isolated individuals (Cosendai & Hörandl 2010). The ability to found populations even with a single individual will provide an advantage to rapidly establish populations. Our results largely confirm Baker's law (Baker 1965; Baker 1967).

## THE RELEVANCE OF GENETIC DIVERSITY

Neither AFLP nor SSR data revealed a pronounced clonal population structure in the tetraploid apomicts, but unique genotypes for almost all individuals (Fig. 2). This result confirms earlier studies on the species (Burnier et al. 2009), but is striking as apomictic reproduction is expected to result in clones, as it has been observed in comparable AFLP studies (Paun et al. 2006; Hörandl & Paun 2007). Since the mutational dynamics is in *R. kuepferi* in both marker systems extremely low (only two private allele in SSR data, only 9 private alleles in AFLPs in the tetraploid populations; Cosendai et al. subm.), a highly facultative apomictic system may best explain the unexpected high genotypic diversity of apomictic plants. The presence of meiosis in parallel to aposporous embryo sac formation has been demonstrated by Burnier et al., (2009) and by Cosendai and Hörandl (2010), and opens the possibility that occasional sexual events maintain recombination and genotypic diversity.

Genetic diversity indices ($F_{ST}$ values) calculated with dominant AFLP data are very similar among populations and reveal that the most of the diversity is distributed within the populations. The comparison of $F_{ST}$ values of diploid sexuals and tetraploid apomicts presents similar results. This surprising result is on the one hand due to reduced genetic diversity of the diploid sexuals, on the other hand to a highly facultative apomixis in the tetraploids. The $F_{ST}$ values of diploid sexuals are above the average reported for outcrossers in dominant markers systems (0.27) and are nearer to values of mixed systems (0.40; Nybom 2004). Since our observations on breeding systems do confirm predominant outcrossing for the diploids, their low genetic diversity might be not explained by frequent selfing or inbreeding, but rather by a reduction of diversity of the complete gene pool of the diploids due to geographic isolation in the past. The restriction of their distribution to refugial area during glacial advances, and the fragmentation and geographical isolation of the four main sexual gene pools (Fig. 1 and supplementary) might have caused genetic bottlenecks and loss of genetic diversity within the sexual populations. Regional gene pools and lack of gene

flow between regions in the AFLP dataset, and a strong isolation-by-distance strongly support this hypothesis. The low $F_{ST}$, $R_{ST}$ and $G_{ST}$ values in microsatellite data support a low genetic differentiation among populations (Wright 1978; Atangana *et al.* 2009), confirming the tendency observed in the dominant marker. However, the SSR data might be also influenced by undersampling of loci. On the other hand, the apomictic populations maintain allelic diversity by polyploidy and individual diversity by facultative sexuality, which may explain that genetic diversity measures equal to sexuals.

Levels of observed heterozygosity in SSRs are in sexuals between mean values of mixed breeding systems (0.51) and outcrossers (0.63), but are clearly higher of values reported for selfers (0.05; (Nybom 2004). This result confirms that reduced genetic diversity and among-population differentiation in sexuals, as observed in $F_{ST}$ values, is likely not due to selfing, but rather has a background in historical geographical fragmentation and isolation (see above). In apomicts, high levels of heterozygosity are to be expected because the bypass of meiosis and mixis maintains maternal genotypes. However, the *R. kuepferi* populations do not reach the maximum value of Ho = 1 as observed in other apomictic populations (e.g., Paun & al., 2006), which confirms the presence of frequent facultative recombination. Allelic diversity reaches a higher maximum value in apomictic populations of *R. kuepferi* which is probably due to a higher allelic diversity and multiple allelism within polyploid cytotypes (Cosendai et al. subm.); however, allelic diversity does not differ significantly from that of the sexuals. The high mixture of the gene pool of the diploids in their tetraploid derivatives associated with a highly dynamic and intense exchange of alleles within populations is still present in the apomictic populations.

We can assume that the geographical parthenogenesis pattern in *R. kuepferi* is largely caused by apomixis by itself (Hörandl 2006; Hörandl et al. 2008; Hörandl 2009). Genotypic diversity does not differ between sexual and apomictic populations in this species. This result does not support the frozen niche variation model, which relies on a broad array of clones that would be able to occupy various ecological niches (Vrijenhoek 2009); however, the equal genetic diversity of apomicts compared to sexuals, combined with rapid colonization my have allowed for a faster occupation of various types of alpine habitats that successively became available after the retreat of glaciers. Our results did not reveal a single widespread clone, and thus contradict the general purpose genotype model, which assumes that a more flexible genotype is able to spread under various ecological conditions. The lack of clonal population structure and equal genotypic diversity between sexuals and apomicts makes also the Red Queen hypothesis inapplicable.

From the different hypothesis reviewed and discussed by Hörandl (2006, 2009; Hörandl et al. 2008) only uniparental reproduction (Baker's law) remains supported as a differential feature. However, under this aspect, the maintenance of genetic diversity in apomicts is probably of highly indirect relevance. Up to now the idea prevailed that only allopolyploids maintain genetic diversity in a GP pattern (*Daphnia*; Haag & Ebert 2004) to deal with the loss of genetic diversity after founder events. As we demonstrated in our previous paper (Cosendai and Hörandl 2010), and Cosendai et al subm., the tetraploids are of autopolyploid origin and exhibit a combination of gene pools from their sexual progenitors. Polyploidy thus enhances maintenance of allelic diversity within individuals and populations even after founder events with single individuals. In contrast to sexual selfing, apomixis avoids a loss of heterozygosity and potential inbreeding depression in small populations, while facultative sexuality maintains genotypic diversity that is probably required to meet environmental variability. In fact, our model suggests a combinatorial effect of polyploidy and facultative apomixis for maintenance of genetic diversity even after the genetic bottleneck situations of multiple founder events. The apparent low seed set of the polyploids can be compensated by the opportunity that potentially one single viable seed can found a population. The large devastated areas in the Alps after the retreat of glaciers offered opportunities for rapid colonizers (Hörandl 2009). In contrast, the diploid sexual populations cannot benefit from colonizing advantages of sexual selfing, and maintain only a restricted genetic diversity in disjunct relic areas after historical fragmentation of their distributional range.

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## TABLES

Table 1. Provenance of materials used in this study. Population numbers correspond to those in Figure 1. Country abbreviations: F, France; I, Italy; A, Austria; CH, Switzerland. Collectors: ACC, Anne-Caroline Cosendai; ACC-AC, Anne-Caroline Cosendai & André Cosendai; CS, Christoph Seger; EH, Elvira Hörandl; MH, Marc Hämmerli; PK, Philippe Küpfer; PS-GS, Peter Schönswetter & Gerald Schneeweiss. In locality, a roman number indicates repeated sampling on the same population in different years (but on different individuals), arabic numbers are herbaria numbers of E. Hörandl (vouchers have been deposited in the herbarium of the University of Vienna, WU).

| Population number | Country | Province | Locality | Ploidy level | Altitude | Coordinates N | Coordinates S | Sampler |
|---|---|---|---|---|---|---|---|---|
| 1 | F | Corse-du-Sud | Corsica | 4x | 1541 m | 042°01'46.4'' | 09°12'34.5'' | ACC |
| 2 | I | Emila-Romagna | Mt Cusna | 4x | 1594 m | 044°18'06.7" | 010°22'26.6" | ACC-AC |
| 3 | F | Var | Mt La Chens I | 2x | 1607 m | 043°44'58.5" | 06°39°29.1" | ACC-AC |
| 4 | F | Var | Mt La Chens II | 2x | 1610 m | 043°44'59.3" | 06°39'25.5" | PK |
| 5 | F | Alpes-Maritimes | Col de Tende | 4x | 1888 m | 044°09'03.0" | 07°33'56.3" | ACC |
| 6 | I | Piemonte | Valle di Pesio I 9589 | 2x | 1700 m | 044°14'40" | 07°37'42" | EH |
| 7 | I | Piemonte | Passo del Duca II 9592 | 2x | 1700 m | 044°11'43.68" | 07°39'33.85" | EH |
| 8 | I | Piemonte | Passo del Duca 9525 / 9534 | NA | 1700 m | 044°11'43.68" | 07°39'33.85" | EH |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 9 | I | Piemonte | Valle di Pesio III 9593 | 2x | 1925 m | 044°11'43.68" | 07°39'33.85" | EH |
| 10 | I | Piemonte | Vallone Cravina 9595 | 2x | 1960 m | 044°13'24.72" | 07°37'11.16" | EH |
| 11 | I | Piemonte | Colle della Perla_I_9596 | 3x, 4x, 5x, 6x | 2080 m | 044°9'11.05" | 07°37'21.14" | EH |
| 12 | I | Piemonte | Colle della Perla II 9597 | 3x, 4x, 5x | 2200 m | 044°9'11.05" | 07°37'21.14" | EH |
| 13 | F | Alpes-Maritimes | Notre Dame de la Fenestre | 4x | 1885 m | 044°05'45.3" | 07°21'34.2" | ACC |
| 14 | F | Alpes-Maritimes | Isola 200 | 4x | 2210 m | 044°11'43.7" | 07°09'19.7" | ACC |
| 15 | I | Piemonte | Colle della Lombarde I 9601 | 2x | 2260 m | 044°12'25.68" | 07°8'51.98" | EH |
| 16 | I | Piemonte | Colle della Lombarde II 9602 | 2x | 2477 m | 044°13'17.60" | 07°9'9.25" | EH |
| 17 | F | Alpes-de-Haute-Provence | Vallette | 2x, 3x | 1820 m | 044°07.5' | 06°33.5' | PK |
| 18 | F | Alpes-Maritimes | Col des Champs I | 3x, 4x | 2080 m | 044°10'33.8" | 06°41'53.0" | ACC-AC |
| 19 | F | Alpes-Maritimes | Col des Champs II | 3x, 4x | 1925 m | 044°09'59.5" | 06°42'28.0" | ACC |
| 20 | F | Alpes-de-Haute-Provence | Col de la Cayolle I | 4x | 2325 m | 044°15'34.4" | 06°44'41.3" | ACC-AC |
| 21 | F | Alpes-de-Haute-Provence | Col de la Cayolle II | 4x, 6x | 2193 m | 044°15'13.6" | 06°44'52.1" | ACC |
| 22 | F | Alpes-de-Haute-Provence | Col d'Allos I | 3x, 4x, 6x | 2080 m | 044°18'03.4" | 06°35'06.0" | ACC-AC |
| 23 | F | Alpes-de-Haute- | Col d'Allos II | 3x, 4x, 6x | 2247 m | 044°22'0" | 06°37'0" | PK |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Provence | | | | | | |
| 24 | F | Hautes-Alpes | Col de Vars | 4x | 2134 m | 044°32'21.3" | 06°42'05.6" | ACC |
| 25 | F | Hautes-Alpes | Col de Raboux | 4x | 1859 m | 044°38'38.4" | 05°58'43.1" | ACC |
| 26 | F | Hautes-Alpes | Queyras LaCroix | 4x | 2000 m | 044°46'0'' | 07°01'0'' | SH |
| 27 | F | Hautes-Alpes | Queyras Montette | 4x | 2000 m | 044°50'0'' | 06°55'0'' | SH |
| 28 | F | Drôme | Vercors II | 2x | 1325 m | 044°50'26.1" | 05°25'23.1" | ACC-AC |
| 29 | F | Drôme | Vercors I | 2x | 1470 m | 044°54'07" | 05°28'039" | PK |
| 30 | F | Hautes-Alpes | Col du Lautaret | 4x | 2060 m | 045°02'40.6" | 06°24'04.1" | ACC |
| 31 | F | Savoie | Mt Cenis | 4x | 2025 m | 045°13'55.4" | 06°53'53.6" | ACC-AC |
| 32 | F | Savoie | Col d'Iseran | 4x | 2768 m | 045°25'09.2" | 07°01'52.9" | ACC-AC |
| 33 | I | Valle d'Aoste | Gran Paradiso | 3x, 4x | 2079 m | 045°37'0.5" | 07°33'12.2" | ACC-AC |
| 34 | F | Savoie | Pt St Bernard | 4x | 2215 m | 045°40'40.4" | 06°52'55.2" | ACC-AC |
| 35 | CH | Valais | Gd St Bernard | 4x | 2380 m | 045°52'09.9" | 07°09'33.3" | ACC-AC |
| 38 | I | Valle d'Aoste | Cervinia | 4x | 2200 m | 045°55'54.6" | 07°38'18.1" | ACC-AC |
| 39 | CH | Valais | Jeizinen | 4x | 2020 m | 046°20'07" | 07°43'85" | ACC |
| 40 | CH | Valais | Lötschental | 4x | 1773 m | 046°26'05.5" | 07°51'48.0" | ACC |
| 41 | CH | Valais | Col du Simplon | 4x | 2019 m | 046°15'03.2" | 08°01'48.2" | ACC |
| 42 | CH | Valais | Furka Pass | 4x | 2162 m | 046°34'45.8" | 08°25'30.9" | ACC |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 43 | CH | Ticino | Lukmanier Pass | 4x | 1946 m | 046°33'49.2" | 08°47'54.7" | ACC |
| 44 | CH | Graubünden | Rheinwald 9603 | 4x | 2100 m | 046°33'16.05" | 09°14'52.32" | EH |
| 45 | CH | Graubünden | Julier Pass | 4x | 2277 m | 046°28'20.9" | 09°44'01.4" | ACC |
| 46 | CH | Graubünden | Albula Pass | 4x | 2312 m | 046.58333° | 09.83333° | ACC |
| 47 | CH | Graubünden | Bernina Pass | 4x, 6x | 2301 m | 046°24'47.3" | 010°01'17.3" | ACC |
| 48 | A | Vorarlberg | Mt Kapall St Anton | 4x | 2269 m | 047°08'49.1" | 010°14'55.3" | ACC |
| 49 | CH | Graubünden | Umbrail Pass | 4x | 2463 m | 046°32'51.3" | 010°26'06.3" | ACC |
| 50 | A | Tirol | Kaunertal | 4x | 2525 m | 046°52'21.8" | 010°42'37.5" | ACC |
| 51 | I | Trento | Tonale Pass | 4x | 2400 m | 046°16'21.27" | 010°34'40.88" | EH |
| 52 | A | Tirol | Timmelsjoch Pass | 4x | 2105 m | 046°55'13.5" | 011°03'10.6" | ACC |
| 53 | A | Tirol | Tuxer Alps | 4x | 2315 m | 047°07' | 011°34' | CS |
| 54 | I | Trento | Rosengarten | 4x | 2500 m | 046°27'19.56" | 011°37'56.51" | EH |
| 55 | I | Trento | Padon Pass | 4x | 2350 m | 046°27'47.80" | 011°53'42.88" | EH |
| 56 | I | Veneto | Mt Dürrenstein | 4x | 2400 m | 046°39'39.24" | 012°10'57.86" | EH |
| 58 | A | Carinthia | Mt Sadnig | 4x | 2200 m | 046°57'42" | 013°0'35" | PS-GS |
| 59 | A | Carinthia | Turracherhöhe | 4x | 2220 m | 046°55'20.47" | 013°52'44.35" | EH |

Table 2 Summary of the primers used in this study, with the conservative sequence of flanking region, the base pair repeat, the recognition size, the total number of alleles found with the primer and the GenBank accession number.

| Locus name | Forward primer sequence (5'– 3') | Reverse primer sequence (5'– 3') | Repeat | Size in bp | Total of alleles | GeneBank accession no |
|---|---|---|---|---|---|---|
| RK_11 | GTTTAAACCTTGCTGGTCCCGAG | CAGTCGGGCGTCATCACTTCAACGGAGAGGGGTGG | (CTT)^22 | 149-204 | 3 | XXX |
| RK_26 | CAGTCGGGCGTCATCAGTTGGATTTCGCATGTTAGG | GTTTGGATTGAGGGAACAACGTCC | (GTTT)^4 | 205-234 | 2 | XXX |
| RK_27 | CAGTCGGGCGTCATCACGAGTCACACAGATCCTGAAG | GTTTGGTCTTCGTTTGCCCATTC | (CT)^12 | 133-217 | 18 | XXX |
| RK_35 | CAGTCGGGCGTCATCAATAAAAGGGTTCACTTCTTTCCAC | GTTTGAAATCATGGAGAGCGGTTTG | (CT)^6...(CT)^21 | 129-152 | 11 | XXX |
| RK_37 | CAGTCGGGCGTCATCCCCATGACCCGAAC | GTTTGACACCGTATTCCGAGGC | (AG)^18 | 126-203 | 13 | XXX |
| RK_38 | CAGTCGGGCGTCATCACACACACACCGTACACAG | GTTTCGAATCCCAACTAGCGGAC | (AC)^10...(AC)^13 | 139-156 | 7 | XXX |

Table 3 AMOVA of AFLPs and SSRs, A; AFLPs values of $F_{ST}$, B; SSRs value of $F_{ST}$, $G_{ST}$ and $R_{ST}$ in bold, d.f., degree of freedom; CI, confidence index; All, all samples were used.

**A**

| AMOVA AFLPs | Source of variation | d.f. | Sum of squares | Variance components | % of variation | 95 % CI | $F_{ST}$ AFLPs Global |
|---|---|---|---|---|---|---|---|
| **All** | Among populations | 71 | 14545.564 | 13.094 | 36.820 | 0.34540 - 0.38246 | **0.368**** |
| | Within populations | 937 | 21048.938 | 22.464 | 63.180 | | |
| | Total | 748 | 24655.899 | 33.259 | | | |
| **2x only** | Among populations | 10 | 2307.004 | 14.497 | 38.500 | 0.35246 - 0.41687 | **0.385**** |
| | Within populations | 152 | 3519.916 | 23.157 | 61.500 | | |
| | Total | 162 | 5826.920 | 37.654 | | | |
| **3x only** | Among populations | 5 | 572.197 | 12.707 | 36.930 | 0.33554 - 0.40202 | **0.369**** |
| | Within populations | 39 | 846.425 | 21.703 | 63.070 | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Total | 44 | 1418.622 | 34.411 | | | |
| **4x only** | Among populations | 42 | 8964.774 | 11.033 | 33.170 | 0.31275 - 0.3500 | **0.332**** |
| | Within populations | 706 | 15691.125 | 22.225 | 66.830 | | |
| | Total | 748 | 24655.899 | 33.259 | | | |
| **2x vs 4x** | Among populations | 53 | 12615.932 | 12.825 | 36.420 | 0.34509 - 0.38274 | **0.364**** |
| | Within populations | 858 | 19211.041 | 22.390 | 63.580 | | |
| | Total | 911 | 31826.973 | 35.216 | | | |

** highly significant P-values were < 0.001

**B**

| **AMOVA SSRs** | **Fit** | **Fis** | **$F_{ST}$** | **Rit** | **Ris** | **$R_{ST}$** | **$G_{ST}$** |
|---|---|---|---|---|---|---|---|
| All among population | 0.158** | 0.0218** | **0.1393**** | 0.2282** | 0.1494** | **0.0926**** | **0.178** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2x among populations | 0.1435** | 0.0146 | **0.1308**** | 0.2461** | 0.1379** | **0.1254**** | **0.114** |
| 3x among populations | 0.1311* | -0.0462* | **0.1695** | 0.2968* | 0.3088* | **-0.017** | **0.316** |
| 4x among populations | 0.1748** | 0.0414** | **0.1392**** | 0.2091** | 0.1464** | **0.0734**** | **0.182** |

* significant p<0.5

** highly significant P<0.05

Table 4 Heterozygosity within three loci of SSRs data, sorted by populations within ploidy levels, N, number of individuals, 2x; diploids, 4x; tetraploids, other ploidy levels including triploids and pentaploids; Pop number, population number of Table 1.

| Pop Number | | N | Rk37 Ho % | N | Rk 35 Ho % | N | Rk 27 Ho % | Mean |
|---|---|---|---|---|---|---|---|---|
| **2x** | | | | | | | | |
| 3 | Mt La Chens I | 4 | 50 | 5 | 40 | 5 | 20 | 36.67 |
| 4 | Mt La Chens II | 23 | 47.83 | 24 | 41.67 | 24 | 8.33 | 32.61 |
| 15 | Col de la Lombarde I 9601 | 22 | 27.27 | 23 | 95.65 | 23 | 69.57 | 64.16 |
| 16 | Col de la Lombarde II 9602 | 9 | 44.44 | 4 | 50 | 10 | 40 | 44.81 |
| 6 | Col Pesio I 9589 | 24 | 95.83 | 24 | 29.17 | 24 | 58.33 | 61.11 |
| 7 | Col de la Duca I 9592 | 3 | 66.67 | 28 | 96.43 | 4 | 50 | 71.03 |
| 28 | Vercors I | 5 | 60 | 5 | 60 | 5 | 60 | 60.00 |
| 29 | Vercors II | 23 | 69.57 | 24 | 29.17 | 24 | 70.83 | 56.52 |
| | | | | | | | | **53.37** |
| | **4x** | | | | | | | |
| 1 | Corsica | 19 | 78.95 | 22 | 90.91 | 22 | 70.83 | 80.23 |
| 2 | Mt Cusna | 2 | 50 | 4 | 100 | 4 | 0 | 50.00 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 45 | Julier Pass | 20 | 45 | 24 | 87.5 | 24 | 79.17 | 70.56 |
| 40 | Lötschental | 21 | 57.14 | 24 | 62.5 | 24 | 36.36 | 52.00 |
| 48 | Kapall St Anton | 17 | 64.71 | 24 | 62.5 | 23 | 21.74 | 49.65 |
| 55 | Padon Pass | 21 | 76.19 | 24 | 54.17 | 24 | 50 | 60.12 |
| 58 | Mt Sadnig | 14 | 50 | 16 | 56.25 | 16 | 6.25 | 37.50 |
| 59 | Turracherhöhe | 20 | 65 | 23 | 17.39 | 24 | 83.33 | 55.24 |
| 53 | Tuxer Alps | 25 | 52 | 24 | 50 | 24 | 52.17 | 51.39 |
| 19 | Col des Champs | 13 | 84.62 | 13 | 61.54 | 13 | 53.85 | 66.67 |
| 33 | Gran Paradiso | 16 | 31.25 | 23 | 56.52 | 22 | 8.7 | 32.16 |
| 12 | Colle dela Perla II 9597 | 14 | 64.29 | 15 | 46.67 | 15 | 93.33 | 68.10 |
| | | | | | | | | **56.13** |

**Other ploidy levels**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 19 | Col des Champs | 11 | 63.64 | 11 | 72.73 | 11 | 45.45 | 60.61 |
| 33 | Gran Paradiso 3x | 1 | 0 | 1 | 100 | 0 | 0 | 33.33 |
| 12 | Colle dela Perla II 9597 | 11 | 72.73 | 11 | 27.27 | 11 | 100 | 66.67 |
| | | | | | | | | **53.54** |

Table 5 Summary of allelic diversity of SSRs data, Ploidy level; 2x; diploids, 3x; triploids, 4x; tetraploids, Mean; mean number of alleles per populations, Mean per locus; mean number of alleles per locus, Sum per locus; total of alleles per locus.

| | | | **Loci** | | | | | **Mean** |
|---|---|---|---|---|---|---|---|---|
| **Population number** | **Ploidy level** | **Locality** | **Rk 37** | **Rk 35** | **Rk 11** | **Rk 26** | **Rk 27** | |
| 3 | 2x | Mt La Chens_I | 3 | 3 | 2 | 2 | 3 | **2.60** |
| 4 | 2x | Mt La Chens_II | 5 | 2 | 2 | 2 | 3 | **2.80** |
| 6 | 2x | Valle di Pesio I 9589 | 10 | 4 | 2 | 2 | 8 | **5.20** |
| 7 | 2x | Passo del Duca II 9592 | 4 | 2 | 2 | 2 | 3 | **2.60** |
| 15 | 2x | Colle della Lombarde I 9601 | 9 | 9 | 2 | 2 | 10 | **6.40** |
| 16 | 2x | Colle della Lombarde II 9602 | 6 | 3 | 2 | 2 | 2 | **3.00** |
| 28 | 2x | Vercors_I | 4 | 4 | 2 | 2 | 2 | **2.80** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 29 | 2x | Vercors_II | 7 | 3 | 2 | 2 | 6 | **4.00** |
| 33 | 3x, 4x | Gran Paradiso | 9 | 5 | 3 | 2 | 5 | **4.80** |
| 12 | 3x, 4x, 5x | Col della Perla II 9597 | 9 | 4 | 3 | 2 | 8 | **5.20** |
| 19 | 3x, 4x, 5x | Col des Champs_II | 8 | 7 | 3 | 2 | 8 | **5.60** |
| 1 | 4x | Corsica | 6 | 3 | 3 | 2 | 2 | **3.20** |
| 2 | 4x | Mt Cusna | 3 | 3 | 3 | 2 | 1 | **2.40** |
| 40 | 4x | Lötschental | 11 | 2 | 3 | 2 | 7 | **5.00** |
| 45 | 4x | Julier Pass | 10 | 3 | 3 | 2 | 10 | **5.60** |
| 48 | 4x | Mt Kapall St Anton | 9 | 3 | 3 | 2 | 7 | **4.80** |
| 53 | 4x | Tuxer Alps | 8 | 3 | 3 | 2 | 4 | **4.00** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 55 | 4x | Padon Pass | 11 | 3 | 3 | 2 | 6 | **5.00** |
| 58 | 4x | Mt Sadnig | 8 | 2 | 3 | 2 | 2 | **3.40** |
| 59 | 4x | Turracherhöhe | 9 | 2 | 3 | 2 | 2 | **3.60** |
| | | **Sum per locus** | **149** | **70** | **52** | **40** | **99** | |
| | | **Mean per locus** | **7.45** | **3.50** | **2.60** | **2.00** | **4.95** | |

Table 6 Comparison of reproductive success of diploid sexuals and tetraploid apomicts after manual outcrossing, spontaneous selfing and manual selfing.

a) Comparison of all three treatments within diploids and tetraploids (ANOVA)

| | df | F | Sign. |
|---|---|---|---|
| Within 2x sexuals | 2 | 29.399 | <0,001 |
| Within 4x apomicts | 2 | 2.433 | 0.0975 |

b) Pairwise comparison of treatments within diploids and tetraploids (significance)

| | outcrossed (2x) | spontan. selfed (4x) | hand-selfed (4x) |
|---|---|---|---|
| outcrossed (4x) | | 0.600 | 0.516 |
| spontan. selfed (2x) | <0.001 | | 0.098 |
| hand-selfed (2x) | <0.001 | 0.972 | |

c) Comparison of treatments between diploids and tetraploids (ANOVA)

| | df | F | Sign. |
|---|---|---|---|
| outcrossed | 1 | 36.387 | <0,001 |
| spontan. selfed | 1 | 0.797 | 0.381 |
| hand-selfed | 1 | 4.401 | 0.044 |

## FIGURES

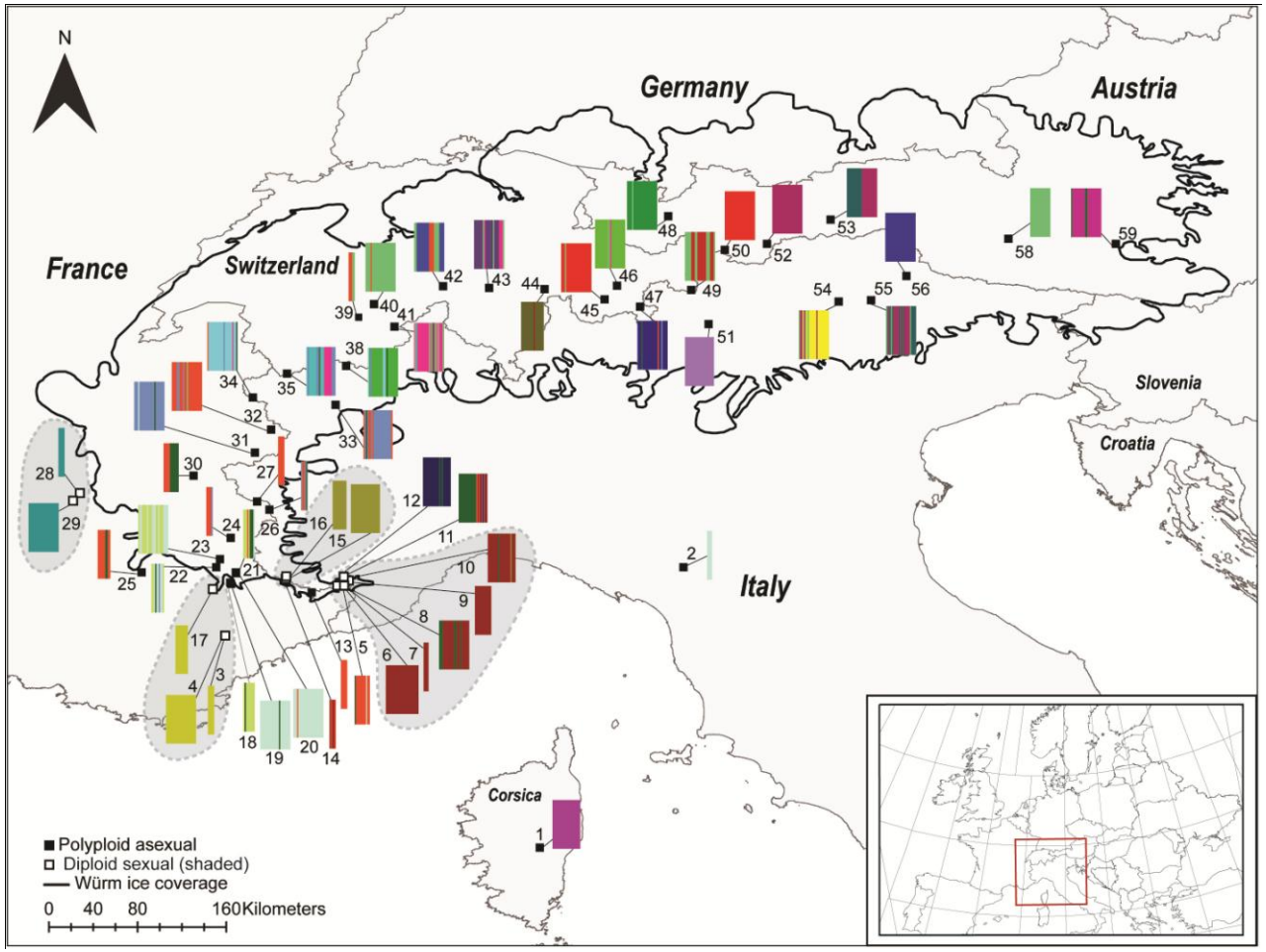



Figure 1 Map of the distribution area of *R. kuepferi*; black squares represent tetraploid populations, white square the diploids populations; number beside the square correspond to population numbers in Table 1. For each population, the results of the BAPS analysis of AFLP data are presented.

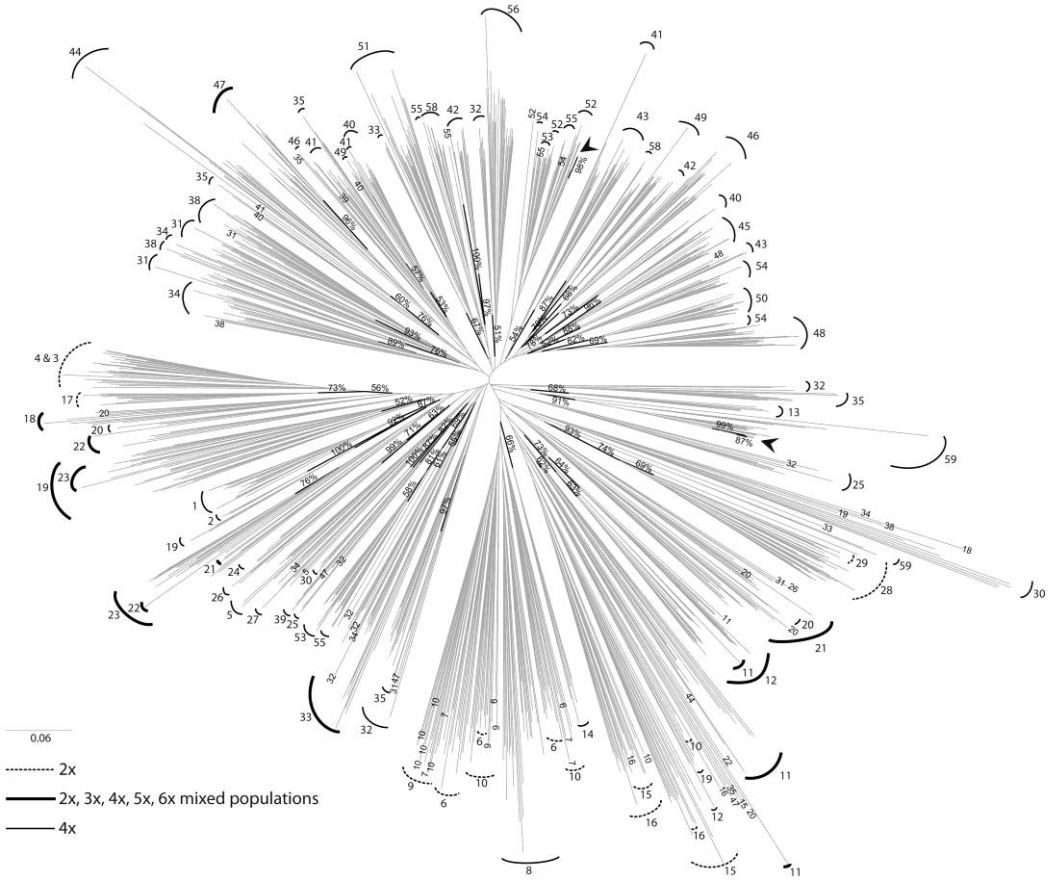



Figure 2 NJ tree of AFLP data; numbers at the tip of the branches or next to brackets indicate the population number (Table 1); branches in dark indicate bootstraps support above 50 %, the bootstrap values are indicated next to the branch; arrows show the position of the clones.

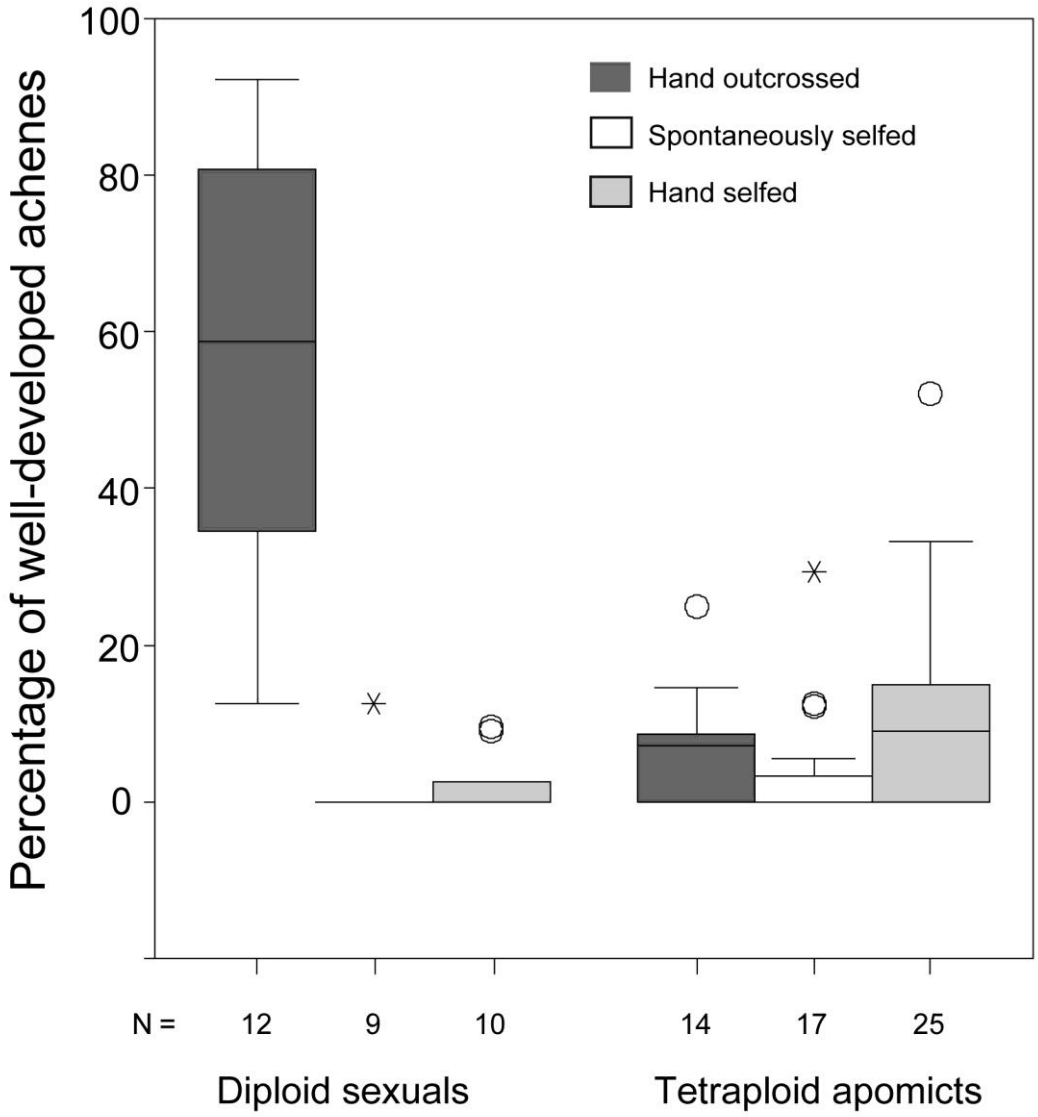



Figure 3 Boxplots of the variation of percentages of well-developed achenes per collective fruit for diploid sexuals and tetraploid apomicts. The box shows the 25th and 75th percentile range and the median value; circles are outliers, asterisks extreme values. N = number of flowers.

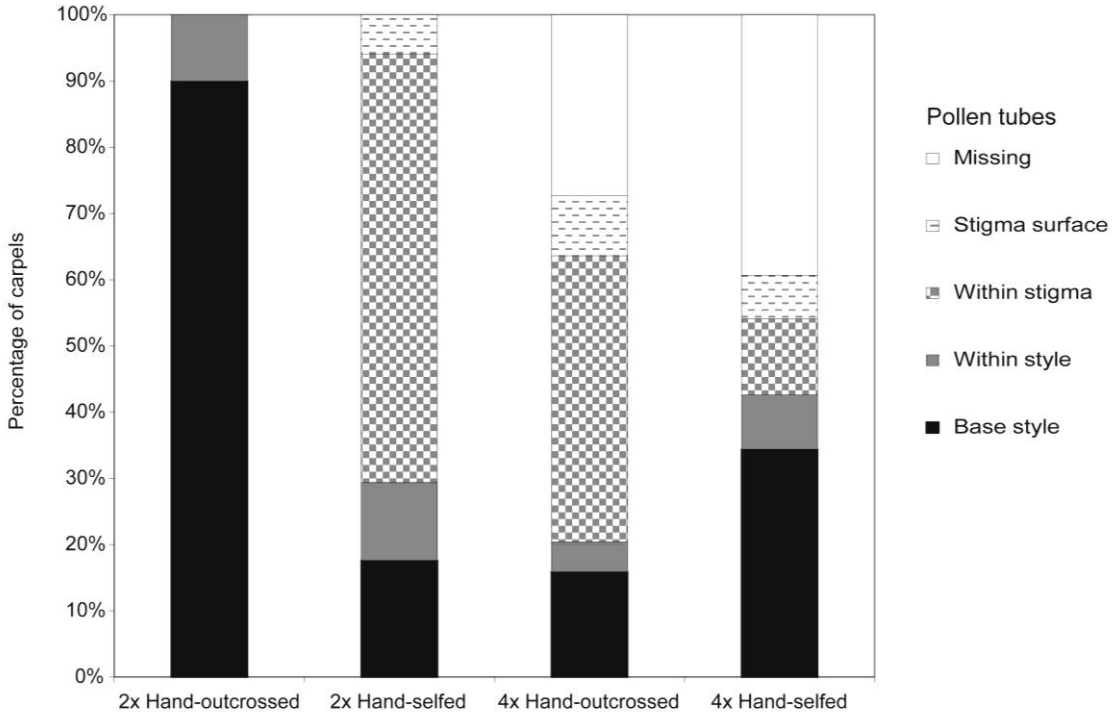
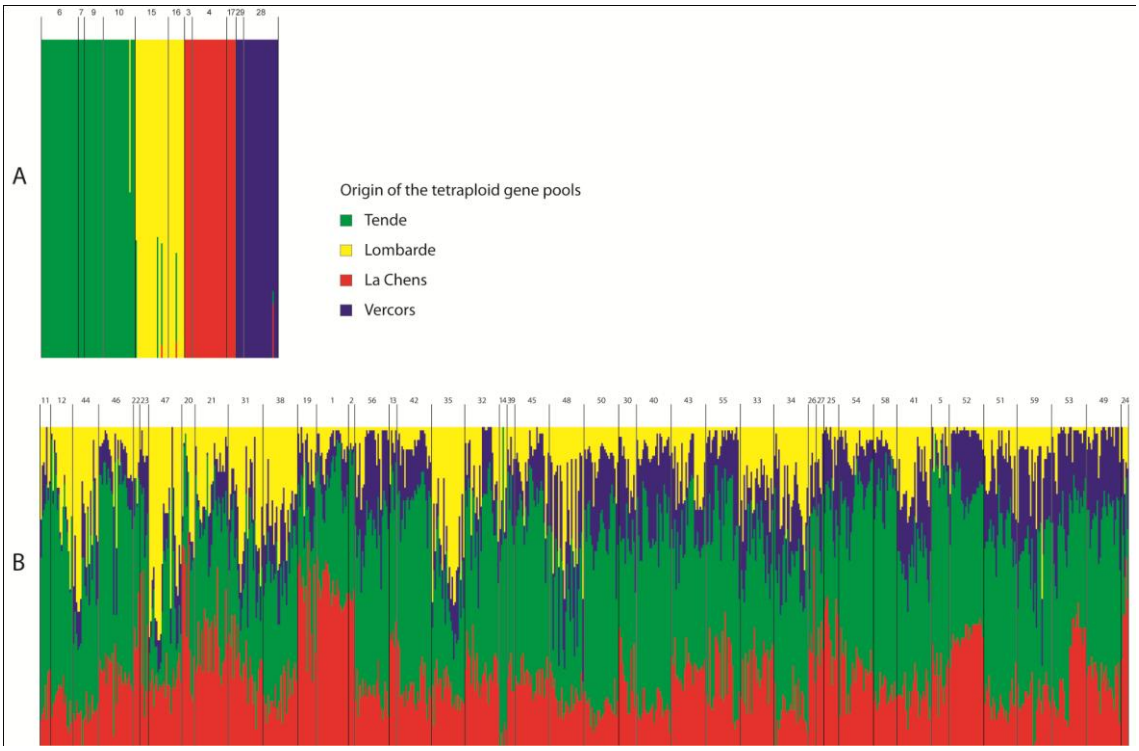
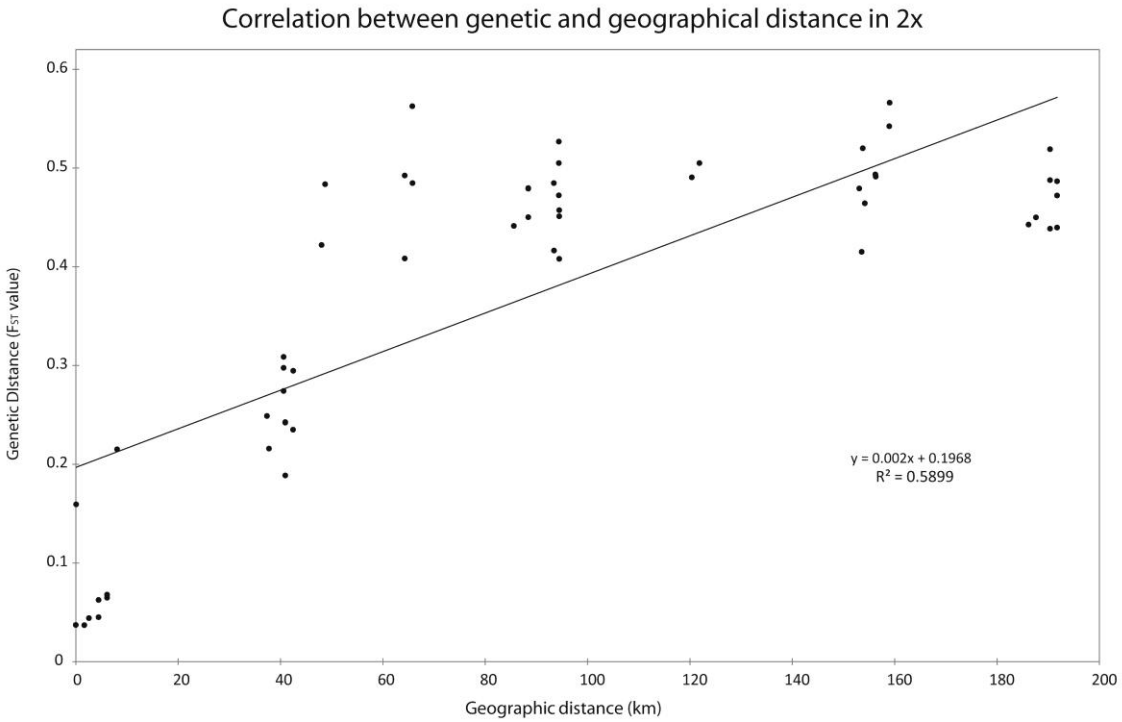


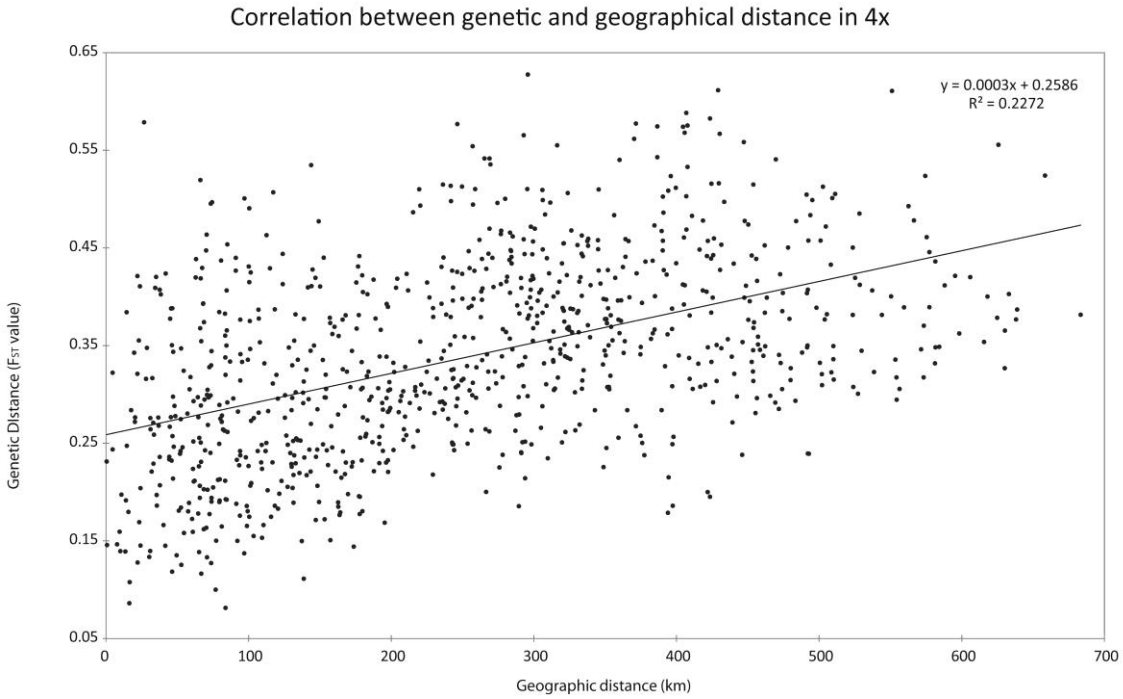

Figure 4 Pollen tube growth in diploids and tetraploids following hand-crossing and hand-selfing. Values are percentages of carpels with pollen tubes, white, pollen tube growth missing; dashed, pollen tube growth at the stigmata surface; squared, tube growth stops within the stigma; grey, tube growth stops in the style, and black, pollen tubes reach the basis of the style and enter the ovary.




Supplementary 1 BAPS analysis of AFLP data in a two step analysis, A; diploid sexual populations with only four partitions; B; tetraploid asexual populations with their gene reshuffling of the diploid gene pools without a fifth group.




Supplementary 2 Mantel test of diploids, showing correlations between geographic distance and genetic $F_{ST}$ distance.




Supplementary 3 Mantel test of tetraploids, showing correlations between geographic distance and genetic $F_{ST}$ distance.

# CONCLUSION

With this PhD, we were able to confirm in this study that *Ranunculus kuepferi* presents a geographical parthenogenesis pattern and that apomictic populations can be successful with a complex system of facultative apomixis combined with autopolyploidy. Autopolyploidy was thought to be a handicap because of disturbances in meiosis and seed formation and the lack of genomic novelty. But, the disadvantage of the disturbed meiosis can be overcome by apospory. The gamethophytic apomixis pathway of seed production combines the advantage of both founding a new population with a single individual (Baker's law) and the capacity of maintaining a high genetic diversity within populations. In contrast, sexual populations present a reduced genetic diversity which might be due to the glaciations of past restricting the diploids in refugial areas which had as consequence a fragmentation of the distribution area and genetic bottleneck effects for populations. For a better understanding of the whole pattern an ecological survey might be needed which would indicate if niche differentiation plays a stronger role in the GP pattern.

# CURRICULUM VITAE

# CURRICULUM VITAE

## PERSONAL DATA

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## EDUCATION

2006 – Present

PhD fellowship funded by FWF (Austrian Research Foundation)
On Geographical parthenogenesis — a multidisciplinary case study on alpine *Ranunculus* species, University of Vienna, Vienna, Austria.
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2000 – 2006

Master of Science in behaviour, ecology and evolution
Special orientation in evolutionary botany at University of Neuchâtel, Switzerland.

1998 – 2000

Medical student
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1993 - 1998

Swiss Maturity diploma (High school diploma; type E, economic), CESSNOV, Yverdon-les-Bains, Switzerland

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## WORKING ACTIVITY WHILE STUDYING

March – June 2010

Scientific researcher, Flow cytometric seed screen an subnivalen Blütenpflanzen (ÖAW), Vienna, Austria

January - September 2006

Assistant editor, *Taxon* (Journal of the International Association for Plant Taxonomy), Vienna, Austria.

Office assistant, IAPT (International Association for Plant Taxonomy), Vienna, Austria.

February - April 2004

Scientific researcher; Novartis, Animal health, Center of Research, St-Aubin, Switzerland; Validation of a screening test using ticks (*Boophilus microplus*).

July -2001- November -2003

Shop assistant, Gourmessa (catering), Migros, Marin, Switzerland.

July - September2000

Waitress, Navigation company of Neuchatel Lake and Beaulac Hotel, Neuchâtel, Switzerland.

July - September 1998; 1999

Assistant Nurse, EMS Claire-Vully (Altersheim), Bellerive, Switzerland.

July - August 1997

Assistant Nurse, Bellevue Hospital (psychatric-center), Yverdon-les-Bains, Switzerland.

July - August 1993; 1995

Shop assistant, Grocer shop, Cudrefin, Switzerland.

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## ADDITIONAL QUALIFICATIONS

## LAB PRACTICAL EXPERIENCE

CTAB extraction, RAPD analysis, AFLPs analyis, Flow cytomertric analyis and microsatellites.

### COMPUTER SKILLS GENERIC

MS-Office, Photoshop, Illustrator, Windows XP, Filemaker Pro, Endnote, PDF Formating, programmiing (Basic-understanding), Flash animation (Basic-understanding), computer security (Basic-understanding), QuarkExpress (journal edition).

### COMPUTER SKILLS SPECIFIC

R, Arlequin, Paup, Structure, BAPS, GeneMarker, Genographer, SplitsTree, Arc GIS.

## LANGUAGE SKILLS

French: mother tongue.
German: fluent both, written and spoken.
English: fluent both, written and spoken.
Italian: basic knowledge.

## RESEARCH INTERESTS

Systematic botany, plant biology, ecology, evolution, geographical parthenogenesis, apomixis, reproductive systems and evolution of sex

## PERSONAL INTERESTS

2006 – present

Membership, IAPT (International Association for Plant Taxonomy).

1998-2006

Fire service volunteer, in Cudrefin, Switzerland, officer and special education in first aid assistance.

1995

Humanitarian trip to Madagascar with Nouvelle Planète.

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## PUBLICATION LIST

## CONFERENCE PARTICIPATION

### POSTER

17th-20th may 2009

ICPHB 2009, International Conference on Polyploidy, Hybridization and Biodiversity, St Malo, France.

30th January – 01st February.2008

Plant Species Concepts and Evolution, University of Neuchâtel, Switzerland.

25th-27th June 2007

History, Evolution & Future of Arctic & Alpine Flora - Botanical Society of Scotland Symposium, St Andrews University, Scotland.

### TALK

2nd-4th July 2008

Xth Symposium of International Organisation of Plant Biosystematists, Strbské Pleso, Vysoke Tatry, Slovakia.

28th - 31st August 2007

6th Biennial Meeting: Biennial conferences of the Systematics Association, Royal Botanic Garden, Edinburgh.

## SCIENTIFIC PUBLICATIONS

2005

Masters thesis, Liens de parenté et origine de *Ranunculus kuepferi* en Corse et dans le sud de la France (Parental relationship of *Ranunculus kuepferi*'s populations in Corsica and south of France; title translated), University of Neuchâtel, Switzerland

2008

Hörandl, Elvira, Anne-Caroline Cosendai and Eva Maria Temsch. Understanding the geographic distributions of apomictic plants: a case for a pluralistic approach. Plant Ecology & Diversity. 1(2):309-320.

# *CURRICULUM VITAE*

2010

Cosendai Anne-Caroline and Elvira Hörand. Cytotype stability, facultative apomixis and geographical parthenogenesis in *Ranunculus kuepferi* (Ranunculaceae). Annals of Botany. 105 (3): 457-470.

2010 Submitted

Anne-Caroline Cosendai, Jan Rodewald and Elvira Hörandl, Origin and distribution of autopolyploids via apomixis in the alpine plant species *Ranunculus kuepferi* (Ranunculaceae)

Vienna,