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**"Mechanisms of cytotype coexistence in the  
autopolyploid complex of  
*Senecio carniolicus* (syn. *Jacobaea carniolica*,  
Asteraceae)"**

verfasst von / submitted by

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R. Flatscher

This thesis is dedicated to my long-time colleague and friend  
Ruth Flatscher who left us forever in 2014.



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## CO-AUTHOR STATEMENT

This thesis encompasses a series of three articles published in peer-reviewed journals where I am first or joint first author. The thesis was carried out in the context of a comprehensive, interdisciplinary research project funded by the Austrian Science Fund (FWF) and with participation of numerous collaborators. In this context, several additional papers have been published to which I contributed to various extents, predominantly in terms of fieldwork (data preparation, analyses and manuscript preparation to a lesser extent). Abstracts are included in the appendix.

Chapter 1 provides a short overview of relevant aspects of polyploidy, an introduction into the study species (including also some results of the overall project, which were not in central focus of the thesis) and a description of the research topics. Additionally, the discussion gives a synopsis of the three articles, followed by a short summary.

In the following, I declare my contribution to the individual papers (Chapter 2, 3 and 4):

### *Article 1*

**Sonnleitner M**, Flatscher R, Escobar García P, Rauchová J, Suda J, Schneeweiss GM, Hülber K, Schönswetter P. 2010. Distribution and habitat segregation on different spatial scales among diploid, tetraploid and hexaploid cytotypes of *Senecio carniolicus* (Asteraceae) in the Eastern Alps. *Annals of Botany* 106(6): 967–977.

- o planning and organizing of field trips
- o collecting of plant material for ploidy determination, genetic and morphological analyses; vegetation relevés and recording of metadata (with collaborators)
- o data management (preparing database, data input, GIS processing, etc.)
- o data analyses and preparing figures and tables (with K. Hülber)
- o literature survey, drafting and co-writing of the manuscript

### *Article 2*

**Sonnleitner M**, Weis B, Flatscher R, Escobar García P, Suda J, Krejčíková J, Schneeweiss GM, Winkler M, Schönswetter P, Hülber K. 2013. Parental ploidy strongly affects offspring fitness in heteroploid crosses among three cytotypes of autopolyploid *Jacobaea carniolica* (Asteraceae). *PLoS ONE* 8: e78959.

- o design of field and germination experiments
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- o performing in situ pollination experiments and harvesting of seeds (with collaborators)
- o processing of seeds and performing germination experiments (with B. Weis)
- o data management
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## ABSTRACT

The ways and means how closely related species accomplish to coexist in certain natural habitats is a key question in ecology and ultimately influences biodiversity. Polyploidy, a common phenomenon in vascular plants and an important mechanism of sympatric speciation, offers unique opportunities for plant coexistence in close proximity. The polyploid complex of *Senecio carniolicus* s.l. is an excellent model system for studying various aspects of cytotype coexistence.

The first paper of this thesis comprises a detailed analysis of the cytotype distribution throughout the Eastern Alps on different spatial scales. Among c. 3000 investigated individuals, we found a remarkably high diversity of DNA ploidy levels ranging from diploid to enneaploid. The three main cytotypes (diploid, tetraploid, hexaploid) showed a distinct spatial segregation on the macro-scale, however with large overlapping areas resulting in frequent cytotype mixtures. Coexisting cytotypes formed multiple contact zones including all possible combinations of the main cytotypes, however most frequently between diploids and hexaploids. Despite frequent cytotype co-occurrence on the micro-scale, intermediate ploidy levels (putative hybrids) were rarely detected (c. 1% of investigated individuals), suggesting strong pre- and/or postzygotic reproductive barriers. Additionally, microhabitat descriptors revealed significant habitat differences among the three main cytotypes according to altitude, exposition and vegetation coverage.

The second paper aims at unravelling reproductive interactions among different cytotypes in contact zones. The extent of hybridizations and the performance of progeny with intermediate ploidy levels play a crucial role in the evolutionary dynamics of contact zones (“triploid bridge” vs. “triploid block”) and the integrity of lineages. We performed reciprocal *in situ* cross-pollinations within and among main cytotypes. Harvested seeds were subjected to DNA ploidy determination via flow cytometry and greenhouse germination experiments for performance analyses of seedlings. Evaluating seed viability and survival of hybrid seedlings in comparison with intra-cytotype offspring revealed significantly inferior performance for diploid-polyploid hybrid offspring, whereas inter-polyploid crosses performed at least intermediate compared with their respective parental intra-cytotype crosses. Here, no effect of the direction of crosses could be detected, rendering the

presence of maternal or paternal effects unlikely. Growth traits of seedlings showed differences between reciprocal crosses, partly expressing maternal effects. The observed strong selection against hybrids involving diploids, and the full interfertility of tetraploids and hexaploids likely has strong implications on the stability of contact zones. Therefore, we can assume that diploid-polyploid contact zones are more stable (realized particularly in frequently occurring diploid-hexaploid contact zones) compared with tetraploid-hexaploid contact zones.

A further mechanism of plant coexistence is the partitioning of ecological requirements relaxing resource competition. In polyploid complexes, niche differentiation is a common prezygotic isolation mechanism between cytotypes and is addressed for *S. carniolicus* s.l. in the third paper. Multivariate analyses of ecological indicator values (Landolt values) assigned to accompanying species revealed clearly differentiated ecological niches for diploids, tetraploids and hexaploids. The observed niche shift followed a complex ecological gradient mainly with respect to temperature, light and soil parameters. Moreover, niche differentiation was more strongly pronounced where cytotypes co-occurred with one or two other cytotypes. Such niche displacement may facilitate stable coexistence of cytotypes but could also be a sign of niche homogenization in the course of parapatric cytotype distribution. In general, niche displacement seems to be essential for the understanding of ecological processes involving polyploids.

In conclusion, cytotypes of *S. carniolicus* are well differentiated from each other in many respects. Reproductive and ecological interference among cytotypes provide explanations for the spatial patterns of contact zones. The frequent occurrence of diploid-hexaploid contact zones likely can be traced back to almost lacking reproductive interactions and strong niche differentiation. In contrast, tetraploid-hexaploid contact zones, although rare and restricted to narrow stripes, exhibit high evolutionary dynamics, where gene flow mediated by hybrids may influence one or both lineages in the long run.

## ZUSAMMENFASSUNG

Mechanismen, die die Koexistenz von nah verwandten Arten in natürlichen Lebensräumen ermöglichen, sind von zentralem Interesse in der Ökologie und beeinflussen letztlich auch die Biodiversität. Polyploidie, ein häufiges Phänomen bei Gefäßpflanzen und ein wichtiger Mechanismus der sympatrischen Artbildung, erlaubt Zytotypen mit unterschiedlicher Anzahl an Chromosomensätzen gemeinsam auf engem Raum vorzukommen. Der Polyploidkomplex *Senecio carniolicus* s.l. ist eine geeignete Modellgruppe, um verschiedene Aspekte der Koexistenz von Zytotypen zu untersuchen.

Das erste Manuskript dieser Arbeit beinhaltet eine detaillierte Analyse der Verbreitungsmuster der Zytotypen von *S. carniolicus* in den Ostalpen auf unterschiedlichen räumlichen Ebenen. In ca. 3000 untersuchten Individuen fanden wir ein bemerkenswert breites Spektrum an Ploidiestufen, von diploid bis enneaploid. Die drei Hauptzytotypen (diploid, tetraploid, hexaploid) zeigten eine deutliche räumliche Strukturierung auf der Makroebene (gesamte Ostalpen), jedoch mit ausgedehnten Arealen, in denen verschiedene Zytotypen gemeinsam vorkamen. Die meisten Kontaktzonen wurden von diploiden und hexaploiden Individuen gebildet. Trotz dieser häufigen Koexistenz auf kleinstem Raum konnten Individuen mit intermediärer Ploidiestufe, d.h. mutmaßliche Hybriden, nur selten nachgewiesen werden (ca. 1 % der untersuchten Individuen), was auf starke prä- und/oder postzygotische Fortpflanzungsbarrieren hindeutet. Darüber hinaus zeigten Mikrohabitat-Parameter signifikante Habitatunterschiede zwischen den drei Hauptzytotypen, hauptsächlich in Bezug auf Seehöhe, Exposition und Vegetationsdeckung.

Das zweite Manuskript zielt darauf ab, reproduktive Interaktionen zwischen Zytotypen in Kontaktzonen zu analysieren. Das Ausmaß der Hybridisierungen und die Fitness der hybridogenen Nachkommenschaft spielen eine entscheidende Rolle bei der evolutionären Dynamik von Kontaktzonen ("triploid bridge" vs. "triploid block") und der Integrität genetischer Linien. Wir führten reziproke *in situ* Kreuzbestäubungen innerhalb und zwischen diploiden, tetraploiden und hexaploiden Zytotypen von *S. carniolicus* durch. Ein Teil der geernteten Samen wurde der Ploidie-Bestimmung mittels Durchflusszytometrie zugeführt, die anderen unter kontrollierten Bedingungen im Glashaus zur Keimung gebracht, um die Fitness der F1-Generation bestimmen zu können. Die Auswertung der Keimfähigkeit von

hybridogenen Samen und des Überlebens von Hybridsämlingen im Vergleich mit Nachkommen aus homoploiden Kreuzungen zeigte eine signifikant niedrigere Fitness für diploid-polyploide Hybrid-Nachkommen, während Kreuzungen zwischen Polyploiden im Vergleich zu den Nachkommen der homoploiden Kreuzungen ihrer Eltern gleich hohe oder höhere Fitness zeigten. Die Richtung der Kreuzungen (welcher Elternteil hatte die niedrigere/höhere Ploidie) hatte dabei keine Auswirkung auf Keimfähigkeit und Überleben der Keimlinge, was das Vorhandensein maternaler oder paternaler Effekte unwahrscheinlich macht. Im Gegensatz dazu zeigten an Sämlingen erhobene Wachstumsparameter sehr wohl Unterschiede zwischen reziproken Kreuzungen, die zum Teil auf maternale Effekte schließen lassen. Die starke Selektion gegen Hybriden mit einem diploiden Elternteil einerseits und die volle Interfertilität von Tetraploiden und Hexaploiden andererseits lassen einen starken Einfluss auf die Stabilität von Kontaktzonen vermuten. Daher können wir annehmen, dass diploid-polyploide Kontaktzonen im Vergleich zu tetraploid-hexaploiden Kontaktzonen evolutiv stabiler sind, was vermutlich auch die Häufigkeit von diploid-hexaploiden Kontaktzonen erklärt.

Ein weiterer Mechanismus der Koexistenz nah verwandter Taxa ist die (teilweise) Differenzierung der ökologischen Nischen, um den Ressourcenwettbewerb zu verringern. Nischendifferenzierung ist ein häufig beobachteter präzygotischer Isolationsmechanismus zwischen Zytotypen innerhalb von Polyploidkomplexen, der für *S. carniolicus* s.l. in der dritten Publikation behandelt wird. Multivariate Analysen ökologischer Indikatorwerte (Landolt-Zahlen), die den Begleitarten zugeordnet wurden, zeigten deutlich differenzierte ökologische Nischen für Diploide, Tetraploide und Hexaploide. Die Unterschiede in der Einnischung folgten einem komplexen ökologischen Gradienten vor allem in Bezug auf Temperatur, Licht und Bodenparameter. Darüber hinaus wurde bei sympatrischem Vorkommen von zwei oder mehreren Zytotypen eine stärkere Nischendifferenzierung beobachtet als in parapatrischen Populationen. Eine solche Nischenverschiebung kann einerseits Kontaktzonen zwischen verschiedenen Zytotypen stabilisieren, andererseits könnte es aber auch als Zeichen der Nischenhomogenisierung in parapatrischen Populationen interpretiert werden. Im Allgemeinen scheint „niche displacement“ für das Verständnis ökologischer Prozesse in heteroploiden Kontaktzonen wesentlich zu sein und sollte in zukünftigen Untersuchungen mitberücksichtigt werden.

Die vorliegende Studie zeigt, dass die Zytotypen von *S. carniolicus* in verschiedener Hinsicht gut differenziert sind. Reproduktive und ökologische Beziehungen zwischen den Zytotypen erklären die räumlichen Muster in den Kontaktzonen. Das häufige Auftreten von diploid-hexaploiden Kontaktzonen kann wahrscheinlich auf die nahezu fehlenden reproduktiven Interaktionen und die starke Nischendifferenzierung zurückgeführt werden. Im Gegensatz dazu zeigen tetraploid-hexaploide Kontaktzonen, obwohl sie selten und räumlich stark begrenzt sind, eine hohe evolutionäre Dynamik, wobei der von Hybriden vermittelte Genfluss eine oder beide Linien auf lange Sicht beeinflussen kann.



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## CHAPTER 1: GENERAL INTRODUCTION

### i. Polyploidy

#### **Polyploidy – an ubiquitous phenomenon in nature**

Polyploidy is the inheritable possession of more than two homologous sets of chromosomes in each cell nucleus, inherently multiplying the genome size of an organism. Since the discovery in the beginning of the 20<sup>th</sup> century (Lutz, 1907; Gates, 1909) the view on importance and ubiquity of polyploidy has changed fundamentally and polyploidization is now widely recognized as an evolutionarily significant process that has shaped the genome structure of all eukaryotes (Otto and Whitton, 2000; Wendel, 2000; Soltis *et al.*, 2009; Madlung, 2013) and probably is the single most important mode of sympatric speciation (Coyne and Orr, 2004). Polyploidy research traditionally has been strongly focused on plants, where the prevalence of polyploidy in angiosperms and ferns is proven (Soltis *et al.*, 2009). In animals, polyploidy has long been regarded as a rare event facing severe problems with e.g. the complex mode of sexual reproduction (Mable, 2004); however, it turned out to be more frequent than previously assumed. In fact, polyploidy occurs across all major taxonomic groups (Otto and Whitton, 2000; Gregory and Mable, 2005) and is relatively common in some of them (especially in fish and amphibians; Mable *et al.*, 2011).

Depending on the mode of origin, two major forms of polyploidy can be distinguished: allopolyploidy and autopolyploidy (Kihara and Ono, 1926). Allopolyploidy combines interspecific hybridization with polyploidization, whereas autopolyploids result from genome doubling within a single species thus combining two homologous genomes (Stebbins, 1950). This distinction is however not absolute, as it merely represents two extremes of a continuum of genetic divergence of the progenitors (Stebbins, 1947) and is additionally subjected to inconsistencies in taxonomic treatments. In practice, the assignment to either allo- or autopolyploids is not always straightforward, even with molecular tools (Winkler *et al.*, 2017). The mode of chromosome pairing during meiosis (bivalents in allopolyploids, multivalents in autopolyploids) traditionally serves as a distinction criterion (Müntzing, 1936; Jackson and Casey, 1982). The relative evolutionary importance of allopolyploidy vs. autopolyploidy has long been under debate, generally favouring allopolyploids, whereas autopolyploids usually have been seen as ephemeral phenomena (Müntzing, 1936; Stebbins,

1971; Ramsey and Schemske, 1998). In recent years, established autopolyploid complexes have been increasingly discovered, emphasizing their significance for evolution (e.g. Soltis *et al.*, 2003; Tate *et al.*, 2005; Soltis *et al.*, 2007).

Polyploidization confers a drastic change in the genome accompanied with a state of instability often followed by severe genomic rearrangements (e.g. Wendel, 2000; Weiss-Schneeweiss *et al.*, 2013). Neopolyploids surviving this initial phase may experience immediate changes of the phenotype, which offer unique opportunities for evolutionary transitions. Increased genetic variation, particularly in allopolyploids, can foster further modifications of the genome, not available to their diploid progenitors. In general, polyploidy implies well-documented advantages and disadvantages (Comai, 2005). Advantages of being polyploid mainly refer to heterosis and gene redundancy as immediate consequences of genome duplication. Heterosis often is expressed as transgressive performance of a polyploid compared with the corresponding diploids (Birchler *et al.*, 2010), whereas gene redundancy can protect from deleterious mutations (Gu *et al.*, 2003) and may confer the possibility of diversifying gene functions (Wendel, 2000; Adams and Wendel, 2005; Madlung and Wendel, 2013). Disadvantages of polyploidy are connected to direct consequences of nuclear and cell enlargement, the disposition of polyploids to produce aneuploid cells caused by meiotic and mitotic dysfunction, changes in gene expression and epigenetic instability (Comai, 2005). Finally, some changes in polyploids can be either advantageous or disadvantageous or even detrimental, particularly alterations in the transcriptome and epigenetic regulation mechanisms (e.g. Wang *et al.*, 2004; Hegarty and Hiscock, 2008). Immediate effects of polyploidization are less pronounced in autopolyploids since autopolyploidization is primarily a neutral process (Parisod *et al.*, 2010). Heterosis e.g. is not evident in autopolyploids (Birchler *et al.*, 2003), however, increased genetic material may provide the potential for genome reorganization and changes in gene expression in the longer term and genome flexibility especially under changing environments (Parisod *et al.*, 2010).

In general, established polyploids have been found to inhabit different and larger geographic ranges than diploids, often including more extreme environments such as high altitudes and latitudes (Stebbins, 1984; Otto and Whitton, 2000; Brochmann *et al.*, 2004) due to expected increased ecological tolerance, plasticity or greater adaptive potential for polyploids (Otto

and Whitton, 2000; Levin, 2002). Yet, a general valid link between distribution and ploidy cannot be drawn so far, mainly due to uneven geographical sampling and inconsistencies in the geographic distributions of diploids and polyploids in certain taxonomic groups (Husband *et al.*, 2013).

### **Polyploidy in flowering plants**

As already mentioned above, polyploidy is common across the angiosperm phylogenetic tree (Tate *et al.*, 2005) and considered as a major driving force for evolution in plants (Chen *et al.*, 2007; Soltis *et al.*, 2009). Whereas for a long time polyploidy has been notoriously underestimated accounting for 30–35% of land plants (Stebbins, 1971), nowadays it is believed that all flowering plants faced at least two rounds of whole genome duplications in their evolutionary history (Jiao *et al.*, 2011). Ancient polyploidy dates back to the period of early angiosperm evolution and might have substantially contributed to the major radiations of angiosperms since the Early Cretaceous (De Bort *et al.*, 2005; Fawcett *et al.*, 2009; Fawcett *et al.*, 2013). Beside the fact that all angiosperms are ancient polyploids, about 35% of vascular species are recent polyploids, i.e. they have formed since the origin of their genus (Wood *et al.*, 2009). Although the number of extant polyploid lineages differs strongly among taxonomic groups (Otto and Whitton, 2000), this phenomenon substantially contributes to plant diversity.

### **Polyploid formation and establishment**

In rare cases polyploidy is achieved by somatic doubling in meristematic tissues or sporophytes, however, the most frequent pathway of polyploidization is via the union of unreduced gametes (Ramsey and Schemske, 1998). There are two possibilities of formation, either as a one-step process by fusion of two unreduced ( $2n$ ) gametes generating a tetraploid embryo at once, or in two steps via backcrosses of triploid hybrids (i.e. “triploid bridge hypothesis”) with diploid ancestors (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998). Both modes of polyploid formation depend on the frequency of unreduced gamete production, where 0.1%–2.0% of gametes are expected to be unreduced, with even increased rates in hybrids and under environmental stress (Ramsey, 2007; Parisod *et al.*, 2010). As the one-step mechanism relies on the simultaneous occurrence of two stochastic events, this pathway is considered quite rare in natural populations (Husband, 2004). In the

two-step process, the union of one  $2n$  and one  $n$  gamete produces triploids. These triploid hybrids, if they are at least partially fit and produce functional (often unreduced,  $n = 3x$ ) gametes, can form tetraploids by backcrossing with diploid ancestors (Ramsey and Schemske, 1998; Husband, 2004). Hence, triploids play an important role in polyploid origin; they can serve as a bridge to polyploid formation, which has been confirmed by theoretical and empirical studies (Felber, 1991; Husband, 2004). Alternatively, a “triploid block”, caused by dosage effects in the endosperm, may prevent the formation of fit and fertile triploid hybrids counteracting polyploid establishment (Köhler *et al.*, 2010).

Newly formed polyploids, independent of whether they are of hybrid origin or not, necessarily emerge in sympatry with their lower ploid ancestors and face a severe extinction risk by means of minority cytotype exclusion (Levin, 1975; Felber, 1991; Baack, 2005). One hurdle is to overcome the fitness loss caused by ineffective matings with the progenitor, though, at least partially fit hybrids may promote polyploid establishment via recurrent formation of polyploid individuals (Ramsey and Schemske, 1998; Soltis and Soltis, 1999). Instantaneous reproductive isolation with the ancestor, together with reproductive assurance, such as self-fertilization, apomixis or vegetative propagation may counterbalance frequency dependent disadvantage of polyploids (Baack, 2005). Immediate ecological differentiation, which lowers the competitive pressure between neopolyploids and their diploid relatives is regarded as one of the most significant features for successful establishment (Otto, 2007) facilitated by increased vigour, phenotypic plasticity and high adaptive potential as a direct consequence of polyploidization (Parisod *et al.*, 2010). Thus, the long-term fate of a polyploid depends on the evolution of (various) reproductive isolation mechanisms (see next chapter) and the ability to adapt to a certain habitat (Otto and Whitton, 2000).

### **Polyploidy in natural populations – cytotype coexistence in contact zones**

Polyploids, particularly autopolyploids, morphologically often resemble their diploid ancestors and have therefore been frequently overlooked in the past. Only since high-throughput technology for ploidy determination, such as flow cytometry (FCM), is available, large-scale ploidy screening is feasible (Suda and Trávníček, 2006; Kron *et al.*, 2007; Suda and Pyšek, 2010) and new ploidy levels are detected in many plant taxa (e.g. Mahelka *et al.*, 2005; Mandáková and Münzbergová, 2006; Mráz *et al.*, 2008; Koutecký *et al.*, 2012). Ploidy

variation within species is reported from 12–13% of angiosperms (Wood *et al.*, 2009), however this is likely underestimated and will increase with more intense sampling. Additionally, spatial distribution of intraspecific cytotypes, even on small scales, can nowadays be assessed via FCM (Suda and Pyšek, 2010). Of particular interest are sites where cytotypes of different ploidies co-occur sympatrically. Such cytotype mixtures can extend over large areas (e.g. Burton and Husband, 1999; Halverson *et al.*, 2008) or form narrow contact zones sometimes involving only a few populations or individuals (e.g. Husband and Schemske, 2000; Baack, 2004; Kolář *et al.*, 2009; Trávníček *et al.*, 2010; Hülber *et al.*, 2015). These contact zones are regarded “natural laboratories for evolutionary studies” (Hewitt, 1988) as they provide insights into mechanisms of polyploid origin and coexistence. Two principle types of contact zones can be distinguished (Petit *et al.*, 1999), (i) primary contact zones are sites of *de novo* polyploidy formation, where neopolyploids emerge as descendants of diploid ancestors, and (ii) secondary contact zones are areas where cytotypes of different ploidy regain contact after a period of allopatric distribution (e.g. in the course of postglacial range expansion). Discrimination between primary and secondary contact, however, is not always straightforward without genetic evidence. Cytotypes in secondary contact zones are usually more distantly related than in primary contact zones (Husband *et al.*, 2013), though the mechanisms shaping the dynamics in such zones might be similar.

Contact zones, independent of type, can be transient stages of evolution, e.g. when one cytotype outcompetes the other, or rather stable through time (Petit *et al.*, 1999). The dynamics of contact zones are strongly governed by the interactions of coexisting cytotypes and the evolution of pre- and postzygotic isolation mechanisms, which limit gene flow and therefore allow cytotypes to co-exist (Petit *et al.*, 1999; Moyle *et al.*, 2004; Widmer *et al.*, 2009). Reproductive isolation, usually achieved via concurrence of various mechanisms, is essential for the process of speciation (Coyne and Orr, 1998). Spatial segregation and ecological differentiation are among the most frequently observed prezygotic isolation mechanisms and probably play a key role in cytotype coexistence (Levin, 2002; Oswald and Nuismer, 2011; Ramsey and Ramsey, 2014). Habitat differentiation can also be observed in secondary contact zones (e.g. Baack, 2004; Duchoslav *et al.*, 2010; Mráz *et al.*, 2012; Zozomová-Lihová *et al.*, 2015), and on various spatial scales (Trávníček *et al.*, 2011).

Additional prezygotic mechanisms may act, preventing gene flow between cytotypes, e.g. assortative mating via pollinator discrimination (Segraves and Thompson, 1999), flowering time displacement and predominance of selfing or apomixis (Petit *et al.*, 1999). Further isolation may happen in the phase between pollination and fertilization, e.g. reduced pollen germination on stigmata, dysfunctional pollen tube growth, which confer conspecific pollen precedence (Howard *et al.*, 1998; Howard, 1999).

Whereas the above mentioned mechanisms prevent pollination or fertilization, postzygotic isolation acts on embryo formation and hybrid fitness (Petit *et al.*, 1999). Polyploidization per se may cause instantaneous reproductive isolation of varying degree between polyploids and their diploid or lower-ploid ancestors (Ramsey and Schamske, 1998). Although inter-cytotype hybrids are thought to be rarely formed in natural populations, they – depending on their fitness – strongly influence the integrity of coexisting lineages (Barton and Hewitt, 1985). Postzygotic isolation often is a consequence of genome imbalance in the endosperm caused by mismatched amounts of DNA (“triploid block” or more general “inter-ploidy block”; Marks, 1966; Köhler *et al.*, 2010; Greiner and Oberprieler, 2012). In fact, deviations from the proper ratio of two maternal (2m) and one paternal (1p) genomes in the endosperm frequently cause seed abortion (Brink and Cooper, 1947; Johnston *et al.*, 1980; Lin, 1984). The direction of crosses, whether the higher ploidy part is the mother or the father, necessarily alters the contribution of maternal and paternal genomes, where maternal excess leads to increased cellularization and inhibits proliferation of the endosperm (producing small embryos), while paternal excess has the opposite effect (Haig and Westoby, 1991; Scott *et al.*, 1998). The underlying mechanisms, which are based on complex genetic, genomic and regulatory processes such as imprinting of genes (Haig and Westoby, 1991), affect survival of seedlings and the fitness of inter-cytotype hybrids. Numerous investigations in diploid-polyploid systems exhibit different strength of the interploidy block and provide indications for tolerance of genomic imbalance to some extent (Burton and Husband, 2000; Peckert and Chrtek, 2006; Castro *et al.*, 2011; Koutecký *et al.*, 2012; Sutherland and Galloway, 2017). The degree of reproductive isolation as well as vigour and fertility of inter-cytotype hybrids are among the key factors shaping the dynamics of contact zones and determine the lineage evolution of participating cytotypes (Barton and Hewitt, 1985).

## Role of polyploidy for speciation

In angiosperms, at least 15% of speciation events are assumed to involve polyploidy (Wood *et al.*, 2009) and polyploidization is seen as the single most important mechanism of sympatric speciation (Otto and Whitton, 2000). What features enable polyploidy as speciation mechanism? First, polyploidization is a drastic mutation accompanied by profound genetic and genomic changes, which equips the neopolyploid with innovative phenotypical features leading to morphological and/or ecological transition (Adams and Wendel, 2005; Moore and Purugganan, 2005; Hegarty and Hiscock, 2008). Second, polyploidization can lead to instantaneous reproductive isolation from the lower-ploid ancestor (Ramsey and Schemske, 1998). Third, duplicated genes may have favourable effects on further lineage evolution and speciation like increased adaptation ability to different habitats (Wood *et al.*, 2009). These combined attributes of polyploids often result in complete reproductive barriers, which results in the formation of new species (Dobzhansky, 1937). The relevance of polyploidy for evolutionary success of a taxonomic group reflected in speciation rates is still debated with several lines of evidence favouring and doubting a major impact on lineage diversification (Weiss-Schneeweiss *et al.*, 2013). The evidence for a dramatic radiation of angiosperms following ancient whole genome duplication (Soltis *et al.*, 2009), the largely irreversible nature of polyploidy, which accumulates gene redundancies over time (Meyers and Levin, 2006), together with the observed high percentage of contemporary polyploids (Wood *et al.* 2009) in some species-rich groups are indicative for high diversification rates in polyploid angiosperms. In contrast, other authors found no correlation between species richness and polyploidy (Wood *et al.* 2009) and argue that polyploidization events are overrepresented at the tips of phylogenetic trees and face higher extinction risks (Mayrose *et al.*, 2011). Additionally, the probability of polyploidization in already polyploid lineages and the net speciation rate in polyploids seem to be low (Mayrose *et al.*, 2011). Even if polyploidy often represents a dead end, there is strong evidence for high polyploid diversification rates under specific climatic circumstances in the past (Fawcett *et al.*, 2009) but also within more recently originated polyploid *Gossypium* and *Glycine* species (Wendel and Cronn, 2003; Schlueter *et al.*, 2004). Despite extensive effort and significant progress in knowledge about consequences of polyploidy, still no conclusive statement can be provided about the evolutionary success of polyploids in general. It seems

that the success of polyploidization strongly relies on stochastic events with significantly varying responses to genome doubling (and hybridization) between or even within species (Madlung, 2013). Furthermore, recent integration of molecular and phylogenetic studies provides no clear picture about specific differences of evolutionary success between autopolyploids and allopolyploids (Madlung, 2013).

In general, the treatment of polyploid lineages as separate taxonomic species is rather inconsistent (Barker *et al.* 2016), which likely has implications on estimates of speciation rates and numbers of polyploid species within genera. Since species delimitation still strongly relies on morphology, well-separated polyploid lineages are often not recognized as taxonomic species, because they, particularly autopolyploids, morphologically often strongly resemble their progenitors (Soltis *et al.*, 2007). Thus, it can be assumed that many autopolyploid lineages still are hidden as cryptic species within diploid populations.

## **ii. Study system**

*Senecio carniolicus* Willdenow (syn. *Jacobaea carniolica* (Willdenow) Schrank) is a member of the large Asteraceae family and belongs to a monophyletic clade of mainly European mountain plants, informally called *Incani* s.l., within *Senecio* sect. *Jacobaea* (Miller) Gray (Pelser *et al.*, 2002; Pelsner *et al.*, 2003; Pelsner *et al.*, 2004). The taxonomic status of *S. carniolicus* in the literature and plant databases is denoted inconsistently. In some cases *S. carniolicus* still is treated as a subspecies of the western Alpine *S. incanus* (e.g. Aeschimann *et al.*, 2004; Fischer *et al.*, 2008), which, however, is genetically clearly distinct and is now regarded as a sister species (Pelser *et al.*, 2003; Escobar García *et al.*, 2012). As a taxonomic consequence of a comprehensive study, in which this thesis was imbedded, *S. carniolicus* has been split into four independent species, namely *S. carniolicus* s.str., *S. disjunctus*, *S. noricus* and *S. insubricus* (Flatscher *et al.*, 2015; Appendix 4). To warrant readability and consistency with the respective publications, the four taxa are referred to as “cytotypes” or “lineages” in the following chapters. Some authors recommend the genus *Jacobaea* (Miller) for *Senecio* sect. *Jacobaea* (Miller) Gray based on the molecular phylogenies of Pelsner *et al.* (2002; 2003), however the morphological characterization of *Jacobaea* could not be entirely

resolved so far (Pelser *et al.*, 2004). This may explain why both names are currently in use in pertinent literature.

*Senecio carniolicus* s.l. is a common perennial herb, which grows mainly in the alpine to subnival belt of the Eastern Alps from the Alpi Lepontine and the Prealpi Luganesi in the west to the easternmost central Alps of Austria (Suda *et al.*, 2007) and the Carpathians. *Senecio carniolicus* s.l. is usually restricted to siliceous bedrock (Ellenberg, 1996) with a few exceptions and inhabits a wide spectrum of habitats, such as alpine grasslands, dwarf shrub communities, stable screes, moraines, rock crevices and wind-exposed fellfields (Beger, 1928; Pignatti and Anzalone, 1982), ranging from the timberline up to altitudes of 3300 m a.s.l. (Reisigl and Pitschmann, 1958).

*Senecio carniolicus* s.l. has long been recognized as a morphologically highly variable species, whereby the south-westernmost populations, which have been viewed morphologically intermediate with the western *S. incanus* (Chater and Walters, 1976), were treated as *S. incanus* subsp. *insubricus* (Chenevard) Braun-Blanquet. The latter has therefore been assumed to be a tetraploid hybrid between the western Alpine diploid *S. incanus* and the presumed uniformly hexaploid *S. carniolicus* (Chenevard, 1906; Ozenda *et al.*, 1988). However, molecular data unambiguously show that *S. incanus* is clearly distinct from *S. carniolicus* s.l. with no traces of gene flow, and *S. incanus* subsp. *insubricus* is most closely related to *S. carniolicus* s.l. (Escobar García *et al.*, 2012). Additionally, based on flow cytometric and karyological studies, Suda *et al.* (2007) uncovered an intricate polyploid complex in the eastern Alpine distribution range of *S. carniolicus* s.l. comprising three main cytotypes, diploids ( $2n = 2x = 40$ ), tetraploids ( $2n = 4x = 80$ ), and hexaploids ( $2n = 6x = 120$ ).

Later molecular investigations by Escobar García *et al.* (2012) revealed two distinct lineages on the diploid level. These results clearly reject the assumption of populations regarded as *S. incanus* subsp. *insubricus* as tetraploid intermediate between *S. incanus* and *S. carniolicus* s.l. Moreover, the delimitation of the two diploid lineages does not correspond with the former boundary between *S. incanus* subsp. *insubricus* and *S. carniolicus* s.l. but runs further east along the Isel and Drau valleys and finally led to a revised circumscription of the taxon (Flatscher *et al.*, 2015).

Molecular analyses of the origin of the polyploid lineages, based on nuclear ITS sequences and AFLP data, revealed an autopolyploid origin of the tetraploids and hexaploids (Winkler

*et al.*, 2017). The eastern diploid lineage gave rise to (eastern) tetraploids, which in turn led to hexaploids by an additional autopolyploidization step. Considering western tetraploids (the western part of disjunct tetraploid distribution; refer to Chapter 2), the ancestry could not be resolved unambiguously. Two possible scenarios are put forward by Winkler *et al.* (2017): (i) allopolyploid origin from eastern and western diploid lineages (ii) autopolyploid origin from eastern diploids. The authors suggest the latter scenario as the most plausible, supported by ribotype sharing with eastern diploids and strong genetic connectivity within tetraploids as well as with hexaploids, pointing to common ancestry.

In general, autopolyploid derivatives are regarded to resemble their diploid ancestors due to gene homology, in particular morphologically (Soltis *et al.*, 2007), therefore autopolyploids were often not recognized as separate taxa. However, in the *S. carniolicus* polyploid complex substantial morphological variation exists, which corresponds with the four identified lineages. Morphological differences refer mainly to indumentum density, growth height, leaf shape and characters of the flowering part; details are given in the determination key (Flatscher *et al.*, 2015). Even though morphology shows some overlap between lineages, with some experience they can be determined in the field with high accuracy.

### **iii. Research topics**

This thesis was conducted within the framework of an interdisciplinary project with two complementary aims: (i) Inferring the origin and differentiation of cytotype mixtures with different methodological approaches and (ii) exploring isolation mechanisms allowing maintenance of cytotype mixtures in the *S. carniolicus* polyploid complex.

This thesis comprises the following main topics:

(i) Conducting an extensive cytogeographic survey across the whole eastern Alpine distribution range of the study species to provide the basis for further investigations. A detailed recording of the geographical and spatial distribution of cytotypes on a macro scale allows for phylogeographic inferences and detecting cytotype mixtures; patterns of cytotype coexistence on meso- and micro-scales provide insights into the nature and dynamics of contact zones, particularly via the existence of secondary (contact) cytotypes. Additionally, the survey aimed at characterizing habitat preferences of cytotypes.

(ii) One of the most influential mechanisms of cytotype coexistence in contact zones is their reproductive interference. The degree of interfertility of cytotypes of different ploidy strongly determines the evolutionary dynamics of contact zones and influences the long-term integrity of participating lineages (Barton and Hewitt, 1985). We performed reciprocal *in situ* crossing experiments among three main cytotypes and subsequent germinations of harvested seeds to test for differences in cross-compatibility by evaluating four fitness components of early life stages, encompassing hybrid formation and seedling performance.

(iii) Since reproductive isolation among cytotypes of different ploidy levels is rarely complete, additional prezygotic isolation mechanisms often are at work (e.g. Segraves and Thompson, 1999; Husband and Schemske, 2000; Kennedy *et al.*, 2006). Ecological differentiation is regarded as an important factor of polyploid speciation (e.g. Felber-Girard *et al.*, 1996; Ståhlberg and Hedrén, 2009; Duchoslav *et al.*, 2010). Ecological indicator values (Landolt values; Landolt *et al.*, 2010) of vascular plant species, accompanying target individuals of *S. carniolicus* s.l., were used to identify ecological parameters responsible for habitat differentiation between cytotypes on the microhabitat level. Additionally we tested for niche displacement induced by the presence of a second cytotype, i.e. whether the ecological niches of homoploid sites differ from heteroploid sites.

#### iv. Discussion

Polyploidy and its consequences for evolutionary processes in plants have been studied since almost a century now and still provide challenging topics (Ramsey and Ramsey, 2014; Soltis *et al.*, 2014). Methodological advances in terms of ploidy screening, new molecular methods or statistical and modelling approaches encouraged polyploidy research and led to a wealth of case studies across the plant kingdom. Nevertheless, conclusions concerning the evolutionary power of polyploidy valid for all investigated systems can hardly be drawn and many questions still have to be answered (Soltis *et al.*, 2010; Madlung and Wendel, 2013). The present study provides multifaceted insights into the polyploid study system *Senecio carniolicus* s.l.

A detailed knowledge of the geographical and spatial distribution of cytotypes constituting a polyploid complex is a prerequisite for studying polyploid evolution including spatio-

ecological aspects. Since flow-cytometric DNA ploidy determination (FCM) of dried leaf samples (Suda and Trávníček, 2006) enables time- and cost saving large-scale screening of whole populations and across distribution ranges ploidy variations (especially autopolyploidy) within species are discovered more often and spatial structures of contact zones can be characterized.

The impetus for a large-scale investigation of *S. carniolicus* resulted from the study of Suda *et al.* (2007), which unravelled a complex picture of ploidy variation within the previously thought uniformly hexaploid species for the first time. With an extended sampling effort including approximately 5000 individuals for DNA ploidy determination together with almost 3000 vegetation relevés and recordings of geographical data we created a detailed cytogeography and the basis for further ecological, molecular and morphological studies (see Chapter 2).

Surprisingly, the general distribution of *S. carniolicus* main cytotypes (diploids, tetraploids and hexaploids) largely resembled the results from Suda *et al.* (2007) despite a 12-fold increased sampling size. On a large scale, cytotypes were spatially structured, however, with extensive overlapping areas creating multiple contact zones. Diploid and hexaploid cytotypes were widespread and occurred in large parts of the Eastern Alps, whereby smaller areas were inhabited by only one of the two cytotypes, a pattern which largely can be explained by Pleistocene persistence and postglacial range dynamics. Tetraploids displayed a rather restricted distribution forming two spatially disjunct (>180 km) patches which correspond with predicted glacial refugia (Schönswetter *et al.*, 2003; Tribsch and Schönswetter, 2003; Schönswetter *et al.*, 2005). In contrast to hexaploids, which inhabit areas most heavily glaciated during the Last Glacial Maximum (van Husen, 2000), the assumption of high colonization success of polyploids (Stebbins, 1984) is not met for tetraploid *S. carniolicus*. Alternatively, other processes, e.g. interspecific competition, habitat preferences or physiological reasons limit their spread.

Accordingly, we can conclude that today's large-scale spatial pattern of the members of the *S. carniolicus* complex is largely a product of their Pleistocene history and postglacial range dynamics rather than of their polyploidization history itself. In course of these postglacial range expansions and colonization routes populations of different ploidy partially regained contact after a period of parapatry, now observed as secondary contact zones. In our study,

we found multiple narrow contact zones across large parts of the distribution range in the Eastern Alps contrasting single broad(er) contact zones reported from other species complexes (e.g. Burton and Husband, 1999; Castro *et al.*, 2012; Zozomová-Lihová *et al.*, 2015). Almost half (44%) of the investigated *S. carniolicus* sites contained cytotype mixtures. Within this mixed-ploidy sampling sites, cytotypes were spatially aggregated on a micro-scale, which might be explained by a correlation between spatial proximity and microhabitat similarity combined with inherent habitat differentiation of cytotypes but also, at least partly, by dispersal limitation. Contact zones in *S. carniolicus* are multiple and narrow – as known from many other polyploid complexes, e.g. *Chamerion angustifolium* (Husband and Schemske, 2000) or *Knautia arvensis* (Kolář *et al.*, 2009) – and provide ideal opportunities to study structuring and interactions between cytotypes, which may be similar in primary and secondary contact zones. The mechanisms maintaining the coexistence of cytotypes of different ploidy are manifold and the focal topic of this study.

Although *S. carniolicus* cytotypes frequently occur in close proximity and in all possible combinations, the observed secondary (putative hybrid) cytotypes were diverse (high cytotype diversity in general) but rare (c. 1% altogether). This suggests the presence of strong crossing barriers among the main cytotypes. The most striking result here was the complete absence of tetraploids in diploid-hexaploid mixed populations (confirmed by in-depth studies of Schönswetter *et al.*, 2007; Hülber *et al.*, 2009), the most frequent cytotype combination (almost one third of 100 investigated sites). A closer look into the cytotype composition of populations at this point of knowledge led to the assumption that secondary cytotypes are more likely the outcome of crosses involving unreduced gametes rather than hybrids between main cytotypes. The by far most common secondary cytotype was the pentaploid, which in ten out of 27 cases occurred in otherwise pure tetraploid but also in mixed populations. More detailed investigations were performed by Hülber *et al.* (2015) to unravel spatial and niche patterns as well as gene flow and introgression in contact zones. They confirm a non-random spatial pattern of cytotypes at a micro-scale and the absence of gene flow between diploids and polyploids. In contrast, within tetraploid-hexaploid contact zones intermediate pentaploids were common in a narrow strip of immediate contact and found to mediate gene flow between participating lineages. However, the general scarcity of

pentaploids (c. 0.7% in 100/5000 surveyed populations/individuals) can be explained by ecological differentiation resulting in spatially restricted and rare contact zones.

Microhabitat descriptors recorded for c. 3000 *S. carniolicus* individuals across the Eastern Alps revealed a strong ecological segregation among main cytotypes according to altitudinal distribution, microhabitat exposition and the degree of surrounding vegetation cover. Diploids occur predominantly at higher altitudes in often wind-blown habitats with sparse vegetation cover, whereas hexaploids prefer habitats in lower altitudes and densely overgrown habitats with deep soils. The tetraploids occupy intermediate positions and more often prefer northern expositions. Generally, overlaps are large. In a further study ecological niches were characterized in more detail (see Chapter 4).

Essential for the evolutionary dynamics in contact zones, disregarding whether they are of primary or secondary origin, is the degree of reproductive isolation or, in other words, the viability and fertility of hybrid offspring from crosses between cytotypes (Petit *et al.* 1999).

Polyploidy itself often provokes instantaneous reproductive isolation between the newly formed polyploid and the diploid ancestors (Ramsey and Schemske, 1998). Far less is known about reproductive isolation in secondary contact zones and between different polyploid levels (but see: Kolář *et al.*, 2009; Greiner and Oberprieler, 2012; Sutherland and Galloway, 2017). Performing reciprocal crossing experiments among all three main cytotypes of *S. carniolicus* aimed at unravelling the degree of postzygotic isolation between (eastern) diploids and polyploids but also within polyploids. We tested the effect of parental ploidy on four early life stage fitness parameters of F1-individuals: *seed viability*, *survival of seedlings*, *duration of seedling development* and *size of seedlings* by comparing offspring of heteroploid and parental homoploid crosses. Assuming no selection against hybrids, F1-individuals descending from heteroploid crosses should perform intermediate compared with pooled parental crosses.

Our experiments clearly revealed that reproductive success between cytotypes strongly depends on the ploidy of the parents. Strong selection against hybrid offspring stemming from diploid-polyploid parents was expressed by low seed viability and strongly reduced survival of seedlings, whereas crosses within polyploids showed full interfertility and well performing offspring. The observed pattern of cross-compatibility meets the predictions from genome dosage effects where a 2:1 ratio of maternal to paternal genomes in the

endosperm is required for normal seed formation (Haig and Westoby, 1991; Scott *et al.*, 1998). Hence, the strongly reduced fitness of diploid-polyploid hybrid seedlings is based on a larger deviation (3-fold and 2-fold for crosses of diploids with hexaploids and tetraploids, respectively) from the homoploid parental genome ratio in the endosperm compared with inter-polyploid offspring (0.67-fold deviation), where virtually no interploidy block was expressed. Reproductive success of crosses between (sexual) autotetraploids and their diploid ancestors varies strongly among taxonomic groups, ranging from complete (Peckert and Chrtek, 2006), partial (Scott *et al.*, 1998; Burton and Husband, 2000) to nearly absent (e.g. Koutecký *et al.*, 2012) interfertility, but diploid-hexaploid crosses – like in our experiments – always failed to produce viable offspring (Scott *et al.*, 1998; Castro *et al.*, 2011). Knowledge of inter-polyploid-fertility is much scarcer, however, it has been observed that a deviation from the “normal”  $2m : 1p$  genome ratio can be tolerated to some extent. Inter-polyploid block seems to be relaxed at least in some autopolyploid complexes (Greiner and Oberprieler, 2012; Laport *et al.*, 2016; Sutherland and Galloway, 2017). Results from crossing experiments in *Campanula rotundifolia* s.l. largely resemble our results from the *S. carniolicus* aggregate; in both studies postzygotic isolation between tetraploids and hexaploids was strongly reduced compared to diploid-tetraploid crosses (Sutherland and Galloway, 2017).

The most straightforward explanation for the failure of diploid-polyploid crosses are developmental defects caused by the triploid block or more general inter-ploidy block (Marks, 1966; Köhler *et al.*, 2010). Alternatively, differences in cross-compatibility among cytotypes may also be a consequence of genetic distance, which is larger between diploid and polyploid *S. carniolicus* than within polyploids (Winkler *et al.*, 2017).

Crosses between diploids and hexaploids, those cytotypes that most frequently co-occur in natural populations, produced almost no viable offspring, thus representing the strongest postzygotic barrier in *S. carniolicus* cytotypes. This result is in line with complete absence of tetraploid hybrids in 29 mixed populations across the entire distribution range in the Eastern Alps as well as in a detailed survey of a diploid-hexaploid contact zone on Mt Sadnig (Hülber *et al.*, 2009). In experimental crosses between diploid and polyploid *S. carniolicus* we observed a multilateral mentor effect (i.e. increased selfing in the presence of foreign pollen; Richards, 1997), which is also known from a few other heteroploid systems (e.g. Peckert and

Chrtek, 2006; Koutecký *et al.*, 2011). This mentor effect possibly countervails the fitness disadvantage of co-occurring lineages caused by foreign pollen (stigma clogging). Factors counteracting fitness disadvantage of the pollen donor due to pollen loss remain elusive. Taking into account nearly complete postzygotic barriers, a supporting mentor effect combined with strong ecological differentiation (see Chapter 4) keeps diploid and hexaploid lineages well separated. Therefore, contact zones can be assumed as evolutionarily stable, which may also explain their ubiquity.

Reproductive isolation between diploids and tetraploids was slightly weaker but still only a few seedlings survived the early life stages. Despite strongly reduced fitness and fertility, triploids might play a determinant role for the maintenance of mixed-ploidy populations (Felber and Bever, 1997). Unfortunately, we have no experimental data concerning performance and fertility of triploid hybrids under natural conditions. Triploids recorded in our range-wide survey (see Chapter 2) were likely the product of matings with unreduced gametes within pure diploid populations, accordingly, triploid hybrids seem to be absent in natural contact zones (detailed study from Mt Rosenstock; Hülber *et al.*, 2015) suggesting a similarly strong reproductive isolation as in diploid-hexaploid contact zones. The scarceness of diploid-tetraploid contact zones is causally linked to the restricted overall distribution of the tetraploid cytotype (see Chapter 2).

The situation within polyploids is contrary. Experimental crosses between tetraploids and hexaploids revealed virtually no reproductive barrier – seed viability and seedling performance parameters were at least intermediate between parental treatments. Therefore, dynamics in tetraploid-hexaploid contact zones differ fundamentally from diploid-polyploid ones. In the survey of 100 populations (see Chapter 2), we found a very low frequency of pentaploids (~0.7%), which on one hand reflects a general scarcity of tetraploid-hexaploid contact zones, on the other hand makes clear that the applied sampling design could not capture the whole truth. An in-depth study recording all target individuals in a tetraploid-hexaploid contact zone on Turracher Höhe (Hülber *et al.*, 2015) showed that pentaploids are locally abundant along a narrow cline in the immediate vicinity of their fully interfertile parents. Depending on frequency, viability, fertility, gene-flow patterns, etc. of hybrids the effect on stability of the contact zone and lineage integrity of parental cytotypes may differ substantially (Felber and Bever, 1997; Burton and Husband, 2000). In the case of

*S. carniolicus* locally abundant pentaploids produce aneuploid but at least partly fertile gametes (FCM of pollen produced by pentaploid individuals revealed consistent 2.5 fold ploidy; unpublished data) mediating introgressive gene flow between tetraploid and hexaploid lineages (Hülber *et al.*, 2015). Results from AFLP fingerprinting suggest a unidirectional gene flow from tetraploids to hexaploids, whereas preliminary data from crossing experiments (reciprocal crosses between tetraploid, pentaploid and hexaploid cytotypes; unpublished data) do not support this pattern. Further detailed investigations are underway. An assumed asymmetric gene flow between lineages could lead to introgression enhancing genetic diversity, transfer of adaptive traits or create new traits favouring niche evolution of the receptor lineage in the long run, impeding the other lineage (Martin *et al.*, 2006; Whitney *et al.*, 2010). The ecological niche of the pentaploid cytotype is more similar to the niche of hexaploids albeit somewhat narrower and overlapping with both parental niches. The lack of transgressive niche characteristics probably accounts for the narrow distribution of hybrids (Hülber *et al.*, 2015).

The ploidy of F1 seeds/seedlings of inter-cytotype crosses partly differed from the expectation of being intermediate. Whereas tetraploid-hexaploid crosses produced almost uniformly pentaploid offspring as expected, diploid-polyploid crosses resulted in a remarkably high cytotype diversity, congruent with observations in experimental crosses in *Campanula rotundifolia* s.l. (Sutherland and Galloway, 2017). F1 ploidy, apart from real hybrids, traces back either to self-fertilization (“mentor effect”) or to the involvement of unreduced gametes. It is well known that unreduced gametes play an important role in primary hybrid zones in the course of the origin and establishment of polyploids (Ramsey and Schemske, 1998), however, their evolutionary implications in secondary contact zones are difficult to predict. According to crossing results in *S. carniolicus*, unreduced gametes seem to play a pronounced role in the presence of heteroploid pollen; however, cytotype distribution in natural contact zones did not resemble this result indicating either low in situ formation and/or survival rates.

In *S. carniolicus* s.l. as in many other polyploid study systems postzygotic isolation is not always complete and additional prezygotic isolation mechanisms (e.g. Segaves and Thompson, 1999; Husband and Schemske, 2000; Nuismer and Cunningham, 2005; Thompson

and Merg, 2008) support the long-term coexistence of polyploid lineages with their ancestor, ultimately facilitating sympatric polyploid speciation.

Habitat differentiation, probably the most important prezygotic isolation mechanism, is well known from many polyploidy complexes (Lumaret *et al.*, 1987; Felber-Girard *et al.*, 1996; Zozomová-Lihová *et al.*, 2015; Laport *et al.*, 2016), yet often investigated on a rather coarse spatial scale. Since mechanisms responsible for local co-occurrence of cytotypes likely act on a microhabitat level, we aimed at identifying microclimatic parameters reflecting differences in ecological niches of cytotypes (see Chapter 4). The first survey of habitat preferences revealed significant differences concerning vegetation cover, rock cover, relative altitude and exposition among the three main cytotypes (see Chapter 2) with an extensive overlap. Landolt indicator values (Diekmann, 2003; Landolt *et al.*, 2010) assigned to vascular plant species accompanying target *S. carniolicus* individuals as surrogate for microhabitat descriptors confirmed habitat differentiation among cytotypes. Niche optima were positioned along a complex ecological gradient, however, overlapping to some degree. Eastern diploids occur mainly in open, wind exposed, cold and well-drained habitats opposed by hexaploids, which are predominately found in densely vegetated habitats with deeper, more humus and nutrient rich soils and higher temperatures. Habitat characteristics of tetraploids are intermediate and western diploids tolerate the driest conditions of *S. carniolicus* cytotypes. The niches of eastern diploids and their polyploid derivatives show a directionality possibly reflecting phenotypic changes in polyploids corresponding with the order of autopolyploidization events. Compared with diploids, hexaploids, though morphologically very variable, are generally taller (Flatscher, 2010), which inherently is connected with higher resource requirements and competitive ability. This is in line with frequent assumptions of increased body size and competitiveness correlated with higher DNA content (Levin, 2002; Parisod *et al.*, 2010). Causality of such ecological and phenotypical relationships is difficult to assess and not generally applicable. However, we can say that niche differentiation is present among *S. carniolicus* cytotypes and likely has played a role in lineage separation, although it is unclear whether this dates back to polyploidization per se or accumulated over time.

Additionally, we hypothesized that niche differentiation is stronger in areas of sympatry (co-occurrence of more than one cytotypes) compared with single cytotype occurrences (i.e.

niche displacement; Peers *et al.*, 2013) based on the concept of character displacement, which predicts character divergence resulting from competition thus minimizing competition (Schluter, 2000; Dayan and Simberloff, 2005; Pfennig and Pfennig, 2009). Our results confirm the hypothesis of niche displacement in sympatry, which strongly depends on the involved cytotype (see Chapter 4). The observed shift in niche optima was always symmetrical rendering competitive superiority of one of the involved cytotypes unlikely (Cooley, 2007). Most strikingly, the niche of hexaploids was shifted when co-occurring with tetraploids and western diploids, but not so with eastern diploids. Complete reproductive isolation (see Chapter 3) and the generally strong habitat differentiation between hexaploids and eastern diploids obviously reduce the selective pressure for further niche shifts, whereas niches of western diploids and hexaploids overlap strongly forcing further niche differentiation. Fully interfertile hexaploids and tetraploids, which have partly overlapping ecological niches, exhibit niche displacement in sympatry as a consequence of competitive interaction. Despite very similar niches, tetraploids and western diploids are not subjected to niche displacement, probably due to their rare co-occurrence. In contrast, niches of tetraploids and eastern diploids, the putative ancestors, still are very similar and further differentiating. This interesting picture of varying strength of niche displacement provides insights into evolutionary processes in contact zones. Niche displacement may be an important and frequent process facilitating cytotype coexistence in secondary contact zones and probably influences niche evolution of lineages in general. However, long-term consequences of niche displacement are not known, because comparisons of pure vs. mixed populations in polyploid systems are rare (but see Grace and Wetzel, 1981; Ståhlberg and Hedrén, 2009; Peers *et al.*, 2013).

## **v. Summary**

This thesis aimed at contributing to the knowledge concerning the role of polyploidy as a trait, which fosters the coexistence of closely related lineages and ultimately contributes to speciation (Wood *et al.*, 2009). The evolutionary significance of autopolyploidy has long been doubted because of its inherent “just more of the same” nature (Parisod *et al.*, 2010). The present study offers an instructive example of lineage diversification in an autopolyploid complex.

A detailed cytogeography of the autopolyploid complex of *S. carniolicus* revealed one of the highest diversities of ploidy levels (diploid to enneaploid) observed within perennial and predominantly outcrossing plant species, however, only diploids, tetraploids and hexaploids (main cytotypes) were widespread across the eastern Alps. The main cytotypes displayed a complex spatial pattern, which likely has been shaped by historical processes (Tribusch and Schönschwetter, 2003), and gave rise to multiple secondary contact zones with locally co-occurring cytotypes.

Experimental studies revealed that, concerning reproductive interaction, among three main cytotypes of *S. carniolicus* s.l. contact zones with entirely different characteristics exist. An almost complete postzygotic isolation governed by a strong inter-ploidy block (Marks, 1966; Köhler *et al.*, 2010) between polyploids and their diploid progenitors, complemented by ecological niche differentiation most likely support a long-term stability of contact zones at least under stable ecological conditions, thus explaining the high number of diploid-hexaploid cytotype mixtures in nature. Whereas interaction between diploids and polyploids is rather limited, contact zones between polyploids are, although rare and spatially restricted to narrow stripes, evolutionary highly active. Genetically less well separated tetraploids and hexaploids are fully interfertile, supporting evidence for a relaxed inter-ploidy block in crosses between polyploid cytotypes (Greiner and Oberprieler, 2012; Laport *et al.*, 2016; Sutherland and Galloway, 2017). At least partially fit hybrids mediate gene flow between parental cytotypes in one or both directions and therefore will be crucial for further lineage evolution and the fate of contact zones (Barton and Hewitt, 1985). Motivated by these results, in depth studies of tetraploid-pentaploid-hexaploid contact zones have been conducted. Molecular data (Hülber *et al.*, 2015) and artificial crosses (Peskoller *et al.*, unpublished) indicate that pentaploids produce partially fertile gametes (including aneuploidy) enabling backcrosses with parents. To draw conclusions about the direction of gene flow and its long-term consequences further investigations are required.

Habitat differentiation, which is regarded as a common prezygotic isolation mechanism in polyploid complexes (e.g. Felber-Girard *et al.*, 1996; Ståhlberg and Hedrén, 2009; Duchoslav *et al.*, 2010) also plays a role in the *S. carniolicus* complex. Additionally, we found niche displacement (Peers *et al.*, 2013), i.e. ecological niches of cytotypes were shifted more pronouncedly in sympatry with other cytotypes than in allopatry, reflecting competition

patterns. Niche displacement, though mainly investigated in invasive species so far (e.g. Treier *et al.*, 2009), may prove as relevant process for stabilizing contact zones and lineage diversification in polyploids.

Reproductive isolation and niche differentiation are among the most important mechanisms responsible for the long-term maintenance of contact zones (Petit *et al.*, 1999) and sufficiently explain the frequent occurrence of diploid-polyploid contact zones. Despite lacking reproductive isolation and similar niche differentiation (compared with diploid-polyploid contact zones) of tetraploids and hexaploids, contact zones are rarely found. This may be caused either by an insufficient sampling density, or by other isolation mechanisms like assortative mating via pollinator preferences (Segraves and Thompson, 1999; Kennedy *et al.*, 2006) and/or flowering time differences (Husband and Schemske, 2000). Since pollinators in alpine habitats show a rather generalistic behaviour (Olesen and Jordano, 2002), their influence likely is marginal. Flowering times generally overlap strongly among the main cytotypes (personal observation); however, subtle differences may exist in the immediate contact of cytotypes (respective data awaiting analyses).

This thesis was part of a multifaceted study of the autopolyploid *S. carniolicus* complex, comprising extensive field studies, molecular and morphological analyses, as well as experimental approaches. Combined results of these investigations – genetical, ecological, reproductive and morphological differentiation – provide strong support for autopolyploidy as important speciation mechanism and justified a taxonomic revision. *S. carniolicus* s.l. is an illustrative example of detecting cryptic species influencing alpha-diversity estimations. Concerning the fact that this study uncovered one of the newly described species (*S. noricus*) as an endemic for Austria, in-depth studies of autopolyploid complexes may have an impact on conservation issues.

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## CHAPTER 2: CYTOTYPE DISTRIBUTION, HYBRIDS AND HABITAT SEGREGATION

### Article 1

**Sonnleitner M**, Flatscher R, Escobar García P, Rauchová J, Suda J, Schneeweiss GM, Hülber K & Schönswetter P. 2010. Distribution and habitat segregation on different spatial scales among diploid, tetraploid and hexaploid cytotypes of *Senecio carniolicus* (Asteraceae) in the Eastern Alps. *Annals of Botany* 106(6): 967–977

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hexaploid *S. carniolicus*



tetraploid *S. carniolicus*



diploid *S. carniolicus*

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## Distribution and habitat segregation on different spatial scales among diploid, tetraploid and hexaploid cytotypes of *Senecio carniolicus* (Asteraceae) in the Eastern Alps

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- **Background and Aims** The spatial distribution of cytotypes can provide valuable insights into evolutionary patterns of polyploid complexes. In a previous study the macro-scale distribution of the three main cytotypes in *Senecio carniolicus* (Asteraceae) within the Eastern Alps was characterized. Employing a roughly 12-fold extended sampling, the present study focuses on unravelling patterns of cytotype distribution on the meso- and microscale and on correlating those with ecological properties of the growing sites.
- **Methods** DAPI flow cytometry of dried samples was used to determine DNA ploidy level in 5033 individuals from 100 populations spread over the entire Eastern Alpine distribution area of *S. carniolicus*. Descriptors of microhabitats as well as spatial data were recorded in the field, and analysed with a mixed-effects ANOVA.
- **Key Results** Extensive variation in DNA ploidy levels (2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x) was detected. Of the main cytotypes, diploids and hexaploids were widespread and had strongly overlapping distributions resulting in the frequent occurrence of cytotype mixtures (half of the investigated populations), whereas tetraploids were disjunctly distributed and occurred in the south-west and the east of the species' distribution area. In spite of the frequent co-occurrence of cytotypes, only 1 % of the samples belonged to secondary cytotypes (3x, 5x, 7x, 8x, 9x). Diploids, tetraploids and hexaploids were altitudinally segregated, but with broad overlap. Similarly, highly significant differences in vegetation and rock cover as well as microhabitat exposure were found between the main cytotypes.
- **Conclusions** *Senecio carniolicus* shows a remarkable diversity of cytotypes. The distribution of the three main cytotypes (2x, 4x, 6x) has been shaped by Pleistocene glaciations to different extents. Whereas tetraploids are nearly entirely restricted to refugia, hexaploids colonized areas that were extensively glaciated. Diploid and hexaploid individuals often co-occur in mixed populations, where they are spatially and ecologically segregated at both the meso-scale (altitudinal differentiation, exposure of the growing site) and the micro-scale (cover of vegetation and bare rock). With regard to the ecological parameters investigated, the tetraploid cytotype occupies an intermediate position. The rareness of secondary cytotypes suggests the presence of strong pre- or post-zygotic mating barriers.

**Key words:** Contact zones, cytotype mixture, Eastern Alps, flow cytometry, habitat segregation, ploidy level, polyploidy, refugia, *Senecio carniolicus*.

### INTRODUCTION

Polyploidization, the multiplication of complete chromosome sets, has played a fundamental role in the evolution and diversification of angiosperms (Soltis *et al.*, 2009). Estimates of its frequency have experienced a steady upward trend, although current discussions are more focused on how many rounds of polyploidization various lineages might have undergone rather than on estimating the percentage of polyploid

angiosperm species (Soltis *et al.*, 2003). Major radiations in the angiosperm tree of life have been temporally and perhaps causally linked to key polyploidization events (Fawcett *et al.*, 2009; Soltis *et al.*, 2009). Formerly believed to be 'a hindrance to the evolutionary success of higher plants' (Stebbins, 1971), polyploidy is now recognized as an important diversifying force in evolutionary history, and one of the important mechanisms of sympatric speciation in land plants (Otto and Whitton, 2000).

Successful long-term establishment of a newly arisen polyploid results in cytotype mixtures, which may either be a transitional state or stable through time. In the latter case, they may form narrow contact zones, eventually comprising only a few populations, as evidenced in *Chamaerion angustifolium* (Husband and Schemske, 2000), *Knautia arvensis* (Kolář *et al.*, 2009), *Melampodium* (Stuessy *et al.*, 2004) and *Ranunculus adoneus* (Baack, 2004). Cytotype mixtures extending over large areas have been less frequently reported, for example for *Galax urceolata* (Burton and Husband, 1999) or *Solidago altissima* (Halverson *et al.*, 2008), and sometimes involved apomixis as in *Arnica cordifolia* (Kao, 2008). They are expected to be maintained by different means of reproductive isolation that prevent the minority cytotype from losing too many gametes in crosses with the majority cytotype, which eventually would lead to its extirpation (minority cytotype exclusion principle; Levin, 1975; Husband, 2000). Reproductive isolation is often conferred by the interaction of several mechanisms, which reduce or even inhibit gene flow between potentially interbreeding populations (Widmer *et al.*, 2009). Pre-zygotic isolating mechanisms such as adaptation to different pollinators, flowering time divergence, ecological or habitat differentiation, as well as the predominance of selfing or apomixis prevent pollination or fertilization and thus the formation of hybrid zygotes (Petit *et al.*, 1999). In contrast, post-zygotic isolating mechanisms concern the viability and reproductive success of hybrid offspring (Orr and Presgraves, 2000; Husband and Sabara, 2004; Rieseberg and Willis, 2007).

Theoretical considerations and empirical studies suggest that the role of inter-cytotype hybrids in mixed stands of different cytotypes is ambiguous. On the one hand, offspring from inter-cytotype crosses are often sterile, producing non-functional, unbalanced gametes, especially when hybridization results in odd ploidy levels (Levin, 1975). Additionally, backcrossing of polyploid plants with either parent most often leads to non-viable progeny due to endosperm malfunction (Köhler *et al.*, 2010). This is often referred to as ‘triploid block’ (Felber, 1991), although comparable outcomes may also be expected for higher-ploidy level crosses. In this case hybridization weakens both parental lineages and potentially drives the rarer cytotype to extinction (Levin, 1975; Husband, 2000). On the other hand, odd-ploid hybrids may not only produce functional gametes with complete chromosome complements (Felber and Bever, 1997; Burton and Husband, 2001), but are also likely to generate an enhanced number of unreduced gametes. Backcrosses with diploid parental lineages may act as additional sources for the recurrent formation of polyploids (Burton and Husband, 2001). Under these conditions, hybrids may mediate the coexistence and stabilize the equilibrium between co-occurring cytotypes (Felber and Bever, 1997).

A good system for studying various aspects of stable cytotype mixtures and incipient speciation processes is *Senecio carniolicus*. This common and abundant high mountain species of the Eastern Alps and the Carpathians was long considered to be uniformly hexaploid, but recent investigations (Suda *et al.*, 2007) revealed a complex pattern with three main cytotypes (di-, tetra- and hexaploid). Although a considerable number of the investigated sample sites contained cytotype mixtures, only very few odd-ploid individuals

(pentaploids, heptaploids) were found, indicating that there are effective mechanisms preventing the formation of inter-cytotype hybrids. Further support for this hypothesis arises from the absence of tetraploids – the potential hybridogenic offspring – from many sample sites containing mixtures of both diploid and hexaploid individuals including Mt Hoher Sadnig, Austria, where roughly 500 individuals have been investigated (Schönswetter *et al.*, 2007; Hülber *et al.*, 2009). To date, mechanisms maintaining cytotype mixtures in *S. carniolicus* are not fully understood, but there is evidence for habitat segregation between diploid and hexaploid cytotypes. Whereas hexaploids are linked to communities with denser vegetation, diploids are growing in open, rocky habitats (Hülber *et al.*, 2009), the greater abundance of which at higher altitudes is probably responsible for the previously found altitudinal separation of cytotypes (Schönswetter *et al.*, 2007). As these hypotheses were based on data collected from a single mountain with diploids and hexaploids only, they need to be tested on a wider geographical scale and also including tetraploids, the third main cytotype.

Here, we explore the cytotype distribution of *S. carniolicus sensu lato* in the Eastern Alps on different spatial scales, i.e. macro-scale (entire Eastern Alps), meso-scale (within each of the 100 investigated single mountains) and micro-scale (immediate environment of each of the roughly 3000 individuals). Employing a moderately increased number of populations and a roughly 12-fold extended sampling of individuals as compared with Suda *et al.* (2007), we test for spatial segregation on all three spatial scales across the entire Eastern Alpine distribution area also including the tetraploid cytotype. Geographical and environmental descriptors representing the meso-scale position (altitude, geographical coordinates) and the ecological micro-site conditions (e.g. rock and vegetation cover) were recorded to address the following questions: (1) Does the macro-scale pattern of the distribution of the main cytotypes (2x, 4x, 6x) change with an increased number of investigated individuals, i.e. are areas which were previously thought to be cytologically uniform in fact heterogeneous? (2) Can the hypothesis of habitat segregation between diploids and hexaploids on the meso- (altitude) and on the micro-scale (dense vegetation and high rock cover, respectively), as established from an in-depth study on a single mountain (Schönswetter *et al.*, 2007; Hülber *et al.*, 2009), be corroborated on a range-wide scale? If at all, how are tetraploids segregated from diploids and hexaploids? (3) Which secondary cytotypes (3x, 5x, >6x) exist, how frequent are they and does the combination of associated main cytotypes give an indication for their origin (hybridogenic or via unreduced gametes)? The results presented form the basis for future molecular investigations exploring the evolution of the intricate polyploid complex of *S. carniolicus*.

## MATERIAL AND METHODS

### *Study species*

*Senecio carniolicus* Willd. (Asteraceae) is a common mountain species endemic to the Eastern European Alps and the Carpathians. It is a herbaceous perennial that inhabits a variety of habitats on siliceous bedrock, such as grasslands,

dwarf shrub communities, stable screes, moraines, rock crevices and fellfields, ranging from the treeline up to altitudes of 3300 m a.s.l. (Reisigl and Pitschmann, 1958).

#### Plant material and recording of environmental descriptors

In summer 2008, 100 sampling sites were visited in Switzerland, Italy, Austria and Slovenia, covering the entire distribution of *S. carniolicus* in the Eastern Alps (Fig. 1, Appendix). The Carpathian distribution was not included because Suda *et al.* (2007) found exclusively hexaploid populations in that area. Leaves of approx. 30 individuals per sampling site were collected spanning the entire local altitudinal range and habitat types and were dried in silica gel. We aimed to collect samples as representative as possible for the population, i.e. areas with high densities of individuals were sampled more intensively than areas with less frequent occurrences. Additionally, we did not favour specific individuals (flowering or particularly big plants) but chose individuals at random. This strategy combined with our inability to distinguish cytotypes in the field at the time of collecting ensured an unbiased sample. Herbarium specimens for each sampling site were deposited in the herbarium of the University of Vienna (WU).

We recorded environmental parameters describing the micro-site, namely exposure and inclination, as well as percentage coverage of rock (> 1 cm) and flowering plants (in the following termed vegetation cover) within a distance of 0.2 m from each sampled individual (in the following referred to as central individuals). Geographical coordinates were recorded

with a GPS system, and GPS-corrected barometric calibration was used for altitude. Furthermore, we determined the number of additional *S. carniolicus* individuals in the same plot (referred to as additional individuals) and sampled – where available – two individuals for ploidy-level estimation.

#### Flow cytometry

DNA ploidy levels of silica-dried leaf tissue were determined using DAPI flow cytometry as described by Suda *et al.* (2007), but using *Pisum sativum* as the sole internal reference standard. Mean fluorescence values of standard and sample never differed more than 3.4-fold, thus being well within the range deemed acceptable by Suda and Leitch (2010). *Vicia faba* was not suitable for flow cytometric analysis of 9x plants because the similarity in genome size with the standard resulted in overlapping peaks. Pooled samples of three *Senecio* individuals were usually analysed. In the case of mixed-ploidy samples or low-quality histograms (i.e. coefficients of variation, CVs, of G0/G1 *Senecio* peaks > 5 %, high background or a low number of intact nuclei forming the peaks), each individual was re-analysed separately. Analyses of all minority cytotypes were repeated 2–5 times on different days to minimize potential instrument instability.

#### Statistical analyses

The following environmental descriptors were used in the analyses: altitude, inclination, exposure, as well as cover of

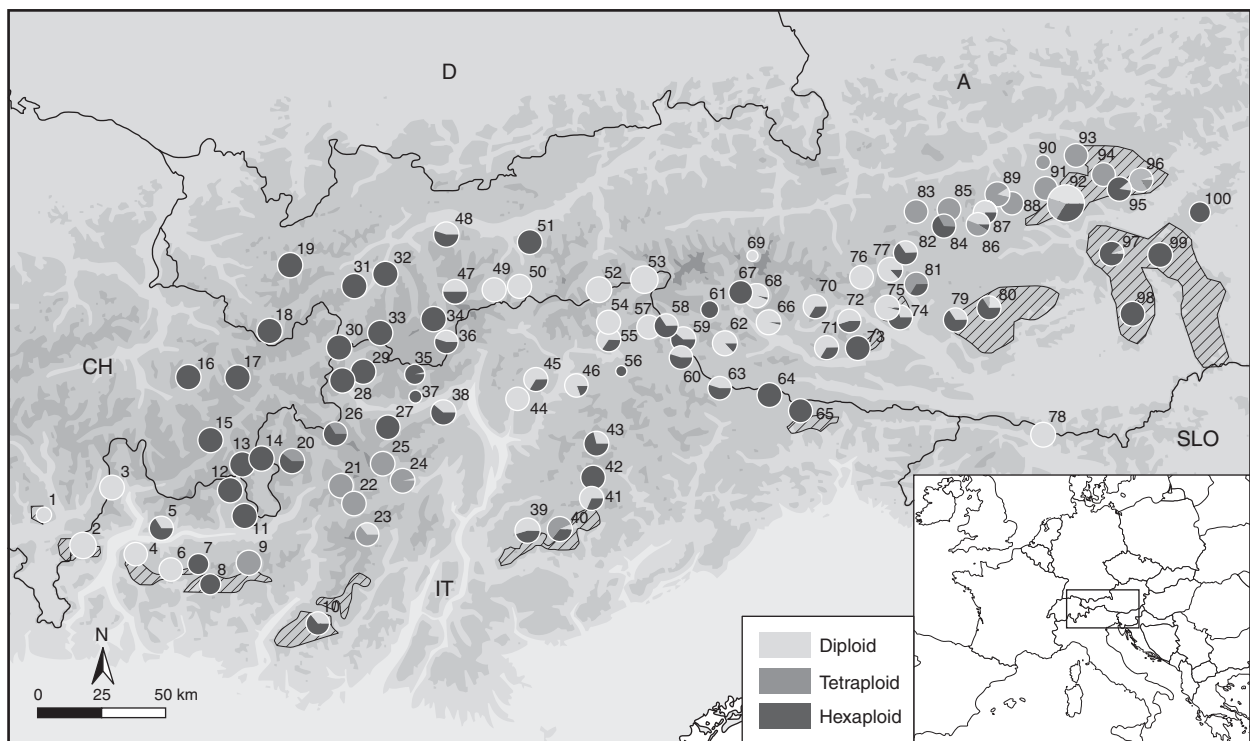


FIG. 1. Distribution of the three main cytotypes of *Senecio carniolicus* in the Eastern Alps based on a sampling of 2914 individuals from 100 sample sites (labelled 1–100). The size of each pie chart is proportional to the number of sampled individuals. Hatched areas indicate presumed glacial refugia on siliceous bedrock (modified from Tribsch and Schönschetter, 2003 and Schönschetter *et al.*, 2005). Details of the populations are given in the Appendix.

rock, flowering plants and cryptogams. Exposure was determined in degrees ( $0-360^\circ$ ) and subsequently transformed ('Northing', 'Easting') to values between 0 (North or East, respectively) and 1 (South or West, respectively). In order to account for different altitudinal ranges of the alpine and subnival zones in different parts of the Alps, relative altitude was used, which is defined as the altitudinal distance from the local tree line (extracted from topographical maps with scales of 1:25 000 or 1:50 000). Except for altitude, each descriptor was arc-sine transformed to approximate a normal distribution. To test for significant differences among the three main cytotypes, a mixed-effects ANOVA was applied separately to each environmental descriptor. We used linear fixed-effects models computed by the glmer-function included in the lme4 library (Bates and Sarkar, 2008) of R (R Development Core Team, 2008). We used the environmental descriptor as response, the main cytotypes as categorical predictor and the sampling site as grouping variable to calculate random intercepts. With this approach we accounted for potential spatial autocorrelation among individuals from the same sample site. For all models a Gaussian error distribution was assumed. Degrees of freedom of the models were defined as the number of observations minus the number of estimated parameters (i.e. 3) minus 1, assuming that all levels of the grouping variable consume one degree of freedom. Differences in the number of additional individuals among cytotypes were tested using the same model structure, but assuming a Poisson error distribution.

In order to evaluate potential clustering of cytotypes within sampling sites, pairwise Euclidean distances among individuals of each mixed population were calculated using GPS coordinates and altitude. The proportion of pairwise distances between plants of the same cytotype was computed relative to the total number of distances within each of the following distance classes:  $<10$  m, 10–100 m, 100–1000 m and  $>1000$  m. Finally, presence or absence of additional individuals (see above) was used as a criterion to define the lowest distance class (0–0.2 m).

## RESULTS

### *Occurrence, distribution and frequency of cytotypes*

DNA ploidy levels were estimated from 5033 plants (2914 central individuals and 2119 additional individuals; Appendix, and Supplementary Data Table S1, available online). Flow cytometric analyses mostly yielded high-resolution histograms, with average sample CV of 3.37 % (range 1.08–7.71 %) and average standard CV of 2.63 % (range 1.03–6.46 %). The arbitrary threshold of 5.0 % was achieved in 92.6 and 98.0 % of sample and standard runs, respectively. Only small between-day variation (4.1 % maximum) was observed when the same sample was re-analysed. Collectively, these measures of quality indicate that the recorded fluorescence values are stable and allow a reliable estimation of DNA ploidy levels.

Eight distinct groups of fluorescence intensities were obtained (see Supplementary Data Table S2, Figs S2 and S3). Most samples matched the fluorescence values reported by Suda *et al.* (2007) for karyologically counted diploid

( $2n = 2x = 40$ ), tetraploid ( $2n = 4x = 80$ ) and hexaploid ( $2n = 6x = 120$ ) individuals. Five additional groups corresponded to DNA-pentaploids, DNA-heptaploids and previously unknown DNA-triploids, putative DNA-octoploids, and DNA-enneaploids. The relative monoploid genome size varied only weakly among the ploidy levels (see Supplementary Data Table S2). Slightly lower fluorescence values per monoploid genome observed in DNA-octoploids together with their rarity (only two individuals found in one population) leave room for some uncertainty in the inferred ploidy. Most plants belonged to DNA-hexaploids (49.6 %), whereas DNA-diploids and DNA-tetraploids made up 33.8 and 15.6 % of the samples, respectively. Secondary cytotypes were found in 27 sample sites: six DNA-triploids, 37 DNA-pentaploids, four DNA-heptaploids, two DNA-octoploids and five DNA-enneaploids, totalling 54 individuals (1.07 %). For simplicity, we will refer to the different cytotypes from here on without using the prefix 'DNA-'.

The distribution of the three main cytotypes of *S. carniolicus* is complex, showing some degree of large-scale spatial segregation with large areas of overlap and hence frequent cytotype mixture (Fig. 1). Di- and hexaploid individuals are widespread throughout most of the distribution range. Hexaploids are absent from the western-most Hohe Tauern and from the south-western and south-eastern distribution margin around Lago di Como and in the Karawanken/Karavanke, respectively, where only diploids occur. The Central Alps around the upper Inn valley are exclusively populated by hexaploids and correspond to a large break in the distribution of diploids. Tetraploids were found in two disjunct areas, whose centres are located in the Niedere Tauern in the East and in the Ortler and Adamello massifs in the west. Roughly half of the sample sites (44 %) contained more than one main cytotype. All possible combinations of cytotypes were found with  $2x/6x$  mixtures being most frequent (28 % of sample sites), whereas  $2x/4x$  and  $4x/6x$  sample sites were rare (3 and 5 %, respectively). In 8 % of the sample sites, all three main cytotypes occurred. Spatial co-occurrence patterns of cytotypes within selected mixed populations are shown in the Supplementary Data (Fig. S1).

Many sampled individuals, irrespective of ploidy level, had no conspecific neighbours within a 0.2-m radius, but in sample plots with open habitats or vegetation gaps numerous additional individuals were sometimes encountered. The number of additional individuals did not differ ( $P = 0.371$ ) between tetraploids and hexaploids (both with median 0 additional individuals), whereas diploids had significantly more neighbours (1 individual as median) compared with other main cytotypes ( $P < 0.001$  for both).

Secondary cytotypes were scattered over the distribution area both in mixed and in otherwise pure sample sites. Their occurrence depended on the composition of main cytotypes within sample sites (Table 1). Among secondary cytotypes, pentaploids were most frequent (37 individuals in 14 populations) and occurred with the exception of a single population (PLE; for details see Appendix) exclusively in populations comprising tetraploids. More than one-quarter of all pentaploids were found in otherwise purely tetraploid populations. Triploids were considerably rarer (six individuals in four populations) and were only found in sample sites where diploid individuals were present, either in pure diploid populations

TABLE 1. Abundance of secondary cytotype individuals of *Senecio carniolicus* in relation to the presence of di-, tetra- and hexaploid individuals within sampling sites

| Main cytotypes within sampling sites | No. of sampling sites | No. of individuals | No. of secondary cytotype individuals (no. of sampling sites) |            |            |           |            |
|--------------------------------------|-----------------------|--------------------|---------------------------------------------------------------|------------|------------|-----------|------------|
|                                      |                       |                    | Triploid                                                      | Pentaploid | Heptaploid | Octoploid | Enneaploid |
| Diploid                              | 15                    | 711                | 3 (3)                                                         |            |            |           |            |
| Di-, tetraploid                      | 3                     | 162                |                                                               | 4 (1)      |            |           |            |
| Di-, tetra-, hexaploid               | 8                     | 467                | 3 (1)                                                         | 8 (4)      | 1 (1)      |           |            |
| Di-, hexaploid                       | 29                    | 1595               |                                                               | 3 (1)      | 1 (1)      | 2 (1)     | 2 (2)      |
| Tetraploid                           | 10                    | 403                |                                                               | 10 (5)     |            |           |            |
| Tetra-, hexaploid                    | 5                     | 229                |                                                               | 12 (3)     |            |           |            |
| Hexaploid                            | 30                    | 1466               |                                                               |            | 2 (2)      |           | 3 (3)      |
| Total                                | 100                   | 5033               | 6 (4)                                                         | 37 (14)    | 4 (4)      | 2 (1)     | 5 (5)      |

or in mixed populations comprising all three main cytotypes. Hepta-, octo- and enneaploid plants were exclusively found in association with hexaploids.

Close relationships of each secondary cytotype to a single main cytotype were also encountered on the micro-scale. Within a distance of 0.2 m, triploids were exclusively associated with diploids (three individuals) and pentaploids grew close to themselves (ten individuals) and/or to tetraploids (20 individuals) with the exception of a single individual located close to a diploid. Similarly, hepta-, octo- or enneaploids occurred next to individuals with a ploidy level equal to or higher than hexaploid (seven individuals).

#### Ecological differentiation among cytotypes

Cytotypes were spatially clustered within mixed sampling sites. While 99.98 % of all pairwise distances < 0.2 m involved individuals of the same ploidy level ( $n = 2088$  comparisons), this proportion decreased to 93.6 % ( $n = 513$ ) in the distance class < 10 m, 78.5 % ( $n = 2401$ ) for 10–100 m, 58.3 % ( $n = 12\,358$ ) for 100–1000 m and 37.0 % ( $n = 4879$ ) for distances > 1000 m.

Pairwise comparisons among the three main cytotypes revealed highly significant differences for most of the environmental descriptors investigated (Fig. 2). Diploids, tetraploids and hexaploids differed significantly in vegetation and rock cover as well as in the relative altitude ( $P < 0.001$  in all cases) while northing discriminated between tetraploids and the other cytotypes but not between diploids and hexaploids. Local inclination, cryptogam cover and East/West exposure had no significant explanatory value. Cover of rock and of vegetation displayed an inverse relationship and revealed largely clear differences between diploid and hexaploid individuals (Fig. 3). Most diploids occupied rocky micro-sites with low vegetation cover, whereas hexaploids were generally associated with high vegetation cover. Tetraploids displayed an intermediate behaviour with regard to rock cover, while vegetation cover was similar to those of hexaploids. Both diploids and hexaploids covered a large altitudinal range with hexaploids centred at lower altitudes than diploids which ascended to the highest relative altitudes sampled. Tetraploids showed a narrower vertical distribution but highest average values. Furthermore, tetraploids were more frequent on northerly exposed slopes, whereas di- and hexaploids appeared to be more indifferent with respect to exposure.

#### DISCUSSION

Diploids, tetraploids and hexaploids, the main cytotypes encountered in *Senecio carniolicus*, are partially segregated on the macro-scale, i.e. throughout the whole Alpine range, but overlap strongly. The resulting frequent occurrence of cytotype mixtures enables us to explore patterns and mechanisms of spatial and ecological segregation within roughly half of the 100 investigated sample sites (meso-scale) as well as with respect to the immediate vicinity of about 3000 investigated individuals (micro-scale).

#### The macro-scale: cytogeography across the Eastern Alps

The distribution pattern of diploid, tetraploid and hexaploid cytotypes throughout the Eastern Alps was found to be remarkably complex (Suda *et al.*, 2007). The approximately 12-fold increase of overall sample size here compared with the previous study resulted only in minor changes in the large-scale pattern of cytotype distribution (e.g. in the Niedere Tauern, thought to be dominated by tetraploids, diploids are widespread, but occur nearly exclusively south of the main chain), but the proportion of mixed sample sites, mostly di- and hexaploids, rose from about one-third to nearly a half (Fig. 1). Range disruption and survival of at least the last cold stage of the Pleistocene in disjoint refugia appear to have played an important role in shaping the current cytotype distribution. Areas previously suggested to be glacial refugia based on molecular data as well as on species distribution patterns (Tribusch and Schönschwetter, 2003; Schönschwetter *et al.*, 2005) match regions with high main cytotype diversity. Additionally, the two main regions comprising only diploids (populations 1–4 and 6 from the south-western part and population 78 from Karawanken/Karavanke; Fig. 1) overlap with presumed refugia, a pattern in line with early concepts of presumably relictual diploids being mostly found in unglaciated areas (Ehrendorfer, 1958). The seemingly contradictory exclusive occurrence of hexaploids in the only weakly glaciated (van Husen, 1987) refugial area of the easternmost margin of the Alpine distribution (populations 98–100) fits well with the early recognized poverty of its alpine flora (Scharfetter, 1909) and is probably related to the current scarcity of suitable habitats for the high-altitude adapted (see below) diploid and tetraploid cytotypes, which might have become extirpated due to habitat loss as vegetation zones ascended after the

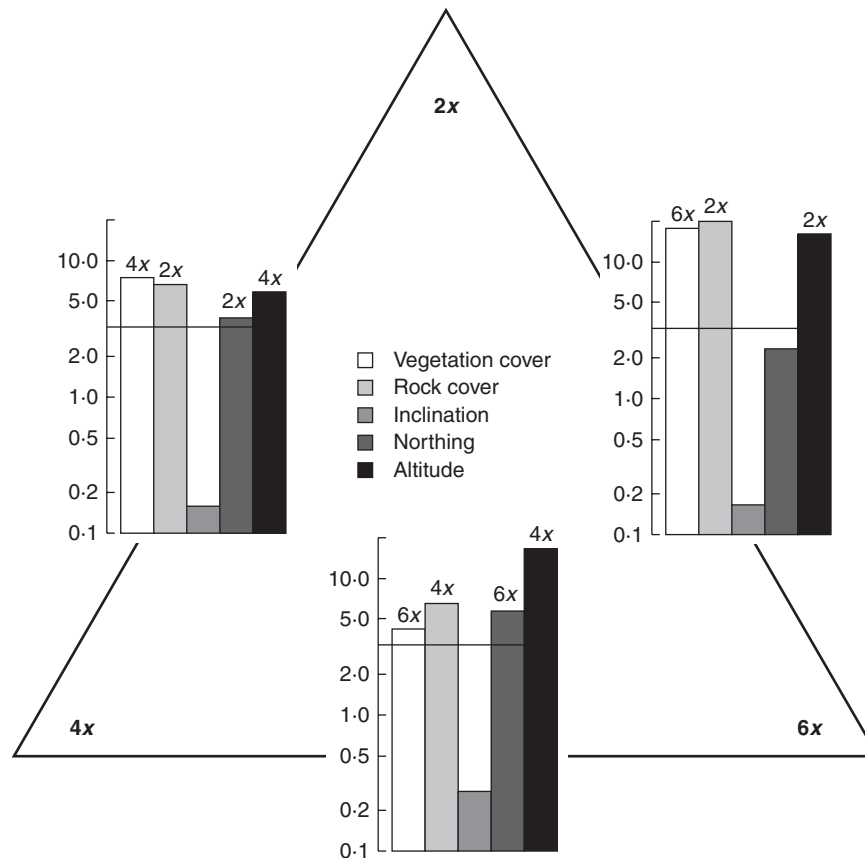


FIG. 2. Pairwise comparisons of five environmental descriptors among the three main cytotypes of *Senecio carniolicus*. Bars represent the absolute value of the *t*-statistic derived from linear mixed-effects models as a measure of the difference between the cytotypes. Black lines correspond to a significance level of  $P = 0.001$ . Above the bars the cytotype with the significantly greater values of the descriptor is given.

last glaciation. Furthermore, both disjoint distribution areas of the tetraploid cytotype, separated by a gap of  $>180$  km, correspond to putative glacial refugia (Schönschwetter *et al.*, 2003, 2005; Tribsch and Schönschwetter, 2003) and are characterized by a large number of rare or endemic plant taxa (Schneeweiss and Schönschwetter, 1999; Tribsch, 2004). Consequently, the distribution pattern of this cytotype was probably shaped by extensive habitat loss during cold stages of the Pleistocene and weak colonization ability after the last glacial period. This contradicts widespread assumptions about colonizing and competitive success of polyploids as compared with diploids (Stebbins, 1984, 1985; Otto and Whitton, 2000; Otto *et al.*, 2007). It is unclear which traits of the tetraploids are limiting their success in dispersal and population establishment. Ongoing experiments suggest delayed germination and lower seedling viability of tetraploids (M. Sonnleitner and R. Flatscher, unpubl. res.), which may at least in part be responsible for their weaker colonizing ability. In contrast to tetraploids, the hexaploid cytotype is the most widespread and the one exclusively found in the north-western part of the distribution range, which was the most intensively glaciated area of the Eastern Alps during the Last Glacial Maximum (van Husen, 1987), in agreement with the above-mentioned hypotheses of polyploids being superior colonizers of novel habitats as compared with diploids. Underlying

factors include greater plasticity and adaptation potential due to gene redundancy and the massive genomic restructuring processes triggered by whole-genome duplication (reviewed by Otto and Whitton, 2000; Wendel, 2000; Hegarty and Hiscock, 2008; Leitch and Leitch, 2008), but the precise nature of the factors responsible for the higher colonization ability of the hexaploids, such as better dispersal capabilities or higher competitiveness, remains to be established.

#### Segregation on meso- and micro-scales

The high frequency of mixed-cytotype populations offers the possibility to test for spatial patterns on smaller geographical scales. Previous studies within a single model population in the Austrian Alps have provided evidence for spatial segregation of diploid and hexaploid cytotypes on meso- as well as micro-scales (Schönschwetter *et al.*, 2007; Hülber *et al.*, 2009). The present study allows a generalization of these patterns for the entire Eastern Alps and provides a first characterization of the habitat requirements of the tetraploid cytotype.

Individuals sharing the same cytotype are spatially strongly aggregated in mixed sampling sites. Whereas virtually no cytotype mixture was encountered within a radius of 0.2 m, variation increased with distance and in the distance class of  $>1000$  m only slightly more than one-third of all pairwise

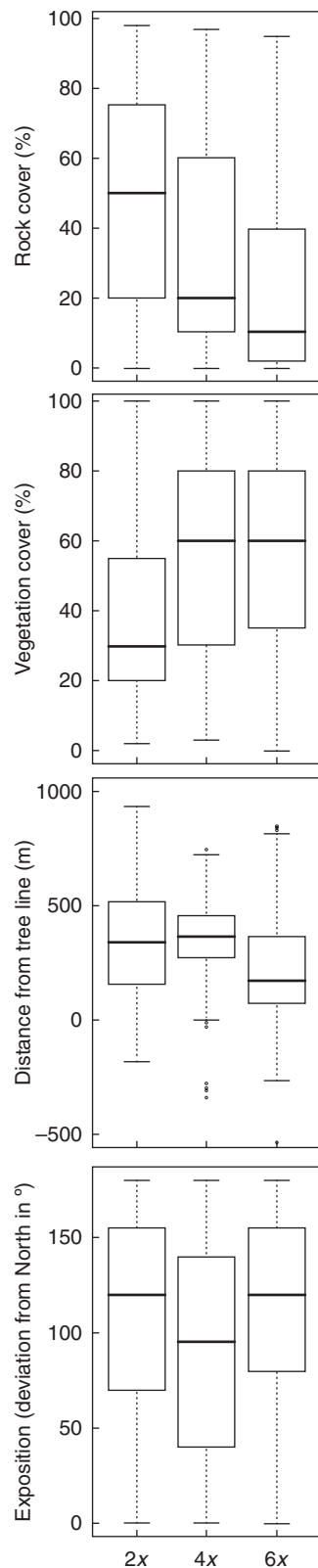


FIG. 3. Comparison of the main cytotypes of *Senecio carniolicus* with respect to rock cover, vegetation cover, altitudinal distance to the local tree line and north/south exposure of the microhabitat. Boxes span the range between the 25th and 75th percentile with indicated median, and whiskers extend to 1.5-fold the interquartile range. Outliers are represented by open circles.

distances involved individuals of the same ploidy level. This pattern may be explained by a correlation between spatial proximity and microhabitat similarity, i.e. neighbouring individuals are more likely to share a similar environment than more distant individuals. Alternatively, clustering of individuals may be caused by dispersal limitation. Although the fruits of *S. carniolicus* are anemochorous, dense flocks of seedlings around adult plants are frequently observed in the field (M. Sonnleitner and R. Flatscher, pers. obs.). This is in line with dispersal kernels of plant diaspores usually reaching their greatest density in the immediate surroundings of the mother plant (Nathan, 2006).

Di- and hexaploid cytotypes of *S. carniolicus* were suggested to be adapted to open, rocky microhabitats and to dense grass swards or dwarf shrub communities, respectively (Schönswetter *et al.*, 2007; Hülber *et al.*, 2009). Our results confirm this as a general pattern over the entire distribution area (Fig. 2). The low-growing diploids are mainly found in rocky habitats with sparse and prostrate vegetation or single grass tussocks and in open, heavily cryoperturbed fellfields (as described by Ellenberg, 1996). In contrast, the larger and potentially more competitive hexaploids populate habitats characterized by high vegetation and low rock cover. These habitat preferences correlate with distinct characteristics of the two cytotypes, especially differences in plant height and leaf length (R. Flatscher *et al.*, unpubl. res.). Our results are in line with other case studies reporting higher productivity and competition ability of polyploids as compared with their diploid progenitors (Lumaret *et al.*, 1987; Lindner and Garcia, 1997; Petit and Thompson, 1997). Consequently, polyploids were often shown to inhabit more nutrient-rich communities with dense vegetation, whereas diploids tend towards more open habitats (Ståhlberg, 2009) where competition for essential resources is reduced and abiotic stress becomes the limiting factor.

Diploid and hexaploid cytotypes are centred at different altitudes, albeit with considerable overlap (Figs 2 and 3) and, in accordance with previous findings (Schönswetter *et al.*, 2007), diploids generally ascend higher than hexaploids (see next paragraph for a discussion on the tetraploids). The observed altitudinal differentiation could either be a direct consequence of altitude and related changes in abiotic parameters such as lower temperatures, lower CO<sub>2</sub> partial pressure, higher irradiation and UV stress (Körner, 2003). Alternatively, it may reflect that, as a general rule, the extent of open habitats with sparse vegetation increases with altitude while dense grasslands and dwarf shrub vegetation decline (Körner, 2003). This correlation probably reconciles the two competing hypothesis for segregation of di- and hexaploid cytotypes of *S. carniolicus*, i.e. altitude (Schönswetter *et al.*, 2007) and openness of the vegetation (Hülber *et al.*, 2009).

Definition of the ecological niche of the tetraploids is not straightforward. Their altitudinal niche of the tetraploids is not straightforward. Their altitudinal amplitude was narrower and significantly higher than those of the other two cytotypes (Figs 2 and 3). With regard to rock and to vegetation cover, tetraploid *S. carniolicus* occupies an intermediate position between di- and hexaploids. Vegetation cover is similarly high, but still statistically significantly smaller than that found in hexaploids, and rock cover is significantly lower than in diploids (Figs 2 and 3). However, tetraploids are more frequent on or sometimes even restricted to north-facing

slopes, whereas di- and hexaploids seemed to be more indifferent towards exposure direction (Figs 2 and 3). Furthermore, tetraploids have a tendency towards slightly base-rich soils (P. Escobar García *et al.*, pers. obs.) whereas diploids and hexaploids are strictly acidophilic. The single exception is the disjunct diploid population 78 in the Karawanken/Karavanke range, where *S. carniolicus* grows on dolomite bedrock, which may be a product of local adaptation processes facilitated by its isolated position.

#### *Secondary cytotypes: occurrence and possible evolutionary implications*

Secondary cytotypes, i.e. tri-, penta-, hepta-, octo- and enneaploids, were encountered in low frequencies only (approx. 1 % altogether). This number is comparable with previous results of Suda *et al.* (2007) but based here on a more than 12-fold increased sample size; tri-, octo- and enneaploids are reported here for the first time for *S. carniolicus*. It should be noted that ploidy levels in our study were inferred from nuclear DNA amounts (DNA ploidy levels; Suda *et al.*, 2006) only because the rarity of secondary cytotypes in the field prevents their cytological investigation. *S. carniolicus* represents the most salient example of high intraspecific ploidy variation (occurrence of eight DNA ploidy levels) ever recorded in a sexually reproducing angiosperm with monocentric chromosomes. Disregarding aneuploid series (e.g. Lewis *et al.*, 1967; Murray and Young, 2001), no more than six different euploid chromosomal levels have previously been observed for the same species (Marhold *et al.*, 2010). Note, however, that the number of cytotypes found in a taxon is of course dependent on the adopted taxonomic framework. Future potential recognition of several taxa within *S. carniolicus* will reduce the number of intraspecific cytotypes. Regardless, the present data clearly demonstrate that ploidy diversity in natural populations is much higher than originally believed and that representative cytotype screening (most conveniently done using flow cytometry) can reshape our perception of the magnitude and dynamics of genome duplication in the wild.

Crosses involving unreduced gametes and hybridization between different cytotypes are commonly put forward as mechanisms to explain the formation of secondary cytotypes (Ramsey and Schemske, 1998). As in other groups (Ramsey, 2007), unreduced gametes are rarely formed in *S. carniolicus* (up to 0.6 % as judged from pollen diameter; Fössinger, 2010). Nevertheless, a significant role of unreduced gametes in the origin of secondary cytotypes is suggested by (1) the tight association of secondary cytotypes with their putative parental main cytotypes (3x with 2x; 5x with 4x; >6x with 6x) on the micro-scale (see Results), (2) the existence of 7x, 8x and 9x cytotypes as well as (3) the occurrence of triploids in geographically isolated purely diploid populations (populations 6, 49, 76) and of pentaploids in purely tetraploid populations (ten of 27 populations). The latter two cases may, however, also be the result of hybridization, involving a higher-ploid parent not sampled, no longer existing or growing on a different mountain massif (i.e. long-distance pollen transfer). Further data will be necessary to establish

the relative contributions of these mechanisms to the origin of secondary cytotypes in *S. carniolicus*.

## CONCLUSIONS AND OUTLOOK

Even if in *S. carniolicus* the 12-fold increase in the number of investigated individuals does not significantly affect the cyto-geographical pattern described previously (Suda *et al.*, 2007), only large data sets such as that presented here with a sampling scheme which is both intensive (many plants per site) and extensive (many sites throughout the whole distribution area; Halverson *et al.*, 2008) allow for a synthetic approach addressing questions about polyploid origin, their morphological, ecological or reproductive differentiation as well as the mechanisms of polyploid speciation.

We have shown that the three main cytotypes of *S. carniolicus* (2x, 4x, 6x) exhibit a complex distribution pattern with an unusually high proportion of contact areas with varying spatial extent. As causes for this distribution pattern we suggest historical processes connected to Pleistocene climatic fluctuations on the macro-scale and ecological divergence on the meso- and micro-scales. As habitat segregation does not prevent the occurrence of different cytotypes in close spatial proximity, other pre- or post-zygotic isolating mechanisms need to be invoked to explain the obviously strong reproductive isolation. These are the subject of current ongoing research that combines molecular data, to enable the reconstruction of spatio-temporal evolutionary diversification of *S. carniolicus*, with experimental approaches, to explore the mechanisms maintaining cytotype mixtures.

## SUPPLEMENTARY DATA

Supplementary Data are available online at [www.aob.oxfordjournals.org](http://www.aob.oxfordjournals.org) and consist of the following. Table S1: Relative fluorescence intensities of DAPI-stained nuclei of 5033 silica gel-dried leaf samples of *Senecio carniolicus* from 100 sample localities. Table S2: Mean relative fluorescence intensities (per monoploid genome) of individual ploidy levels of *Senecio carniolicus* in the present study and in previous work (Suda *et al.*, 2007). Fig. S1: Spatial distribution of individuals of *Senecio carniolicus* within selected mixed populations. Fig. S2: Mean relative fluorescence intensities of DNA-diploid, DNA-triploid, DNA-tetraploid, DNA-pentaploid and DNA-hexaploid samples of *Senecio carniolicus*. Fig. S3: Mean relative fluorescence intensities of DNA-hexaploid, DNA-heptaploid, DNA-octoploid and DNA-enneaploid samples of *Senecio carniolicus*.

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Italy: Autonome Provinz Bozen – Südtirol/Provincia Autonoma di Bolzano – Alto Adige (63-01-05/298143); Switzerland: Ufficio della natura e del paesaggio – Cantone Ticino (s.n., dated 26 March, 2008), Amt für Natur und Umwelt – Kanton Graubünden (La 7-73/5807). We thank Hanna Weiss-Schneeweiss for helpful discussions, and Daniela Stawik, Christian Gilli, Bozo Frajman and Manfred Schmucker for help with fieldwork.

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## APPENDIX

Overview of the 100 sample sites where material of *Senecio carniolicus* was collected for the present study. Geographical position (centroid of the individual coordinates) and altitudinal range of sampled individuals are given as well as DNA ploidy levels with the number of individuals per ploidy level (*n*) and the total number (*N*) of individuals investigated per sample site. Herbarium vouchers stored in WU are labelled with the population code. Country abbreviations: A, Austria; CH, Switzerland; IT, Italy; SLO, Slovenia.

| Code | Locality (Country)             | Geographical position |                | Altitudinal<br>range (m a.s.l.) | <i>n</i> samples per DNA ploidy level |    |    |    |    |    |    |    |   | <i>N</i> |
|------|--------------------------------|-----------------------|----------------|---------------------------------|---------------------------------------|----|----|----|----|----|----|----|---|----------|
|      |                                | Latitude (°N)         | Longitude (°E) |                                 | 2x                                    | 3x | 4x | 5x | 6x | 7x | 8x | 9x |   |          |
| 1    | Cima dell Uomo (CH)            | 46-231                | 8-940          | 2249–2358                       | 16                                    |    |    |    |    |    |    |    |   | 16       |
| 2    | Pizzo di Gino (IT)             | 46-123                | 9-144          | 1864–2226                       | 63                                    |    |    |    |    |    |    |    |   | 63       |
| 3    | Campanile di Val Marina (IT)   | 46-333                | 9-294          | 2134–2495                       | 49                                    |    |    |    |    |    |    |    |   | 49       |
| 4    | Monte Legnone (IT)             | 46-093                | 9-417          | 2122–2591                       | 47                                    |    |    |    |    |    |    |    |   | 47       |
| 5    | Passo Locino (IT)              | 46-188                | 9-549          | 2110–2553                       | 19                                    |    |    |    |    | 32 |    |    |   | 51       |
| 6    | Monte Verrobbio (IT)           | 46-039                | 9-602          | 2044–2083                       | 41                                    | 1  |    |    |    |    |    |    |   | 42       |
| 7    | Cima Cadelle (IT)              | 46-059                | 9-743          | 2112–2482                       |                                       |    |    |    |    | 43 |    |    |   | 43       |
| 8    | Laghi Gemelli (IT)             | 45-986                | 9-805          | 2142–2330                       |                                       |    |    |    |    | 21 |    |    |   | 21       |
| 9    | Pizzo di Coca (IT)             | 46-064                | 10-003         | 2174–2724                       |                                       |    |    | 52 |    | 1  |    |    |   | 53       |
| 10   | Monte Colombine (IT)           | 45-845                | 10-359         | 2095–2212                       | 22                                    |    |    |    |    | 39 |    |    |   | 61       |
| 11   | Bocchetta Vicima (IT)          | 46-229                | 9-968          | 2242–2689                       |                                       |    |    |    |    | 61 |    |    |   | 61       |
| 12   | Bocchetta della Forbici (IT)   | 46-326                | 9-904          | 2175–2673                       |                                       |    |    |    |    | 65 |    |    |   | 65       |
| 13   | Diavolezza, Munt Pers (CH)     | 46-420                | 9-971          | 2127–3162                       |                                       |    |    |    |    | 66 |    |    |   | 66       |
| 14   | Monte Vago (IT)                | 46-439                | 10-070         | 2313–3055                       |                                       |    |    |    |    | 71 |    |    | 1 | 72       |
| 15   | Piz Nair (CH)                  | 46-507                | 9-804          | 2355–2991                       |                                       |    |    |    |    | 64 |    |    |   | 64       |
| 16   | Sandhubel (CH)                 | 46-732                | 9-687          | 2346–2697                       |                                       |    |    |    |    | 68 |    |    |   | 68       |
| 17   | Flüela Schwarzhorn (CH)        | 46-733                | 9-650          | 2347–3093                       |                                       |    |    |    |    | 52 |    |    |   | 52       |
| 18   | Hohes Rad (AT)                 | 46-899                | 10-112         | 2120–2910                       |                                       |    |    |    |    | 52 |    |    |   | 52       |
| 19   | Arlensattel (AT)               | 47-137                | 10-221         | 2073–2162                       |                                       |    |    |    |    | 60 |    |    |   | 60       |
| 20   | Monte Verva (IT)               | 46-431                | 10-228         | 2098–2967                       |                                       |    |    | 18 | 5  | 37 |    |    |   | 60       |
| 21   | Monte di Gavia (IT)            | 46-342                | 10-483         | 2404–3077                       |                                       |    |    | 41 | 1  |    |    |    |   | 42       |
| 22   | Monte Serodine (IT)            | 46-276                | 10-550         | 2476–2629                       |                                       |    |    | 52 |    |    |    |    |   | 52       |
| 23   | Cresta del Belvedere (IT)      | 46-164                | 10-615         | 2305–2581                       | 14                                    |    |    | 29 |    |    |    |    |   | 43       |
| 24   | Cima Valletta (IT)             | 46-356                | 10-803         | 2260–2821                       | 1                                     |    |    | 58 | 4  |    |    |    |   | 63       |
| 25   | Cima Cavaion (IT)              | 46-420                | 10-701         | 2386–2826                       |                                       |    |    | 56 | 1  |    |    |    |   | 57       |
| 26   | Stilfser Joch (IT)             | 46-528                | 10-453         | 2539–3078                       |                                       |    |    | 14 |    | 41 |    |    |   | 55       |
| 27   | Laaser Spitze (IT)             | 46-550                | 10-729         | 2272–2850                       |                                       |    |    |    |    | 59 |    |    |   | 59       |
| 28   | Watles (IT)                    | 46-719                | 10-494         | 2161–2551                       |                                       |    |    |    |    | 60 |    |    |   | 60       |
| 29   | Steinmandlköpf, Mittereck (IT) | 46-749                | 10-598         | 2347–2864                       |                                       |    |    |    |    | 53 |    |    |   | 53       |
| 30   | Piz Lad (IT)                   | 46-840                | 10-475         | 2227–2740                       |                                       |    |    |    |    | 67 | 1  |    | 1 | 69       |
| 31   | Fisser Joch, Brunnenkopf (AT)  | 47-061                | 10-562         | 2520–2670                       |                                       |    |    |    |    | 53 | 1  |    |   | 54       |
| 32   | Hohe Aifnerspitze (AT)         | 47-103                | 10-724         | 2265–2705                       |                                       |    |    |    |    | 44 |    |    |   | 44       |
| 33   | Riffeljoch (AT)                | 46-891                | 10-694         | 2380–2950                       |                                       |    |    |    |    | 54 |    |    |   | 54       |
| 34   | Gaislachkogel (AT)             | 46-937                | 10-976         | 2290–2955                       |                                       |    |    |    |    | 51 |    |    | 1 | 52       |
| 35   | Schröfwand (IT)                | 46-739                | 10-874         | 2398–2730                       | 2                                     |    |    |    |    | 42 |    |    |   | 44       |
| 36   | Festkogel (AT)                 | 46-855                | 11-041         | 2170–3040                       | 25                                    |    |    |    |    | 24 |    |    |   | 49       |
| 37   | Vermoispitze (IT)              | 46-659                | 10-875         | 2486–2875                       |                                       |    |    |    |    | 9  |    |    |   | 9        |
| 38   | Naturnser Hochwart (IT)        | 46-601                | 11-022         | 2201–2605                       | 21                                    |    |    |    |    | 44 |    | 2  | 1 | 68       |
| 39   | Monte Ziolera (IT)             | 46-170                | 11-450         | 2035–2747                       | 40                                    |    |    |    |    | 28 |    |    |   | 68       |

Continued

## Continued

| Code | Locality (Country)               | Geographical position |                |                              | <i>n</i> samples per DNA ploidy level |    |    |    |    |    |    |    |     | <i>N</i> |
|------|----------------------------------|-----------------------|----------------|------------------------------|---------------------------------------|----|----|----|----|----|----|----|-----|----------|
|      |                                  | Latitude (°N)         | Longitude (°E) | Altitudinal range (m a.s.l.) | 2x                                    | 3x | 4x | 5x | 6x | 7x | 8x | 9x |     |          |
| 40   | Cima D'Asta (IT)                 | 46-172                | 11-616         | 1969–2847                    | 4                                     |    | 42 | 2  | 22 |    |    |    | 70  |          |
| 41   | Cavallazza Piccola (IT)          | 46-282                | 11-782         | 2020–2316                    | 26                                    |    |    |    | 19 |    |    |    | 45  |          |
| 42   | Col Margherita (IT)              | 46-356                | 11-799         | 2091–2514                    |                                       |    |    |    | 63 |    |    |    | 63  |          |
| 43   | Pre de Ciapel (IT)               | 46-476                | 11-818         | 2359–2553                    | 16                                    |    |    |    | 46 | 1  |    |    | 63  |          |
| 44   | Sarner Scharte (IT)              | 46-647                | 11-410         | 2035–2435                    | 47                                    |    |    |    |    |    |    |    | 47  |          |
| 45   | Schrotthorn (IT)                 | 46-715                | 11-507         | 2252–2589                    | 46                                    |    |    |    | 20 |    |    |    | 66  |          |
| 46   | Plose (IT)                       | 46-690                | 11-720         | 2134–2408                    | 54                                    |    |    |    | 15 |    |    |    | 69  |          |
| 47   | Schrankogel (AT)                 | 47-035                | 11-091         | 2190–3135                    | 26                                    |    |    |    | 31 |    |    |    | 57  |          |
| 48   | Rietzer Gießkogel (AT)           | 47-242                | 11-052         | 2230–2795                    | 24                                    |    |    |    | 35 |    |    |    | 59  |          |
| 49   | Habicht (AT)                     | 47-041                | 11-299         | 2600–2740                    | 49                                    | 1  |    |    |    |    |    |    | 50  |          |
| 50   | Nöblachjoch, Eggersteller (AT)   | 47-052                | 11-434         | 2180–2270                    | 49                                    |    |    |    |    |    |    |    | 49  |          |
| 51   | Patscherkofel, Viggarspitze (AT) | 47-212                | 11-492         | 2000–2295                    |                                       |    |    |    | 51 |    |    |    | 51  |          |
| 52   | Saurüssel (AT)                   | 47-033                | 11-839         | 2520–2885                    | 63                                    |    |    |    |    |    |    |    | 63  |          |
| 53   | Rauchkofel (IT)                  | 47-069                | 12-090         | 2247–2797                    | 68                                    |    |    |    |    |    |    |    | 68  |          |
| 54   | Speikboden (IT)                  | 46-915                | 11-896         | 2204–2513                    | 53                                    |    |    |    |    |    |    |    | 53  |          |
| 55   | Sambock (IT)                     | 46-851                | 11-894         | 2175–2396                    | 52                                    |    |    |    | 19 |    |    | 1  | 72  |          |
| 56   | Kronplatz (IT)                   | 46-737                | 11-957         | 2255–2281                    |                                       |    |    |    | 14 |    |    |    | 14  |          |
| 57   | Antholzer Scharte (IT)           | 46-892                | 12-109         | 1970–2600                    | 59                                    |    |    |    |    |    |    |    | 59  |          |
| 58   | Almerhorn (AT)                   | 46-896                | 12-198         | 2070–2810                    | 24                                    |    |    |    | 38 |    |    |    | 62  |          |
| 59   | Riepenspitz (IT)                 | 46-846                | 12-288         | 2114–2771                    | 19                                    |    |    |    | 41 |    |    |    | 60  |          |
| 60   | Toblacher Pfannhorn (IT)         | 46-779                | 12-276         | 2147–2661                    | 31                                    |    |    |    | 27 |    |    |    | 58  |          |
| 61   | Donnerstein (AT)                 | 46-948                | 12-431         | 2675–2685                    |                                       |    |    |    | 22 |    |    |    | 22  |          |
| 62   | Gölbner (AT)                     | 46-826                | 12-505         | 2408–2946                    | 44                                    |    |    |    | 6  |    |    |    | 50  |          |
| 63   | Col Quaternà (IT)                | 46-665                | 12-471         | 2028–2506                    | 32                                    |    |    |    | 30 |    |    |    | 62  |          |
| 64   | Monte Peralba (IT)               | 46-633                | 12-729         | 2279–2383                    |                                       |    |    |    | 74 |    |    |    | 74  |          |
| 65   | Monte Crostis (IT)               | 46-571                | 12-888         | 2028–2283                    |                                       |    |    |    | 53 |    |    |    | 53  |          |
| 66   | Schleinitz (AT)                  | 46-896                | 12-730         | 2193–2698                    | 43                                    |    |    |    | 2  |    |    |    | 45  |          |
| 67   | Kalser Höhe (AT)                 | 47-006                | 12-599         | 2219–2363                    |                                       |    |    |    | 33 |    |    |    | 33  |          |
| 68   | Schönleitenstiz (AT)             | 46-992                | 12-680         | 2174–2759                    | 32                                    |    |    |    | 4  |    |    |    | 36  |          |
| 69   | Kapruner Törl (AT)               | 47-137                | 12-668         | 2346–2632                    | 6                                     |    |    |    |    |    |    |    | 6   |          |
| 70   | Sadnig (AT)                      | 46-945                | 12-993         | 2202–2744                    | 31                                    |    |    |    | 23 |    |    |    | 54  |          |
| 71   | Scharnik (AT)                    | 46-793                | 13-041         | 2418–2656                    | 36                                    |    |    |    | 13 |    |    |    | 49  |          |
| 72   | Polinik (AT)                     | 46-889                | 13-167         | 1986–2767                    | 35                                    |    |    |    | 24 |    |    |    | 59  |          |
| 73   | Dolzer, Gaugen (AT)              | 46-789                | 13-203         | 2090–2195                    |                                       |    |    |    | 50 |    |    |    | 50  |          |
| 74   | Gmeineck (AT)                    | 46-891                | 13-428         | 1419–2575                    | 12                                    |    |    |    | 29 |    |    |    | 41  |          |
| 75   | Reißeck (AT)                     | 46-932                | 13-364         | 2516–2743                    | 28                                    |    |    |    | 2  |    |    |    | 30  |          |
| 76   | Ankogel (AT)                     | 47-045                | 13-238         | 2615–2868                    | 45                                    | 1  |    |    |    |    |    |    | 46  |          |
| 77   | Großer Hafner (AT)               | 47-066                | 13-393         | 2280–3007                    | 29                                    |    |    |    | 3  |    |    |    | 32  |          |
| 78   | Belščica (AT / SLO)              | 46-443                | 14-147         | 2000–2050                    | 53                                    |    |    |    |    |    |    |    | 53  |          |
| 79   | Rosennock (AT)                   | 46-874                | 13-721         | 1879–2424                    | 18                                    |    | 1  | 1  | 27 |    |    |    | 47  |          |
| 80   | Bretthöhe (AT)                   | 46-910                | 13-902         | 1997–2312                    | 12                                    |    | 5  |    | 21 | 1  |    |    | 39  |          |
| 81   | Wandspitze (AT)                  | 47-010                | 13-526         | 2066–2605                    |                                       |    | 17 | 3  | 8  |    |    |    | 28  |          |
| 82   | Balonspitze (AT)                 | 47-123                | 13-480         | 2219–2828                    | 17                                    |    |    |    | 36 |    |    |    | 53  |          |
| 83   | Seekarspitze (AT)                | 47-272                | 13-544         | 2196–2410                    |                                       |    | 39 |    |    |    |    |    | 39  |          |
| 84   | Zechnerkarstspitze (AT)          | 47-215                | 13-690         | 2120–2421                    |                                       |    | 12 | 4  | 17 |    |    |    | 33  |          |
| 85   | Trockenbrotscharte (AT)          | 47-273                | 13-720         | 1919–2379                    |                                       |    | 38 | 3  |    |    |    |    | 41  |          |
| 86   | Preber (AT)                      | 47-215                | 13-868         | 2293–2733                    | 27                                    |    | 20 |    | 3  |    |    |    | 50  |          |
| 87   | Predigstuhl (AT)                 | 47-257                | 13-912         | 2081–2540                    | 30                                    |    | 14 |    | 6  |    |    |    | 50  |          |
| 88   | Deneck (AT)                      | 47-284                | 14-053         | 2044–2426                    |                                       |    | 36 |    |    |    |    |    | 36  |          |
| 89   | Großer Knallstein (AT)           | 47-318                | 13-978         | 2193–2598                    | 8                                     |    | 48 |    |    |    |    |    | 56  |          |
| 90   | Hochrettelstein (AT)             | 47-424                | 14-232         | 2120–2227                    |                                       |    | 12 |    |    |    |    |    | 12  |          |
| 91   | Hohenwart (AT)                   | 47-328                | 14-237         | 1875–2374                    |                                       |    | 43 |    |    |    |    |    | 43  |          |
| 92   | Schießeck (AT)                   | 47-272                | 14-337         | 1997–2294                    | 64                                    | 3  | 21 | 1  | 32 |    |    |    | 121 |          |
| 93   | Großer Bösenstein (AT)           | 47-441                | 14-408         | 1953–2406                    |                                       |    | 38 | 2  |    |    |    |    | 40  |          |
| 94   | Gamskogel (AT)                   | 47-366                | 14-546         | 1929–2329                    |                                       |    | 38 | 3  |    |    |    |    | 41  |          |
| 95   | Großer Ringkogel (AT)            | 47-311                | 14-628         | 1943–2277                    | 16                                    |    |    | 3  | 40 |    |    |    | 59  |          |
| 96   | Seckauer Zinken (AT)             | 47-337                | 14-740         | 1903–2339                    | 6                                     |    | 40 | 4  | 6  |    |    |    | 56  |          |
| 97   | Zirbitzkogel (AT)                | 47-080                | 14-565         | 1848–2223                    | 4                                     |    | 2  |    | 28 |    |    |    | 34  |          |
| 98   | Saualpe (AT)                     | 46-856                | 14-654         | 1948–2115                    |                                       |    |    |    | 30 |    |    |    | 30  |          |
| 99   | Ameringkogel (AT)                | 47-062                | 14-824         | 1891–2183                    |                                       |    |    |    | 44 |    |    |    | 44  |          |
| 100  | Roßbachkogel (AT)                | 47-207                | 15-045         | 1763–1877                    |                                       |    |    |    | 27 |    |    |    | 27  |          |

## SUPPLEMENTARY DATA

TABLE S1. Relative fluorescence intensities of DAPI-stained nuclei (with *Pisum sativum* ‘Ctirad’ used as internal reference standard for FCM) of 5033 silica gel-dried leaf samples of *Senecio carniolicus* from 100 sample localities. Numbers of individuals upon which the presented mean values are based are given in the Appendix in the main text.

|      |                                 | Relative fluorescence intensity (mean ± SD) |       |               |               |               |       |               |       |
|------|---------------------------------|---------------------------------------------|-------|---------------|---------------|---------------|-------|---------------|-------|
| Code | Locality (Country)              | 2x                                          | 3x    | 4x            | 5x            | 6x            | 7x    | 8x            | 9x    |
| 1    | Cima dell Uomo (CH)             | 0.837 ± 0.004                               |       |               |               |               |       |               |       |
| 2    | Pizzo di Gino (IT)              | 0.817 ± 0.010                               |       |               |               |               |       |               |       |
| 3    | Campanile di Val Marina (IT)    | 0.833 ± 0.009                               |       |               |               |               |       |               |       |
| 4    | Monte Legnone (IT)              | 0.813 ± 0.013                               |       |               |               |               |       |               |       |
| 5    | Passo Locino (IT)               | 0.825 ± 0.014                               |       |               |               | 2.230 ± 0.028 |       |               |       |
| 6    | Monte Verrobbio (IT)            | 0.820 ± 0.007                               | 1.219 |               |               |               |       |               |       |
| 7    | Cima Cadelle (IT)               |                                             |       |               |               | 2.200 ± 0.033 |       |               |       |
| 8    | Laghi Gemelli (IT)              |                                             |       |               |               | 2.178 ± 0.056 |       |               |       |
| 9    | Pizzo di Coca (IT)              |                                             |       | 1.502 ± 0.019 |               | 2.273         |       |               |       |
| 10   | Monte Colombine (IT)            | 0.802 ± 0.018                               |       |               |               | 2.211 ± 0.050 |       |               |       |
| 11   | Bocchetta Vicima (IT)           |                                             |       |               |               | 2.242 ± 0.018 |       |               |       |
| 12   | Bocchetta della Forbici (IT)    |                                             |       |               |               | 2.238 ± 0.017 |       |               |       |
| 13   | Diavolezza, Munt Pers (CH)      |                                             |       |               |               | 2.216 ± 0.035 |       |               |       |
| 14   | Monte Vago (IT)                 |                                             |       |               |               | 2.255 ± 0.025 |       |               | 3.307 |
| 15   | Piz Nair (CH)                   |                                             |       |               |               | 2.211 ± 0.026 |       |               |       |
| 16   | Sandhubel (CH)                  |                                             |       |               |               | 2.221 ± 0.044 |       |               |       |
| 17   | Flüela Schwarzhorn (CH)         |                                             |       |               |               | 2.216 ± 0.031 |       |               |       |
| 18   | Hohes Rad (AT)                  |                                             |       |               |               | 2.191 ± 0.025 |       |               |       |
| 19   | Arlensattel (AT)                |                                             |       |               |               | 2.211 ± 0.028 |       |               |       |
| 20   | Monte Verva (IT)                |                                             |       | 1.491 ± 0.010 | 1.813 ± 0.060 | 2.194 ± 0.045 |       |               |       |
| 21   | Monte di Gavia (IT)             |                                             |       | 1.486 ± 0.023 | 1.840         |               |       |               |       |
| 22   | Monte Serodine (IT)             |                                             |       | 1.521 ± 0.014 |               |               |       |               |       |
| 23   | Cresta del Belvedere (IT)       | 0.820 ± 0.007                               |       | 1.497 ± 0.024 |               |               |       |               |       |
| 24   | Cima Valletta (IT)              | 0.809                                       |       | 1.498 ± 0.023 | 1.899 ± 0.035 |               |       |               |       |
| 25   | Cima Cavaion (IT)               |                                             |       | 1.499 ± 0.021 | 1.824         |               |       |               |       |
| 26   | Stilfser Joch (IT)              |                                             |       | 1.469 ± 0.029 |               | 2.154 ± 0.049 |       |               |       |
| 27   | Laaser Spitze (IT)              |                                             |       |               |               | 2.236 ± 0.016 |       |               |       |
| 28   | Watles (IT)                     |                                             |       |               |               | 2.215 ± 0.038 |       |               |       |
| 29   | Steinmandlköpfl, Mittereck (IT) |                                             |       |               |               | 2.217 ± 0.024 |       |               |       |
| 30   | Piz Lad (IT)                    |                                             |       |               |               | 2.216 ± 0.031 | 2.494 |               | 3.338 |
| 31   | Fisser Joch, Brunnenkopf (AT)   |                                             |       |               |               | 2.224 ± 0.023 | 2.421 |               |       |
| 32   | Hohe Aifnerspitze (AT)          |                                             |       |               |               | 2.162 ± 0.039 |       |               |       |
| 33   | Riffljoch (AT)                  |                                             |       |               |               | 2.158 ± 0.042 |       |               |       |
| 34   | Gaislachkogel (AT)              |                                             |       |               |               | 2.152 ± 0.026 |       |               | 3.311 |
| 35   | Schröfwand (IT)                 | 0.819 ± 0.003                               |       |               |               | 2.224 ± 0.020 |       |               |       |
| 36   | Festkogel (AT)                  | 0.788 ± 0.012                               |       |               |               | 2.162 ± 0.046 |       |               |       |
| 37   | Vermoispitze (IT)               |                                             |       |               |               | 2.239 ± 0.051 |       |               |       |
| 38   | Naturnser Hochwart (IT)         | 0.805 ± 0.011                               |       |               |               | 2.220 ± 0.029 |       | 2.780 ± 0.056 | 3.353 |
| 39   | Monte Ziolera (IT)              | 0.802 ± 0.016                               |       |               |               | 2.220 ± 0.037 |       |               |       |
| 40   | Cima D'Asta (IT)                | 0.814                                       |       | 1.535 ± 0.022 | 1.844 ± 0.058 | 2.197 ± 0.038 |       |               |       |
| 41   | Cavallazza Piccola (IT)         | 0.811 ± 0.008                               |       |               |               | 2.238 ± 0.035 |       |               |       |
| 42   | Col Margherita (IT)             |                                             |       |               |               | 2.210 ± 0.034 |       |               |       |
| 43   | Pre de Ciapel (IT)              | 0.807 ± 0.010                               |       |               |               | 2.208 ± 0.034 | 2.483 |               |       |
| 44   | Sarner Scharte (IT)             | 0.803 ± 0.012                               |       |               |               |               |       |               |       |
| 45   | Schrotthorn (IT)                | 0.791 ± 0.023                               |       |               |               | 2.192 ± 0.050 |       |               |       |
| 46   | Plose (IT)                      | 0.810 ± 0.012                               |       |               |               | 2.229 ± 0.028 |       |               |       |

|     |                                  |               |               |               |               |               |       |
|-----|----------------------------------|---------------|---------------|---------------|---------------|---------------|-------|
| 47  | Schrankogel (AT)                 | 0.796 ± 0.013 |               |               |               | 2.168 ± 0.042 |       |
| 48  | Rietzer Griebkogel (AT)          | 0.798 ± 0.007 |               |               |               | 2.197 ± 0.035 |       |
| 49  | Habicht (AT)                     | 0.783 ± 0.010 | 1.172         |               |               |               |       |
| 50  | Nöblachjoch, Eggersteller (AT)   | 0.789 ± 0.010 |               |               |               |               |       |
| 51  | Patscherkofel, Viggarspitze (AT) |               |               |               |               | 2.189 ± 0.035 |       |
| 52  | Saurüssel (AT)                   | 0.788 ± 0.006 |               |               |               |               |       |
| 53  | Rauchkofel (IT)                  | 0.796 ± 0.014 |               |               |               |               |       |
| 54  | Speikboden (IT)                  | 0.805 ± 0.009 |               |               |               |               |       |
| 55  | Sambock (IT)                     | 0.792 ± 0.025 |               |               |               | 2.199 ± 0.038 | 3.252 |
| 56  | Kronplatz (IT)                   |               |               |               |               | 2.207 ± 0.033 |       |
| 57  | Antholzer Scharte (IT)           | 0.805 ± 0.013 |               |               |               |               |       |
| 58  | Almerhorn (AT)                   | 0.812 ± 0.012 |               |               |               | 2.172 ± 0.029 |       |
| 59  | Riepenspitz (IT)                 | 0.812 ± 0.029 |               |               |               | 2.159 ± 0.021 |       |
| 60  | Toblacher Pfannhorn (IT)         | 0.816 ± 0.007 |               |               |               | 2.165 ± 0.024 |       |
| 61  | Donnerstein (AT)                 |               |               |               |               | 2.142 ± 0.019 |       |
| 62  | Gölbner (AT)                     | 0.810 ± 0.006 |               |               |               | 2.145 ± 0.016 |       |
| 63  | Col Quaternà (IT)                | 0.810 ± 0.012 |               |               |               | 2.162 ± 0.031 |       |
| 64  | Monte Peralba (IT)               |               |               |               |               | 2.095 ± 0.050 |       |
| 65  | Monte Crostis (IT)               |               |               |               |               | 2.117 ± 0.038 |       |
| 66  | Schleinitz (AT)                  | 0.769 ± 0.004 |               |               |               | 2.154 ± 0.026 |       |
| 67  | Kalser Höhe (AT)                 |               |               |               |               | 2.125 ± 0.036 |       |
| 68  | Schönleiten Spitze (AT)          | 0.741 ± 0.007 |               |               |               | 2.032 ± 0.047 |       |
| 69  | Kapruner Törl (AT)               | 0.754 ± 0.001 |               |               |               |               |       |
| 70  | Sadnig (AT)                      | 0.760 ± 0.009 |               |               |               | 2.130 ± 0.028 |       |
| 71  | Scharnik (AT)                    | 0.742 ± 0.014 |               |               |               | 2.138 ± 0.021 |       |
| 72  | Polinik (AT)                     | 0.726 ± 0.019 |               |               |               | 2.066 ± 0.057 |       |
| 73  | Dolzer, Gaugen (AT)              |               |               |               |               | 2.070 ± 0.029 |       |
| 74  | Gmeineck (AT)                    | 0.750 ± 0.015 |               |               |               | 2.084 ± 0.022 |       |
| 75  | Reißeck (AT)                     | 0.755 ± 0.013 |               |               |               | 2.088 ± 0.008 |       |
| 76  | Ankogel (AT)                     | 0.760 ± 0.007 | 1.110         |               |               |               |       |
| 77  | Großer Hafner (AT)               | 0.758 ± 0.020 |               |               |               | 2.163 ± 0.020 |       |
| 78  | Belščica (AT / SLO)              | 0.778 ± 0.011 |               |               |               |               |       |
| 79  | Rosennock (AT)                   | 0.762 ± 0.014 |               | 1.468         | 1.799         | 2.116 ± 0.033 |       |
| 80  | Bretthöhe (AT)                   | 0.754 ± 0.011 |               | 1.472 ± 0.035 |               | 2.134 ± 0.043 | 2.590 |
| 81  | Wandspitze (AT)                  |               |               | 1.479 ± 0.033 | 1.787 ± 0.049 | 2.127 ± 0.074 |       |
| 82  | Balonspitze (AT)                 | 0.740 ± 0.008 |               |               |               | 2.058 ± 0.018 |       |
| 83  | Seekarspitze (AT)                |               |               | 1.427 ± 0.019 |               |               |       |
| 84  | Zechnerkarspitze (AT)            |               |               | 1.431 ± 0.010 | 1.767 ± 0.014 | 2.046 ± 0.043 |       |
| 85  | Trockenbrotscharte (AT)          |               |               | 1.440 ± 0.026 | 1.804 ± 0.059 |               |       |
| 86  | Preber (AT)                      | 0.746 ± 0.012 |               | 1.429 ± 0.016 |               | 2.046 ± 0.018 |       |
| 87  | Predigstuhl (AT)                 | 0.752 ± 0.018 |               | 1.434 ± 0.013 |               | 2.068 ± 0.034 |       |
| 88  | Deneck (AT)                      |               |               | 1.447 ± 0.028 |               |               |       |
| 89  | Großer Knallstein (AT)           | 0.728 ± 0.015 |               | 1.431 ± 0.014 |               |               |       |
| 90  | Hochrettelstein (AT)             |               |               | 1.487 ± 0.041 |               |               |       |
| 91  | Hohenwart (AT)                   |               |               | 1.453 ± 0.011 |               |               |       |
| 92  | Schießeck (AT)                   | 0.765 ± 0.011 | 1.138 ± 0.016 | 1.458 ± 0.019 | 1.797         | 2.121 ± 0.054 |       |
| 93  | Großer Bösenstein (AT)           |               |               | 1.455 ± 0.018 | 1.790 ± 0.015 |               |       |
| 94  | Gamskogel (AT)                   |               |               | 1.449 ± 0.025 | 1.779 ± 0.036 |               |       |
| 95  | Großer Ringkogel (AT)            | 0.759 ± 0.009 |               |               | 1.770 ± 0.010 | 2.143 ± 0.034 |       |
| 96  | Seckauer Zinken (AT)             | 0.758 ± 0.009 |               | 1.434 ± 0.025 | 1.790 ± 0.021 | 2.104 ± 0.054 |       |
| 97  | Zirbitzkogel (AT)                | 0.759         |               | 1.460 ± 0.013 |               | 2.130 ± 0.017 |       |
| 98  | Sauualpe (AT)                    |               |               |               |               | 2.124 ± 0.032 |       |
| 99  | Ameringkogel (AT)                |               |               |               |               | 2.139 ± 0.038 |       |
| 100 | Roßbachkogel (AT)                |               |               |               |               | 2.077 ± 0.015 |       |

TABLE S2. Mean relative fluorescence intensities (per monoploid genome) of individual ploidy levels of *Senecio carniolicus* (relative to *Pisum sativum* ‘Ctirad’) in the present study and in our previous work (Suda *et al.* 2007). Note the stability of fluorescence values in polyploids, indicating the absence of genome downsizing.

| DNA ploidy level | Mean fluorescence per monoploid genome (Suda et al. 2007) | Mean fluorescence per monoploid genome (present study) |
|------------------|-----------------------------------------------------------|--------------------------------------------------------|
| 2x               | 0.395                                                     | 0.393                                                  |
| 3x               | -                                                         | 0.387                                                  |
| 4x               | 0.375                                                     | 0.367                                                  |
| 5x               | 0.367                                                     | 0.361                                                  |
| 6x               | 0.359                                                     | 0.359                                                  |
| 7x               | 0.360                                                     | 0.357                                                  |
| 8x               | -                                                         | 0.348                                                  |
| 9x               | -                                                         | 0.368                                                  |

FIG. S1. The spatial distribution of individuals of *Senecio carniolicus* within selected mixed populations illustrates that cytotypes can be largely separated from each other with only a small contact zone (population 20) as well as strongly intermingled (population 86). These patterns did not obviously depend on the combination of cytotypes occurring at a particular population. Diploid, tetraploid and hexaploid individuals are given as yellow, red and blue dots, respectively. Numbers refer to the population number in the Appendix in the main text.

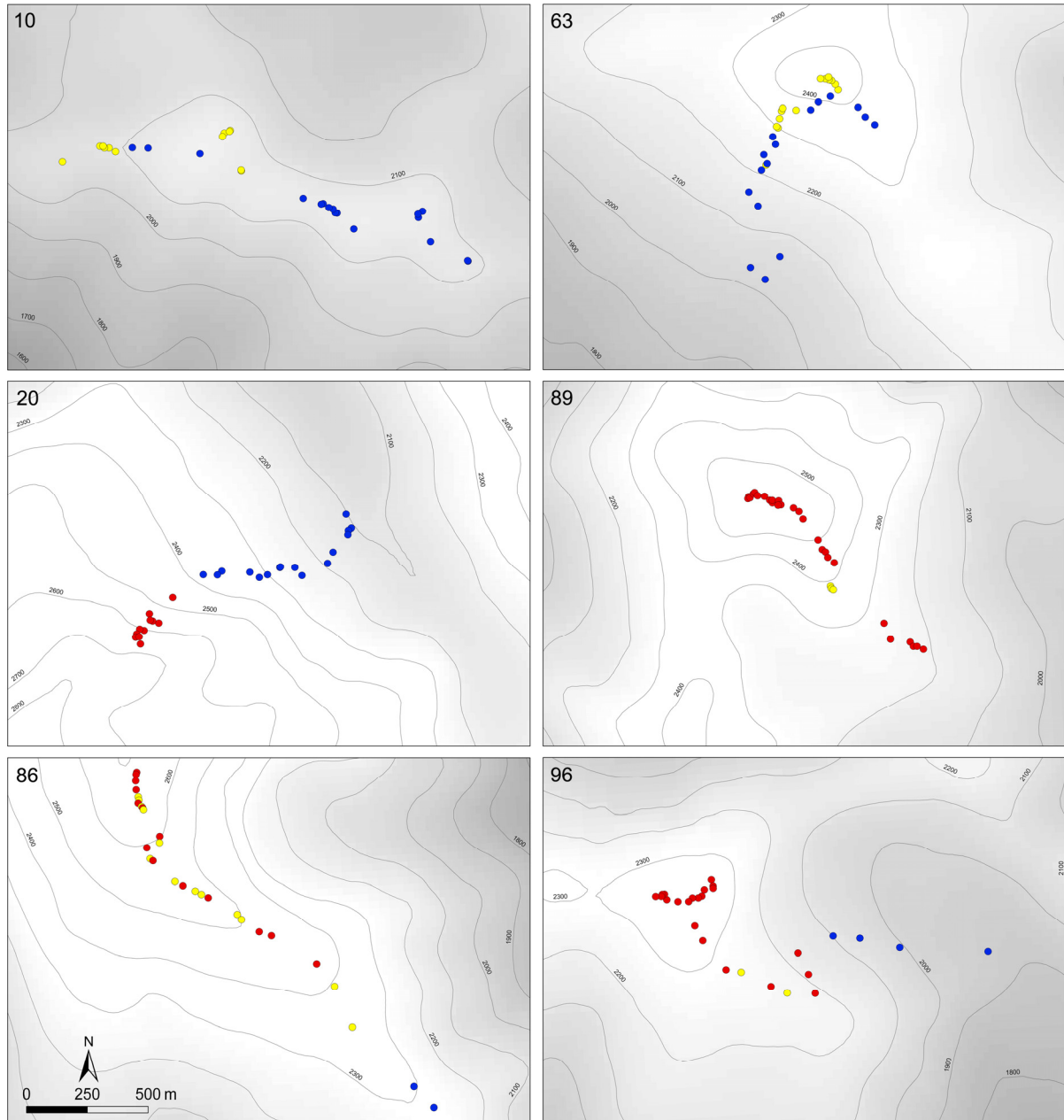


FIG. S2. Mean relative fluorescence intensities of DNA-diploid, DNA-triploid, DNA-tetraploid, DNA-pentaploid and DNA-hexaploid samples of *Senecio carniolicus* (using *Pisum sativum* 'Ctirad',  $2C = 9.09$  pg as a unit value). Note the marked discontinuity in fluorescence values resulting in distinct separation of putative triploid and pentaploid plants.

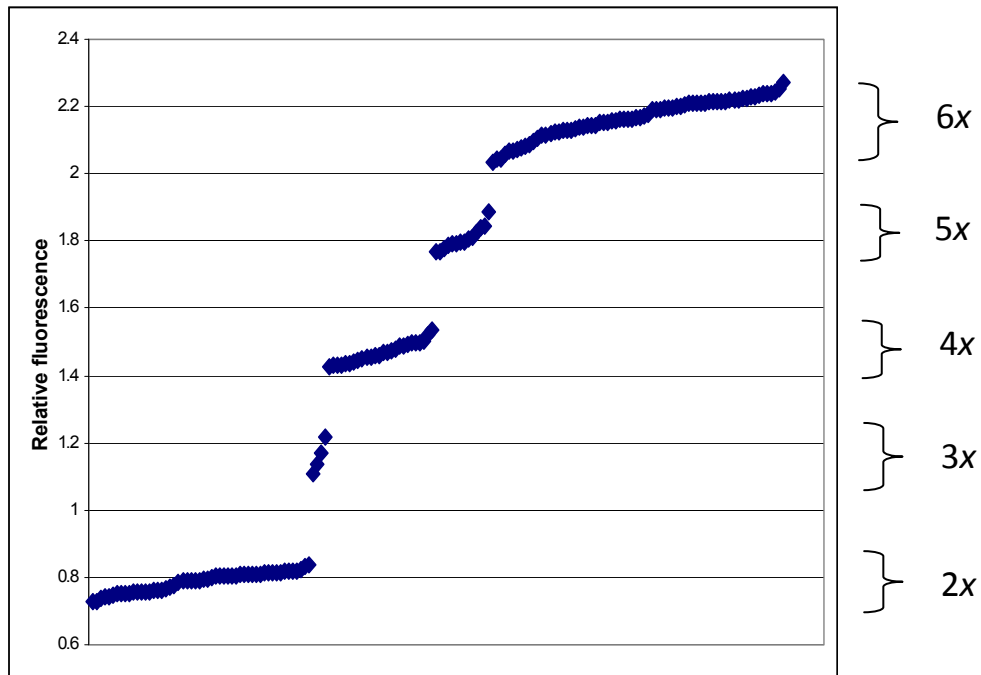
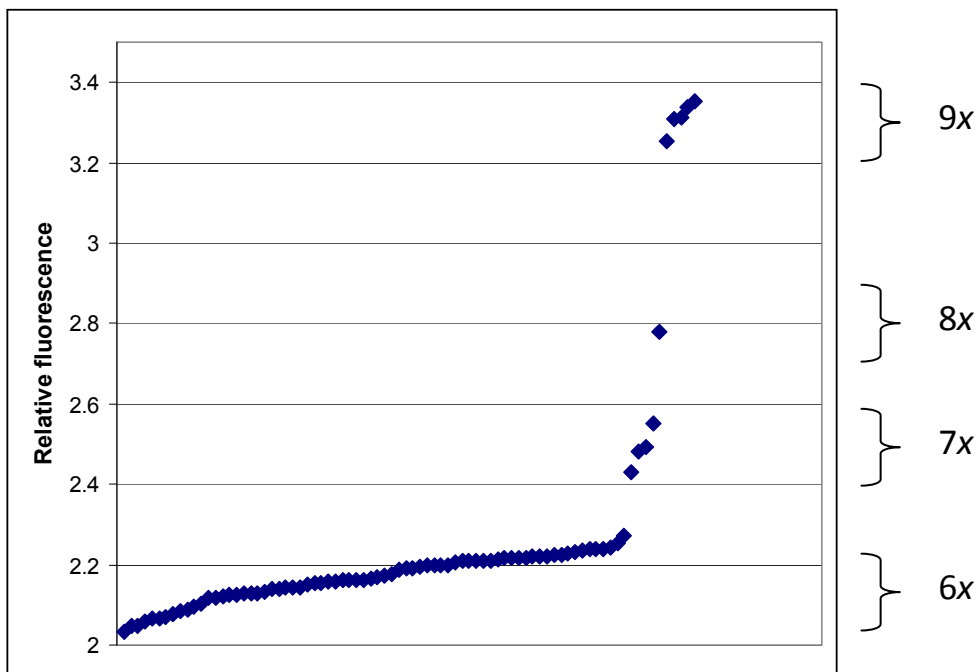


FIG. S3. Mean relative fluorescence intensities of DNA-hexaploid, DNA-heptaploid, DNA-octoploid and DNA-enneaploid samples of *Senecio carniolicus* (using *Pisum sativum* 'Ctirad',  $2C = 9.09$  pg as a unit value). Note the marked discontinuity in fluorescence values between different DNA ploidy levels.



## CHAPTER 3: REPRODUCTIVE INFERENCE AMONG CYTOTYPES

### Article 2

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Field experiments: *in situ* pollinations and germination

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# Parental Ploidy Strongly Affects Offspring Fitness in Heteroploid Crosses among Three Cytotypes of Autopolyploid *Jacobaea carniolica* (Asteraceae)

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## Abstract

Reproductive interactions among cytotypes in their contact zones determine whether these cytotypes can co-exist and form stable contact zones or not. In autopolyploids, heteroploid cross-compatibilities might depend on parental ploidy, but tests of this hypothesis in autopolyploid systems with more than two ploidies are lacking. Here, we study *Jacobaea carniolica*, which comprises diploid, tetraploid, and hexaploid individuals regularly forming contact zones. Seeds obtained from *in situ* cross-pollinations within and among cytotypes were subjected to DNA flow cytometry and greenhouse germination experiments. Hybrid fitness and parental effects on hybrid fitness were tested with regression models comparing fitness parameters of early life stages. Irrespective of the direction of crosses, seed viability and seedling survival in diploid-polyploid crosses were substantially lower than in tetraploid-hexaploid crosses. In contrast, seedling growth traits indicated neither transgressive character expression nor any selection against hybrid offspring. Congruent with a model of genome dosage effects, these traits differed between reciprocal crosses, especially of diploids and tetraploids, where trait values resembled those of the maternal parent. The strong effect of parental ploidy on offspring fitness in heteroploid crosses may cause contact zones involving exclusively polyploid cytotypes to be less stable over longer terms than those involving diploids and polyploids.

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## Introduction

Polyploidisation, i.e. the multiplication of the whole genome, is among the most important evolutionary mechanisms in plants, accounting for 15 and 31% of speciation events in angiosperms and ferns, respectively [1]. Even though genome multiplication per se is widely accepted as a possible mechanism for instantaneous speciation [2], it usually does not confer complete reproductive isolation of the polyploid from its lower-ploid ancestors. Therefore, other adaptive or neutral processes are necessary to enable coexistence of both cytotypes [3]. Much research has focussed on the early phases of polyploid establishment, such as the frequency-dependent mating disadvantage of newly emerged polyploids (minority cytotype exclusion principle; [4,5]), the role of fitness advantages [6,7] or niche differentiation [8,9], and on cytotype interfertility among established polyploids and/or their ancestors [10–17].

Minority cytotype exclusion and fitness differences among cytotypes are not only significant for neopolyploid establishment, but are assumed to be equally important in secondary contact

zones [11]. Such areas where cytotypes regain contact after a period of divergence in allopatry are common [18] and frequently arose in the course of (postglacial) range expansions [19–21]. They are evolutionary melting pots enhancing local diversity [22] and may ultimately even promote speciation [23].

The extent of reproductive isolation among cytotypes determines the long-term integrity of lineages in any contact zone [24]. Mechanisms of reproductive isolation include ecological niche differentiation [25–27], flowering time divergence [26], pollinator preferences [28,29], pollen competition and genetic/genomic incompatibilities preventing zygote and/or endosperm formation [30–32], or negative effects on vitality and fertility of hybrid offspring from seed formation to fertility in the F<sub>1</sub> or even later generations [11]. Frequently, several mechanisms act in concert allowing the coexistence of closely related lineages [33,34]. Reproductive isolation mechanisms between a polyploid and its lower-ploid ancestor(s) have been studied both in allopolyploids [35,36] and in autopolyploids [14,27,33,37,38]. As these systems often comprise only a single polyploid cytotype (tetraploid or hexaploid, but see: [15,39]), little is known whether reproductive

isolation mechanisms and/or their strength differ in dependency of the ploidy.

Postzygotic isolation may be conferred by genome imbalance in the endosperm [40] frequently leading to seed abortion in heteroploid crosses [41,42]. A ratio of two maternal (2 m) and one paternal (1p) genomes in the endosperm is critical for seed maturation in many species [5,43–45], whereas the embryo itself is often more resistant to differences in parental ploidies [46]. Mechanisms underlying this dosage effect include cytoplasmatic effects and/or genomic imprinting [42], the latter referring to the epigenetic phenomenon of functional non-equivalence of maternal and paternal genes despite their identical DNA sequence [40]. Since the magnitude of deviation from a balanced endosperm depends on parental ploidy (e.g. two-fold versus three-fold in diploid-tetraploid versus diploid-hexaploid crosses), the extent of offspring fitness reduction might also be expected to vary between different heteroploid crosses, but this hypothesis has been insufficiently tested so far. Furthermore, the direction of the deviation from a balanced endosperm can have complementary phenotypic consequences [42,47]. Specifically, an excess of maternal genomes induces accelerated endosperm cellularisation and delayed mitosis, resulting in small embryos, whereas paternal excess leads to delayed cellularisation and accelerated mitosis in the endosperm producing large embryos [46]. This will affect seed size and thus offspring fitness and may even cause seed abortion, resulting in cross-incompatibility termed triploid [5] or, more generally, inter-ploidy block [39]. Consequently, the fitness of hybrid offspring might also depend on the direction of heteroploid crosses (e.g., refs. [14,16]).

Comprehensive knowledge of heteroploid cross-compatibility including hybrid fitness is, hence, important to understand the dynamics of contact zones with respect to, for instance, the spatial configuration of contact zones, competition avoidance of cytotypes or gene-flow between cytotypes potentially leading to the formation of new hybrids or the elimination of one cytotype in natural habitats [15,24,48]. Our study presents the results of reciprocal *in situ* cross-pollinations in the alpine plant *Jacobaea carniolica* (Asteraceae). This group comprises three main cytotypes (diploids, tetraploids, hexaploids), which form contact zones in every conceivable combination [49]. The occurrence of pentaploids in tetraploid-hexaploid contact zones and the lack of tetraploids in diploid-hexaploid contact zones [48,49] suggest ploidy-dependent differences in cross-compatibility, but these inferences might be biased as early life stages are hard to find in nature and, therefore, have not been considered in the previous investigations. Here, we test for differences in cross-compatibility in all combinations of heteroploid crosses evaluating four fitness components of early life stages (encompassing hybrid formation and seedling performance). Specifically, we aim to answer the following questions: (1) What is the extent of cross-compatibility between cytotypes and which fitness components are most strongly affected by reproductive incompatibility? (2) Is there any evidence for selection against hybrids? Are the characters of hybrids intermediate, parental-like or transgressive? (3) Do the magnitude and/or the direction of deviation from a balanced endosperm affect seed formation and/or seedling fitness?

## Materials and Methods

### Ethics Statement

Field studies were carried out on private land with oral permissions of the owners (Schwarzenberg'sche Forstverwaltung and Jacques Lemans GmbH, St. Veit an der Glan). Field studies did not involve endangered or protected species.

### Study Species

*Jacobaea carniolica* (Willd.) Schrank (syn. *Senecio carniolicus* Willd.) is a herbaceous perennial common on acidic bedrock in the alpine to subnival belt of the Eastern Alps and the Carpathians. The polyploid complex comprises mainly diploids, tetraploids and hexaploids in the Eastern Alps and only hexaploids in the Carpathians [49,50]. In contrast to the majority of polyploid complexes, *J. carniolica* does not form a single contact zone containing otherwise geographically well separated cytotypes (e.g., refs. [51–54]), but occurs in various combinations of cytotypes throughout major parts of the Eastern Alps [49,50]. Of 100 investigated sample sites, diploids and hexaploids, tetraploids and hexaploids, and diploids and tetraploids co-occurred at 28, five and three sites, respectively, and all three cytotypes co-occurred in eight sample sites. Molecular genetic evidence suggests that the polyploid cytotypes are autopolyploid derivatives of a diploid lineage distributed in the easternmost Alps ([55], M. Winkler et al., unpublished data). Strong genetic divergence between the ancestral eastern diploid lineage and its polyploid derivatives renders ongoing polytopic origin of the polyploids unlikely (M. Winkler et al., unpublished data), which is in line with a consistent morphological differentiation of diploids and polyploids [56]. Despite substantial habitat segregation [48], individuals of different cytotypes commonly occur in close spatial proximity (less than one meter; [49]), potentially enabling *in situ* heteroploid pollination. In addition, flowering times overlap strongly (M. Sonnleitner, pers. obs.) enabling artificial crossing throughout the flowering time of all three cytotypes and generalistic behaviour of alpine pollinators [57] precludes a strong pre-pollination isolation.

### Artificial Crosses

Reciprocal *in situ* cross-pollinations were conducted during summer of 2009 east of Turracher Höhe (Gurktaler Alps, Austria, N 46.91 E 13.92 at c. 2250 m a.s.l.), where pairwise contact zones of the three main cytotypes occur. Using individuals from a single population avoids that effects of ploidy, the focus of this study, are confounded with those of geographic differentiation due to, for instance, adaption to different ecoclimatic conditions. Each cytotype was either outcrossed with itself (i.e. homoploid crosses) or with other cytotypes (heteroploid crosses), or self-pollinated to test for self-incompatibility (SI; Table 1). The ploidy of all crossing partners was determined via flow cytometry (FCM) of silica-dried leaf material following Sonnleitner et al. [49]. Each inflorescence serving either as pollen receptor or as pollen donor was bagged before anthesis using a small-meshed tissue to prevent uncontrolled pollen transfer. As the floret morphology in *Jacobaea* precluded emasculation, self-pollination could not be avoided, but was found to be low (see Results); as cytotypes did not differ in their selfing levels, the quantitative effects of selfing on the measured fitness parameters (via, for instance pollen competition or interference) are expected to be similar for all cytotypes. Hand-pollinations were done by rubbing anthetic flower heads of mother plants and pollen donor plants [14,58], or using a brush in case of selfing. Inflorescences were kept in bags for seven to eight weeks and harvested at seed maturity. After harvest, cypselas (termed “seeds” in the following for simplicity) were stored at 4°C. Seeds were visually classified as viable (firm and plump pericarp) and non-viable (empty pericarp). Prior dissections of soaked seeds were used to assess the accuracy of this classification. To discriminate hybridogenic and selfed seeds, determination of the ploidy is essential. As the ploidy status of tiny seeds currently cannot be determined in a non-destructive way, viable seeds of each mother plant were split into two groups of equal size designated for

**Table 1.** *In situ* cross-pollinations (left) among diploid, tetraploid and hexaploid individuals of *Jacobaea carniolica* and consecutive germination of a subset of the seed yield of heteroploid crosses (right) in the climate chamber.

| Treatment <sup>1</sup> | no. mother plants | no. florets (ovules) | seed viability % | no. of seeds cytotype % | % of cytotype seeds by ploidy (hybrids given in bold) |             |             |             |      | no. of exposed seeds | surviving seedlings no./% | % of surviving seedlings according to estimated ploidies (hybrids given in bold) |             |             |            |             |      |       |
|------------------------|-------------------|----------------------|------------------|-------------------------|-------------------------------------------------------|-------------|-------------|-------------|------|----------------------|---------------------------|----------------------------------------------------------------------------------|-------------|-------------|------------|-------------|------|-------|
|                        |                   |                      |                  |                         | 2x                                                    | 3x          | 4x          | 5x          | 6x   |                      |                           | 7x/8x                                                                            | 2x          | 3x          | 4x         | 5x          | 6x   | 7x/8x |
| 2x2x                   | 39                | 3955                 | 11.1             | 121                     | 21.5                                                  | <b>69.4</b> | 9.1         | –           | –    | –                    | 195                       | 58/29.7                                                                          | <b>41.4</b> | 3.4         | 1.7        | –           | –    | 5.2   |
| 4x2x                   | 37                | 5878                 | 13.8             | 123                     | –                                                     | <b>69.1</b> | 30.9        | –           | –    | –                    | 212                       | 63/29.7                                                                          | –           | <b>52.4</b> | 30.1       | 3.2         | –    | 14.3  |
| 2x6x                   | 40                | 4290                 | 2.9              | 43                      | 72.1                                                  | –           | <b>23.3</b> | 4.6         | –    | –                    | 56                        | 16/28.6                                                                          | 81.2        | –           | –          | 6.3         | –    | 12.5  |
| 6x2x                   | 36                | 5692                 | 7.6              | 57                      | –                                                     | –           | <b>31.6</b> | 28.1        | 40.3 | –                    | 102                       | 40/39.2                                                                          | –           | –           | <b>5.0</b> | 27.5        | 57.5 | –     |
| 4x6x                   | 34                | 5624                 | 39.4             | 154                     | –                                                     | –           | –           | <b>95.4</b> | –    | 0.7                  | 286                       | 181/63.3                                                                         | –           | –           | 1.1        | <b>90.1</b> | 1.1  | –     |
| 6x4x                   | 39                | 6107                 | 38.5             | 186                     | –                                                     | –           | –           | <b>92.5</b> | 5.9  | 1.6                  | 365                       | 192/52.6                                                                         | –           | –           | –          | <b>88.5</b> | 3.7  | 6.8   |
| 2x2x                   | 40                | 3858                 | 52.2             | 103                     | 100                                                   | –           | –           | –           | –    | –                    | 405                       | 149/36.8                                                                         | –           | –           | –          | –           | –    | –     |
| 4x4x                   | 36                | 5614                 | 50.5             | 122                     | –                                                     | –           | 100         | –           | –    | –                    | 334                       | 100/29.9                                                                         | –           | –           | –          | –           | –    | –     |
| 6x6x                   | 38                | 5822                 | 32.6             | 154                     | –                                                     | –           | –           | –           | 100  | –                    | 354                       | 207/58.5                                                                         | –           | –           | –          | –           | –    | –     |
| 2xSI                   | 31                | 3095                 | 4.6              | –                       | –                                                     | –           | –           | –           | –    | –                    | 92                        | 39/42.4                                                                          | –           | –           | –          | –           | –    | –     |
| 4xSI                   | 29                | 4268                 | 7.7              | –                       | –                                                     | –           | –           | –           | –    | –                    | 130                       | 35/26.9                                                                          | –           | –           | –          | –           | –    | –     |
| 6xSI                   | 28                | 4966                 | 1.3              | –                       | –                                                     | –           | –           | –           | –    | –                    | 54                        | 29/53.7                                                                          | –           | –           | –          | –           | –    | –     |

<sup>1</sup>maternal parent is given first; SI, selfing treatments.  
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germination and for cytotype determination via FCM, respectively.

### Flow Cytometry of Seeds (FCM)

Ploidy of dry seeds was estimated using 4',6-diamidino-2-phenylindole (DAPI) FCM. Basically, we adopted the methodology described previously [50] with the following modifications: (i) the samples were run on a flow cytometer *c.* 30 s after the addition of the staining solution (longer incubation period resulted in a lower histogram quality), (ii) *Pisum sativum* 'Ctirad' [59] was used as the sole internal reference standard, (iii) fluorescence intensity of 3000 particles was recorded, (iv) manual gating of the embryo and the endosperm peaks was occasionally applied (particularly in analyses with a relatively low yield of intact nuclei). Two seeds were usually analysed together. The flow histograms mostly consisted of three peaks: (i) nuclei of the internal reference standard (usually having the lowest coefficient of variation, CV), (ii) embryo nuclei and (iii) endosperm nuclei. The peak corresponding to endosperm nuclei was usually distinctly smaller than that of embryo nuclei; in rare cases it was completely lacking. The quality threshold (maximum acceptable CV values) for the embryo peak was relaxed to 8%.

### Germination Experiment

If available, ten potentially viable seeds per mother plant, resulting in a total of 2585 seeds, were germinated on moist filter paper in petri dishes exposed in a climate chamber (Heraeus Vötsch NPS1500 S-CTC) using settings closely resembling *in situ* conditions: day/night 14/10 hrs, temperature 15/5°C; temperature adaptation within 1 hr, 90% constant relative humidity (rH), approximately 370  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  light (Osram powerstar HQ I-R 250W/NDL neon lights) during day time. Petri dishes were controlled, watered and repositioned daily to avoid edge effects. Seedlings with fully expanded cotyledons were transplanted into multipot plates, each pot containing *c.* 150 ml of substrate (coco fibre, sand, Osmocote<sup>TM</sup> depot-fertiliser, proportions approximately 90:9:1) and further cultivated in acclimatisation chambers with 60% rH, *c.* 22°C temperature, automatic watering and a day-length of 14 hrs. Every fourth day the vitality of seedlings was controlled and the appearance of primary leaves was recorded. At the end of the experiment (i.e. approximately seven weeks after exposure), for each rosette the largest diameter and the largest perpendicular to it, the number of leaves and the maximum leaf length were measured and one of the cotyledons was sampled for ploidy determination via FCM (initial analyses found no incidence of endopolyploidy in cotyledons: data not shown).

### Statistical Analyses

The effects of ploidy of crossing partners (i.e. treatments) on the reproductive success were analysed by means of four fitness components of  $F_1$ -individuals: (1) *Seed viability* represents the state of seeds classified as viable/non-viable; (2) *Survival of seedlings* represents the vitality of individuals exposed in the climate chamber at the end of the experiment coded as alive/dead; (3) *The time from exposure of seeds to the appearance of the primary leaf* given in days; and (4) *the size of seedlings* described by the first axis (72.3% of total variation) of a principal component analysis (PCA) using the number of leaves, the length of the longest leaf and the two rosette diameters measured at the end of the experiment. Offspring of heteroploid crosses may show intermediate ploidy due to hybridisation, the maternal ploidy due to selfing, or increased ploidy due to the involvement of unreduced gametes [12,32]. Whereas seed viability comprised the overall seed yield only individuals with intermediate ploidy (referred to as hybrid

seedlings in the following) were used to estimate the other fitness components of seedlings.

We analysed the effects of parental ploidy on the four fitness components described above by means of linear mixed-effects models (LMM, in case of size of hybrid seedlings) or generalised linear mixed-effects models (GLMM, in case of the three other fitness components). Models relate one fitness component as response to the treatment as fixed-effect predictor. Since for these analyses data of more than one seed or seedling from each pollinated individual were used, we accounted for the potential effects of this dependence by using mother plant as a grouping variable and allowing for a random intercept for each group. For the binomially distributed data of seed viability and survival of hybrid seedlings we used the canonical logit link-function. To model the time to emergence of the primary leaf and the size of hybrid seedlings, Poisson (log-link) and Gaussian (identity-link) distributions of errors, were assumed, respectively. Parameters were estimated by restricted maximum likelihood in case of the LMM and based on the Laplacian approximation in case of the logistic and Poisson GLMM. For the Gaussian models the degrees of freedom were calculated as  $n_{\text{observations}} - n_{\text{fixed effects}} - n_{\text{random effects}} + n_{\text{random terms}}$ .

We tested the expectation that in case of no selection against hybrid offspring the fitness of  $F_1$ -individuals of heteroploid crosses, measured by seed viability and seedling survival, is intermediate to that of the parental cytotypes or at least not lower than the worse performing parental cytotype. Thus, by using Helmert contrasts, each heteroploid crossing treatment was compared to the pooled data of homoploid crosses of the parental cytotypes (in the following referred to as parental treatments), e.g.  $2x4x$  (maternal parent given first) was compared to pooled  $2x2x$  and  $4x4x$ . In case of hybrid fitness not intermediate between parental treatments we evaluated if hybrid fitness differed from that of the worse performing parental cytotype. Furthermore, we tested whether phenotypic fitness traits, time to primary leaf and seedling size, are intermediate, parental-like or transgressive using the same approach as described above, but in case of non-intermediacy fitness parameters were compared with the more similar parental treatment. Finally, to detect maternal or paternal effects on hybrid fitness, treatments comprising the same parental cytotypes but with reversed direction of crosses were tested, e.g.  $2x4x$  was compared to  $4x2x$ .

As the ploidy of seeds that failed to germinate and of dead or dying seedlings usually cannot be determined, the ploidy of seedlings is only known if they survived until the end of the experiment when leaf/cotyledon material was collected for FCM. Thus, it is unknown whether an individual that has died during the experiment originated from selfing or hybridisation, which makes a straightforward estimation of the survival of hybrid seedlings impossible. Therefore, we assumed an equal percentage of hybrid seeds in the germination experiment and among the seeds analysed with FCM. A subset of the size  $n_{\text{Hyb}_{\text{dead}}}$  of dead individuals was randomly selected and defined as hybrids in the survival models following the formula

$$n_{\text{Hyb}_{\text{dead}}} = (n_{\text{Total}} * p) - n_{\text{Hyb}_{\text{surv}}} \quad (1)$$

where  $n_{\text{Total}}$  represents the number of seeds exposed in the germination experiment,  $p$  is the proportion (ranging from 0 to 1) of hybrid seeds in the FCM analysis, and  $n_{\text{Hyb}_{\text{surv}}}$  and  $n_{\text{Hyb}_{\text{dead}}}$  are the numbers of surviving and dead hybrid seeds in the germination experiment, respectively. Coefficients and z-values given for survival models represent means of 100 repetitions of this random association of dead progeny to mother plants and

subsequent model fitting. P-values of models of seedling survival were calculated using the mean of z-values.

All statistical analyses were computed in R 2.13.0 [60]. LMM and GLMM were fitted using the `glmer`-function implemented in the `lme4`-package [61].

## Results

A total of 427 plants were pollinated in the crossing experiments (see Table 1 for an overview of the crossing and germination experiment). Each diploid, tetraploid and hexaploid mother plant had on average 101, 157 and 160 florets, respectively. Seed viability was strongly affected by the ploidy of crossing partners (Table 1). Diploid-polyploid crosses showed significantly lower seed viability than both the pooled (Figure 1, Table 2) and the worse performing parental treatment (Table 3). In contrast, seed viability of tetraploid-hexaploid crosses was intermediate between the parental treatments (Table 2). The direction of crosses had no significant effect on the seed viability for any combination of cytotypes (Table 4). Seed viability in all SI-treatments was low (<10%), indicating a low selfing capability of all cytotypes (Table 1).

The proportion of hybrids (seeds and seedlings with intermediate ploidy) of total progeny in heteroploid crosses was strongly affected by their parental cytotypes (Table 1). Tetraploid-hexaploid, diploid-tetraploid and diploid-hexaploid crosses yielded the highest, intermediate and lowest numbers of hybrids, respectively. The proportion of seeds and seedlings derived from selfing (showing the maternal ploidy) was high in all diploid-polyploid and low in tetraploid-hexaploid crosses. Progeny with irregular ploidy due to the involvement of unreduced gametes was mainly produced in diploid-hexaploid crosses, particularly with maternal hexaploids (6x2x with a siring success of unreduced diploid pollen of 28.1%; Table 1), but rare in tetraploid-hexaploid crosses (siring success of unreduced gametes of less than 2%; Table 1). Homoploid treatments yielded only seeds of the parental ploidy. In diploid-tetraploid crosses it is not possible to distinguish with FCM triploids of hybridogenic origin (2x4x) from triploids produced via selfing of diploids with unreduced pollen. Likewise, tetraploids stemming from selfing cannot be discriminated from hybridogenic tetraploids involving unreduced diploid pollen (4x2x treatment; Table 1). The overall reproductive success, i.e. the proportions of hybrid seedlings alive at the end of the experiment related to the total number of ovules/florets pollinated, was 1.37/2.18% in 2x4x/4x2x, <0.01/0.15% for 2x6x/6x2x and 23.37/18.53% for 4x6x/6x4x crosses. Nuclear DNA amounts of offspring corresponded well with those of parental plants of the same ploidy level.

Survival of diploid-polyploid hybrid seedlings was significantly lower than the average survival of seedlings emerging from parental treatments (Figure 1, Table 2). This is particularly evident in 2x6x crosses, where not a single tetraploid hybrid survived until the end of the experiment. Hybrids with a diploid parent most often performed inferior to the worse performing parent (only 4x2x did not significantly differ from 4x4x; Table 3). Pentaploids originating from tetraploid-hexaploid crosses either showed intermediate (6x4x) or parental-like (4x6x resembling 6x6x) survival compared to the parental treatments. Again, the direction of crosses did not influence the survival of hybrid offspring (Table 4).

The time to develop the primary leaf in pentaploid seedlings was intermediate to that of parental treatments (Figure 1, Table 2) with no effect of crossing direction (Table 4). In contrast, triploid seedlings developed significantly faster (2x4x) or slower (4x2x) than the expected intermediate level (Table 2). However, hybrids

between diploids and tetraploids did not differ significantly from the respective maternal treatment (Table 3). Only two out of 32 surviving hybrid seedlings of the 6x2x and none of the 2x6x treatment developed a primary leaf rendering statistical tests impossible.

The size of hybrid seedlings was larger (2x4x) or smaller (4x2x, 4x6x) than the average of the parental treatments (Figure 1, Table 2). The size of triploid hybrids thus reflects their developmental speed, since faster-growing seedlings also reach larger size. The 4x2x seedlings were significantly smaller than plants derived from both parental treatments (Table 3), representing the only case of transgressive character expression. The direction of crosses significantly influenced the size of triploid and pentaploid hybrid seedlings, which was larger when the lower ploidy acted as maternal parent (Table 4).

## Discussion

### Parental Cytotypes Determine Success of Heteroploid Crosses

Reproductive interactions of cytotypes of *Jacobaea carnolica* in secondary contact zones containing diploids and their autopolyploid derivatives (i.e. tetraploids and hexaploids) differed strongly depending on the cytotypes involved. Seed viability and survival of seedlings resulting from crosses of diploids with polyploids were significantly reduced as compared to the offspring of homoploid parental treatments, indicating strong selection against hybrid offspring. In particular, cross-pollinations between diploids and hexaploids, i.e. the cytotypes co-occurring most frequently throughout the Eastern Alps [49,50], often within a few decimetres [48], produced only a few seeds with very low or even no germination success and seedling survival (Figure 1, Table 2). Reproductive isolation between diploids and tetraploids was slightly weaker than for diploid-hexaploid crosses with respect to seed viability and seedling survival (Figure 1). In both diploid-tetraploid and diploid-hexaploid crosses, the few surviving seedlings showed no evidence for phenotypic inferiority compared to homoploid seedlings, with the possible exception of seedling size in 4x2x crosses (Figure 1, Table 3). In contrast to heteroploid crosses involving diploids, reproductive success between polyploid cytotypes was high; seed viability and/or seedling performance parameters were either intermediate between parental treatments or equal to the better performing parental treatment (Figure 1, Table 2). We found no evidence for transgressive character expression in favour of hybrids, at least in early life stages, since hybrid fitness in none of the treatments significantly exceeded the fitness of the better performing parental cytotypes (Table 3).

Reproductive success of heteroploid crosses between diploids and (sexual) autopolyploid tetraploids varies strongly among taxonomic groups ranging from complete interfertility (*Hieracium echinoides* [62]) via reduced fitness and fertility (*Arabidopsis thaliana* [46], *Chamerion angustifolium* [11], *Solanum chacoense* [63]) to almost complete failure of crosses (*Centaurea phrygia* [38], *Cyrtanthus breviflorus* [64], *Plantago media* [65]). In contrast, crosses between diploids and hexaploids consistently fail (*Arabidopsis thaliana* [46], *Aster amellus* [16]). However, there is much less known about reproductive success of heteroploid crosses in polyploid complexes encompassing more than one polyploid cytotype. Whereas diploids, tetraploids and hexaploids of the *Leucanthemum pluriflorum* clan [39], closely related diploid, tetraploid and octoploid species of *Castilleja* spp. and diploid to dodecaploid taxa belonging to the *Aster occidentalis* complex [66,67] showed complete or nearly complete interfertility (but low germination of seeds resulting from heteroploid crosses in *Castilleja* [67]), diploid, tetraploid and

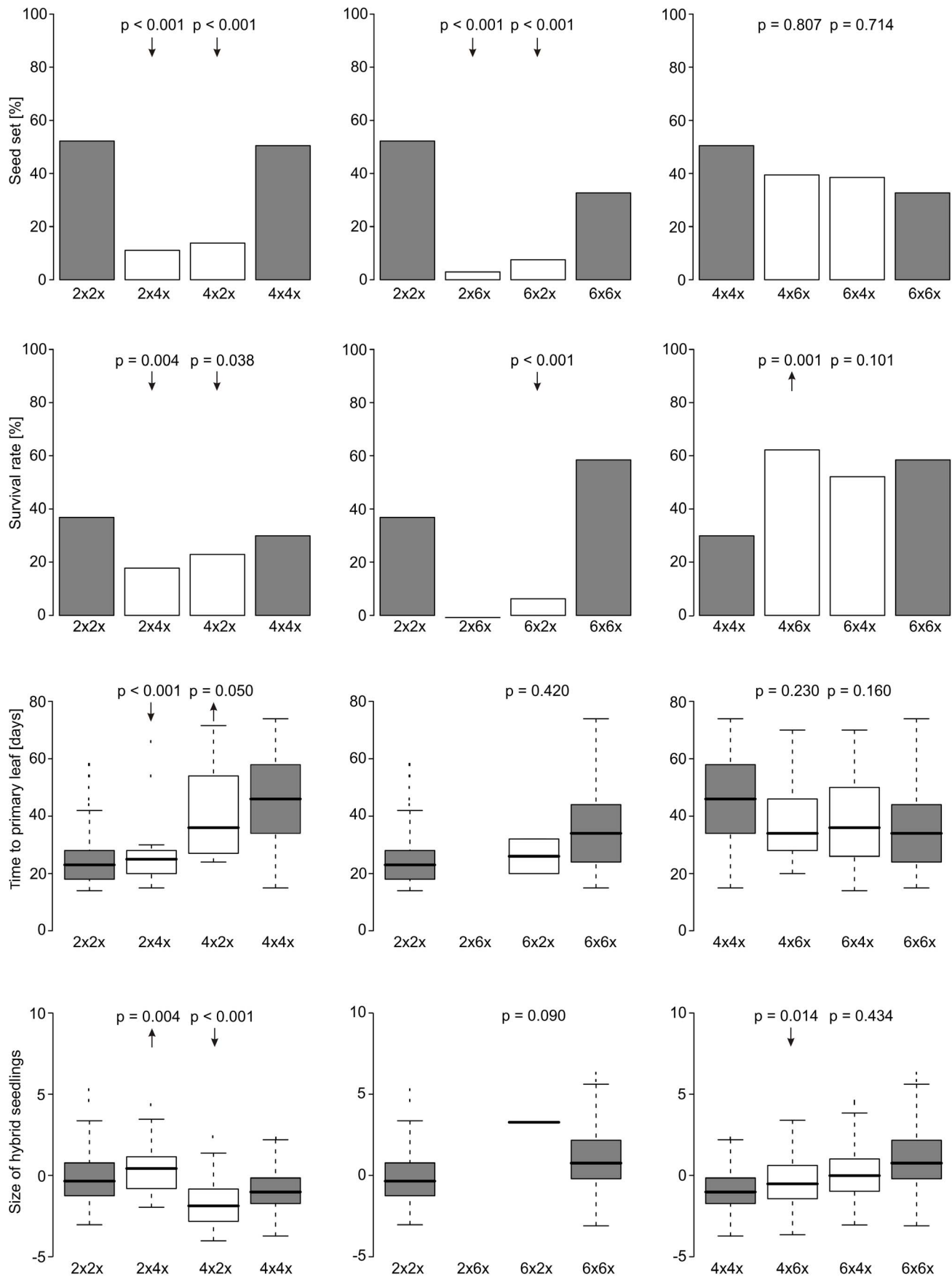


**Figure 1. Fitness of progeny.** Comparison of the fitness of progeny derived from homoploid (grey bars) or heteroploid crosses (white bars) of the three main cytotypes of *Jacobaea carniolica*. Seed set and survival rate represent the proportion of viable seeds and the proportion of seedlings alive at the end of the experiment, respectively. For heteroploid crosses only hybrids (ploidy intermediate to parental ploidies) were considered, except for seed set. P-values derived from (generalised) linear mixed-effects models indicate significance of deviations of heteroploid progeny (e.g. 2x4x, 4x2x; maternal parent is given first) from the intermediate value of parental homoploid crosses (e.g. 2x2x, 4x4x); arrows indicate the direction of significant deviations.

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hexaploid cytotypes of *Mimulus glaberratus* failed to cross or produced infertile offspring [68]. However, for these systems the origin of polyploids is not fully resolved and may involve allopolyploidy. Thus, to our knowledge, *J. carniolica* is the first natural entirely autopolyploid system where reproductive interactions among three cytotypes have been comprehensively investigated by performing the full set of *in situ* reciprocal crosses.

Postmating isolation mechanisms in *J. carniolica* provide a strong barrier to gene flow between diploids and polyploids and might explain the rarity of intermediate cytotypes in nature despite a high number of contact zones [49]. As diploids and polyploids frequently co-occur in close proximity [48,49] and have largely

overlapping flowering periods (M. Sonnleitner et al., pers. obs.), additional mechanisms are expected to be at work that prevent fitness reduction due to the loss of gametes in infertile heteroploid crosses. Assortative mating by means of pollinator discrimination could reduce such losses, but generalistic behaviour of alpine pollinators [57] renders this effect unlikely. Alternatively, apomixis or autogamy frequently accompany polyploidisation and are considered to enforce lineage integrity [69]. In polyploid *J. carniolica*, however, we neither found evidence for apomixis (based on FCM profiles of seed analyses, i.e. the endosperm/embryo ploidy ratio, and amplified fragment length polymorphisms; M. Winkler et al., unpublished data) nor for a breakdown of self-

**Table 2.** Test for intermediacy of fitness components of heteroploid crosses of *Jacobaea carniolica*.

| Comparison of treatments <sup>1,2</sup>         | no. total <sup>3</sup> | no. hybrids <sup>4</sup> | coefficient ± SE | z- or t- value | p-value <sup>5</sup> |
|-------------------------------------------------|------------------------|--------------------------|------------------|----------------|----------------------|
| <b>Seed viability</b>                           |                        |                          |                  |                |                      |
| (2x2x,4x4x) ↔ 2x4x                              | 13427/115              | –                        | –0.82±0.08       | –9.97          | <b>&lt;0.001</b>     |
| (2x2x,4x4x) ↔ 4x2x                              | 15350/113              | –                        | –0.83±0.09       | –9.30          | <b>&lt;0.001</b>     |
| (2x2x,6x6x) ↔ 2x6x                              | 13970/118              | –                        | –1.22±0.10       | –12.74         | <b>&lt;0.001</b>     |
| (2x2x,6x6x) ↔ 6x2x                              | 15372/114              | –                        | –1.10±0.11       | –9.75          | <b>&lt;0.001</b>     |
| (4x4x,6x6x) ↔ 4x6x                              | 17060/108              | –                        | –0.03±0.11       | –0.24          | 0.807                |
| (4x4x,6x6x) ↔ 6x4x                              | 17543/113              | –                        | –0.03±0.09       | –0.37          | 0.714                |
| <b>Survival of hybrid seedlings</b>             |                        |                          |                  |                |                      |
| (2x2x,4x4x) ↔ 2x4x                              | 874                    | 135                      | –0.29±0.10       | –2.91          | <b>0.004</b>         |
| (2x2x,4x4x) ↔ 4x2x                              | 883                    | 147                      | –0.18±0.09       | –2.08          | <b>0.038</b>         |
| (2x2x,6x6x) ↔ 2x6x                              | –                      | –                        | –                | –              | –                    |
| (2x2x,6x6x) ↔ 6x2x                              | 791                    | 32                       | –0.95±0.29       | –3.30          | <b>&lt;0.001</b>     |
| (4x4x,6x6x) ↔ 4x6x                              | 950                    | 273                      | 0.28±0.09        | 3.12           | <b>0.001</b>         |
| (4x4x,6x6x) ↔ 6x4x                              | 1014                   | 338                      | 0.13±0.08        | 1.64           | 0.101                |
| <b>Time to primary leaf in hybrid seedlings</b> |                        |                          |                  |                |                      |
| (2x2x,4x4x) ↔ 2x4x                              | 326/86                 | 26/16                    | –0.10±0.02       | –4.10          | <b>&lt;0.001</b>     |
| (2x2x,4x4x) ↔ 4x2x                              | 331/87                 | 31/17                    | 0.05±0.02        | 1.96           | <b>0.050</b>         |
| (2x2x,6x6x) ↔ 2x6x                              | –                      | –                        | –                | –              | –                    |
| (2x2x,6x6x) ↔ 6x2x                              | 416/75                 | 2/2                      | –0.05±0.07       | –0.81          | 0.420                |
| (4x4x,6x6x) ↔ 4x6x                              | 486/95                 | 160/30                   | –0.02±0.01       | –1.20          | 0.230                |
| (4x4x,6x6x) ↔ 6x4x                              | 495/101                | 169/36                   | –0.02±0.02       | –1.40          | 0.160                |
| <b>Size of hybrid seedlings</b>                 |                        |                          |                  |                |                      |
| (2x2x,4x4x) ↔ 2x4x                              | 260/83                 | 23/15                    | 0.33±0.11        | 2.91           | <b>0.004</b>         |
| (2x2x,4x4x) ↔ 4x2x                              | 268/87                 | 31/19                    | –0.38±0.10       | –3.75          | <b>&lt;0.001</b>     |
| (2x2x,6x6x) ↔ 2x6x                              | –                      | –                        | –                | –              | –                    |
| (2x2x,6x6x) ↔ 6x2x                              | 346/71                 | 1/1                      | 0.96±0.57        | 1.70           | 0.090                |
| (4x4x,6x6x) ↔ 4x6x                              | 456/94                 | 158/30                   | –0.18±0.07       | –2.46          | <b>0.014</b>         |
| (4x4x,6x6x) ↔ 6x4x                              | 467/100                | 169/36                   | 0.05±0.07        | 0.78           | 0.434                |

<sup>1</sup>maternal parent is given first;

<sup>2</sup>pooled data of the homoploid crosses of the parental cytotypes were compared to heteroploid crosses by means of (generalised) linear mixed-effects models;

<sup>3</sup>number of observations (i.e. number of pollinated florets of all treatments in a comparison) and number of groups (i.e. number of pollinated plants) used in the models;

<sup>4</sup>number of flow-cytometrically verified hybridogenic seedlings and number of pollinated plants from which these hybrids originate (see text for details);

<sup>5</sup>p-values ≤0.05 are given in bold.

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**Table 3.** Test for parental-like fitness components of heteroploid crosses of *Jacobaea carniolica*.

| Comparison of treatments <sup>1</sup>           | no. total <sup>2</sup> | coefficient $\pm$ SE | z- or t- value | p-value <sup>3</sup> |
|-------------------------------------------------|------------------------|----------------------|----------------|----------------------|
| <b>Seed viability</b>                           |                        |                      |                |                      |
| 2x4x $\leftrightarrow$ 4x4x                     | 9569/75                | $-2.37 \pm 0.31$     | -7.69          | <b>&lt;0.001</b>     |
| 4x2x $\leftrightarrow$ 4x4x                     | 11492/73               | $-2.41 \pm 0.34$     | -7.00          | <b>&lt;0.001</b>     |
| 2x6x $\leftrightarrow$ 6x6x                     | 10112/78               | $-3.14 \pm 0.37$     | -8.53          | <b>&lt;0.001</b>     |
| 6x2x $\leftrightarrow$ 6x6x                     | 11514/74               | $-2.79 \pm 0.46$     | -6.12          | <b>&lt;0.001</b>     |
| <b>Survival of hybrid seedlings</b>             |                        |                      |                |                      |
| 2x4x $\leftrightarrow$ 4x4x                     | 469                    | $-0.70 \pm 0.34$     | -2.09          | <b>0.037</b>         |
| 4x2x $\leftrightarrow$ 4x4x                     | 478                    | $-0.38 \pm 0.28$     | -1.35          | 0.177                |
| 2x6x $\leftrightarrow$ 2x2x                     | –                      | –                    | –              | –                    |
| 6x2x $\leftrightarrow$ 2x2x                     | 437                    | $-2.24 \pm 0.82$     | -2.72          | <b>0.007</b>         |
| 4x6x $\leftrightarrow$ 4x4x                     | 596                    | $1.56 \pm 0.30$      | 5.22           | <b>&lt;0.001</b>     |
| 4x6x $\leftrightarrow$ 6x6x                     | 616                    | $0.15 \pm 0.33$      | 0.44           | 0.659                |
| <b>Time to primary leaf in hybrid seedlings</b> |                        |                      |                |                      |
| 2x4x $\leftrightarrow$ 2x2x                     | 220/55                 | $-0.02 \pm 0.07$     | 0.32           | 0.748                |
| 4x2x $\leftrightarrow$ 4x4x                     | 137/48                 | $-0.13 \pm 0.08$     | -1.62          | 0.106                |
| <b>Size of hybrid seedlings</b>                 |                        |                      |                |                      |
| 2x4x $\leftrightarrow$ 2x2x                     | 165/52                 | $-0.59 \pm 0.35$     | 1.69           | 0.094                |
| 4x2x $\leftrightarrow$ 4x4x                     | 126/50                 | $-0.78 \pm 0.33$     | -2.37          | <b>0.020</b>         |
| 4x6x $\leftrightarrow$ 4x4x                     | 253/61                 | $0.39 \pm 0.24$      | 1.59           | 0.114                |

<sup>1</sup>maternal parent is given first; calculated only for significant comparisons in Table 2;<sup>2</sup>number of progeny and number of pollinated plants used in the (generalised) linear mixed-effects models;<sup>3</sup>p-values <0.05 are given in bold.

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incompatibility (Table 1). Instead, we observed a multilateral mentor effect [70], i.e. an increased selfing in the presence of heteroploid pollen, compared to SI-treatments (Table 1), especially

in diploid-hexaploid crosses, where the majority of seeds originated from selfing rather than hybridisation. Similar mentor effects have also been documented in other diploid and polyploid

**Table 4.** Influence of the direction of heteroploid cross-pollinations in *Jacobaea carniolica* on the fitness of the progeny.

| Comparison of treatments <sup>1</sup>           | no. <sup>2</sup> | coefficient $\pm$ SE | z- or t- value | p-value <sup>3</sup> |
|-------------------------------------------------|------------------|----------------------|----------------|----------------------|
| <b>Seed viability</b>                           |                  |                      |                |                      |
| 2x4x $\leftrightarrow$ 4x2x                     | 9833/76          | $-0.02 \pm 0.33$     | -0.08          | 0.940                |
| 2x6x $\leftrightarrow$ 6x2x                     | 9982/76          | $0.45 \pm 0.43$      | 1.04           | 0.299                |
| 4x6x $\leftrightarrow$ 6x4x                     | 11731/73         | $-0.02 \pm 0.34$     | -0.05          | 0.963                |
| <b>Survival of hybrid seedlings</b>             |                  |                      |                |                      |
| 2x4x $\leftrightarrow$ 4x2x                     | 279              | $-0.32 \pm 0.34$     | -0.93          | 0.352                |
| 2x6x $\leftrightarrow$ 6x2x                     | –                | –                    | –              | –                    |
| 4x6x $\leftrightarrow$ 6x4x                     | 588              | $0.44 \pm 0.33$      | 1.31           | 0.190                |
| <b>Time to primary leaf in hybrid seedlings</b> |                  |                      |                |                      |
| 2x4x $\leftrightarrow$ 4x2x                     | 57/33            | $0.43 \pm 0.10$      | 4.31           | <b>&lt;0.001</b>     |
| 2x6x $\leftrightarrow$ 6x2x                     | –                | –                    | –              | –                    |
| 4x6x $\leftrightarrow$ 6x4x                     | 329/66           | $-0.01 \pm 0.06$     | -0.23          | 0.816                |
| <b>Size of hybrid seedlings</b>                 |                  |                      |                |                      |
| 2x4x $\leftrightarrow$ 4x2x                     | 54/34            | $-2.20 \pm 0.50$     | -4.41          | <b>&lt;0.001</b>     |
| 2x6x $\leftrightarrow$ 6x2x                     | –                | –                    | –              | –                    |
| 4x6x $\leftrightarrow$ 6x4x                     | 327/66           | $0.68 \pm 0.21$      | 3.17           | <b>0.002</b>         |

<sup>1</sup>maternal parent is given first;<sup>2</sup>number of seeds/hybridogenic seedlings and pollinated plants used in the (generalised) linear mixed-effects models;<sup>3</sup>p-values <0.05 are given in bold.

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taxa [71,72], possibly counteracting fitness loss in the recipient of heteroploid pollen due to stigma clogging. Factors to remedy fitness loss in the pollen donor due to pollen loss remain, however, elusive.

Full interfertility of tetraploids and hexaploids suggests the absence of postmating isolation mechanisms between the polyploid cytotypes. Depending on vigour and fertility, pentaploids, which are restricted to areas of immediate contact (M. Sonnleitner & M. Winkler, pers. obs.), may determine the dynamics of such contact zones [73]. These include competition with parental cytotypes (pentaploid seedlings perform consistently better than tetraploid ones: Figure 1), mediating gene flow between the polyploid cytotypes, potentially affecting their ecological niches, or the establishment of a moving contact zone due to unidirectional backcrossing, which ultimately may expand the distribution of one and narrow the distribution of the other cytotype [74].

Our results might be biased because they were derived from a single sampling site only. However, the emerged pattern of cross-compatibility matches the co-occurrence pattern of cytotypes in the Eastern Alps [49] suggesting that the observed pattern of cross-compatibilities likely are valid for the entire distribution range.

### Dosage Effect

The observed pattern of cross-compatibility in heteroploid crosses of *J. carniolica*, evaluated by means of seed viability and seedling survival, can be explained by genome dosage effects. It matches the general expectation of a positive correlation between the magnitude of detrimental effects on seedling development and the deviation from the homoploid parental genome ratio of 2 m : 1p [46]. Thus, the almost complete failure of reciprocal diploid-hexaploid crosses reflects a 3-fold deviation (0.67 m : 1p, 6 m : 1p), low hybrid viability in diploid-tetraploid crosses corresponds to a 2-fold deviation (1 m : 1p, 4 m : 1p), and the lowest deviation (0.67-fold) in tetraploid-hexaploid crosses (1.33 m : 1p, 3 m : 1p) does not result in any fitness loss of hybrids. Similar patterns were observed in *Arabidopsis*, where crosses of diploids with tetraploids produced viable embryos, whereas crosses of diploids with hexaploids resulted in embryo abortion [46] (no crosses were, however, conducted between tetraploids and hexaploids). There is evidence for relaxed reproductive barriers between tetraploids and hexaploids (i.e. presence of pentaploids) as compared to diploids and polyploids in *Centaurea* [72] and *Knautia* [15]. These results are in line with the observation that deviations from the 2 m : 1p genome ratio in the endosperm can be tolerated to some extent [46].

An alternative explanation for different compatibilities among cytotypes may be sought in allelic differences. The diploid cytotype is genetically more distant from the polyploids than tetraploids and hexaploids are from each other (M. Winkler et al., unpublished data). Therefore, Bateson-Dobzhansky-Muller incompatibilities [75], pollen-pistil incompatibilities and/or impaired pollen tube growth [76], or nuclear-cytoplasmic interactions [77] may play a role as well.

In contrast to seed viability and seedling survival, seedling growth, estimated from time to first leaf and size of seedlings, was affected by the direction of crosses. This was particularly pronounced in crosses of diploids and tetraploids (Figure 1, Table 4). Triploids emerging from crosses of 2x4x grew faster and reached larger sizes than the average of parental treatments, whereas triploids emerging from 4x2x grew slower and remained smaller than the average of parental treatments. This pattern meets the expectations of complementary offspring phenotypes for reciprocal crosses in that paternal excess will result in large embryos [42,46,47], which may allow faster growth and larger

size, whereas small embryos due to maternal excess grow more slowly and remain smaller. An alternative explanation for these differences are cytoplasmic effects resulting in hybrid trait values similar to those of the maternal parent [78]. This is consistent not only with trait values in offspring from diploid-tetraploid crosses, but would also explain the smaller seedlings in 4x6x crosses (Figure 1, Table 4) despite paternal excess.

### Unreduced Gametes

The progeny derived from experimental crosses among cytotypes of *J. carniolica* showed a remarkable diversity of ploidies as observed in heteroploid crosses of other species [32,62,72]. Apart from intermediate ploidy (hybrid offspring) and maternal ploidy (self-fertilised offspring), the offspring of heteroploid crosses also contained ploidies best explained by the involvement of unreduced gametes. Almost all unreduced gametes involved in successful heteroploid crosses originated from the diploid parent, mainly in the form of unreduced pollen. Unreduced gametes have also been reported from other polyploid systems (e.g., refs. [32,72,79]), but their evolutionary impact is difficult to predict because the frequency of unreduced gametes differs strongly among taxa [80,81], among individuals and even within them [82,83]. In *J. carniolica*, mating success of unreduced gametes appears to depend on the presence/absence of heteroploid pollen. For instance, a high proportion of seeds (16 out of 57) originated from 6x2x crosses were pentaploid and, thus, implied the involvement of unreduced diploid pollen, but no evidence for unreduced gametes was found in homoploid control treatments. Very few pentaploids were found in natural diploid-hexaploid populations (3 out of 1595 individuals; [49]), which may indicate low *in situ* formation and/or low survival rates (no seedlings were investigated by Sonnleitner et al. [49]).

### Conclusions

Although our conclusions are drawn from the analysis of plants originating from a single mixed-ploidy site, the degree of postzygotic reproductive isolation seems to vary strongly among cytotypes of *J. carniolica*. A likely underlying cause is the dosage effect in the endosperm [46], because cross-compatibility decreases with increasing deviation from the balanced ratio of maternal to paternal genome and trait values of early seedling stages are largely congruent with differences in seed size expected for reciprocal heteroploid crosses. Thus, the isolation between diploids and polyploids might be an immediate consequence of polyploidisation, although allelic differentiation and/or reinforcement in secondary contact zones [84] cannot be excluded. Irrespective of the underlying mechanisms, cross-compatibility patterns among cytotypes, together with ecological segregation and postglacial (re)colonisation history, will affect the distribution of cytotypes. This is evident from the frequent syntopic occurrence of the nearly fully incompatible diploids and hexaploids compared to the rare co-occurrence of the fully compatible tetraploids and hexaploids, where pentaploid hybrids will determine the evolutionary dynamics of contact zones. The dependence of heteroploid cross-compatibility on the parental ploidy described here for the autopolyploid complex of *J. carniolica* determines the long-term stability of contact zones and their roles in autopolyploid diversification and speciation.

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## Author Contributions

Conceived and designed the experiments: MS PS KH. Performed the experiments: MS BW RF PE JS JK KH. Analyzed the data: MS BW JS JK MW KH. Wrote the paper: MS GMS PS KH. Read and approved manuscript: MS BW RF PE JS JK GMS MW PS KH.

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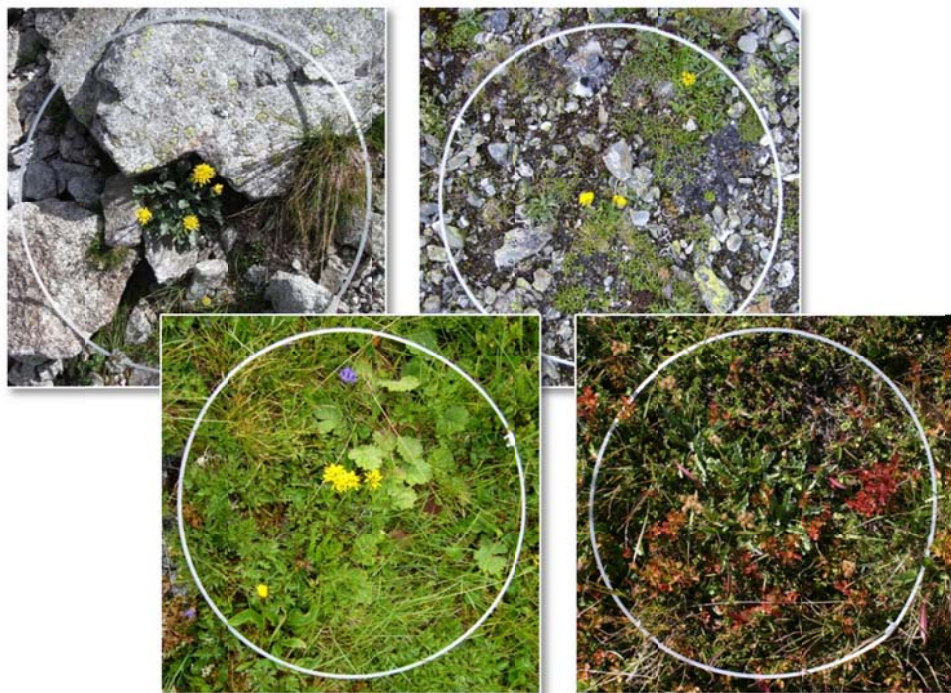
## CHAPTER 4: ECOLOGICAL DIFFERENTIATION AND NICHE DISPLACEMENT

### Article 3

**Sonnleitner M**, Hülber K, Flatscher R, Escobar García PE, Winkler M, Suda J, Schönswetter P, Schneeweiss GM. 2016. Ecological differentiation of diploid and polyploid cytotypes of *Senecio carniolicus* sensu lato (Asteraceae) is stronger in areas of sympatry. *Annals of Botany* 117(2): 269–276.

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Habitat types of *S. carniolicus* cytotypes

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## Ecological differentiation of diploid and polyploid cytotypes of *Senecio carniolicus sensu lato* (Asteraceae) is stronger in areas of sympatry

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• **Background and Aims** Ecological differentiation is recognized as an important factor for polyploid speciation, but little is known regarding whether the ecological niches of cytotypes differ between areas of sympatry and areas where single cytotypes occur (i.e. niche displacement).

• **Methods** Ecological niches of four groups of *Senecio carniolicus sensu lato* (*s.l.*) (western and eastern diploid lineages, tetraploids and hexaploids) were characterized via Landolt indicator values of the accompanying vascular plant species and tested using multivariate and univariate statistics.

• **Key Results** The four groups of *S. carniolicus s.l.* were ecologically differentiated mainly with respect to temperature, light and soil (humus content, nutrients, moisture variability). Niche breadths did not differ significantly. In areas of sympatry hexaploids shifted towards sites with higher temperature, less light and higher soil humus content as compared with homoploid sites, whereas diploids and tetraploids shifted in the opposite direction. In heteroploid sites of tetraploids and the western diploid lineage the latter shifted towards sites with lower humus content but higher aeration.

• **Conclusions** Niche displacement can facilitate the formation of stable contact zones upon secondary contact of polyploids and their lower-ploid ancestors and/or lead to convergence of the cytotypes' niches after they have attained non-overlapping ranges. Niche displacement is essential for understanding ecological consequences of polyploidy.

**Key words:** autopolyploidy, biodiversity, coexistence, contact zones, ecological differentiation, range-wide niche displacement, *Senecio carniolicus s.l.*, speciation.

### INTRODUCTION

Polyploidy is a major force in plant diversification and speciation (Wood *et al.*, 2009; Weiss-Schneeweiss *et al.*, 2013). Polyploidization can confer instantaneous reproductive isolation and is therefore regarded as the most frequent mode of sympatric speciation (Otto and Whitton, 2000). However, reproductive isolation is rarely complete, hampering independent evolution of the polyploid. It is well recognized, especially from modelling results, that dispersal limitation (Baack, 2005), high levels of selfing (Levin, 1983) and particularly ecological differentiation (Fowler and Levin, 1984; Levin, 2002; Johnson *et al.*, 2003; Oswald and Nuismer, 2011; Ramsey and Ramsey, 2014) are important factors allowing polyploids to persistently survive in sympatry with their lower-ploid ancestors, thus contributing to polyploid speciation.

Although habitat segregation is recognized as a major factor allowing different cytotypes to coexist (e.g. Lumaret *et al.*, 1987; Thompson and Lumaret, 1992; Felber-Girard *et al.*, 1996; Ståhlberg and Hedrén, 2009; Duchoslav *et al.*, 2010), little is known regarding whether the ecological niches of cytotypes differ between areas of sympatry and areas where single cytotypes occur. Habitat requirements of ecologically similar taxa might differ more pronouncedly when occurring in sympatry than in allopatry (Grace and Wetzel, 1981; Peers *et al.*, 2013), as is well known for phenotypic characters (i.e. character displacement: Dayan and Simberloff, 2005; Pfennig and Pfennig, 2009). In fact, the resulting niche displacement (Peers *et al.*, 2013) is expected to reflect a complex pattern of displacement in morphological, ecophysiological and/or behavioural characters. There is limited evidence suggesting niche

displacement in polyploid complexes (Felber-Girard *et al.*, 1996; Ståhlberg and Hedrén, 2009) but these studies were based on geographically restricted sampling with a low number of populations harbouring diploids and polyploids. Range-wide tests of niche displacement in polyploid complexes are lacking so far.

An excellent system to study ecological differentiation on a range-wide scale is *Senecio carniolicus sensu lato* (*s.l.*) (syn. *Jacobaea c.*; Asteraceae), a common plant in various alpine to nival habitats on siliceous bedrock in the eastern European Alps. This alpine polyploid complex consists of two geographically non-overlapping diploid lineages (Escobar García *et al.*, 2012) as well as one tetraploid and one hexaploid cytotype (Sonnleitner *et al.*, 2010; Fig. 1) that originated from the eastern diploid lineage (Sonnleitner *et al.*, 2013). Although recently described as four separate species (Flatscher *et al.*, 2015), we here refer to them as lineages or cytotypes to facilitate comparisons with previous studies (Schönswetter *et al.*, 2007; Sonnleitner *et al.*, 2010, 2013; Hülber *et al.*, 2015). Based on coarse estimates of site conditions, cytotypes are ecologically segregated, but overlap broadly (Sonnleitner *et al.*, 2010). The degree of separation and the discriminative ecological gradients, however, remain unknown. Throughout the distribution range of *S. carniolicus s.l.* in the eastern Alps, sites inhabited by a single cytotype (hereafter ‘homoploid sites’) as well as sites harbouring two or three cytotypes in sympatry (hereafter ‘heteroploid sites’) occur frequently (Suda *et al.*, 2007; Sonnleitner *et al.*, 2010; Fig. 1). The widespread co-occurrence of cytotypes might be facilitated by niche displacement, but this has not yet been tested. Here, we aim (1) to identify the ecological factors responsible for the previously identified cytotype differentiation (Sonnleitner *et al.*, 2010) and (2) to test whether realized niches in heteroploid sites differ from those in homoploid sites as expected under a model of niche displacement. To this end, we used Landolt indicator values for vascular plant species surrounding target individuals of *S. carniolicus* to characterize the ecological niches of the four *S. carniolicus* groups (two diploid lineages, tetraploids, hexaploids) on the microhabitat level. Although originally developed for the Swiss flora (Landolt, 1977), Landolt indicator values have subsequently been extended to the entire Alps (Landolt *et al.*, 2010). They represent qualitative assessments of ecological variables and hence are appropriate to characterize environmental conditions when their explicit measurement is not feasible because of, for instance, the remoteness and/or high number of sampling sites. Furthermore, they are less prone to between-year fluctuations in environmental conditions that may yield biased measurements, especially in cases of long-lived perennial plants of alpine regions (Diekmann, 2003).

## MATERIALS AND METHODS

### Fieldwork

Ecological niches of western and eastern diploid lineages and of the polyploids (tetraploids and hexaploids) were analysed based on samples from 99 sites (i.e. mountains) across the entire distribution area in the European Alps (Fig. 1) as described previously (Sonnleitner *et al.*, 2010). Individuals were selected to represent the entire local altitudinal range and all occupied

habitat types. As at the time of the fieldwork recognition of cytotypes based on morphological characters was not possible, cytotypes are expected to be included without bias. Cytotype assignment of each sampled individual was taken from Sonnleitner *et al.* (2010). Individuals showing secondary ploidy levels (triploid, pentaploid, heptaploid, octoploid and enneaploid, together comprising 1.07 % of analysed individuals) were not considered in this study, resulting in a total data set of 2826 individuals (i.e. on average 29 individuals per site; Supplementary Information Table S1).

### Statistical analyses

Environmental conditions were characterized by mean Landolt indicator values of accompanying vascular plant species (Landolt *et al.*, 2010) calculated for circular plots of 0.2 m radius around each target individual. Landolt indicator values describe ecological requirements of species in terms of climate parameters (temperature, T; continentality, K; light, L) and of soil parameters (moisture, F; reaction, R; nutrients, N; humus content, H; aeration, D; moisture variability, W), all ranging from 1 (low) to 5 (high). For species indifferent to particular indicator values, we used the mean of the concerned indicator values at the respective sites; *S. carniolicus* was omitted from the calculation.

### Multivariate analyses

Canonical correspondence analysis (CCA; ter Braak, 1986) was used to test for shifts of niche optima and differences in niche breadth among groups of *S. carniolicus*. CCA allows for non-linear and even non-monotonic (unimodal) relationships between species occurrences and environmental gradients, and performs well with skewed species distributions, with situations where not all environmental factors determining species composition are known, and even with highly correlated variables (Palmer, 1993). We constrained a matrix of Landolt indicator values, which were scaled to zero mean and unit variance prior to the ordination, by one of two design matrices. These design matrices, where columns represent the membership of a given individual to one of the groups without and with differentiation into homoploid and heteroploid sites, were used to test for overall niche differences among western diploids, eastern diploids, tetraploids and hexaploids, and for niche differences between homoploid and heteroploid sites of the same group (western diploids, eastern diploids, tetraploids and hexaploids), respectively. Because of only two sites inhabited exclusively by eastern diploids – one of which was only represented by six individuals – no comparisons between homoploid and heteroploid sites of this lineage were made.

Additionally, we identified effects of co-occurring cytotypes (the two diploid lineages do not co-occur: Escobar García *et al.*, 2012) by using design-matrices representing the presences/absences of cytotypes within sites. The target cytotype and each potentially co-occurring cytotype were represented by one column each in the design matrix. The target cytotype was defined as present only in homoploid sites (i.e. the target cytotype co-occurs only with itself). This analysis was performed

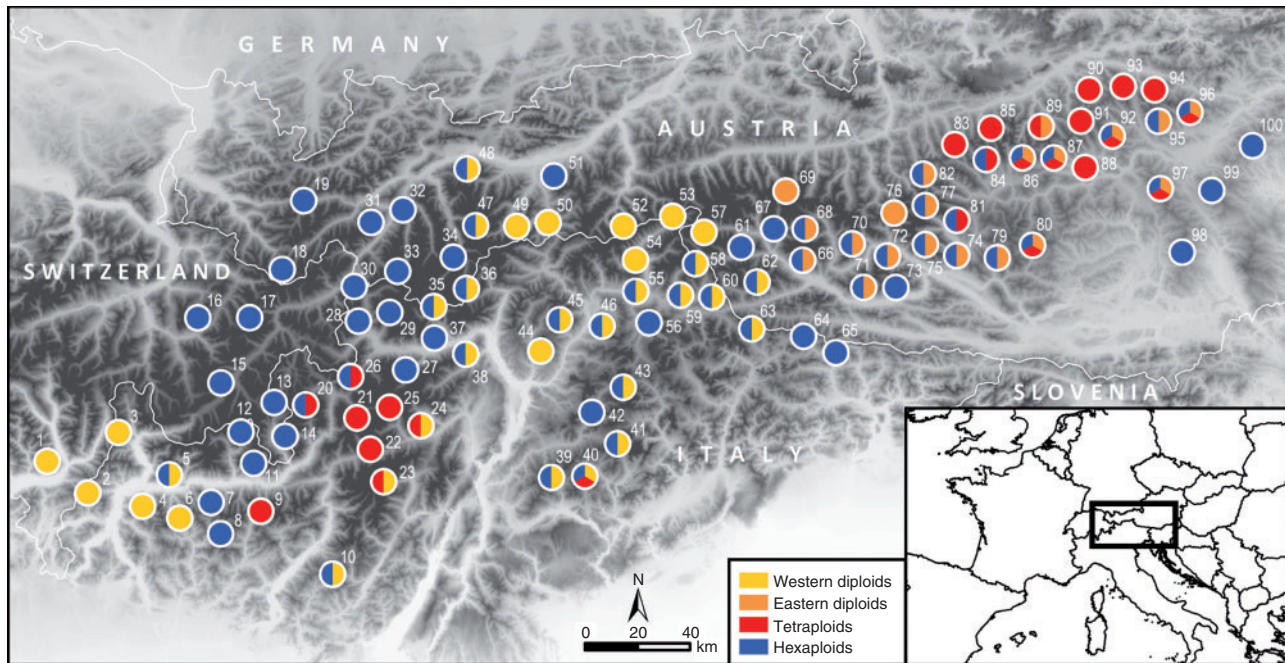


FIG. 1. Distribution of western diploids, eastern diploids, tetraploids and hexaploids of *Senecio carnolicus* in the eastern European Alps. Numbers correspond to site codes in Table S1 (Supplementary Information).

separately for individuals of each target cytotype except for eastern diploids.

Following Treier *et al.* (2009) we defined niche shifts as differences in niche optima among western diploids, eastern diploids, tetraploids and hexaploids, and niche breadth as the area of an ellipse representing a graphical summary of the cloud of individuals of each group (western diploids, eastern diploids, tetraploids and hexaploids). Niche shifts were calculated as the Euclidean distance between the centroids of the cloud of individuals of each group weighted by the Eigenvalues of ordination axes. Differences in niche breadth were calculated as differences in the area of ellipses defined by the niche optimum and the standard deviation of the projections of points on the ellipse axis, which were drawn orthogonally with maximal dispersion (Treier *et al.*, 2009). CCAs were computed with the maximum number of axes, but only the first and second axes were considered for the calculation of niche shift and breadth. Ten thousand Monte Carlo randomizations were applied by randomly assigning individuals to environmental conditions (i.e. by randomizing the rows in the design matrix). The significance of niche shifts and differences in niche breadth were evaluated by comparing the observed values with the distribution of simulated values in one-sided (observed > simulated) and two-sided tests (observed <> simulated), respectively.

To avoid the inflation of Type I errors due to multiple comparisons we adjusted the threshold for *P* values to be interpreted as significant according to the Dunn–Šidák correction:  $P = 1 - 0.95^{(1/n)}$ , where *n* is the number of comparisons. Thus, for six pairwise comparisons among eastern diploids, western diploids, tetraploids and hexaploids, and three comparisons between homoploid and heteroploid sites for western diploids,

tetraploids and hexaploids the threshold was set to 0.008 and 0.017, respectively.

#### Univariate analyses

To test for significant differences among western diploids, eastern diploids, tetraploids and hexaploids, linear mixed-effects models optimizing the restricted maximum-likelihood (following Laird and Ware, 1982) were applied separately to each Landolt indicator value. We used each indicator value as response, the groups as categorical predictor and the site as grouping variable to calculate random intercepts. With this approach we accounted for potential spatial clustering among individuals from the same site (Bolker *et al.*, 2009; Dullinger and Hülber, 2011). For all models we assumed a Gaussian error distribution. Degrees of freedom of the models were defined as the number of observations minus the number of estimated parameters (i.e. 4) minus 1, assuming that the grouping variable consumed only one degree of freedom.

To identify ecological gradients along which niches are displaced between homoploid and heteroploid sites a randomization procedure was applied. In pairwise comparisons of cytotypes (western diploids, tetraploids, hexaploids), for each cytotype 500 individuals from homoploid sites and 500 individuals from heteroploid sites, where the two compared cytotypes co-occur, were randomly chosen (with replacement) and differences in Landolt indicator values between individuals from homoploid and heteroploid sites were calculated. Statistical significance was assessed using two-sided *t*-tests adjusting for multiple comparisons by using Dunn–Šidák correction.

All analyses were performed with R 2.13.0 (<http://www.R-project.org/>). Mixed-effects models were calculated using the

function lmer included in the package lme4 (<http://cran.r-project.org/web/packages/lme4/lme4.pdf>). Niche shifts and niche breadth were evaluated using the ade4 package (Dray and Dufour, 2007).

## RESULTS

### *Ecological differentiation among groups*

The four groups of *S. carniolicus* (western diploids, eastern diploids, tetraploids and hexaploids) were ecologically differentiated. Linear mixed-effects models revealed significantly different positions of western diploids, eastern diploids, tetraploids and hexaploids along the temperature (T) gradient, with eastern diploids occupying the coldest sites, followed by tetraploids and western diploids at intermediate sites, and hexaploids at the warmest sites (Fig. 2). The same order of these four groups – eastern diploids and hexaploids occupying opposite ends of a gradient with western diploids and tetraploids in intermediate positions – was found for humus content (H), soil nutrients (N) and soil moisture variability (W), with differences among groups being stronger for humus content (H) than for soil nutrients (N) and soil moisture variability (W). A similar differentiation but in reverse order was found along the light (L) gradient, with tetraploids and western diploids occupying the same range along the gradient. For soil aeration (D) hexaploids showed the lowest values, followed by tetraploids and both diploids at equally high values. The four groups showed similar requirements for soil moisture (F) except western diploids, which inhabited drier micro-habitats. This is in line with high continentality (K) values for western diploids. No clear differentiation of groups was found for soil reaction (R) except between western diploids and hexaploids.

The Monte Carlo approach based on CCA revealed significant differences in niche optima of the four groups except for western diploids compared with tetraploids (Table 1). Groups were separated along an environmental gradient combining light (L), soil aeration (D), soil nutrients (N), humus content (H), temperature (T) and soil moisture variability (W; Supplementary Information Figs S1 and S2). Eastern diploids and hexaploids were located at the extreme ends of this gradient, while western diploids and tetraploids occupied intermediate positions. The latter two were separated, albeit not significantly, along a moisture (F) and continentality (K) gradient. In contrast to niche optima, no significant differences were found for niche breadth (Table 1).

### *Ecological differentiation between homoploid and heteroploid sites*

Ecological niches of cytotypes in heteroploid sites were displaced compared with homoploid sites. In a CCA (Fig. 3; Supplementary Information Fig. S3) niche optima of cytotypes in homoploid and heteroploid sites were significantly different for western diploids ( $P = 0.005$ ) and for tetraploids ( $P = 0.019$ ) but not for hexaploids ( $P = 0.168$ ; significance assessed via randomizations). However, compared with homoploid sites, niches of hexaploids in heteroploid sites tended towards higher temperature (T) and lower soil moisture (F), while tetraploids were displaced roughly in the opposite direction along the

gradient. Western diploid individuals occurred at sites with higher light (L) and soil aeration (D) in heteroploid compared with homoploid sites. These shifts resulted in stronger niche separation in the presence of other cytotypes, i.e. the pattern predicted by niche displacement. When looking at mutual effects of two cytotypes, the niches of hexaploids were displaced in response to the presence of tetraploids as well as of western diploids (Table 2), both of which responded to the presence of hexaploids. In contrast, tetraploids and western diploids did not interfere with each other, but tetraploids showed a stronger differentiation if co-occurring with eastern diploids. No changes in niche breadth were detected among homoploid and heteroploid sites (western diploids:  $P = 0.169$ ; tetraploids:  $P = 0.036$ ; hexaploids:  $P = 0.682$ ).

Significant differences in the direction of niche displacement were detected for single indicator values (Supplementary Information Fig. S4). In the presence of western diploid or tetraploid cytotypes, hexaploids shifted towards sites with higher temperature (T), less light (L) and higher humus content (H), whereas western diploids and tetraploids shifted towards lower temperature (T), more intensive light (L) and lower soil humus content (H). In heteroploid sites with tetraploids, western diploids shifted towards sites with lower humus content (H) but higher aeration (A).

## DISCUSSION

### *Ecological differentiation among cytotypes*

Niche differentiation is considered an important prezygotic isolation mechanism fostering polyploid establishment (Levin, 1983; Fowler and Levin, 1984; Thompson and Lumaret, 1992; Levin, 2003). Although climatic niches are often shared on a broad scale (Glennon *et al.*, 2014; but see Thompson *et al.*, 2014), ecological differentiation is amply documented on various scales (e.g. Johnson *et al.*, 2003; Duchoslav *et al.*, 2010; McIntyre, 2012). Using Landolt indicator values (Landolt *et al.*, 2010) as a surrogate for fine-scale environmental parameters, we show that the four groups – corresponding to three cytotypes – constituting *S. carniolicus* s.l. are indeed differentiated along a complex ecological gradient (Fig. 2, Supplementary Information Figs S1, S2; Table 1). Eastern diploids are found in open, wind-exposed, cold habitats with well-drained substrates, whereas hexaploids occupy habitats with deeper, more humus- and nutrient-rich soils and higher temperatures; habitats of tetraploids show intermediate ecological characteristics. Western diploids occur in drier conditions than these three groups (Supplementary Information Fig. S1).

The ecological differentiation of eastern diploids and their polyploid derivatives exhibits a clear directionality, reflecting the order of polyploidization events, i.e. tetraploids are autopolyploid derivatives of eastern diploids and hexaploids originated from tetraploids (western diploids were not involved in the ancestry of polyploids; Escobar García *et al.*, 2012; Sonnleitner *et al.*, 2013). Directional niche shift is probably the result of directional phenotypic change (e.g. taller growth of polyploids; Bennett, 1971), even though the relationship between phenotype and genome size (which correlates with ploidy level in *S. carniolicus*; Sonnleitner *et al.*, 2010) is not always monotonic (Balao *et al.*, 2011). The phenotypic traits

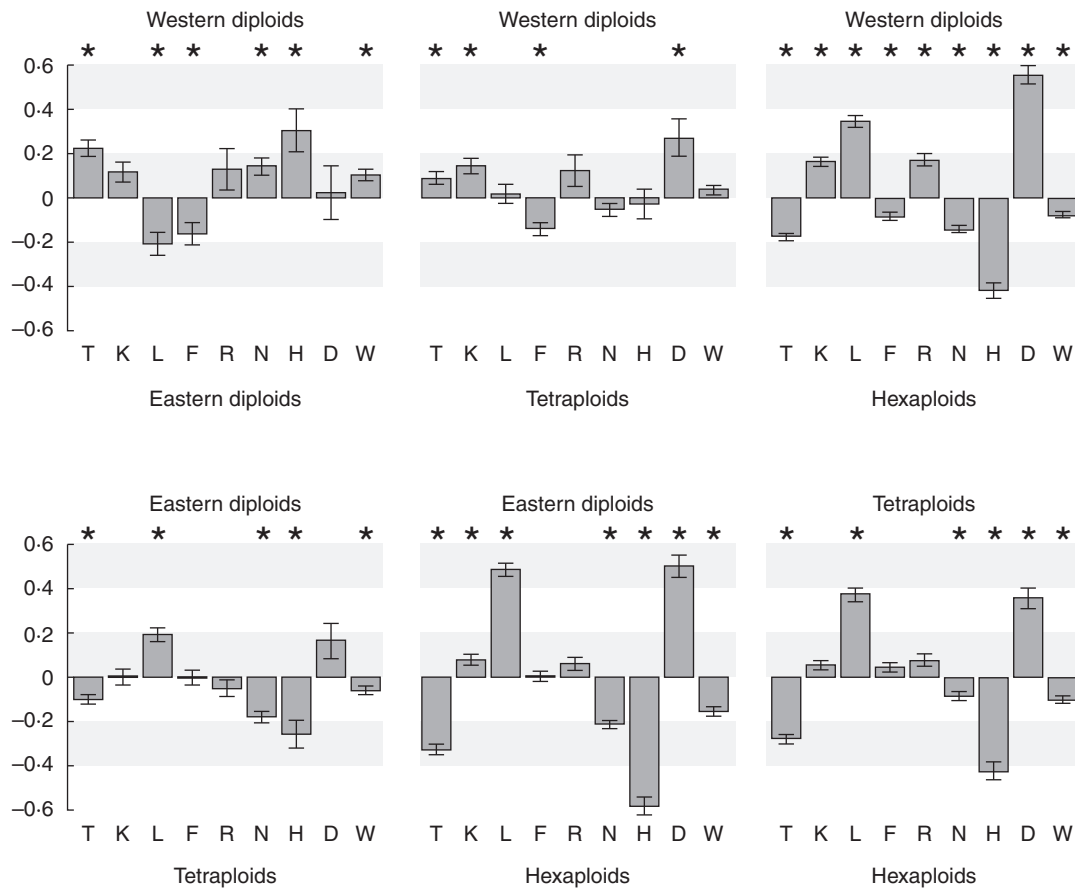


FIG. 2. Niche differentiation among western diploids ( $n = 612$ ), eastern diploids ( $n = 305$ ), tetraploids ( $n = 499$ ) and hexaploids ( $n = 1410$ ) of *Senecio carniolicus* derived from regression analyses of mean Landolt indicator values (Landolt *et al.*, 2010) for temperature (T), continentality (K), light (L), soil moisture (F), soil reaction (R), soil nutrients (N), humus content (H), soil aeration (D) and soil moisture variability (W). Bars show fixed-effects coefficients ( $\pm$ SE) of linear mixed-effects models representing differences in mean indicator values between two groups and point towards the group with the higher value; asterisks indicate significant differences.

TABLE 1. Comparison of niche optima (niche shift) and ecological tolerance (niche breadth) among groups of *Senecio carniolicus* in the Alps derived from canonical correspondence analysis

|                                     | Shift*           | Breadth |
|-------------------------------------|------------------|---------|
| Western diploids – eastern diploids | <b>0.001</b>     | 0.480   |
| Western diploids – tetraploids      | 0.394            | 0.534   |
| Western diploids – hexaploids       | <b>0.003</b>     | 0.866   |
| Eastern diploids – tetraploids      | <b>0.006</b>     | 0.514   |
| Eastern diploids – hexaploids       | <b>&lt;0.001</b> | 0.147   |
| Tetraploids – hexaploids            | <b>&lt;0.001</b> | 0.508   |

\*  $P$  values of significant differences among groups after Dunn–Šidák correction for multiple comparisons are given in bold.

underlying the directional ecological differentiation of polyploid *S. carniolicus* are unknown, but probably include stouter habit (Flatscher *et al.*, 2015) conferring higher competitive ability (Levin, 2002; Parisod *et al.*, 2010) in habitats with denser vegetation cover (Sonnleitner *et al.*, 2010). In addition, polyploid *S. carniolicus* may have higher nutritional demands, which would be more easily met in habitats with deeper and more nutrient-rich soil (Supplementary Information Fig. S1). In

polyploid *S. carniolicus* these same traits may be (co-)responsible for the lack of increased niche breadth, presumed to be common in polyploids (Parisod *et al.*, 2010).

#### Displacement, but no narrowing of niches in sympatry

Although character displacement is recognized as an important mechanism for enabling closely related species to coexist (Dayan and Simberloff, 2005; Pfennig and Pfennig, 2009; Beans, 2014), it has rarely been invoked in the context of cytotype co-occurrence (Van Dijk *et al.*, 1992; Van Dijk and Bijlsma, 1994). We show that the niches of cytotypes in a heteroploid species are affected by the presence/absence of other cytotypes, i.e. that niche displacement (Peers *et al.*, 2013) occurs. Specifically, niche optima between homoploid and heteroploid sites were shifted roughly along the same environmental gradients that determine the cytotypes' niches, leading to stronger ecological differentiation of co-occurring cytotypes (Fig. 3, Supplementary Information Fig. S3). Previously, patterns consistent with niche displacement have been found in the grass *Anthoxanthum alpinum* in a narrow contact zone of ecologically non-differentiated diploids and autotetraploids (Felber-Girard

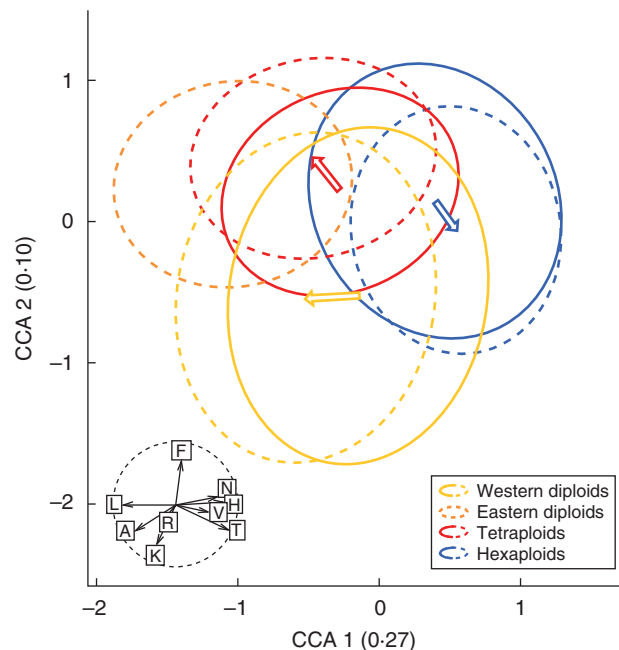


FIG. 3. Niche displacement between homoploid (solid outline) and heteroploid sites (dashed outline) of western diploids ( $n_{\text{homoploid}} = 337$ ,  $n_{\text{heteroploid}} = 275$ ), eastern diploids ( $n_{\text{heteroploid}} = 270$ ), tetraploids ( $n_{\text{homoploid}} = 294$ ,  $n_{\text{heteroploid}} = 205$ ) and hexaploids ( $n_{\text{homoploid}} = 856$ ,  $n_{\text{heteroploid}} = 554$ ) of *Senecio carnolicus* derived from canonical correspondence analysis. Homoploid sites of eastern diploids were not included due to their low number. Differentiation of niche optima was defined as the Euclidean distance among centroids of ellipses weighted by axis inertia. The correlation circle ( $r = 1$ ) shows the environmental variables used as constraints in the ordination, i.e. the Landolt indicator values for temperature (T), continentality (K), light (L), soil moisture (F), soil reaction (R), soil nutrient (N), humus content (H), soil aeration (D) and soil moisture variability (W) averaged for all vascular plant species surrounding *S. carnolicus* individuals. Axes inertias (i.e. eigenvalues of constrained axes) are given in parentheses. Arrows indicate the direction of shifts in niche optima from homoploid to heteroploid sites.

TABLE 2. Comparison of niche optima (niche shift) and ecological tolerance (niche breadth) between homoploid and heteroploid sites for groups of *Senecio carnolicus*

|                       | Shift*           | Breadth |
|-----------------------|------------------|---------|
| Western diploids:     |                  |         |
| with tetraploids      | 0.105            | 0.053   |
| with hexaploids       | <b>&lt;0.001</b> | 0.581   |
| Tetraploids:          |                  |         |
| with western diploids | 0.493            | 0.594   |
| with eastern diploids | <b>0.001</b>     | 0.765   |
| with hexaploids       | <b>&lt;0.001</b> | 0.750   |
| Hexaploids:           |                  |         |
| with western diploids | <b>0.001</b>     | 0.471   |
| with eastern diploids | 0.042            | 0.447   |
| with tetraploids      | <b>&lt;0.001</b> | 0.601   |

\* *P* values of significant differences after Dunn–Šidák correction for multiple comparisons are given in bold.

*et al.*, 1996) as well as in contact zones of the diploid and autotetraploid orchid *Dactylorhiza maculata* s.l. with evidence for wider ecological amplitudes in homoploid compared with heteroploid populations (Ståhlberg and Hedrén, 2009). However, both studies cover only one or two local contact zones.

Patterns consistent with niche displacement can also result from variation in resource availability between homo- and heteroploid populations, ecological sorting or chance (Grant, 1975; Arthur, 1982). In *S. carnolicus*, homoploid and heteroploid sites are found over large parts of the distribution range and are not geographically separated (Fig. 1), rendering variation in resource availability or processes of ecological sorting as the sole causes for the observed divergence unlikely. Using a randomization procedure (i.e. comparing the actual shift against a null distribution), we confirmed that niche shifts are stronger than expected by chance.

The occurrence of niche displacement in *S. carnolicus* depended on the cytotypes involved, but always was symmetrical, making competitive superiority of either partner (Cooley, 2007) as the underlying cause unlikely. Hexaploids showed significant displacement in sympatry with western diploids and tetraploids, but not with eastern diploids (Table 2; this may be responsible for the lack of statistical significance in the CCA) despite their frequent co-occurrence (Schönswetter *et al.*, 2007; Hülber *et al.*, 2009; Sonnleitner *et al.*, 2010). As hexaploids and eastern diploids are reproductively isolated via strongly reduced seed set and seed viability (Sonnleitner *et al.*, 2013) and showed well-differentiated niches (Fig. 2, Table 1), selective pressure for stronger niche differentiation may be low. Tetraploids showed significant displacement with hexaploids and eastern diploids, but not with western diploids (Table 2), despite having similar ecological requirements (Fig. 2), which might be due to their rare co-occurrence (Sonnleitner *et al.*, 2010).

In contrast to previously studied plants (Felber-Girard *et al.*, 1996; Ståhlberg and Hedrén, 2009), niche displacement in *S. carnolicus* includes shifts in niche optima but no significant change in niche breadth (Table 2). Niche breadths may remain constant if resource use expands into or increases in portions of the fundamental niche that are not or are only rarely realized in homoploid populations (e.g. Peers *et al.*, 2013). Due to our sampling design (on average 29 *S. carnolicus* individuals per location) it cannot be ruled out that rarely occupied habitats have remained under-sampled and that our estimates of niche breadth are therefore conservative.

A consequence of niche displacement is that upon secondary contact polyploids and their lower-ploid ancestors can form stable contact zones more easily than expected from the cytotypes' ecological niches, especially if cytotypes still compete for resources or are prone to fitness loss due to the formation of inferior hybrids in heteroploid crosses, as probably is the case for *S. carnolicus*, where pollination barriers are lacking and polyploid cytotypes are interfertile (Sonnleitner *et al.*, 2013). Hence, niche displacement may be (co-)responsible for secondary contact zones being common (Petit *et al.*, 1999). An alternative yet not mutually exclusive consequence of niche displacement can be that overall ecological niches of polyploids and their lower-ploid ancestors converge after cytotypes have attained largely or completely non-overlapping distributions. This may contribute to the observed similarity of climatic niches of diploid plants and their polyploid derivatives (Glennon *et al.*, 2014), or the perceived niche expansion of invasive polyploids in their non-native ranges (te Beest *et al.*, 2011). Testing any of these hypotheses is currently not possible because studies on ecological differentiation among cytotypes

usually do not discriminate between homoploid and heteroploid populations. As is evident from the present study, taking niche displacement into account will be essential for understanding ecological consequences of polyploidy, a major force generating biodiversity.

## SUPPLEMENTARY INFORMATION

Supplementary data are available online at [www.aob.oxfordjournals.org](http://www.aob.oxfordjournals.org) and consist of the following. Table S1: an overview of the sample sites and DNA ploidy level of sampled individuals. Figures S1–S4: detailed information concerning niche differentiation of the four groups of *S. carniolicus* and niche displacement within groups between homoploid and heteroploid sites.

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## Supplementary Information

TABLE S1. Sample sites used to test for ecological differentiation among groups of *Senecio carniolicus*. Geographic position (centroid of the individual coordinates) and altitudinal range of sampled individuals are given together with the number of individuals per main DNA ploidy level and the total number of individuals investigated per sample site. Population no 78 has been omitted from analyses because it is the only one on calcareous bedrock. Herbarium vouchers stored in WU are labelled with the site code. Country abbreviations: A, Austria; CH, Switzerland; IT, Italy; SLO, Slovenia (modified from Sonnleitner et al. 2010)

| Code | Locality (Country)              | Geographic position |                    |                                 | Number of samples |    |    |    |
|------|---------------------------------|---------------------|--------------------|---------------------------------|-------------------|----|----|----|
|      |                                 | Latitude<br>(° N)   | Longitude<br>(° E) | Altitudinal<br>range (m a.s.l.) | 2x                | 4x | 6x | N  |
| 1    | Cima dell Uomo (CH)             | 46.231              | 8.940              | 2249–2358                       | 12                |    |    | 12 |
| 2    | Pizzo di Gino (IT)              | 46.123              | 9.144              | 1864–2226                       | 35                |    |    | 35 |
| 3    | Campanile di Val Marina (IT)    | 46.333              | 9.294              | 2134–2495                       | 29                |    |    | 29 |
| 4    | Monte Legnone (IT)              | 46.093              | 9.417              | 2122–2591                       | 28                |    |    | 28 |
| 5    | Passo Locino (IT)               | 46.188              | 9.549              | 2110–2553                       | 10                |    | 20 | 30 |
| 6    | Monte Verrobbio (IT)            | 46.039              | 9.602              | 2044–2083                       | 24                |    |    | 24 |
| 7    | Cima Cadelle (IT)               | 46.059              | 9.743              | 2112–2482                       |                   |    | 22 | 22 |
| 8    | Laghi Gemelli (IT)              | 45.986              | 9.805              | 2142–2330                       |                   |    | 21 | 21 |
| 9    | Pizzo di Coca (IT)              | 46.064              | 10.003             | 2174–2724                       |                   | 31 |    | 31 |
| 10   | Monte Colombine (IT)            | 45.845              | 10.359             | 2095–2212                       | 12                |    | 18 | 30 |
| 11   | Bocchetta Vicima (IT)           | 46.229              | 9.968              | 2242–2689                       |                   |    | 30 | 30 |
| 12   | Bocchetta della Forbici (IT)    | 46.326              | 9.904              | 2175–2673                       |                   |    | 30 | 30 |
| 13   | Diavolezza, Munt Pers (CH)      | 46.420              | 9.971              | 2127–3162                       |                   |    | 33 | 33 |
| 14   | Monte Vago (IT)                 | 46.439              | 10.070             | 2313–3055                       |                   |    | 34 | 34 |
| 15   | Piz Nair (CH)                   | 46.507              | 9.804              | 2355–2991                       |                   |    | 30 | 30 |
| 16   | Sandhubel (CH)                  | 46.732              | 9.687              | 2346–2697                       |                   |    | 30 | 30 |
| 17   | Flüela Schwarzhorn (CH)         | 46.733              | 9.650              | 2347–3093                       |                   |    | 28 | 28 |
| 18   | Hohes Rad (AT)                  | 46.899              | 10.112             | 2120–2910                       |                   |    | 30 | 30 |
| 19   | Arlensattel (AT)                | 47.137              | 10.221             | 2073–2162                       |                   |    | 30 | 30 |
| 20   | Monte Verva (IT)                | 46.431              | 10.228             | 2098–2967                       |                   | 11 | 17 | 28 |
| 21   | Monte di Gavia (IT)             | 46.342              | 10.483             | 2404–3077                       |                   | 24 |    | 24 |
| 22   | Monte Serodine (IT)             | 46.276              | 10.550             | 2476–2629                       |                   | 31 |    | 31 |
| 23   | Cresta del Belvedere (IT)       | 46.164              | 10.615             | 2305–2581                       | 6                 | 19 |    | 25 |
| 24   | Cima Valletta (IT)              | 46.356              | 10.803             | 2260–2821                       | 1                 | 28 |    | 29 |
| 25   | Cima Cavaion (IT)               | 46.420              | 10.701             | 2386–2826                       |                   | 28 |    | 28 |
| 26   | Stilfser Joch (IT)              | 46.528              | 10.453             | 2539–3078                       |                   | 9  | 18 | 27 |
| 27   | Laaser Spitze (IT)              | 46.550              | 10.729             | 2272–2850                       |                   |    | 30 | 30 |
| 28   | Watles (IT)                     | 46.719              | 10.494             | 2161–2551                       |                   |    | 34 | 34 |
| 29   | Steinmandlköpfl, Mittereck (IT) | 46.749              | 10.598             | 2347–2864                       |                   |    | 33 | 33 |
| 30   | Piz Lad (IT)                    | 46.840              | 10.475             | 2227–2740                       |                   |    | 29 | 29 |
| 31   | Fisser Joch, Brunnenkopf (AT)   | 47.061              | 10.562             | 2520–2670                       |                   |    | 29 | 29 |
| 32   | Hohe Aifnerspitze (AT)          | 47.103              | 10.724             | 2265–2705                       |                   |    | 30 | 30 |
| 33   | Riffljoch (AT)                  | 46.891              | 10.694             | 2380–2950                       |                   |    | 29 | 29 |
| 34   | Gaislachkogel (AT)              | 46.937              | 10.976             | 2290–2955                       |                   |    | 29 | 29 |
| 35   | Schröfwand (IT)                 | 46.739              | 10.874             | 2398–2730                       | 1                 |    | 21 | 22 |
| 36   | Festkogel (AT)                  | 46.855              | 11.041             | 2170–3040                       | 14                |    | 15 | 29 |
| 37   | Vermoispitze (IT)               | 46.659              | 10.875             | 2486–2875                       |                   |    | 8  | 8  |
| 38   | Naturnser Hochwart (IT)         | 46.601              | 11.022             | 2201–2605                       | 13                |    | 18 | 31 |
| 39   | Monte Ziolera (IT)              | 46.170              | 11.450             | 2035–2747                       | 16                |    | 14 | 30 |
| 40   | Cima D'Asta (IT)                | 46.172              | 11.616             | 1969–2847                       | 2                 | 18 | 11 | 31 |
| 41   | Cavallazza Piccola (IT)         | 46.282              | 11.782             | 2020–2316                       | 20                |    | 9  | 29 |
| 42   | Col Margherita (IT)             | 46.356              | 11.799             | 2091–2514                       |                   |    | 31 | 31 |
| 43   | Pre de Ciapel (IT)              | 46.476              | 11.818             | 2359–2553                       | 7                 |    | 21 | 28 |
| 44   | Sarner Scharte (IT)             | 46.647              | 11.410             | 2035–2435                       | 30                |    |    | 30 |
| 45   | Schrotthorn (IT)                | 46.715              | 11.507             | 2252–2589                       | 23                |    | 10 | 33 |
| 46   | Plose (IT)                      | 46.690              | 11.720             | 2134–2408                       | 24                |    | 6  | 30 |

|     |                                  |        |        |           |    |    |    |    |
|-----|----------------------------------|--------|--------|-----------|----|----|----|----|
| 47  | Schrankogel (AT)                 | 47.035 | 11.091 | 2190–3135 | 14 |    | 15 | 29 |
| 48  | Rietzer Griebkogel (AT)          | 47.242 | 11.052 | 2230–2795 | 13 |    | 17 | 30 |
| 49  | Habicht (AT)                     | 47.041 | 11.299 | 2600–2740 | 30 |    |    | 30 |
| 50  | Nöblachjoch, Eggersteller (AT)   | 47.052 | 11.434 | 2180–2270 | 30 |    |    | 30 |
| 51  | Patscherkofel, Viggarspitze (AT) | 47.212 | 11.492 | 2000–2295 |    |    | 30 | 30 |
| 52  | Saurüssel (AT)                   | 47.033 | 11.839 | 2520–2885 | 30 |    |    | 30 |
| 53  | Rauchkofel (IT)                  | 47.069 | 12.090 | 2247–2797 | 30 |    |    | 30 |
| 54  | Speikboden (IT)                  | 46.915 | 11.896 | 2204–2513 | 29 |    |    | 29 |
| 55  | Sambock (IT)                     | 46.851 | 11.894 | 2175–2396 | 22 |    | 9  | 31 |
| 56  | Kronplatz (IT)                   | 46.737 | 11.957 | 2255–2281 |    |    | 7  | 7  |
| 57  | Antholzer Scharte (IT)           | 46.892 | 12.109 | 1970–2600 | 30 |    |    | 30 |
| 58  | Almerhorn (AT)                   | 46.896 | 12.198 | 2070–2810 | 10 |    | 21 | 31 |
| 59  | Riepenspitz (IT)                 | 46.846 | 12.288 | 2114–2771 | 13 |    | 20 | 33 |
| 60  | Toblacher Pfannhorn (IT)         | 46.779 | 12.276 | 2147–2661 | 14 |    | 17 | 31 |
| 61  | Donnerstein (AT)                 | 46.948 | 12.431 | 2675–2685 |    |    | 16 | 16 |
| 62  | Gölbner (AT)                     | 46.826 | 12.505 | 2408–2946 | 27 |    | 4  | 31 |
| 63  | Col Quaternà (IT)                | 46.665 | 12.471 | 2028–2506 | 13 |    | 16 | 29 |
| 64  | Monte Peralba (IT)               | 46.633 | 12.729 | 2279–2383 |    |    | 33 | 33 |
| 65  | Monte Crostis (IT)               | 46.571 | 12.888 | 2028–2283 |    |    | 30 | 30 |
| 66  | Schleinitz (AT)                  | 46.896 | 12.730 | 2193–2698 | 30 |    | 2  | 32 |
| 67  | Kalser Höhe (AT)                 | 47.006 | 12.599 | 2219–2363 |    |    | 30 | 30 |
| 68  | Schönleiten Spitze (AT)          | 46.992 | 12.680 | 2174–2759 | 28 |    | 2  | 30 |
| 69  | Kapruner Törl (AT)               | 47.137 | 12.668 | 2346–2632 | 6  |    |    | 6  |
| 70  | Sadnig (AT)                      | 46.945 | 12.993 | 2202–2744 | 20 |    | 13 | 33 |
| 71  | Scharnik (AT)                    | 46.793 | 13.041 | 2418–2656 | 19 |    | 11 | 30 |
| 72  | Polinik (AT)                     | 46.889 | 13.167 | 1986–2767 | 17 |    | 13 | 30 |
| 73  | Dolzer, Gaugen (AT)              | 46.789 | 13.203 | 2090–2195 |    |    | 30 | 30 |
| 74  | Gmeineck (AT)                    | 46.891 | 13.428 | 1419–2575 | 8  |    | 22 | 30 |
| 75  | Reißeck (AT)                     | 46.932 | 13.364 | 2516–2743 | 28 |    | 1  | 29 |
| 76  | Ankogel (AT)                     | 47.045 | 13.238 | 2615–2868 | 29 |    |    | 29 |
| 77  | Großer Hafner (AT)               | 47.066 | 13.393 | 2280–3007 | 27 |    | 3  | 30 |
| 78  | Belščica (AT / SLO)              | 46.443 | 14.147 | 2000–2050 | 30 |    |    | 30 |
| 79  | Rosennock (AT)                   | 46.874 | 13.721 | 1879–2424 | 9  |    | 20 | 29 |
| 80  | Bretthöhe (AT)                   | 46.910 | 13.902 | 1997–2312 | 7  | 5  | 17 | 29 |
| 81  | Wandspitze (AT)                  | 47.010 | 13.526 | 2066–2605 |    | 17 | 7  | 24 |
| 82  | Balonspitze (AT)                 | 47.123 | 13.480 | 2219–2828 | 9  |    | 20 | 29 |
| 83  | Seekarspitze (AT)                | 47.272 | 13.544 | 2196–2410 |    | 30 |    | 30 |
| 84  | Zechnerkarspitze (AT)            | 47.215 | 13.690 | 2120–2421 |    | 10 | 16 | 26 |
| 85  | Trockenbrotscharte (AT)          | 47.273 | 13.720 | 1919–2379 |    | 28 |    | 28 |
| 86  | Preber (AT)                      | 47.215 | 13.868 | 2293–2733 | 13 | 14 | 3  | 30 |
| 87  | Predigstuhl (AT)                 | 47.257 | 13.912 | 2081–2540 | 15 | 8  | 7  | 30 |
| 88  | Deneck (AT)                      | 47.284 | 14.053 | 2044–2426 |    | 26 |    | 26 |
| 89  | Großer Knallstein (AT)           | 47.318 | 13.978 | 2193–2598 | 3  | 27 |    | 30 |
| 90  | Hochrettelstein (AT)             | 47.424 | 14.232 | 2120–2227 |    | 12 |    | 12 |
| 91  | Hohenwart (AT)                   | 47.328 | 14.237 | 1875–2374 |    | 29 |    | 29 |
| 92  | Schießeck (AT)                   | 47.272 | 14.337 | 1997–2294 | 28 | 16 | 23 | 67 |
| 93  | Großer Bösenstein (AT)           | 47.441 | 14.408 | 1953–2406 |    | 28 |    | 28 |
| 94  | Gamskogel (AT)                   | 47.366 | 14.546 | 1929–2329 |    | 27 |    | 27 |
| 95  | Großer Ringkogel (AT)            | 47.311 | 14.628 | 1943–2277 | 5  |    | 26 | 31 |
| 96  | Seckauer Zinken (AT)             | 47.337 | 14.740 | 1903–2339 | 2  | 21 | 4  | 27 |
| 97  | Zirbitzkogel (AT)                | 47.080 | 14.565 | 1848–2223 | 2  | 2  | 26 | 30 |
| 98  | Saualpe (AT)                     | 46.856 | 14.654 | 1948–2115 |    |    | 30 | 30 |
| 99  | Ameringkogel (AT)                | 47.062 | 14.824 | 1891–2183 |    |    | 30 | 30 |
| 100 | Roßbachkogel (AT)                | 47.207 | 15.045 | 1763–1877 |    |    | 20 | 20 |

FIG. S1. Niche differentiation among western diploids (N=612), eastern diploids (N=305), tetraploids (N=499) and hexaploids (N=1410) of *Senecio carniolicus* derived from Canonical Correspondence Analysis (CCA). Differentiation of niche optima was defined as the Euclidean distance among centroids of ellipses weighted by axis inertia. The area of ellipses represents niche breadth (i.e., ecological tolerance) of groups. The correlation circle ( $r=1$ ) shows the environmental variables used as constraints in the ordination, i.e., the Landolt indicator values (Landolt *et al.*, 2010) for temperature (T), continentality (K), light (L), soil moisture (F), soil reaction (R), soil nutrients (N), humus content (H), soil aeration (D) and soil moisture variability (W) averaged for all vascular plant species surrounding *S. carniolicus* individuals. Axes inertias (i.e., Eigenvalues of constrained axes) are given in parentheses.

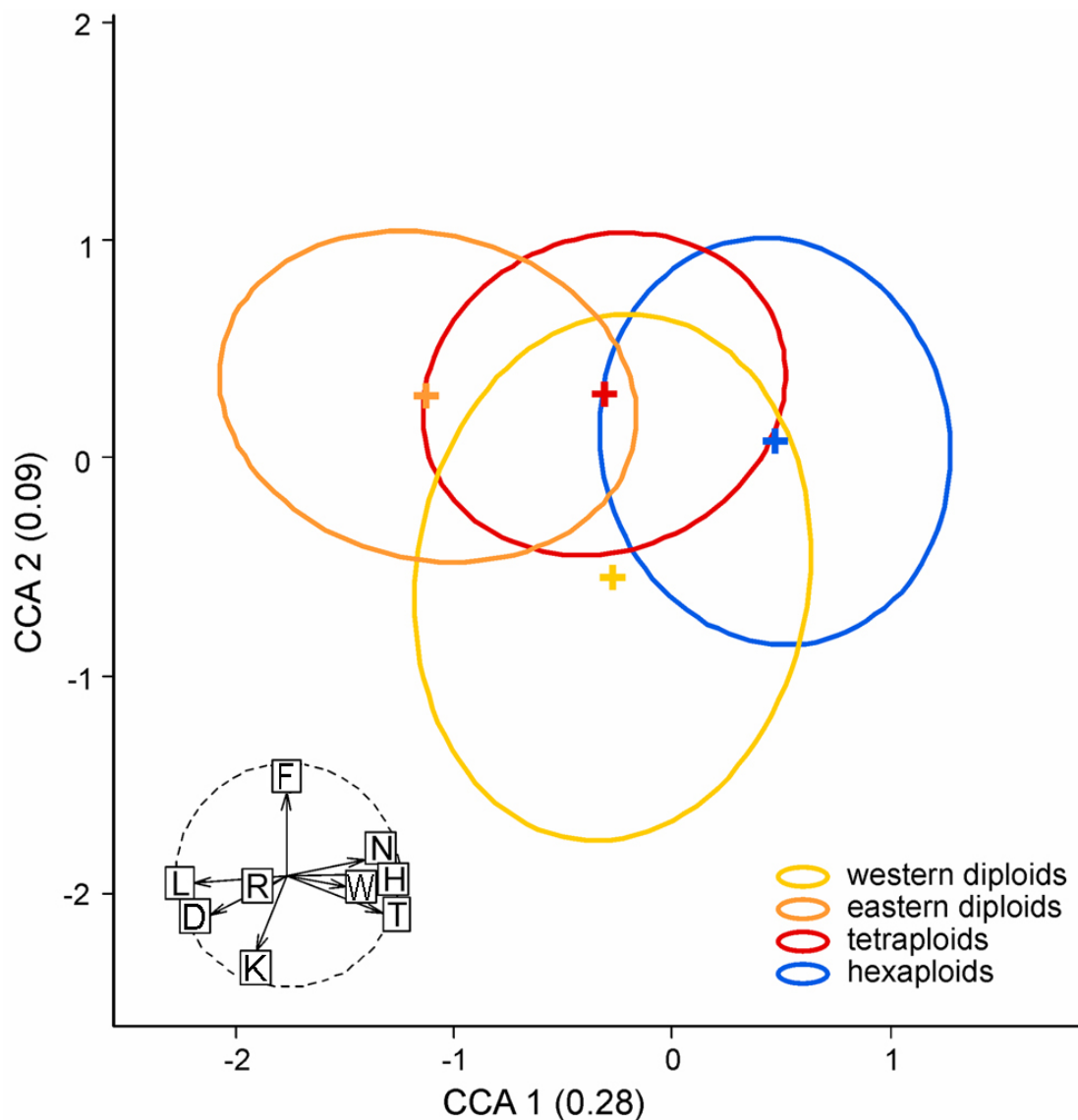


FIG. S2. Niche differentiation among western diploids (N=612), eastern diploids (N=305), tetraploids (N=499) and hexaploids (N=1410) of *Senecio carniolicus* derived from Canonical Correspondence Analysis (CCA). Differentiation of niche optima was defined as the Euclidean distance among centroids of ellipses weighted by axis inertia. Color intensity represents the density of individuals in the ordination space. Centroids (i.e., niche optima) are shown as crosses for each group in each panel, but are enlarged and bold for the focal group.

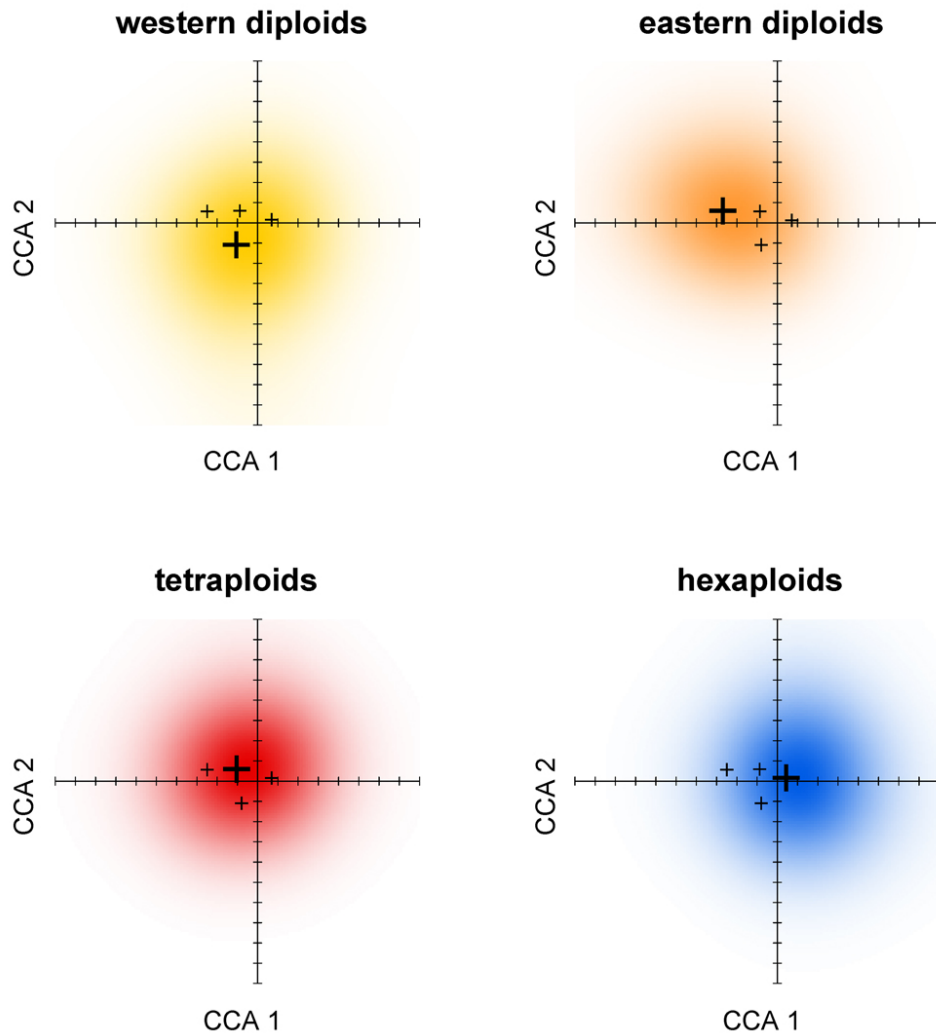


FIG. S3. Niche displacement between homoploid and heteroploid sites of *Senecio carniolicus* derived from Canonical Correspondence Analysis. Color intensity represents the density of individuals in the ordination space. Centroids (i.e., niche optima) for homoploid and heteroploid sites are given as black and white crosses, respectively, in each panel, but are enlarged for the focal site.

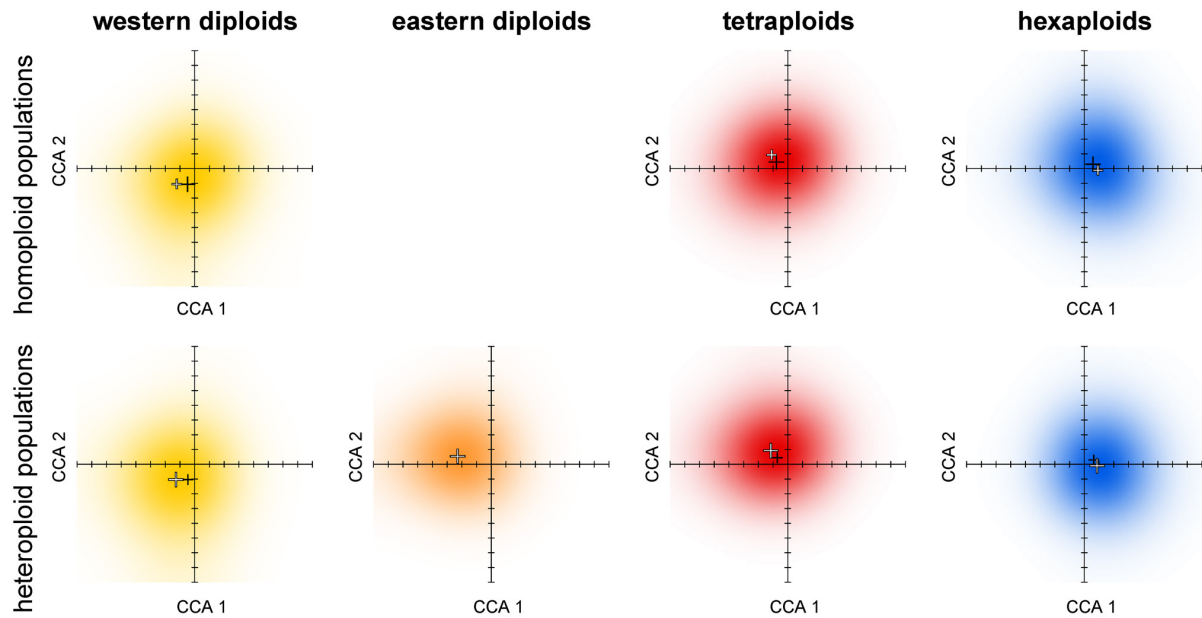
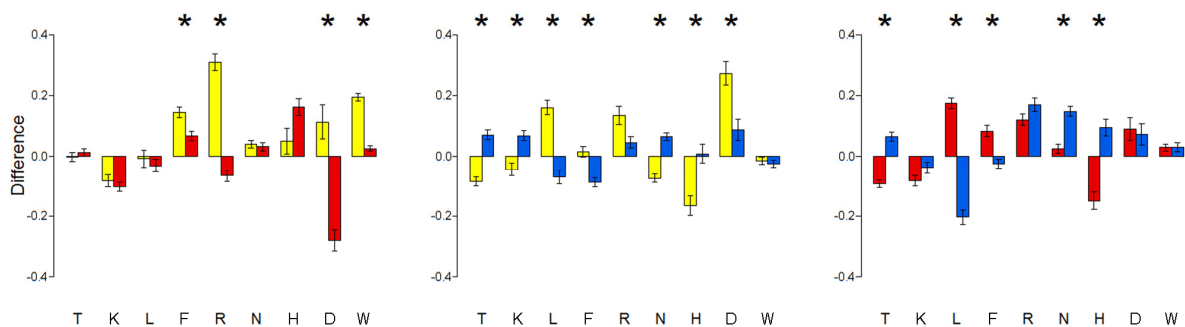


FIG. S4. Pairwise comparison of mean differences ( $\pm$  SE) in mean Landolt indicator values of vascular plant species accompanying randomly selected individuals of *Senecio carniolicus* from homoploid and from those heteroploid sites where the second cytotype is present (N=500 each). Asterisks indicate significant differences (after Dunn-Šidák correction) between western diploids (yellow), tetraploids (red) and hexaploids (blue) in pairwise comparisons. Letters represent indicator values for temperature (T), continentality (K), light (L), soil moisture (F), soil reaction (R), soil nutrients (N), humus content (H), soil aeration (D) and soil moisture variability (W).





## CHAPTER 5: APPENDIX

This chapter contains citations and abstracts of additional papers published in the context of the project “Origin and maintenance of intrapopulational cytotype mixture in an alpine plant species (*Senecio carniolicus*, Asteraceae)” which has been the framework of this thesis.

Schönschwetter P, Lachmayer M, Lettner C, Prehsler D, Rechner S, Reich DS, Sonnleitner M, Wagner I, Hülber K, Schneeweiss GM, Trávníček P, Suda J. 2007. **Sympatric diploid and hexaploid cytotypes of *Senecio carniolicus* (Asteraceae) in the Eastern Alps are separated along an altitudinal gradient.** *Journal of Plant Research* 120: 721–725.

<http://dx.doi.org/10.1007/s10265-007-0108-x>

Abstract: We explored the fine-scale distribution of cytotypes of the mountain plant *Senecio carniolicus* along an altitudinal transect in the Eastern Alps. Cytotypes showed a statistically significant altitudinal segregation with diploids exclusively found in the upper part of the transect, whereas diploids and hexaploids co-occurred in the lower range. Analysis of accompanying plant assemblages revealed significant differences between cytotypes along the entire transect but not within the lower part only, where both cytotypes co-occur. This suggests the presence of ecological differentiation between cytotypes with the diploid possessing the broader ecological niche. No tetraploids were detected, indicating the presence of strong crossing barriers.

Hülber K, Sonnleitner M, Flatscher R, Berger A, Dobrovsky R, Niessner S, Nigl T, Schneeweiss GM, Kubešová M, Rauchová J, Suda J, Schönschwetter P. 2009. **Ecological segregation drives fine-scale cytotype distribution of *Senecio carniolicus* in the Eastern Alps.** *Preslia* 81: 309–319.

Abstract: In order to uncover patterns and processes of segregation of co-existing cytotypes, we investigated a zone in the Eastern Alps (Austria) where diploid and hexaploid individuals of the alpine herb *Senecio carniolicus* Willd. (Asteraceae) co-occur. Linking the fine-scale distribution of cytotypes to environmental and spatial factors revealed segregation along an ecological gradient, which was also reflected in the cytotype-associated plant assemblages. Compared to diploids, hexaploids are found in more species-rich and denser communities. This may be due to their better competitive ability and lower tolerance of abiotic stress compared to the diploids. The lack of any intermediate cytotypes suggests the presence of strong reproductive isolation mechanisms, whose nature is, however, elusive.

Escobar García P, Winkler M, Flatscher R, Sonnleitner M, Krejčíková J, Suda J, Hülber K, Schneeweiss GM, Schönschwetter P. 2012. **Extensive range persistence in peripheral and interior refugia characterizes Pleistocene range dynamics in a widespread Alpine plant species (*Senecio carniolicus*, Asteraceae).** *Molecular Ecology* 21: 1255–1270.

<http://dx.doi.org/10.1111/j.1365-294X.2012.05456.x>

Abstract: Recent evidence suggests that survival of arctic-alpine organisms in peripheral or interior glacial refugia are not mutually exclusive and may both be involved in shaping an organism's Pleistocene history, yet potentially at different time levels. Here, we test this hypothesis in a

high-mountain plant (diploid lineage of *Senecio carniolicus*, Asteraceae) from the Eastern European Alps, in which patterns of morphological variation and current habitat requirements suggest survival in both types of refugia. To this end, we used AFLPs, nuclear and plastid DNA sequences and analysed them, among others, within a graph theoretic framework and using novel Bayesian methods of phylogeographic inference. On the basis of patterns of genetic diversity, occurrence of rare markers, distribution of distinct genetic lineages and patterns of range connectivity both interior refugia in the formerly strongly glaciated central Alps and peripheral refugia along the southern margin of the Alps were identified. The presence of refugia congruently inferred by markers resolving at different time levels suggests that these refugia acted as such throughout several glacial cycles. The high degree of range persistence together with gradual range expansion, which contrasts with the extent of range shifts implied for other Alpine species, is likely responsible for incipient lineage differentiation evident from the genetic data. Replacing a simplistic peripheral vs. interior refugia dualism by more complex models involving both types of refugia and considering different time levels will help identifying common phylogeographic patterns with respect to, for instance, location of refugia and colonization routes and elucidating their underlying genetic and/or ecological causes.

Flatscher R, Escobar García P, Hülber K, Sonnleitner M, Winkler M, Saukel J, Schneeweiss GM, Schönswetter P. 2015. **Underestimated diversity in one of the world's best studied mountain ranges: The polyploid complex of *Senecio carniolicus* (Asteraceae) contains four species in the European Alps.** *Phytotaxa* 213: 1–21.

<http://dx.doi.org/10.11646/phytotaxa.213.1.1>

Abstract: *Senecio carniolicus* (Asteraceae) is an intricate polyploid complex distributed in the European Alps (di-, tetra- and hexaploids) and Carpathians (hexaploids only). Molecular genetic, ecological, and crossing data allowed four evolutionary groups within *S. carniolicus* to be identified. Here, we establish that these four groups (two vicariant diploid lineages, tetraploids and hexaploids) are also morphologically differentiated. As a consequence, we draw taxonomic conclusions by characterizing four species, including the more narrowly circumscribed *S. carniolicus* (lectotypified here), the taxonomically elevated *S. insubricus* comb. nov. (lectotypified here), and the two newly described species *S. disjunctus* and *S. noricus*.

Hülber K, Sonnleitner M, Suda J, Krejčíková J, Schönswetter P, Schneeweiss GM, Winkler M. 2015. **Ecological differentiation, lack of hybrids involving diploids, and asymmetric gene flow between polyploids in narrow contact zones of *Senecio carniolicus* (syn. *Jacobaea carniolica*, Asteraceae).** *Ecology and Evolution* 5: 1224–1234.

<http://dx.doi.org/10.1002/ece3.1430>

Abstract: Areas of immediate contact of different cytotypes offer a unique opportunity to study evolutionary dynamics within heteroploid species and to assess isolation mechanisms governing coexistence of cytotypes of different ploidy. The degree of reproductive isolation of cytotypes, that is, the frequency of heteroploid crosses and subsequent formation of viable and (partly) fertile hybrids, plays a crucial role for the long-term integrity of lineages in contact zones. Here, we assessed fine-scale distribution, spatial clustering, and ecological niches as well as patterns of gene flow in parental and hybrid cytotypes in zones of immediate contact of di-, tetra-, and hexaploid *Senecio carniolicus* (Asteraceae) in the Eastern Alps. Cytotypes were spatially separated also at the investigated microscale; the strongest spatial separation

was observed for the fully interfertile tetra- and hexaploids. The three main cytotypes showed highly significant niche differences, which were, however, weaker than across their entire distribution ranges in the Eastern Alps. Individuals with intermediate ploidy levels were found neither in the diploid/tetraploid nor in the diploid/hexaploid contact zones indicating strong reproductive barriers. In contrast, pentaploid individuals were frequent in the tetraploid/hexaploid contact zone, albeit limited to a narrow strip in the immediate contact zone of their parental cytotypes. AFLP fingerprinting data revealed introgressive gene flow mediated by pentaploid hybrids from tetra- to hexaploid individuals, but not vice versa. The ecological niche of pentaploids differed significantly from that of tetraploids but not from hexaploids.

Winkler M, Escobar García P, Gatringer A, Sonnleitner M, Hülber K, Schönswetter P, Schneeweiss GM. 2017. **A novel method to infer the origin of polyploids from Amplified Fragment Length Polymorphism data reveals that the alpine polyploid complex of *Senecio carniolicus* (Asteraceae) evolved mainly via autopolyploidy.** *Molecular Ecology Resources* (in press).

<http://dx.doi.org/10.1111/1755-0998.12641>

**Abstract:** Despite its evolutionary and ecological relevance, the mode of polyploid origin has been notoriously difficult to be reconstructed from molecular data. Here, we present a method to identify the putative parents of polyploids and thus to infer the mode of their origin (auto- vs. allopolyploidy) from Amplified Fragment Length Polymorphism (AFLP) data. To this end, we use Cohen's *d* of distances between in silico polyploids, generated within a priori defined scenarios of origin from a priori delimited putative parental entities (e.g. taxa, genetic lineages), and natural polyploids. Simulations show that the discriminatory power of the proposed method increases mainly with increasing divergence between the lower-ploid putative ancestors and less so with increasing delay of polyploidization relative to the time of divergence. We apply the new method to the *Senecio carniolicus* aggregate, distributed in the European Alps and comprising two diploid, one tetraploid and one hexaploid species. In the eastern part of its distribution, the *S. carniolicus* aggregate was inferred to comprise an autopolyploid series, whereas for western populations of the tetraploid species, an allopolyploid origin involving the two diploid species was the most likely scenario. Although this suggests that the tetraploid species has two independent origins, other evidence (ribotype distribution, morphology) is consistent with the hypothesis of an autopolyploid origin with subsequent introgression by the second diploid species. Altogether, identifying the best among alternative scenarios using Cohen's *d* can be straightforward, but particular scenarios, such as allopolyploid origin vs. autopolyploid origin with subsequent introgression, remain difficult to be distinguished.

