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Effects of cold stress on reproductive biology and epigenetic profiles of the alpine plant *Ranunculus kuepferi* (Ranunculaceae)

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Abstract

Alpine habitats are shaped by harsh abiotic conditions and cold climates. Plant life in such habitats is challenging, as environmental influence can alter the conditions for development and reproduction.

More specifically, phenotypic plasticity of morphological traits can be influenced by temperature stress. Temperature stress can also affect epigenetic and gene expression profiles, which may have an impact on acclimation and adaptation of the species. Polyploidy seems to affect the DNA methylation profiles, while distribution patterns suggest that it could be advantageous under cold conditions.

Nevertheless, little is known about non-model plants, whether temperature stress can induce methylation changes depending on the cytotypes of the individuals, to what extent a treatment shift can induce epigenetic responses and how they are depicted in phenotypic plasticity and reproduction of the species. Furthermore, it remains vague how cold stress is translated in gene expression changes under different cytotypes and how such a putative response is framed through gene set pathways and epigenetic control.

The perennial alpine plant *Ranunculus kuepferi* was utilized to investigate the correlations of cold stress with polyploidy, mode of reproduction, phenotypic plasticity, epigenetics, gene expression and geographical parthenogenesis. The species is mainly found in the wild with diploid and autotetraploid cytotypes, which are mostly sexual and facultative apomicts, respectively.

Diploid and autotetraploid individuals were placed in two climate chambers and exposed to cold (+7°C day/+2°C night, -1°C cold shocks for three nights per week) and warm (control) (+15°C day/+10°C night) temperature treatments in climate growth chambers for four consecutive flowering periods and shifted from one condition to the other after the first flowering period.

Methylation-sensitive amplified fragment-length polymorphism markers were applied for the first two years, to screen possible genome-wide methylation alterations triggered by temprerature treatments and treatment shifts. For the second year of temperature treatments, morphological traits (height, leaves and flowers) and the proportion of well-developed seeds were measured as fitness indicators, while flow cytometric seed screening (FCSS) was utilized to determine the reproduction mode. Subsequently, comparisons with patterns of methylation-sensitive amplified fragment-length polymorphisms (MS-AFLPs/MSAPs) regarding the same year of treatment were conducted. Finally, for the last year of treatment, both cytotypes were investigated for their gene expression profiles via transcriptome sequencing and qRT-PCR. The datasets

were analyzed for four predefined groups with respect to treatment (Cold/Warm) and ploidy level (Diploid/Tetraploid).

DNA methylation profiles showed temperature sensitivity and propose a ploidy effect for both years of analysis. Likewise, the treatment shift had an impact on both cytotypes, resulting in significantly less epiloci, regardless of the shift's direction. Such correlations of ploidy level and epigenetic profiles may reflect DNA methylation dynamics during cold acclimation. The AMOVA results are in line with the hypothesis of cold stress influencing the epigenetic patterns, while they also depict the DNA methylation dynamics of tetraploids, as a response to temperature treatment shift.

Concerning the phenotypic plasticity of the species under temperature treatments, the potential of acclimation under environmental conditions is underlined, as diploids grow better under warm conditions and tetraploids perform better in cold treatments, while the expressed morphological traits are linked with epigenetic patterns. Moreover, cold stress reduced the reproduction fitness but did not induce apomixis in diploid individuals. These results confirm the different niche preferences of cytotypes in natural populations and empower the geographical parthenogenesis scenario, which is previously proposed for the species.

Cold acclimation of the cytotypes is further indicated by gene expression profiles. Overall, diploid individuals altered more gene set pathways than tetraploid ones, and suppressed pathways involved in ion/cation homeostasis. Gene Set Pathways mostly activated under tetraploids are related to cell wall and plasma membrane. Thus, tetraploids seem to be better acclimated to cold conditions, enabling them to colonize colder climatic areas in the Alps. Finally, an epigenetic background for gene regulation in response to temperature conditions is indicated.

1. Introduction

1.1 Temperature stress

Abiotic and biotic environmental conditions are a major influence, alongside with the genotype, on the expression of phenotypes in plants (West-Eberhard, 2008). Changes in average temperature play a pivotal role in phenology as well as in defense capacity, growth and development of flowering plants, while they are supposed to affect the geographical distribution range of species (*e.g.* Hedhly, 2011; Lau *et al.*, 2018; Fiorucci *et al.*, 2020). Flowering plants evolved various adaptation strategies in order to survive and reproduce under adverse temperature conditions, such as plastic responses, which are highlighted as positive contributions to the colonization of novel habitats (*e.g.* Yeh & Prince, 2004; Sol *et al.*, 2005) and subsequent selection of fitting phenotypes over many generations (*e.g.* Schlichting & Pigliucci, 1998; West-Eberhard, 2003; Chevin & Lande, 2010).

Alpine habitats are shaped by short growth periods and cold spells, eventually with nocturnal frost during flowering time (spring or summer) (Körner, 2021). Such cold temperature stress conditions, which are further defined as chilling (0 – 20° C) or freezing (< 0° C), represent a critical abiotic stress, threatening several growth and developmental procedures and activating adaptive traits (*e.g.* Chinnusamy *et al.*, 2007; Nagy and Grabherr, 2009; Hedhly, 2011; Liu *et al.*, 2017; Körner, 2021). The acquisition of increased freezing tolerance upon prior exposure to non-lethal low temperatures is defined as cold acclimation, which consists of sophisticated mechanisms to endure cold stress and is moderated via structural and functional remodeling (Guy, 1990; Thomashow, 1999; Ding *et al.*, 2019). A reliable body of studies approaches the triggering role of environmental conditions and changes (Angers *et al.*, 2010; Kooke *et al.*, 2015) and the ability of individuals for phenotypic accommodation (West-Eberhard, 2003; Whitman & Agrawal, 2009) as well as acclimation to the new conditions (Chevin & Hoffmann, 2017).

The effect of phenotypic plasticity, *i.e.* the potential of an organism, as a single genotype, to differentially respond to environmental stimuli (Brandshaw, 1965; Schlichting, 1986), on plants does not have an adaptive value *per se*, as the beneficial plastic responses should be transmitted to the next generations (*e.g.* Schlichting, 1986; Kopp and Matuszewski, 2013; Chevin and Hoffmann, 2017). However, plastic responses of sessile organisms, such plants, are considered of great importance concerning the acclimation to environmental conditions (*e.g.* West-Eberhard, 2008; Nicotra *et al.*, 2010; Richards *et al.*, 2017). Such responses can be depicted in morphology, physiology, gene expression, as single changes or a combination of these characteristics (Price *et al.*, 2003; West-

Eberhard, 2008; Whitman and Agrawal, 2009; Munns, 2011; Laland *et al.*, 2015) and estimation of the phenotypic plasticity of several fitness traits would outline the environmental tolerance of the individuals (Merilä and Hendry, 2013; Chevin and Hoffmann, 2017).

For alpine plants, reduction of stem height and dense and cushion-like growth are regarded as acclimation or even adaptation to short vegetation periods, lower temperature and exposure to freezing conditions (Syngelaki *et al.*, 2020b; Körner, 2021). Moreover, cold stress conditions could affect polyploidization and mode of reproduction (Ramsey and Schemske, 1998; Klatt *et al.*, 2018; Syngelaki *et al.*, 2020b).

Furthermore, it is proposed that abiotic environmental parameters, like temperature and light, could have a direct impact on the reproduction modes of plants (Hörandl and Hadacek, 2013; Klatt *et al.*, 2016; Klatt *et al.*, 2018; Ulum *et al.*, 2020), while experimental research suggest that with low temperatures possibly triggering apomictic seed formation, albeit at low frequencies (Klatt *et al.*, 2018). Abiotic stress conditions are also demonstrated to trigger an immediate response of apomictic clonal dandelions, associated with the populations differentiation pattern across latitude (Preite *et al.*, 2015) and linked to methylation patterns and heritability of traits (Verhoeven *et al.*, 2010a,b; Verhoeven and Preite, 2013).

In the past decades, several studies on genetic and epigenetic backgrounds of phenotypic plasticity and the corresponding transcriptional differentiation pose interesting research questions on plasticity under extreme environmental conditions (*e.g.* Dolenson *et al.*, 2017; Richards *et al.*, 2017; Kelly *et al.*, 2019; Syngelaki *et al.*, 2020a, b). Such plasticity of plants to new environmental conditions is correlated further with DNA methylation variation (Mirouze and Paskozwski, 2011; Syngelaki *et al.*, 2020b), which may lead to micro-evolutionary events in plants (Zhang *et al.*, 2013).

Specifically, the effects of cold stress, as depicted in alterations on morphology, physiology, DNA methylation and gene expression (*e.g.* Price *et al.*, 2003; Munns, 2011; Laland *et al.*, 2015; Richards *et al.*, 2017) are depending on timing, combination and intensity of the stress parameters (*e.g.* Suzuki *et al.*, 2014). The genes involved in these procedures can be induced by cold *per se* or by the relative state of dehydration following cold stress (Griffith and Yaish, 2004).

1.2 Polyploidy

Polyploidy represents a heritable condition of organisms, and refers to the possession of more than two complete sets of chromosomes (Comai, 2005). It is considered to have evolutionary consequences on angiosperms, as polyploidization events in plants seem to be correlated with phenotypic innovation and speciation (Madlung, 2013; Soltis *et al.*, 2014).

Successful polyploidization events may have important side effects on general adaptation mechanisms to more extreme climates and to stress responses (Li *et al.*, 2011). Uncovering the putative role of polyploidy in adaptation to extreme conditions, its effects on vigor, physiology, morphology and other adaptive traits can result in increased survival fitness under harsher environments (*e.g.* te Beest *et al.*, 2012; Van de Peer *et al.*, 2017; Wei *et al.*, 2019). Moreover, polyploids are specifically more abundant in high latitudes and regions with colder climates (Rice *et al.*, 2019).

In general, plants in alpine habitats tend to decrease their above-ground vegetative growth and plant height usually due to producing fewer cells, while cell size tends to remain stable, thus forming an alpine dwarfism phenotype (Körner, 2021). This morphology is adaptive to short vegetation periods, and reduces exposure to freezing and overheating (Ladinig *et al.*, 2013).

Hence, it is hypothesized that under cold temperature conditions polyploid plants are adjusting their growth and exposure of reproductive tissues towards the putative adaptive morphology of alpine dwarfism (Körner, 2021) by reducing cell numbers and increasing cell size (te Beest *et al.*, 2012), which requires even less cells for tissue formation.

This strategy can additionally help for rapid sprouting of the polyploid plants directly after snow melting (Körner, 2021). Furthermore, a shift to asexual reproduction modes in some polyploids (Comai, 2005) is suggested to further enhance their adaptation under stress conditions, as freezing temperatures during gamete formation can occur regularly during times of flower development (Körner, 2021). Cold shocks are observed to primarily affect species of average altitudes, which are not explicitly cold-adapted, but reach the alpine zone during upward migration (Hörandl, 2010; Hörandl *et al.*, 2011). Freezing tolerance and degree of damage induced by freezing vary a lot among plant species (Ladinig *et al.*, 2013). Epigenetic modifications are also thought to further improve the adaptation of polyploids to stress conditions (Osborn *et al.*, 2003; Soltis *et al.*, 2014; Körner, 2021), while genome duplication may induce genome-wide transcriptional rewiring linked to various cytogenetic, genetic and epigenetic changes (Madlung and Wendel, 2013).

1.3 Apomixis

Phenotypic plasticity can also occur in mode of reproduction. Apomixis is defined as the asexual reproduction via seeds, *i.e.* agamospermy (Nogler, 1984), it is a heritable trait (*e.g.* Nogler, 1984; Ozias-Akins and van Dijk, 2007) and occurs in 78 families and more than 290 genera of angiosperms (Hojsgaard *et al.*, 2014). Natural origins of apomixis happened mostly during climatic oscillations of the Pleistocene (Carman, 1997; Dobes *et al.*, 2004; Paun *et al.*, 2006). Apomictic plants are mostly perennials and apomixis is usually facultative, meaning that both sexual and apomictic seeds can be produced by the same plant, in variable proportions (Nogler, 1984; Hojsgaard *et al.*, 2014; Schmidt, 2020), while an individual plant may express variation in sexual/apomictic seed formation in different years (Klatt *et al.*, 2018).

The first step toward apomixis is the unreduced female gamete formation from unreduced embryo sacs (apomeiosis).An unreduced embryo sac can be formed from either a somatic cell of the nucellus tissue (apospory) or an unreduced megaspore after a restitutional meiosis of the megaspore mother cell (diplospory) (Asker and Jerling, 1992; Koltunow and Grossniklaus, 2003); thus, meiosis is still present in gametophytic apomixis. Following steps of gametophytic development are the parthenogenetic development of unreduced egg cells, and the formation of endosperm tissue after fertilization of the two polar nuclei with a sperm cell (pseudogamy) or without male contribution (autonomous endosperm) (Koltunow and Grossniklaus, 2003; Hand and Koltunow, 2014).

In flowering plants, apomicts are commonly polyploids/hybrids and genomic changes associated with polyploidization/hybridization events were thought to activate the induction of apomixis, although their role in the origin of apomixis is ambiguous (Carman, 1997; Koltunow and Grossniklaus, 2003; Comai 2005). A successful establishment of polyploids is often connected to a niche differentiation of cytotypes (Levin, 1975; Levin, 2003), which could benefit apomictic polyploid taxa to have a better colonization performance at high latitudes and altitudes, not observed among their diploid and sexual progenitors. Furthermore, several authors have stressed the relative abundance of apomictic populations in higher altitudes and latitudes, while they are able to colonize previously glaciated areas with greater success than the sexual populations (*e.g.* Kearney, 2005; Hörandl, 2006; Hörandl, 2011; Gregor, 2013).

This phenomenon of divergent distribution patterns between apomictic and sexual taxa is called geographical parthenogenesis and is described in plants as well as animals (*e.g.* Vandel, 1928; Bell, 1982; Bierzychudek, 1985; Kearney, 2005; Hörandl, 2006) and it is hypothesized to be associated with the climatic fluctuations during the Pleistocene (*e.g.* Carman, 1997; Paun *et al.*, 2006). The colonization advantage of the apomictic taxa

cannot fully interpret the distribution patterns implied by geographical parthenogenesis (*e.g.* Hörandl *et al.*, 2011), indicating a multifactorial dependency of the phenomenon.

Polyploidy is abundant also in sexual plants (*e.g.* Cifuentes *et al.,* 2010), while growing evidence suggests that apomixis can originate spontaneously, and sometimes occasionally, at low frequencies in diploid wild populations (*e.g.* Kantama *et al.*, 2007; Siena *et al.*, 2008; Aliyu *et al.,* 2010; Schinkel *et al.*, 2016) and in diploid populations under cold temperature stress (Klatt *et al.*, 2018).

Hence, an additional factor must be acting for spontaneous origins of apomixis. Experimental research suggests that abiotic conditions like temperature and light (Hörandl and Hadacek, 2013; Klatt *et al.*, 2018) can directly alter mode of apomictic vs. sexual mode reproduction in plants. Cold treatments are long known to trigger restitutional meiosis and consequently unreduced gamete formation (*e.g.* Gustafsson and Nygren, 1946; Ramsey and Schemske, 1998; De Storme and Geelen, 2013a,b; Bomblies *et al.*, 2015). Recent research on model plants showed that extreme temperatures can have various effects on plant meiosis: a) lowering frequencies of double strand break formation at prophase I, which can affect correct segregation of chromosomes; b) failure of synapsis, c) or failure of spindle formation at meiosis I and II (De Storme and Geelen, 2013b; Bomblies *et al.*, 2015).

Concerning the molecular background, apomixis appears to be a consequence of a temporal or spatial deregulation of genes regulating the sexual pathway (*e.g.* Carman, 1997; Koltunow and Grossniklaus, 2003; Bicknell and Koltunow, 2004; Sharbel *et al.*, 2010; Grimanelli, 2012), but its genetic and epigenetic background and the environmental influence on the expression of that trait are rather complicated and remain enigmatic (Schmidt, 2020).

1.4 Epigenetics and gene expression

Epigenetic studies examine the heritable changes in gene expression and function that cannot be linked to DNA sequence changes (Richards, 2006; Bird, 2007). There are four different mechanisms, *i.e.* sets of molecular processes, which can contribute to epigenetic changes: a) methylation of cytosine residues in the DNA, b) remodeling of chromatin structure through chemical modification of histone proteins, c) histone protein modification that can lead to alteration of DNA wrapping, and d) regulatory processes mediated by small and non-coding RNA molecules (miRNA). These processes do not act independently from each other and can produce epigenetic changes that affect genes expression, by *e.g.* activating, reducing or completely disabling the activity of particular genes (*e.g.* Grant-Downton and Dickinson, 2005; Berger, 2007) (Syngelaki *et al.*, 2020a).

In plants, DNA methylation is the best understood epigenetic mechanism (Jaenisch and Bird, 2003; Bender, 2004) and several studies indicated it exhibits a transgenerational inheritance (*e.g.* Vaughn *et al.*, 2007; Johannes *et al.*, 2009; Finnegan, 2010; Hirsch *et al.*, 2013). DNA methylation is defined as the addition, catalyzed by several methyltransferases, of a methyl group to the 5′ C of a cytosine residue in the DNA sequence and associated with silencing of transposons, imprinting and silencing of both transgenes and endogenous genes (*e.g.* Miura *et al.*, 2001; Grossniklaus *et al.*, 2001; Lippman *et al.*, 2004; Zilberman *et al.*, 2007; Jones, 2012). It is well documented that DNA methylation can be dynamic, as biotic or abiotic environmental stimuli can trigger methylation changes and lead to different DNA methylation profiles (*e.g.* Sherman & Talbert, 2002; Bossdorf *et al.*, 2008; Dowen *et al.*, 2012; Zhang *et al.*, 2013), while DNA methylation could contribute to differentiation of populations according to latitudinal gradients (Preite *et al.*, 2015) (Syngelaki *et al.*, 2020a).

Phenotypic plasticity is thought to be under genetic and epigenetic control (*e.g.* Richards *et al.*, 2017), which are regulated by various epigenetic molecular mechanisms, involving small RNAs, transposable elements, histone modifications and cytosine methylation (Nicotra *et al.*, 2010) and often correlated with transcriptional differentiation (Donelson *et al.*, 2017; Kelly, 2019). The molecular response of plants towards environmental conditions is dynamic and extremely complex, as a typical plant cell possesses more than 30,000 genes (Cramer *et al.*, 2011), while epigenetic modifications, such as DNA methylation, histone modifications and chromatin rearrangement can directly or obliquely regulate gene expression (*e.g.* Bird, 2002; Law & Jacobsen, 2010; Harris *et al.*, 2018; Xiao *et al.*, 2019).

Profound changes in gene expression profiles affect the composition of the transcriptome, proteome and metabolome (*e.g.* Chinnusamy *et al.*, 2007; Jammohammadi *et al.*, 2015), with biochemical, physiological, structural and morphological modifications

(*e.g.* Nagy & Grabherr, 2009; Wani *et al.*, 2013; Wani *et al.*, 2016; Banerjee *et al.*, 2017; Körner, 2021), such as changes in light utilization, ROS production, carbon assimilation, photosynthesis rate, membrane permeability, fluidity and cell wall architecture, being induced (*e.g.* Garsed *et al.,* 1987; Sanghera *et al.*, 2011; Theocharis *et al.*, 2012; Longo *et al., 2017*; Megha *et al.*, 2018). Furthermore, DNA methylation is exhibited to mediate phenotypic plasticity within a single generation (Bossdorf *et al.*, 2010) and between generations (Boyko *et al.*, 2010).

Except for environmental stimuli, methylation profiles further change with genomic stresses, such as hybridization and polyploidization, which could induce DNA methylation changes (Adams and Wendel, 2005; Dong *et al.*, 2006; Grant-Downton and Dickinson, 2005; Verhoeven *et al.*, 2010b). A variation in the DNA methylation profiles of wild populations of polyploids putatively unravels their local adaptation and functional plasticity (Paun *et al.*, 2010; Rois *et al.*, 2013; Schulz *et al.*, 2014) and may be advantageous for the invasion success of some species (Ainouche *et al.*, 2009).

The adaptive dynamics triggered from DNA methylation may have evolutionary consequences to the potential selection of asexual genotypes (Latzel and Klimesova, 2010; Massicotte and Angers, 2012). Strikingly, morphological variation in apomictic plants is linked to methylation variation rather than to genetic variation (Rois *et al.*, 2013). Previous experimental work on apomictic clonal dandelions demonstrated immediate responses of plants to abiotic stress conditions, correlations to methylation patterns and heritability of traits (Verhoeven *et al.*, 2010a; Verhoeven and Preite, 2013).

1.5 *Ranunculus kuepferi*

A suitable model system for studying the correlation of polyploidy, morphology, reproduction mode, DNA methylation and gene expression profiles under temperature treatment is the alpine plant *Ranunculus kuepferi* Greuter & Burdet.

The species is a high-mountain perennial herb, occurring predominantly in diploid and autotetraploid cytotypes and exhibing a gradient distribution mainly across the European Alps and at altitudes between 1300 and 2800 m (Burnier *et al.,* 2009; Cosendai and Hörandl, 2010; Cosendai *et al.,* 2011; Kirchheimer *et al.,* 2016). Concerning the mode of reproduction of the species, diploid plants of *R. kuepferi* are primarily sexual and tetraploid plants are facultative apomictic (aposporous), with varying proportions of sexual and asexual seeds (Burnier *et al.,* 2009; Cosendai *et al.,* 2013; Schinkel *et al.,* 2016).

Seed formation in tetraploids is independent from pollinator visits, as they breakdown self-incompatibility and reproduce independently of pollinators and mating partners (Cosendai *et al.,* 2013). Thus, their reproduction success could be related to adaptation to short vegetation periods, reduced or variable pollinator frequencies and pollen availability in cold climatic conditions (Wagner *et al.,* 2016). Hence, the ability of uniparental reproduction in pseudogamous apomicts, which is a general advantage for colonization, was further supported (Hörandl, 2006; Hörandl, 2010). Moreover, diploid plants are restricted to the warmer Southwestern Alps, whereas autotetraploid plants colonize previously glaciated areas, *i.e.* the Northern, Central and Eastern Alps as well as the northern Apennines and Corsica (Küpfer, 1974; Burnier *et al.,* 2009; Cosendai and Hörandl, 2010; Schinkel *et al.,* 2016), along a pronounced geographical parthenogenesis scenario (Cosendai *et al.,* 2013). Geographical Parthenogenesis in general describes related sexual and asexual organisms having different geographical distributions (Hörandl, 2006). Tetraploid populations exhibit a pronounced niche shift towards higher elevations and colder temperatures in the European Alps (Kirchheimer *et al.,* 2016; Schinkel *et al.,* 2016), which is associated with their reproduction mode and, in that regard, seem to provide asexual taxa with a distributional advantage (Kirchheimer *et al.,* 2018).

Previous studies on the species focused on the origin of cytotypes, the geographical parthenogenesis, the population genetics, the correlations of ploidy and apomixis with alpine environmental gradients, the ecological aspects, the pathways to polyploidy of natural populations and the reproductive and vegetative responses of plants under controlled conditions (Cosendai and Hörandl, 2010; Cosendai *et al.,* 2011; Cosendai *et al.,* 2013; Schinkel *et al.,* 2016; Schinkel *et al.,* 2017; Kirchheimer *et al.,* 2016; Kirchheimer *et al.,* 2018; Klatt *et al.,* 2018).

Moreover, the tetraploid cytotype originated presumably by multiple and recurrent autopolyploidization events (Cosendai *et al.*, 2011; Schinkel *et al.*, 2017), which took place 10 - 80 kyears ago (Kirchheimer *et al.*, 2018). The genetic differentiation between the cytotypes is very low and at a similar level within cytotypes (F_{st}values ~ 0.3 for both cytotypes), while it is not depending on the reproduction mode. Under tetraploid cytotype a high individual genetic diversity is observed, following, thus, the respective frequencies of facultative sexuality (Cosendai *et al.*, 2013). Ecological data suggested temperature as the most decisive environmental factor between cytotypes (Kirchheimer *et al.*, 2016; Kirchheimer *et al.*, 2018).

Concerning the influence of cold treatment on the reproduction mode of both cytotypes, the respective study showed a spontaneous emergence of apomixis among diploid plants that have been cultivated under cold conditions (Klatt *et al.*, 2018). On the other hand, tetraploids remained facultative apomictic under cold conditions but the sexual seed formation is reduced to zero (Klatt *et al.*, 2018). Previous studies on the epigenetic background of the species in natural populations proposed pronounced differences between cytotypes and a correlation of DNA methylation variation with abiotic environmental conditions (Schinkel *et al.*, 2020). Thus, a putative epigenetic background of the niche shift of the tetraploids in the Alps is suggested, with epigenetic variation being associated with elevation in natural populations (Schinkel *et al.*, 2020).

1.6 Aims and Scope

In this thesis, I aimed to shed light on the temperature sensitivity of diploid and tetraploid plants of *R. kuepferi*, through DNA methylation patterns (MSAP profiling), reproduction mode, morphological traits and gene expression patterns. I investigated such unresolved evolutionary questions with experimental approaches from karyology, ecology, genetics and epigenetics, to entangle the effects of temperature treatment on the different ploidy levels and evaluate the phenotypic plasticity of their vegetative and reproductive traits. Thus, the enigmatic phenomenon of geographical parthenogenesis, which was previously proposed for the species (*e.g.* Kircheimer *et al.*, 2018), would be further explored.

Chapter 2 - Effects of temperature treatments on epigenetic patterns

The overall aim of this chapter is to investigate the temperature sensitivity of the DNA methylation patterns of the two main cytotypes (di- and tetraploid) of the perennial species *R. kuepferi*, through their exposure to cold (stress) and warm (control) temperature treatment conditions, simulating natural conditions in the Alps. Cytosine-methylation was examined via the Methylation-Sensitive Amplified Fragment-Length Polymorphisms (MS-AFLP or MSAP) method (Paun *et al.*, 2012), which is described as an informative tool to detect the potential of plants to respond rapidly towards stressful environments (*e.g.* Nicotra *et al.*, 2010). After collection, the plants were placed in climate growth chambers, first acclimated for two years and then rotated (plants from warm were transferred to cold and *vice versa*). These treatment shifts were designed to simulate different seasonal weather conditions in the Alps. The patterns of epigenetic variation were explored separately for diploid and tetraploid individuals for warm/cold within the same year and comparisons for the same individuals after the shift of treatments were performed. Since the work described in this chapter is based on the same individuals under different treatments in consecutive years, results mainly reflect the phenotypic plasticity of perennial plants.

Chapter 3 - Effects of temperature treatments on mode of reproduction and morphological traits and their correlation with DNA methylation

In this chapter, I measured several morphological traits, together with reproductive fitness and the reproduction mode, in two cytotypes of *R. kuepferi*, which were exposed to cold and warm temperature treatment conditions, later to the treatment shift of 2017 (see Chapter 2). Seeds from 63 populations across the entire distribution area in the European Alps were analyzed to calculate the seed sets under each temperature

treatment and to conclude on their reproductive pathways via the Flow Cytometric Seed Screening (FCSS) method (Dolezel *et al.*, 2007), investigating, thus, the effect of treatment on the reproduction of the species and a putative positive correlation between cold temperature and induction of apomixis, as it was reported before by Klatt *et al.* (2018). Furthermore, the phenotypic responses of the two cytotypes under different temperature conditions were quantified and subsequently correlated with the epigenetic data of the same year, in order to disentangle the aspects of the molecular background of environmental response and phenotypic plasticity. Results would provide us insights into the potential of *R. kuepferi* to acclimate to cold conditions during the postglacial establishment of the species in the European Alps.

Chapter 4 - Effects of temperature treatments on gene expression and their correlation with DNA methylation

The diploid and tetraploid plants of *R. kuepferi*, which were exposed to different controlled temperature treatments in Chapter 2 & 3, were further employed to evaluate the gene expression profiles of individuals under cold temperature stress and get further insights into the expression dynamics. The gene expression profiles were further analyzed according to the ploidy level of the individuals and associated with DNA methylation, on a transcriptomic level. The extracted mRNAs from leaf material for three individuals (biological replicates) of each four predefined groups, regarding temperature and ploidy level (Cold Diploids: CD; Cold Tetraploids: CT; Warm Diploids: WD; Warm Tetraploids: WT) were sequenced on a HiSeq 4000 Illumina Sequencer at the Transcriptome and Genome Analysis Laboratory of the Microarray & Deep-Sequencing Core Facility (UMG, Georg-August-Universität, Göttingen, Germany) and further analyzed regarding their differential gene expression, towards a *R. kuepferi* pseudoreference, assembled under Trinity. Moreover, gene annotation for functional inference and RT-PCR validation of selected differentially expressed genes linked to DNA methylation were carried out, for the purpose of inquiring into the regulatory mechanisms of cold acclimation of alpine plants. Principally, I hypothesize that the observed niche shift of the tetraploid cytotype (Kirchheimer *et al.*, 2018) has the physiological background of a better cold acclimation, as well as, the postglacial recolonization of the European Alps.

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Chapter 2

Effects of temperature treatments on cytosine-methylation profiles of diploid and autotetraploid plants of the alpine species *Ranunculus kuepferi* (Ranunculaceae)

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Abstract

The exposure to environmental stress can trigger epigenetic variation, which may have several evolutionary consequences. Polyploidy seems to affect the DNA methylation profiles. Nevertheless, it abides unclear whether temperature stress can induce methylations changes in different cytotypes and to what extent a treatment shift is translated to an epigenetic response. A suitable model system for studying these questions is *Ranunculus kuepferi*, an alpine perennial herb.

Diploid and tetraploid individuals of *R.kuepferi* were exposed to cold [+7 °C day/+2 °C night; frost treatment: -1°C cold shocks for three nights per week] and warm [+15 °C day/+10 °C night] conditions in climate growth chambers for two consecutive flowering periods and shifted from one condition to the other after the first flowering period. Methylation-sensitive amplified fragment-length polymorphism markers were applied for both years, to track down possible alterations induced by the stress treatments.

Patterns of methylation suggested that cytotypes differed significantly in their profiles, independent from year of treatment. Likewise, the treatment shift had an impact on both cytotypes, resulting in significantly less epiloci, regardless the shift's direction. The AMOVAs reflect the DNA methylation dynamics of tetraploids, as a response to temperature's change, and follow up the hypothesis of cold stress affecting the epigenetic variation.

Results suggest that the temperature-sensitivityof DNA methylation patterns shows a highly dynamic phenotypic plasticity in *R. kuepferi*, as both cytotypes responded to temperature shifts. Furthermore, ploidy level has an important effect on epigenetic background variation, which may be correlated with the DNA methylation dynamics during cold acclimation.

2.1 Introduction

Epigenetic studies try to explain the heritable changes in gene expression and function which determine the phenotype of an organism and cannot be linked to DNA sequence changes (Richards, 2006). There are mainly four different epigenetic mechanisms: a) methylation of cytosine residues in the DNA, b) remodeling of chromatin structure through chemical modification of histone proteins, c) histone proteins modification that can lead to extent alteration of DNA wrapping, and d) regulatory processes mediated by small and non-coding RNA molecules (miRNA). These processes do not act independently from each other and can produce epigenetic changes that affect genes expression, by *e.g.* activating, reducing of completely disabling the activity of particular genes (Berger, 2007).

DNA methylation is defined as the addiction, catalyzed by several methyltransferases, of a methyl group to the 5′ C of a cytosine residue in the DNA sequence, which is associated with silencing of transposons, imprinting and silencing of both transgenes and endogenous genes (*e.g.* Grossniklaus *et al.*, 2001; Zilberman *et al.*, 2007; Jones, 2012). In plants, DNA methylation is the best understood epigenetic mechanism and several studies intimate it exhibits a transgenerational inheritance (*e.g.* Vaughn *et al.*, 2007; Johannes *et al.*, 2009; Finnegan, 2010; Hirsch *et al.*, 2013). It is well documented that DNA methylation can be dynamic, as biotic or abiotic environmental stimuli can trigger methylation changes and lead to different DNA methylation profiles (*e.g.*Sherman and Talbert, 2002; Dowen *et al.*, 2012). Except for environmental stimuli, genomic stresses such as hybridization and polyploidization can induce DNA methylation changes (Adams and Wendel, 2005; Jones, 2012; Dong *et al.*, 2006; Grant-Downton and Dickinson, 2005). Furthermore, DNA methylation has been exhibited to mediate phenotypic plasticity within a single generation (Bossdorf *et al.*, 2010) and between generations (Boyko *et al.*, 2010).

Polyploidy is a heritable condition of organisms that refers to the possession of more than two complete sets of chromosomes and is considered tohave evolutionary consequences on angiosperms (Comai, 2005), as polyploidization events in plants seem to be correlated with phenotypic innovation and speciation (Madlung, 2012; Soltis *et al.*, 2014). During the formation of polyploids, several alterations in DNA methylation in allo- and autopolyploids are occurring (Li *et al.*, 2011). As DNA methylation is related with regulation of gene expression (Bird 2002; Yan *et al.*, 2010; Law and Jacobsen, 2010), it is implied the decisive role of epigenetic regulation in regaining the genomic balance and the structural and functional remodeling after polyploidization (Soltis *et al.*, 2010; Hegarty *et al.*, 2011; Madlung and Wendel, 2013; Alonso *et al.*, 2016b). Several studies demonstrate that the epigenetic consequences of polyploidy can lead to gene expression alterations (*e.g.* gene silencing) and genome-wide transcriptional rewiring (Baubec *et al.*,

2010; Li *et al.*, 2011; Song and Chen, 2016). However, most studies deal with polyploid hybrids (allopolyploids). Allopolyploids show more pronounced alterations in gene expression than autopolyploidsdue to effects of a hybrid genome additionally to genome duplication (*e.g.* Chen, 2007). Moreover, DNA methylation alterations can be ignited ad hoc by polyploidization during the first generations following the event (Paun *et al.*, 2007). A variation in the patterns of cytosine methylation regarding wild populations of polyploids putatively unravels their local adaptation and functional plasticity (Paun *et al.*, 2010; Rois *et al.*, 2013; Schulz *et al.*, 2014) and may be advantageous for the invasion success of some species (Ainouche *et al.*, 2009).

Uncovering the putative role that polyploidy plays at adaptation to extreme conditions is correlated with the several effects of polyploidy on vigor, physiology, morphology and other adaptive traits (Li *et al.*, 2011; te Beest *et al.*, 2012). Successful polyploidization events can result in increased survival fitness in harsher environments and may have important side effects on mechanisms, which are related to stress response (Li *et al.*, 2011). Hence, it is hypothesized that polyploidy helpsplants to adjust their growth and exposure of reproductive tissues to cold temperatures by offering a putative adaptive advantage in alpine dwarfism (Körner, 2003), as polyploids achieve it by reducing cell number and increasing cell size (te Beest *et al.*, 2012). This strategy can additionally help for rapid sprouting of the polyploidy plants directly after snow melting (Körner, 2003). According to Comai (2005), a selective advantage of polyploidy can be the way it may affect the mode of reproduction of the organism in absence of sexual mates, by favouring the establishment of asexual reproduction. The perpetuation of asexual reproduction mode on some polyploids is suggested that profits further their adaptation under stress conditions (Körner, 2003).

A suitable model system for studying the correlation of temperature effects, ploidy level and methylation profiles is *Ranunculus kuepferi* Greuter & Burdet, a high-mountain perennial herb. The species occurs mainly with diploid and autotetraploid cytotypes and has a gradient distribution primarily across the European Alps and at altitudes between 1300 – 2800 m (Burnier *et al.*, 2009; Cosendai and Hörandl, 2010; Cosendai *et al.*, 2011; Kirchheimer *et al.*, 2016; Schinkel *et al.*, 2016). The comparison of diploid and autotetraploid cytotypes facilitates the study of effects of genome duplication without side-effects of hybridity. Regarding the reproduction mode of these cytotypes, diploid plants are predominantly sexual, whereas tetraploid plants are facultative apomictic (aposporous), with varying proportions of sexual and asexual seeds (Burnier *et al.*, 2009; Schinkel *et al.*, 2016).

Through the distribution of the species a pronounced geographical parthenogenesis in the European Alps (Cosendai *et al.*, 2013) is indicated: diploid populations are restricted to the south-western Alps, while tetraploid populations colonize previously glaciated

areas in the northern, central and eastern Alps (Küpfer 1974; Burnier *et al.*, 2009; Cosendai and Hörandl, 2010) as well as the northern Apennines and Corsica. Tetraploid populations arise also at higher elevations in the European Alps than diploids and exhibit a pronounced niche shift towards colder temperatures (Kirchheimer *et al.*, 2016; Schinkel *et al.*, 2016). This niche differentiation between diploid and tetraploid cytotypes of *R. kuepferi* is associated with their reproduction mode, and asexual taxa seem to have a distributional advantage (Kirchheimer *et al.*, 2018).

Previous population genetic studiesusing AFLPs throughout the range of the species revealed that sexual and apomictic populations show a very low genetic divergence, as only 3% of AFLP fragments were specific for tetraploids, while all others wereshared with the diploid cytotype (Cosendai *et al.*, 2011). Genetic differentiation and diversity measures within cytotypes were on a similar level (*e.g.* F_{st}s are around 0.3 for both cytotypes; Cosendai *et al.*, 2013). Only diploids showed geographical structure in their refugial areas, comprising six genetic partitions, whereas tetraploid populations exhibited no geographical structure and comprised just three genetic partitions that were derived from the diploids' gene pool (Cosendai *et al.*, 2013). Moreover, a high individual genetic diversity in tetraploids is observed and is in line with multiple origins, allelic diversity and high frequencies of facultative sexuality (Cosendai *et al.*, 2013). A molecular dating revealed that the tetraploid cytotype originated only 10-80 kyears ago (Kirchheimer *et al.*, 2018), probably by multiple and recurrentautopolyploidization events (Cosendai *et al.*, 2011; Schinkel *et al.*, 2017). Previous studies on methylation variation in natural populations suggested pronounced differences between cytotypes, and correlations of methylation variation to climatic gradients according to elevation, but not to spatial distribution (Schinkel *et al.*, 2020). Hence, we hypothesize that the niche shift of tetraploids in the Alps has rather an epigenetic than a genetic background. However, in natural populations it is difficult to entangle various environmental factors, and to discriminate between phenotypic plasticity and heritable traits.

Herein, we employed the two main cytotypes of the perennial species *R.kuepferi* and we exposed plants collected in the Alps under different temperature treatments under controlled conditions, to appraise the putative DNA methylation changes. By assessing methylation variation with methylation-sensitive amplified fragment length polymorphisms (MS-AFLPs) we focus on entangling whether the methylation profiles of vegetative parts differentiate according to ploidy levels, and how the cytotypes are affected by a change in cold/warm conditions. We hypothesize a temperature-sensitivity of methylation patterns, as a potential to respond rapidly to stressful environments. We would expect that cytotypes respond differentially to stress treatments. Since we analyze here the same individuals under different treatments in consecutive years, we test here mainly for phenotypic plasticity of perennial plants. By

differential analysis of types of epiloci we further try to get insights into the dynamics of epigenetic change. A detailed study on correlations of mode of reproduction and methylations will be presented elsewhere.

2.2 Materials & Methods

Plant material and experimental design

Ranunculus kuepferi plants, representing diploid and tetraploid cytotypes were collected at 81 sampling sites throughout the distribution range of the species in the European Alps (Kirchheimer *et al.*, 2016) during the growing seasons of 2013 & 2014. These plants were re-potted in garden soil at the Old Botanical Garden of Göttingen University and overwintered outdoors, while their ploidy level was defined via flow cytometry measurements of silica gel dried leaf material collected in the field (Schinkel *et al.*, 2016). To investigate the implied temperature preferences of the two cytotypes (Kirchheimer *et al.*, 2016), an experiment based on the exposure to different temperature conditions, during the sprouting and flowering period, was designed and conductedfrom 2014 onwards (see Klatt *et al.*, 2018). Hence, a subset of diploid and tetraploid individuals was placed in two climate chambers MC1000E (Snijders Scientific, Tilburg, Netherlands), where cold and warm temperature treatments were implemented, while light regime [photoperiod: 16 hs; 10 hs full light (700 µmol m^{-2}s^{-1})] and all other parameters were kept equal. In the first chamber a cold treatment was applied (+7°C day/+2°C night; frost treatment: -1°C cold shocks for three nights per week), mimicking the typical, harsh high alpine temperature conditions at the habitats of the tetraploid cytotype (Schinkel *et al.*, 2016), while in the second chamber a warm treatment was applied (+15° day/+10°C night). In the cold treatment, the repeated moderate frost treatment is simulating temperature conditions occurring in high mountains and provokes frost injury in reproductive shoots, which subsequently could emerge in full fruit loss (Ladinig *et al.*, 2013); similar damaging effects were observed by Klatt *et al.* (2018).

To elucidate further the effects of temperature treatments on cytotypes, a reciprocal test, by rotating the cold treated plants to the warm and vice versa, was performed. In spring of 2016 (third consecutive flowering period under first treatment) and 2017 (first flowering period after the rotation), leaf material was collected from the plants and was stored in silica gel. The individuals were categorized into four groups regarding their ploidy: Diploids 1, Tetraploids 1, Diploids 2 and Tetraploids 2 (from now on, mentioned as D1, T1, D2 and T2, respectively). The groups that are numbered with 1 were placed first under cold treatment, while the rest of them were first placed under warm treatment. A subset of 100 individuals (25 per group; Supplementary Data Table 1), originated from 57 populations, was selected to proceed with the molecular analysis for

both years.The sampling was randomized and was targeted to cover as precisely as possible the distribution range of the species in the Alps (see map in Supplementary Data Figure 1).

MS-AFLP analysis

The DNA from the leaf material was isolated using the QiagenDNeasy Plant Mini Kit, with a slightly modified protocol. At the second step, 360 µl AP1 Buffer and 40 µl PVP 2.6% were added and incubation time for the elution is prolonged 30 min. The PVP was added to remove polyphenolic compounds from plant DNA extracts as it forms hydrogen bonds with them, can deactivate proteins and hence can inhibit many downstream reactions e.g. PCR (Healey *et al.*, 2014).

In order to investigate their epigenetic response, through the highlighting of the genome-wide patterns of epigenetic variation (*e.g.* Salmon *et al.*, 2008; Massicotte *et al.*, 2011; Herrera *et al.*, 2012), methylation-sensitive amplified fragment-length polymorphisms (MS-AFLPs) were conducted. MS-AFLPs, as a methylation detecting approach, can be applied to non-model plants for which the genome has not been sequenced yet and assess cytosine methylation state in a large number of anonymous loci, which are randomly distributed over the genome (Schrey *et al.*, 2013).

The extracted samples of 100 individuals for each treatment year were screened according to a slightly modified protocol of Paun *et al.* (2012). Restriction and ligation were carried out in two parallel reactions, each one with a different restriction enzyme. The restriction enzymes, which were used, are MspI & HpaII. They are methylation sensitive restriction enzymes, *i.e.* isoschizomers, which recognize the same DNA sequence (CCGG), but differ in the sensitivity regarding the methylation state of C, and used as the frequent cutters, while EcoRI is used as the rare cutter. Ligation products were subjected to pre-selective amplification, whereupon selective amplification was performed with a set of three primer combinations with three selective nucleotides to each primer, used before for an AFLP analysis on the species (Cosendai *et al.*, 2011). Ligation, pre-selective and selective amplification products went through a quality and quantity check on a 1.5% agarose gel and diluted 10-fold dilution prior to pre-selective, selective amplification and fragment analyses, respectively.

The final selective-PCR products were prepared with GeneScan ROX 500 (Thermo Fisher Scientific, Waltham, MA, USA) as the internal size standard and fragment analyzed on the ABI Prism 3700/3730 (Applied Biosystems, Waltham, MA, USA) capillary sequencer. An attempt to increase the precision of the final results was performed, by tracking down genotyping errors and cleaning up the data sets (Bonin *et al.*, 2004). The technical reproducibility of resulting electropherograms was checked by replicating 100% of accessions, *i.e.* duplicates were produced forevery sample used

throughout the MSAP protocol steps, to minimize the false positive fragment peaks. Accounting for the modified lab protocol for our species to overcome the sensitivity of restriction enzymes, the guidelines of ASA for the importance of replicability and reproducibility of scientific work (Wasserstein and Lazar, 2016) and the optimization of fragment detection (Arrigo *et al.*, 2009), the risk of false positive results was minimized.

Fragment scoring

The analysis of electropherograms and fragment scoring were performed using the following scoring pipeline: Peakscanner v.2 (Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA), RawGeno 2.0-1 (Arrigo *et al.*, 2009) and MSAP_calc script (Schulz *et al.*, 2013). We transformed electropherograms of raw data into a binary dominant-marker matrix. Peak Scanner2 was used to determine the height, width & the area of all peaks. The output of the Peak Scanner2 was then imported to RawGeno 2.0-1 to proceed with the binning of detected peaks, the analysis of replication and the filtering of samples of low quality.

RawGeno handles a single dye color at a time, so presence/absence of fragments binary matrices were obtainedfor each of the three dyes (Blue; FAM, Green; HEX, Yellow; NED) and then they were merged in a final binary matrix. Fragments between 50 and 600 base pairs were scored. In order to optimize the dataset, a run of RawGeno with an R script (Arrigo *et al.*, 2009) was performed, which checked stepwise (~5760 steps) the binning and filtering parameters. Going through the resulting table, the optimal combination of the parameters waschosen for each dye (Supplementary Data Table 2) and the respective binary matrices were produced. The selection of parameters represents a balance between quality measures, e.g. the error rate and bin reproducibility, and informativity, measured with the data polymorphism.

The merged binary matrix of optimized dataset for each treatment year was dealt with MSAP_calc script in R (Schulz *et al.*, 2013), to distinguish the four possible methylation conditions as they are described in Schulz *et al.* (2013), using the 'Mixed Scoring 2' approach for scoring the following conditions: I) no methylation (both MspI and HpaII cut the restriction site), II) holo- or hemi-methylation of internal cytosine (^{HMe}CG or ^{Me}CG;MspI cuts the restriction site), III) hemimethylationof external cytosine (^{HMe}CCG; HpaII cuts the restriction site) and IV) holomethylationof external cytosine or of both cytosines or hemi-methylation of both cytosines or mutations(none of them cuts the restriction site). In 'Mixed Scoring 2' condition I was scored as 100 (non-methylated), condition II as 010 (internally-methylated), condition III as 001 (externally methylated), and condition IV as '000' and refers to a non-distinguishable situation, e.g. an ambiguous methylation or a mutation status. Condition IV was, therefore, excluded from further statistical analyses. In 'Mixed Scoring 2' approach both group of fragments

are comprised, so potential inadequacies of methylation- and non-methylation Scoring were avoided and more of the underlying information of the epiloci is utilized (Alonso *et al.*, 2016a).

Statistical analysis

MSAP_calc script returned an epigenetic binary matrix, which presents the methylation condition (externally-, internally- and non-methylated) of each epilocus with the corresponding score and a matrix with descriptive parameters at the group level. The subsequent statistical analyses were mainly performed in R (R Development Core Team 2019) under R Studio (RStudio Team 2016).

The descriptive parametersmatrices were adopted from the MSAP_calc script and used as input for barplots in ggplot2 (Wickham, 2009). In accordance with descriptive statistics, the percentages of each epilocus for each individual were calculated regarding the predefined groups for each year of analysis and the groups with some individuals through the years. These percentages were used to produce the respective boxplots with ggplot2 R package and were arcsine transformed in Excel 2016 to match a normal distribution of data. Based on the arcsine-transformed percentages, multiway ANOVAs regarding pairwise comparisons of the groups (ploidy versus treatments, year *versus* groups) and the non-parametric tests of Wilcoxon and Kruskal-Wallis were computed.

To estimate the epigenetic variances within and among the groups for each treatment year, the groups of same individuals through the treatment years, the cytotypes and the treatments as well as the respective epigenetic phenotypic differentiation (Φ_{ST}), several global and locus-by-locus AMOVAs were conducted, using ARLEQUIN (Excoffier *et al.*, 2010), version 3.5.2.2. Binary matrices were treated before with AFLPdat script (Ehrich, 2006) to get an output file in Arlequin format. Both modes of AMOVA were based on the option for haplotypic data computed with pairwise differences, a gamma value of 0 and a permutation number of 50.000. Measures as F_{ST} (homologous to Φ_{ST}), which describe genetic population structuring, should be equally applied to specify population or group differentiation at the epigenetic level (Bossdorf *et al.*, 2008).

2.3 Results

Epigenetic patterns of each year of treatment

The MS-AFLP analysis was conducted on all (100) individuals for the 2017 treatment and on 99 of them for the 2016 treatment, as RawGeno scoring discarded one individual belonging to T2, because of its low quality. Across the 2016 analysis, 754 fragments were scored, while for 2017, the scorable fragments were 493. Furthermore, in the D1, T1, D2

and T2 groups 401, 562, 485, 559 and260, 258, 266, 232polymorphic markers were detected for 2016 and 2017 analyses, respectively (see Table 1).

In 2016, all diversity measures (percentage of polymorphic loci, private loci and Shannon index) suggest an overall higher diversity for tetraploids, mostly due to internally-methylated and non-methylated markers. In 2017, this trait is reversed as diploids show a higher diversity of markers in these epiloci. All the described differences were consistent under cold treatment and more sporadic under warm treatment (Table 1).

Table 1. Measures of epigenetic diversity within the four groups of *Ranunculus kuepferi* obtained for all the types of epiloci.

	Diploids 1 (D1)	Tetraploids 1 (T1)	Diploids 2 (D2)	Tetraploids 2 (T2)
	2016			
All (754 epiloci)				
Polymorphic epiloci (%)	53.18	74.54	64.32	74.14
Private epiloci	32	43	32	44
Mean Shannon's diversity	0.27	0.45	0.36	0.44
Externally-methylated (255 epiloci)				
Polymorphic epiloci (%)	45.1	65.49	60.39	60.39
Private epiloci	19	23	22	13
Mean Shannon's diversity	0.17	0.38	0.27	0.31
Internally-methylated (254 epiloci)				
Polymorphic epiloci (%)	72.05	67.72	74.41	74.02
Private epiloci	12	7	8	22
Mean Shannon's diversity	0.42	0.43	0.47	0.44
Non-methylated (245 epiloci)				
Polymorphic epiloci (%)	42.04	91.02	57.96	88.57
Private epiloci	1	13	2	9
Mean Shannon's diversity	0.21	0.55	0.34	0.57
	2017			
All (493 epiloci)				
Polymorphic epiloci (%)	52.74	52.33	53.96	47.06
Private epiloci	52	41	96	27
Mean Shannon's diversity	0.21	0.23	0.23	0.23
Externally-methylated (287 epiloci)				
Polymorphic epiloci (%)	55.05	65.51	54.7	58.89
Private epiloci	18	36	26	16
Mean Shannon's diversity	0.22	0.29	0.25	0.32
Internally-methylated (119 epiloci)				
Polymorphic epiloci (%)	48.74	26.89	50.42	30.25
Private epiloci	26	2	38	11
Mean Shannon's diversity	0.17	0.1	0.2	0.12
Non-methylated (87 epiloci)				
Polymorphic epiloci (%)	50.57	43.68	56.32	31.03
Private epiloci	8	3	32	0
Mean Shannon's diversity	0.23	0.19	0.2	0.09

The hypothesis of the ploidy effect is confirmed for both years of analysis, as the three different types of epiloci for diploids and tetraploids differed significantly under the same conditions (Fig. 1). More specifically, in externally-, non- and internally-methylated epiloci the ploidy levels under cold treatment (pairs: T1/D1 for 2016 and T2/D2 for 2017) differ significantly (p-values$_{2016}$ < 0.001, < 0.001, < 0.05 & p-values$_{2017}$ < 0.05, < 0.01, < 0.001 respectively). The ploidy effect is also observed under warm treatment for the non-methylated epiloci of 2016 analysis and externally- as well the internally-methylated epiloci of 2017 analysis (p-value$_{2016}$ =< 0.001 & p-values$_{2017}$ = 0.0373, 0.04599 respectively). A treatment effect within the same year and ploidy level is prominent only for the internally-methylated epiloci of the diploid individuals under 2017 analysis (p-value$_{2017}$ < 0.05).
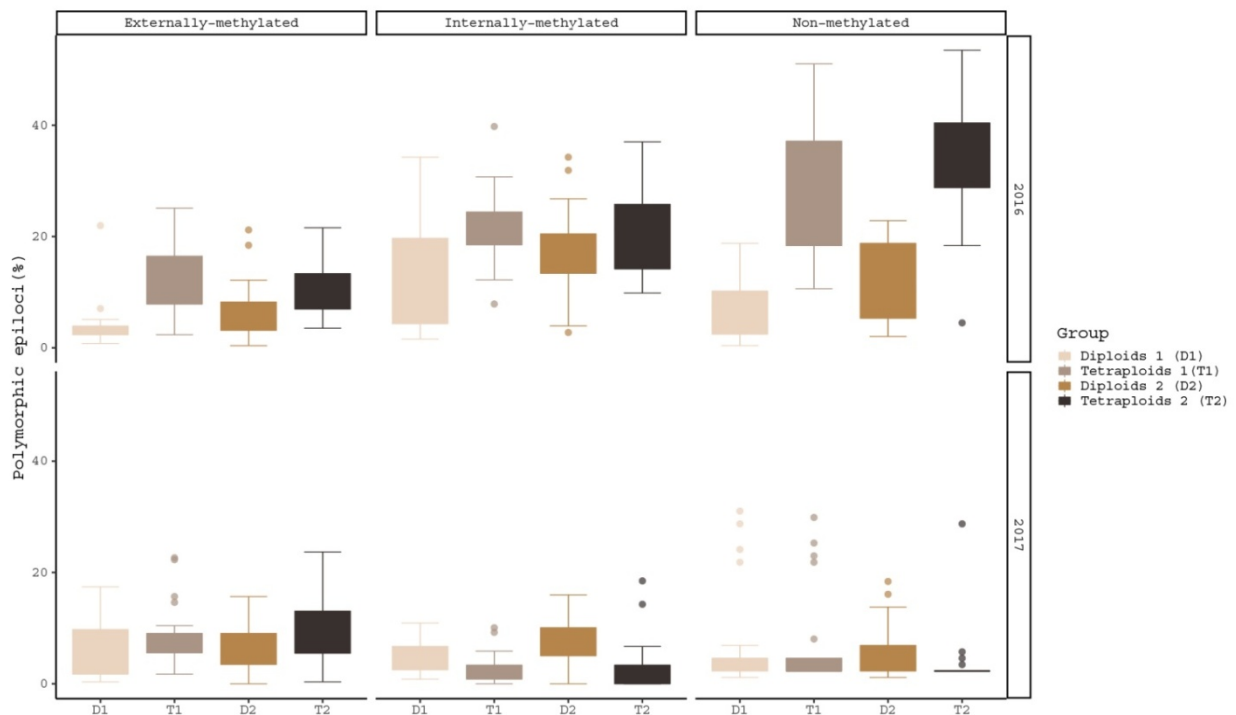
Figure 1. Boxplots of polymorphic epiloci (%) of the four *R. kuepferi* groups for each year of treatment. For test statistics see Table S3.

Correlation of epigenetic patterns under the treatment shift

In order to test how the methylation patterns change with the reversed treatment, pairwise comparisons of same individuals between 2016 and 2017 treatment years were

performed. The treatment shift, as for both directions (Warm to Cold & Cold to Warm), affected the number and the proportion of polymorphic epilocifor the groups with same individuals (Fig. 2).
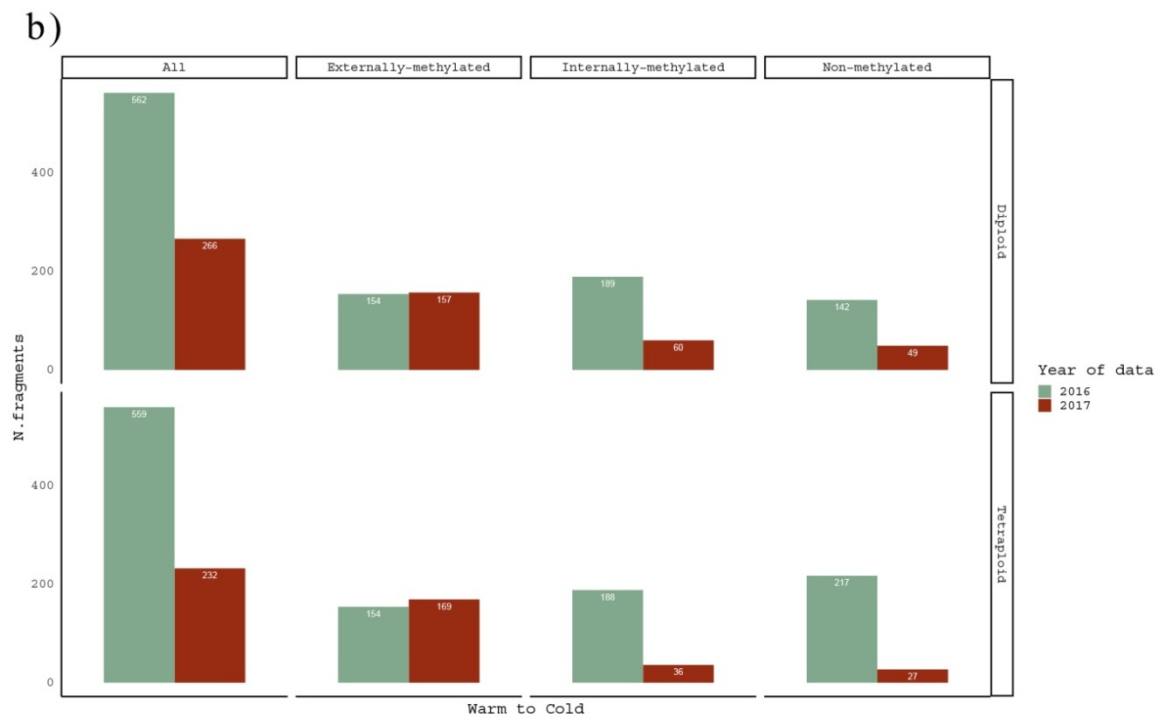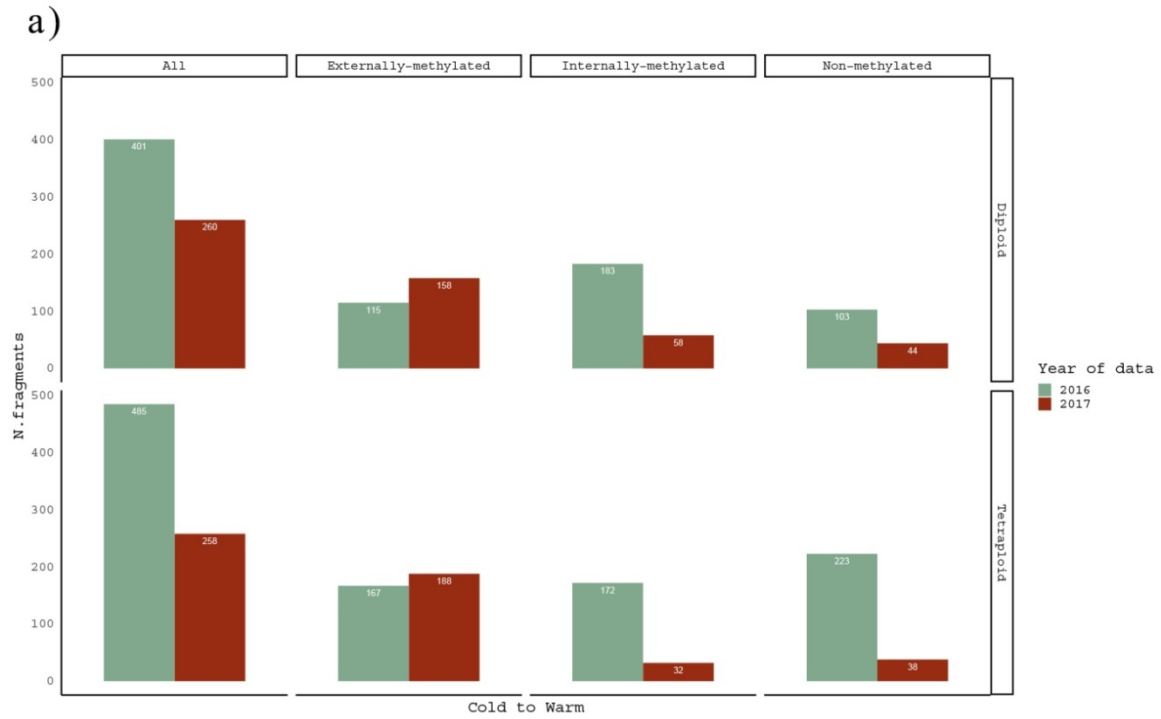
Figure 2. Barplots for comparison of scorable fragments, in absolute numbers (see data in Table 1), for a) Cold to Warm and b) Warm to Cold (*vice versa*) shift, among the same *R. kuepferi* individuals.

Regarding the treatment effect hypothesis through descriptive statistics, boxplots and statistical significant tests propose that both cytotypes changed significantly their methylations under Cold to Warm shift and *vice versa*, for non- and internally-methylated epiloci (Fig. 3).
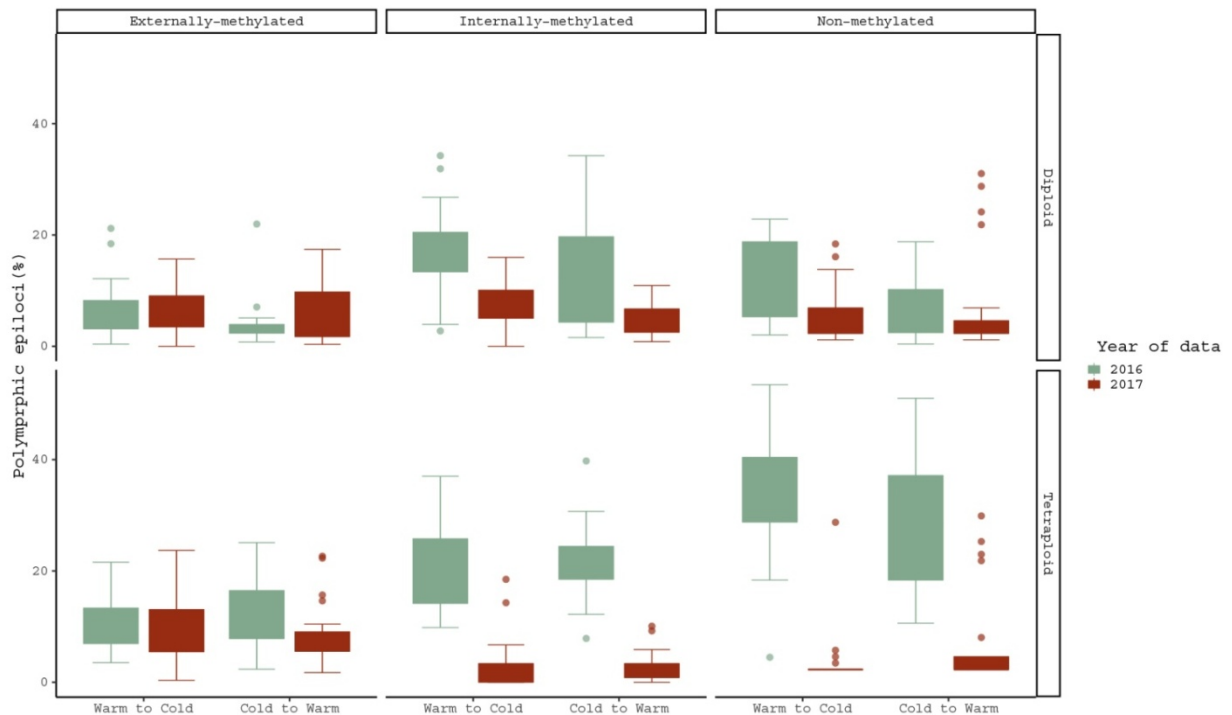
Figure 3. Pairwise comparison of polymorphic epiloci (%) regarding the same *R. kuepferi* individuals, which went through the reciprocal treatment shift from 2016 to 2017. For test statistics see Table S3.

However, the cytotypes do not differ from each other in their response to the temperature changes. Furthermore, in non- and internally-methylated epiloci the pair D1/D2 differs significantly (p-values < 0.001 from Warm to Cold and p-values < 0.005, < 0.001 from Cold to Warm), and as well does the T1/T2 pair (p-values < 0.001 for both directions). Moreover, the corresponding comparison of tetraploids under the shift from Cold to Warm treatment gavesignificant differences also for the case of externally-methylated epiloci (p-value =< 0.005).

Concerning the global AMOVA results, a higher epigenetic variation within than among the groups was found in the diploids for all types of epiloci. Regarding the groups of tetraploids, the epigenetic variation is greater among than within them for non- and internally-methylated epiloci, but not for externally-methylated ones (Table 2). These differences in variation were accounted for both directions of treatment shifts. The Φ_{ST} values are greater than 0.15, except for the externally methylated epiloci of diploids that were alternated from Cold to Warm treatment. Values are ascending from externally- to non- and internally-methylated epiloci.

Similarly, locus-by-locus AMOVA revealed lowest percentages of significantly differentiated epiloci in externally methylated epiloci, followed by non-methylated epiloci, and highest percentages ininternally methylated epiloci, for both ploidy levels and treatment shifts. In addition, under the change from Warm to Cold both ploidy levels exhibit more significantly differentiated epiloci, than the ones for the reciprocal change. For this tendency we observed the exceptions of externally- and internally-methylated epilocifor diploids and tetraploids individuals, respectively (Table 2).

Table 2. Analysis of Molecular Variance (AMOVA) of epiloci, overall and for each type, under both directions of treatment's shift, for the groups of the same individuals of *Ranunculus kuepferi*. 2x: Diploid, 4x: Tetraploid

Epiloci	Overall				Externally-methylated				Internally-methylated				Non-methylated			
Shift's direction	Warm to Cold		Cold to Warm		Warm to Cold		Cold to Warm		Warm to Cold		Cold to Warm		Warm to Cold		Cold to Warm	
Ploidy level	2x	4x	2x	4x	2x	4x	2x	4x	2x	4x	2x	4x	2x	4x	2x	4x
AMOVA global																
Variation among groups (%)	33.38	40.08	31.61	36.01	10.26	22.78	19.28	15.3	48.22	55.93	31.61	57.8	45	57.18	49.54	51.68
Variation within groups (%)	66.62	59.92	68.39	63.99	89.74	77.22	80.72	84.7	51.78	44.07	68.39	42.2	55	42.82	50.46	48.32
Φ_{ST}	0.33	0.4	0.32	0.36	0.1	0.23	0.19	0.15	0.48	0.56	0.32	0.58	0.45	0.57	0.5	0.52
P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
AMOVA locus-by-locus																
Significantly differentiated loci	30	41	24	38	7	19	8	18	16	10	8	12	6	12	6	9
Significantly differentiated loci (%)	30.93	38.68	25	34.86	12.96	30.64	15.38	27.69	66.66	50	33.33	60	31.58	50	30	37.5

2.4 Discussion

In the current study, patterns of epigenetic variation in two cytotypes of *R. kuepferi* along cold (stress) and warm (control) temperature treatments were explored and comparisons for the same individuals after the shift of treatments were performed. The results confirmed the correlation of methylation profiles with the ploidy level, under the same treatment conditions and for both years of analyses. Furthermore, tetraploids had significantly more epiloci than diploids, regarding overall, externally- and non-methylated epiloci under cold treatment of 2016, while for the 2017 analyses the diploid individuals, have more internally-methylated epiloci under both treatments, more non-methylated epiloci under cold treatment and less externally-methylated epiloci under both treatments.

The response to the reciprocal change of treatments is supported by the comparisons of methylation patterns of same individuals before and after the treatment shift. Overall, the treatment shift resulted in a drastic decrease of scorable fragments and, regardless of the shift's direction, a significantly lower number of internally- and non-methylated epiloci. Moreover, the significant higher number of internally-methylatedepiloci under cold treatment, compared to warm in the same year, indicates a temperature-sensitivity of DNA methylation patterns of 2017 among diploids.

Subsequently, we will attempt to interpret all results according to the two factors ploidy level and treatment.

Epigenetic patterns and ploidy level

The observed difference in methylation patterns of the ploidy levels, for both years of analysis, is in contrast to the very low genetic differentiation of cytotypes in wild populations in the AFLP study of Cosendai *et al.* (2011, 2013). Moreover, variation in DNA methylation patterns over short time scales appears to be spontaneous and independent from genetic background (Vidalis *et al.*, 2016). The epigenetic differentiation of cytotypes was also observed in methylation patterns studied in wild populations of *R. kuepferi* (Schinkel *et al.*, 2020). Since tetraploid *R. kuepferi* is autopolyploid, we can also rule out side-effects of hybridity on methylation patterns (*e.g.* Chen, 2007). The consistency of results suggests that polyploidization affects methylation variation independently from genetic background variation and environment. Strikingly, for 2016, tetraploids showed significantly more epiloci than diploids do.Other studies have shown that autopolyploidycan trigger de novo methylation in the model system *Taraxacum officinale* (Verhoeven *et al.*, 2010a) and the endemic species of the Iberian Peninsula, *Dianthus broteri* (Alonso *et al.*, 2016b) as well as increase the global cytosine methylation in six species of the grass species complex Cymbopogon (Lavania *et al.*, 2012).

Cold treatment conditions were quite similar to the natural habitat conditions of the tetraploid *R. kuepferi* plants in the Alps (see also Klatt *et al.*, 2018). They have an extended biogeographical and altitudinal distribution (Cosendai and Hörandl, 2010; Schinkel *et al.*, 2016; Kirchheimer *et al.*, 2016), but neither geographical structure nor isolation-by-distance appearedin their gene pool (Cosendai *et al.*, 2013). The lack of genetic structure in tetraploids is probably due to their recent origin and rapid postglacial colonization of the Alps (Kirchheimer *et al.*, 2018). Kirchheimer *et al.* (2016) suggested that tetraploids' niche optimum is placed in the direction of cooler conditions than the one for diploid cytotype, probably due to the change in the reproductive system of an originally warm-adapted species during postglacial re-colonization of higher regions in the Alps (Kearney, 2005; Hörandl, 2006). Tolerance to cooler conditions allows tetraploids to surmount high elevation barriers and establish new populations throughout a greater distribution range (Kirchheimer *et al.*, 2018). The shift to facultative apomixis may have additionally facilitated rapid colonization (Kirchheimer *et al.*, 2018). Warm treatments rather reflected the natural temperature conditions of the diploids in the southwestern Alps. DNA methylation patterns in the wild populations indeed followed rather a temperature gradient than the biogeographical patternand they seem to correlate with reproduction mode of each cytotype (Schinkel *et al.*,2020). Previous cold/warm treatments by Klatt *et al.* (2018) in climate growth chambers suggested indeed some influence of temperature on mode of reproduction in diploids, and overall a high phenotypic plasticity in this trait. However, this earlier study did not include a reciprocal change of treatments. The potential correlations of reproduction mode and the epigenetic patterns of each cytotype regarding our experiments will be discussed elsewhere, as our main aim of the current study is to decipher the ploidy-treatment linked effects.

Hence, the results of the first treatment in 2016 may still reflect to a great extent the background methylation profiles from the natural habitats, potentially related to differential cold acclimation of diploids and tetraploids.Correlating these background studies with the dynamics of DNA methylation during cold acclimation (Liu *et al.*, 2017), the findings of 2016, which represented the 3rd year (2014 – 2016) of cultivation in growth chambers under the same conditions, are slightly deciphered.

The evolutionary aspects of stress-induced epigenetic variation as well as the epigenetic inheritance have been noteworthy discussed (*e.g.*Wendel *et al.*, 2005; Richards, 2006; Bossdorf *et al.*, 2008; Richards *et al.*, 2017). The exposure to biotic or abiotic environmental stresses can trigger epigenetic changes that seem to persist even after the stress is relieved, resulting in a stress memory that can be stable throughout the lifetime of an organism or even across generations, especially in plants (Richards *et al.*, 2012). The observed cytotype differentiation of methylation in *R. kuepferi* could reflect such a

heritable pattern as ploidy levels, whichare highly stable within natural populations (Cosendai *et al.*, 2010, Schinkel *et al.*, 2016).

Epigenetic patterns and treatment

Regarding the treatment effect on the DNA methylation patterns, only after the reciprocal change of treatments for the same individuals we observed significant changes of the methylation profiles. We suggest that an extreme change of temperature is needed to alter methylation patterns independently from ploidy variation. However, such extreme temperature shifts do occur under natural weather conditions in the Alps, and alpine plants have to be tolerant to temperature extremes between day and night, extreme low temperatures down to -24 °C in higher altitudes, as wellas different weather conditions and seasons (Körner, 2003). The high phenotypic plasticity in methylation patterns, which we observed in *R. kuepferi*, may be responsive to the fluctuating climatic conditions. Several studies insinuate increases in epigenetic variation in response to different environmental factors (Verhoeven *et al.*, 2010b; Dowen *et al.*, 2012; Verhoeven and Preite, 2013; Nicotra *et al.*, 2015; Foust *et al.*, 2016). The 2017 results for *R. kuepferi* may empower the argument of cold-induced DNA methylation changes, as described by *e.g.* Song *et al.* (2015) regarding the alpine to subnivale species *Chorispora bungeana*. Furthermore, in the alpine species *Wahlenbergia ceracea*, adaptive plasticity in methylation was observed in low-elevation plants (Nicotra *et al.*, 2015), while forest trees set off several epigenetic mechanisms, including DNA methylation, to elicit rapid phenotypic variations, which help them respond to environmental changes (Mamadou *et al.*, 2018). We cannot rule out that perennial plants like *R. kuepferi* change their methylation profile over their lifetime independently from environmental influence. However, it is unlikely that such a drastic shift from one year to another would be just an effect of ageing, as it occurred synchronously in plants from different origins. Our plants showed no signs of senescence.

Hereby, concerning the comparison of same individuals for the two years of analysis, fewer fragments were detected after the shift of treatments, *i.e.* 754 for 2016 in contrast to 493 for 2017. The "*Mixed Scoring 2*" approach used in the current study for fragment detection (Schulz *et al.*, 2013) does score "000" for the condition IV, which refers to the uninformative state of fragments' absence due to methylation polymorphisms (Zhang *et al.*, 2007) or restriction site polymorphisms/mutations. Methylation polymorphisms may affect both the external cytosine (*e.g.*^{Me}CCGG, ^{Me}C^{Me}CGG) as well as the internal cytosine (*e.g.*^{HMe}C^{HMe}CGG, ^{Me}C^{Me}CGG), and correct interpretation of changes of methylation status from one to the other is often complicated (Fulnecek and Kovarik, 2014). Remarkably, the loss of epiloci appeared in both cytotypes of *R. kuepferi* and just affected internally-methylated and non-methylated loci. This observation may point at an inability of MspI to cut within the CG context. Hence, we cannot readily interpret our

pattern as demethylation process, but rather as indicative of a high methylation dynamics, or even mutational changes in ce 5-methylcytosin can convert to a thymine via deamination. However, our results suggest that types of epiloci react differentially to abiotic stress, which is in line with involvement of differential methyltransferase families and positions in the genome (*e.g.* Alonso *et al.*, 2016a).

Our speculations refer to the potential of DNA methylation mechanisms to respond to abiotic environmental stress (Richards *et al.*, 2017) and the complex network of them with the other epigenetic mechanisms. It is known that DNA methylation, as an important epigenetic mechanism, is involved in diverse biological processes such as transposon proliferation, genomic imprinting and regulation of gene expression (*e.g.* Law and Jacobsen, 2010) and together with histone modifications and non-histone proteins, encompasses chromatin structure and accessibility (Zhang *et al.*, 2018). The aforementioned phenotypic plasticity in methylations may improve the phenotypic response of plants during acclimation and adaptation to heterogeneous environments (Nicotra *et al.*, 2010).

The lower number of internally- and non-methylated epiloci after the shift, referring to the methylation profiles of the same individuals, regardless the direction of treatment's change and the ploidy level, and the greater number of externally-methylated epiloci of tetraploid individuals, highlight likewise the response to the new abiotic conditionsafter the shift from cold to warm conditions. This epigenetic variation may depict the dynamics of DNA methylation under stress conditions (Bartels *et al.*, 2018; Zhang *et al.*, 2018) and the epigenetic control on the phenotypic plasticity of the species (Zhang *et al.*, 2012; Richards *et al.*, 2017).

Kooke *et al.* (2015) suggested that a DNA demethylation is responsible for variation of phenotypic plasticity by extending its environmental sensitivity. Furthermore, DNA methylation in response to abiotic environmental stress could regulate gene expression (*e.g.* Steward *et al.*, 2002; Shan *et al.*, 2013; Rakei *et al.*, 2016), mostly by global demethylation of genomic DNA, while DNA methylation is believed to play a role in the maintenance of cell stability under stress (Song *et al.*, 2015). However, since the interpretation of our methylation profiles is not straightforward we can just confer a high dynamics from our experiments.

Global AMOVA results for tetraploids reflect these DNA methylation dynamics as a response to the change of temperature, from warm to cold conditionsand *vice versa*. Interestingly, regarding the shift from warm to cold, the higher variation among the tetraploid groups for the non-methylated epiloci may underline the, hypothetically, important role of methylation dynamics under cold treatment. The locus-by-locus AMOVA results, which indicate the epigenetic phenotypic differentiation at each locus,

support the hypothesis of cold stress affecting the epigenetic variation, as there were overall more significantly differentiated epiloci for the shift from warm to cold, also for the diploid groups.

To summarize, the current study demonstrates a ploidy effect on the DNA methylation profiles, mainly under cold treatment, and a significant differentiation of themas a response to the reciprocal temperature treatment experiments. This phenotypic plasticity may affect the potential of the two cytotypes, and therefore also the two different reproduction modes, to tolerate cold stress. Differential response of the different types of epiloci suggests a high epimutational dynamics.

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2.6 Supporting Information

Data availability

The datasets generated for this study can be found in Dryad under: https://doi.org/10.5061/dryad.3n5tb2rcq

Table S1. List of individuals used for the laboratory work. Listed are the sampling ID, ploidy level, treatment, country, province and altitude (Schinkel *et al.,* 2016; Klatt *et al.,* 2018)

Sample ID	Leaf ploidy	Temperature Treatment 2016	Temperature Treatment 2017	Country	Region	Altitude
3/2/3	diploid	cold	warm	France	Provence-Alpes-Côte d'Azur	2291
23/4/1	diploid	cold	warm	France	Provence-Alpes-Côte d'Azur	1616
24/4/3	diploid	cold	warm	France	Provence-Alpes-Côte d'Azur	1925
25/3/2	diploid	cold	warm	France	Provence-Alpes-Côte d'Azur	1435
26/3/3	diploid	cold	warm	France	Provence-Alpes-Côte d'Azur	1456
27/2/1	diploid	cold	warm	France	Rhônes-Alpes	1449
28/3/1	diploid	cold	warm	Italy	Piemonte	1685
28/3/2	diploid	cold	warm	Italy	Piemonte	1685
29/1/2	diploid	cold	warm	Italy	Piemonte	2020
30/1/3	diploid	cold	warm	Italy	Piemonte	1743
31/3/3	diploid	cold	warm	Italy	Piemonte	1937
32/4/2	diploid	cold	warm	Italy	Piemonte	2320
33/3/3	diploid	cold	warm	Italy	Piemonte	2328
112/3/1	diploid	cold	warm	France	Provence-Alpes-Côte d'Azur	1626
112/3/2	diploid	cold	warm	France	Provence-Alpes-Côte d'Azur	1626

Sample ID	Leaf ploidy	Temperature Treatment 2016	Temperature Treatment 2017	Country	Region	Altitude
115/3/3	diploid	cold	warm	France	Provence-Alpes-Côte d'Azur	1891
116/1/1	diploid	cold	warm	France	Provence-Alpes-Côte d'Azur	1953
116/3/3	diploid	cold	warm	France	Provence-Alpes-Côte d'Azur	1953
117/3/2	diploid	cold	warm	France	Provence-Alpes-Côte d'Azur	1632
203/4/3	diploid	cold	warm	France	Provence-Alpes-Côte d'Azur	1840
233/2/1	diploid	cold	warm	France	Provence-Alpes-Côte d'Azur	2185
28B/3/1	diploid	cold	warm	Italy	Piemonte	1685
31B/1/3	diploid	cold	warm	Italy	Piemonte	1937
31B/2/2	diploid	cold	warm	Italy	Piemonte	1937
32B/1/2	diploid	cold	warm	Italy	Piemonte	2320
17/4/3	tetraploid	cold	warm	France	Provence-Alpes-Côte d'Azur	2357
34/2/1	tetraploid	cold	warm	France	Rhônes-Alpes	2120
36/4/1	tetraploid	cold	warm	France	Rhônes-Alpes	2152
37/1/2	tetraploid	cold	warm	Italy	Val d'Aosta	2115
38/1/2	tetraploid	cold	warm	France	Rhônes-Alpes	2182
41/4/3	tetraploid	cold	warm	Italy	Val d'Aosta	2174
42/1/2	tetraploid	cold	warm	Switzerland	Valais	1789
47/3/3	tetraploid	cold	warm	Switzerland	Graubunden	2211

Sample ID	Leaf ploidy	Temperature Treatment 2016	Temperature Treatment 2017	Country	Region	Altitude
51/3/1	tetraploid	cold	warm	Austria	Tirol	2286
53/1/3	tetraploid	cold	warm	Switzerland	Graubunden	2456
54/2/3	tetraploid	cold	warm	Italy	Lombardia	2303
58/1/1	tetraploid	cold	warm	Italy	Trentino Alto Adige/ Südtirol	2117
58/3/3	tetraploid	cold	warm	Italy	Trentino Alto Adige/ Südtirol	2117
74/1/2	tetraploid	cold	warm	Austria	Osttirol	2117
78/3/2	tetraploid	cold	warm	Switzerland	Valais	2000
79/2/3	tetraploid	cold	warm	Switzerland	Graubunden	2280
85/4/1	tetraploid	cold	warm	Austria	Kärnten	2184
88/3/1	tetraploid	cold	warm	Switzerland	Graubunden	2300
103/4/2	tetraploid	cold	warm	Italy	Lombardia	2290
106/4/1	tetraploid	cold	warm	Italy	Trentino Alto Adige/ Südtirol	2142
111/3/2	tetraploid	cold	warm	France	Provence-Alpes-Côte d'Azur	2243
111/3/3	tetraploid	cold	warm	France	Provence-Alpes-Côte d'Azur	2243
116/4/1	tetraploid	cold	warm	France	Provence-Alpes-Côte d'Azur	1953
208/2/1	tetraploid	cold	warm	France	Provence-Alpes-Côte d'Azur	1924

Sample ID	Leaf ploidy	Temperature Treatment 2016	Temperature Treatment 2017	Country	Region	Altitude
3B/1/2	tetraploid	cold	warm	France	Provence-Alpes-Côte d'Azur	2291
3/2/2	diploid	warm	cold	France	Provence-Alpes-Côte d'Azur	2291
3/3/2	diploid	warm	cold	France	Provence-Alpes-Côte d'Azur	2291
23/3/3	diploid	warm	cold	France	Provence-Alpes-Côte d'Azur	1616
24/1/2	diploid	warm	cold	France	Provence-Alpes-Côte d'Azur	1925
25/1/3	diploid	warm	cold	France	Provence-Alpes-Côte d'Azur	1435
26/4/1	diploid	warm	cold	France	Provence-Alpes-Côte d'Azur	1456
27/2/2	diploid	warm	cold	France	Rhônes-Alpes	1449
28/2/3	diploid	warm	cold	Italy	Piemonte	1685
29/4/2	diploid	warm	cold	Italy	Piemonte	2020
30/1/2	diploid	warm	cold	Italy	Piemonte	1743
31/2/3	diploid	warm	cold	Italy	Piemonte	1937
32/3/3	diploid	warm	cold	Italy	Piemonte	2320
33/4/2	diploid	warm	cold	Italy	Piemonte	2328
112/1/2	diploid	warm	cold	France	Provence-Alpes-Côte d'Azur	1626
115/4/2	diploid	warm	cold	France	Provence-Alpes-Côte d'Azur	1891
116/1/3	diploid	warm	cold	France	Provence-Alpes-Côte d'Azur	1953
116/3/1	diploid	warm	cold	France	Provence-Alpes-Côte d'Azur	1953

Sample ID	Leaf ploidy	Temperature Treatment 2016	Temperature Treatment 2017	Country	Region	Altitude
117/3/3	diploid	warm	cold	France	Provence-Alpes-Côte d'Azur	1632
202/3/3	diploid	warm	cold	France	Provence-Alpes-Côte d'Azur	1829
203/2/2	diploid	warm	cold	France	Provence-Alpes-Côte d'Azur	1840
233/2/3	diploid	warm	cold	France	Provence-Alpes-Côte d'Azur	2185
28B/1/2	diploid	warm	cold	Italy	Piemonte	1685
31B/3/3	diploid	warm	cold	Italy	Piemonte	1937
32B/1/1	diploid	warm	cold	Italy	Piemonte	2320
32B/2/1	diploid	warm	cold	Italy	Piemonte	2320
17/4/2	tetraploid	warm	cold	France	Provence-Alpes-Côte d'Azur	2357
34/1/1	tetraploid	warm	cold	France	Rhônes-Alpes	2120
36/2/1	tetraploid	warm	cold	France	Rhônes-Alpes	2152
37/4/1	tetraploid	warm	cold	Italy	Val d'Aosta	2115
40/3/1	tetraploid	warm	cold	Switzerland	Valais	1860
41/2/2	tetraploid	warm	cold	Italy	Val d'Aosta	2174
42/3/2	tetraploid	warm	cold	Switzerland	Valais	1789
45/1/2	tetraploid	warm	cold	Switzerland	Valais	2400
47/2/2	tetraploid	warm	cold	Switzerland	Graubunden	2211
48/4/2	tetraploid	warm	cold	Switzerland	Graubunden	2262
50/2/1	tetraploid	warm	cold	Switzerland	Graubunden	2322

Sample ID	Leaf ploidy	Temperature Treatment 2016	Temperature Treatment 2017	Country	Region	Altitude
53/3/1	tetraploid	warm	cold	Switzerland	Graubunden	2456
54/2/2	tetraploid	warm	cold	Italy	Lombardia	2303
55/3/1	tetraploid	warm	cold	Austria	Tirol	2557
58/1/3	tetraploid	warm	cold	Italy	Trentino Alto Adige/ Südtirol	2117
66/2/2	tetraploid	warm	cold	Italy	Trentino Alto Adige/ Südtirol	2101
79/3/3	tetraploid	warm	cold	Switzerland	Graubunden	2280
82/3/3	tetraploid	warm	cold	Italy	Lombardia	2500
83/3/1	tetraploid	warm	cold	Austria	Osttirol	2271
84/4/2	tetraploid	warm	cold	Austria	Kärnten	2236
90/2/3	tetraploid	warm	cold	Switzerland	Valais	2477
96/2/3	tetraploid	warm	cold	France	Provence-Alpes-Côte d'Azur	2300
106/2/3	tetraploid	warm	cold	Italy	Trentino Alto Adige/ Südtirol	2142
108/3/3	tetraploid	warm	cold	Switzerland	Graubunden	2171
111/2/3	tetraploid	warm	cold	France	Provence-Alpes-Côte d'Azur	2243

Table S2. The parameters, which were chosen for each dye run in RawGeno, with regard to an optimized dataset for each year. Listed are minimum and maximum bin width (bp), Relative Fluorescence Units (RFU), number of bins (nbin), reproducibility threshold (thresh), bin reproducibility (reprd), error rate (ErrBonin) and polymorphy rate (Pol).

Colour dye	Year of data	minbin	maxbin	rfu	thresh	nbin	BinReprod	ErrBonin	Pol
Blue (FAM)	2016	1.5	1.7	175	75	120	31.56	0.15	0.68
	2017	1.6	2.1	100	75	198	6.51	0.05	0.38
Green (HEX)	2016	1.6	1.8	150	75	110	27.85	0.16364	0.74
	2017	1.7	2.1	150	75	137	9.3	0.06	0.42
Yellow (NED)	2016	1.7	1.8	150	75	105	31.67	0.15238	0.73
	2017	1.1	2.1	100	75	126	9.17	0.048	0.33

Table S3. The arcsine transformed percentages of polymorphic epiloci of the 4 groups for 2016 & 2017 treatment years. The data in the current table were used for treatment years' boxplots and for pairwise comparisons of the same *R. kuepferi* individuals through the two treatment years.

Group	Year	SampleID	Externally-methylated	Internally-methylated	Non-methylated
D2	2017	112_1_2	2.73	3.23	3.47
D2	2017	115_4_2	2.22	3.23	1.57
D2	2017	116_1_3	1.86	2.32	2.45
D2	2017	116_3_1	2.86	3.46	3.32
D2	2017	117_3_3	2.42	2.6	1.57
D2	2017	202_3_3	1.96	2.82	2.63
D2	2017	203_2_2	1.96	2.82	2.45
D2	2017	23_3_3	2.14	0	1.57
D2	2017	233_2_3	3.25	2.92	3.61
D2	2017	24_1_2	3.45	0	1.57
D2	2017	25_1_3	2.22	2.72	1.95
D2	2017	26_4_1	2.82	3.01	3.03
D2	2017	27_2_2	2.94	1.93	1.95
D2	2017	28_2_3	3.14	2.47	1.95
D2	2017	28B_1_2	2.36	3.09	3.14
D2	2017	29_4_2	1.48	2.47	0.98
D2	2017	3_2_2	0.34	2.32	2.63
D2	2017	3_3_2	0	0	2.63
D2	2017	30_1_2	2.97	2.47	1.57
D2	2017	31_2_3	2.9	2.32	1.95
D2	2017	31B/32B_2_1	1.86	2.72	1.95
D2	2017	31B_3_3	1.75	3.23	2.92
D2	2017	32_3_3	2.05	2.32	0.98
D2	2017	32B_1_1	3.2	2.72	2.63
D2	2017	33_4_2	2.29	3.23	0.98
T2	2017	106_2_3	3.38	0	1.57
T2	2017	108_3_3	3.14	0	1.57
T2	2017	111_2_3	3.45	0	1.57
T2	2017	17_4_2	0.34	0.76	2.45
T2	2017	36_2_1	3.86	0.76	1.57
T2	2017	37_4_1	3.72	0	1.57
T2	2017	40_3_1	2.42	1.65	1.57
T2	2017	41_2_2	1.32	1.93	1.57
T2	2017	42_3_2	2.29	2.6	1.57
T2	2017	45_1_2	2.9	1.65	1.57
T2	2017	47_2_2	3.11	1.93	1.57

Group	Year	SampleID	Externally-methylated	Internally-methylated	Non-methylated
T2	2017	48_4_2	2.64	1.93	1.57
T2	2017	50_2_1	3.07	3.61	2.23
T2	2017	53_3_1	2.54	1.93	4.05
T2	2017	54_2_2	2.78	1.29	1.57
T2	2017	55_3_1	2.36	0.76	1.57
T2	2017	58_1_3	2.14	2.47	1.57
T2	2017	66_2_2	3.11	0.76	1.57
T2	2017	79_3_3	3.57	3.35	1.95
T2	2017	82_3_3	2.9	0	1.57
T2	2017	83_3_1	2.05	0	1.57
T2	2017	84_4_2	3.25	0	1.57
T2	2017	90_2_3	3.2	0	1.57
T2	2017	96_2_3	3.3	0.76	1.57
D1	2017	112_3_1	3.4	2.32	2.63
D1	2017	112_3_2	3.2	3.09	0.98
D1	2017	115_3_3	3.14	2.82	2.23
D1	2017	116_1_1	2.97	2.6	1.57
D1	2017	116_3_3	2.14	2.32	0.98
D1	2017	117_3_2	1.32	2.6	0.98
D1	2017	203_4_3	2.42	2.32	1.95
D1	2017	23_4_1	0.34	2.32	1.95
D1	2017	233_2_1	1.48	2.14	4.13
D1	2017	24_4_3	1.13	1.93	1.57
D1	2017	25_3_2	1.96	1.93	1.57
D1	2017	26_3_3	3.38	1.65	2.45
D1	2017	27_2_1	3.01	2.82	1.95
D1	2017	28_3_1	3.55	2.32	1.57
D1	2017	28_3_2	1.48	1.93	1.57
D1	2017	28B_3_1	2.05	0.76	3.88
D1	2017	29_1_2	2.69	1.29	4.05
D1	2017	3_2_3	2.22	2.92	2.23
D1	2017	30_1_3	0.65	1.65	1.57
D1	2017	31_3_3	1.75	3.09	3.78
D1	2017	31B/32B_2_2	1.62	1.65	1.57
D1	2017	31B_1_3	1.32	1.65	1.57
D1	2017	32_4_2	1.13	2.47	2.23
D1	2017	32B_1_2	1.32	2.32	1.95
D1	2017	33_3_3	0.34	1.65	1.57
T1	2017	103_4_2	2.86	2.47	1.57
T1	2017	106_4_1	2.69	0.76	1.57
T1	2017	111_3_2	2.9	0.76	3.78

Group	Year	SampleID	Externally-methylated	Internally-methylated	Non-methylated
T1	2017	111_3_3	2.69	0	1.57
T1	2017	116_4_1	3.01	0	1.57
T1	2017	17_4_3	3.38	2.47	2.78
T1	2017	208_2_1	2.22	0	1.57
T1	2017	34_2_1	3.04	2.32	2.23
T1	2017	36_4_1	2.69	1.65	3.83
T1	2017	37_1_2	1.86	0.76	1.57
T1	2017	38_1_2	3.8	2.47	1.57
T1	2017	3B_1_2	2.78	0	1.57
T1	2017	41_4_3	3.81	3.01	1.57
T1	2017	42_1_2	3.45	2.92	2.23
T1	2017	47_3_3	2.42	1.65	1.57
T1	2017	51_3_1	2.64	1.65	1.57
T1	2017	53_1_3	2.48	1.65	1.57
T1	2017	54_2_3	1.32	1.93	1.57
T1	2017	58_1_1	2.73	1.65	1.95
T1	2017	58_3_3	1.96	1.65	1.95
T1	2017	74_1_2	2.73	1.93	1.57
T1	2017	78_3_2	2.42	1.93	3.92
T1	2017	79_2_3	2.42	1.65	4.09
T1	2017	85_4_1	2.36	1.29	1.57
T1	2017	88_3_1	1.75	0.76	1.57
D1	2016	112_3_1	1.43	2.17	2.17
D1	2016	112_3_2	2.33	3.13	3.13
D1	2016	115_3_3	1.97	1.24	1.24
D1	2016	116_1_1	1.43	1.74	1.74
D1	2016	116_3_3	2.08	2.6	2.6
D1	2016	117_3_2	1.59	3.02	3.02
D1	2016	203_4_3	1.86	3.63	3.63
D1	2016	23_4_1	1.59	3.71	3.71
D1	2016	233_2_1	2.25	3.94	3.94
D1	2016	24_4_3	1.86	3.4	3.4
D1	2016	25_3_2	1.59	3.57	3.57
D1	2016	26_3_3	1.73	4.23	4.23
D1	2016	27_2_1	1.97	2.17	2.17
D1	2016	28_3_1	1	2.9	2.9
D1	2016	28_3_2	0.72	1.59	1.59
D1	2016	28B_3_1	2.33	3.5	3.5
D1	2016	29_1_2	1.97	1.43	1.43
D1	2016	3_2_3	2.08	4.12	4.12
D1	2016	30_1_3	1.59	3.45	3.45
D1	2016	31_3_3	1.23	3.89	3.89

Group	Year	SampleID	Externally-methylated	Internally-methylated	Non-methylated
D1	2016	31B/32B_2_2	2.65	4	4
D1	2016	31B_1_3	3.78	1.74	1.74
D1	2016	32_4_2	1.97	3.2	3.2
D1	2016	32B_1_2	2.08	3.2	3.2
D1	2016	33_3_3	1.86	3.67	3.67
T1	2016	103_4_2	3.87	3.2	3.2
T1	2016	106_4_1	3.5	3.79	3.79
T1	2016	111_3_2	3.92	2.76	2.76
T1	2016	111_3_3	2.76	3.73	3.73
T1	2016	116_4_1	3.09	3.69	3.69
T1	2016	17_4_3	1.59	4.38	4.38
T1	2016	208_2_1	3.22	3.9	3.9
T1	2016	34_2_1	2.6	3.95	3.95
T1	2016	36_4_1	3.13	3.65	3.65
T1	2016	37_1_2	3.59	3.73	3.73
T1	2016	38_1_2	2.25	3.69	3.69
T1	2016	3B_1_2	2.65	3.61	3.61
T1	2016	41_4_3	3.31	3.67	3.67
T1	2016	42_1_2	3.5	3.35	3.35
T1	2016	47_3_3	3.4	3.59	3.59
T1	2016	51_3_1	3.19	3.84	3.84
T1	2016	53_1_3	3.06	3.89	3.89
T1	2016	54_2_3	3.54	3.59	3.59
T1	2016	58_1_1	2.85	4.12	4.12
T1	2016	58_3_3	3.16	3.73	3.73
T1	2016	74_1_2	3.87	3.2	3.2
T1	2016	78_3_2	3.16	3.94	3.94
T1	2016	79_2_3	2.33	3.89	3.89
T1	2016	85_4_1	3.22	3.61	3.61
T1	2016	88_3_1	2.71	3.8	3.8
D2	2016	112_1_2	1.23	1.74	1.74
D2	2016	115_4_2	2.81	3.37	3.37
D2	2016	116_1_3	1.23	2.81	2.81
D2	2016	116_3_1	1.59	3.37	3.37
D2	2016	117_3_3	1.73	3.57	3.57
D2	2016	202_3_3	0.38	2.08	2.08
D2	2016	203_2_2	2.71	3.98	3.98
D2	2016	23_3_3	1.86	3.69	3.69
D2	2016	233_2_3	3.06	3.45	3.45
D2	2016	24_1_2	2.33	3.73	3.73
D2	2016	25_1_3	2.47	3.61	3.61

Group	Year	SampleID	Externally-methylated	Internally-methylated	Non-methylated
D2	2016	26_4_1	2.54	2.76	2.76
D2	2016	27_2_2	3.75	2.81	2.81
D2	2016	28_2_3	2.47	3.87	3.87
D2	2016	28B_1_2	2.17	4.16	4.16
D2	2016	29_4_2	2.4	3.52	3.52
D2	2016	3_2_2	3.61	3.29	3.29
D2	2016	3_3_2	3.06	3.65	3.65
D2	2016	30_1_2	3.16	3.35	3.35
D2	2016	31_2_3	2.81	3.69	3.69
D2	2016	31B_3_3	2.17	4.23	4.23
D2	2016	32_3_3	1.59	2.54	2.54
D2	2016	32B/31B_2_1	2.54	3.65	3.65
D2	2016	32B_1_1	2.65	3.89	3.89
D2	2016	33_4_2	3.19	3.71	3.71
T2	2016	106_2_3	2.71	4.01	4.01
T2	2016	108_3_3	2.4	4.07	4.07
T2	2016	111_2_3	3.56	3.1	3.1
T2	2016	17_4_2	1.97	2.98	2.98
T2	2016	36_2_1	3.76	3.35	3.35
T2	2016	37_4_1	2.9	3.82	3.82
T2	2016	40_3_1	3.37	3.35	3.35
T2	2016	41_2_2	3.16	3.73	3.73
T2	2016	42_3_2	3.42	3.32	3.32
T2	2016	45_1_2	3.06	3.45	3.45
T2	2016	47_2_2	3.22	3.35	3.35
T2	2016	48_4_2	3.5	3.61	3.61
T2	2016	50_2_1	2.76	3.8	3.8
T2	2016	53_3_1	2.71	3.92	3.92
T2	2016	54_2_2	2.81	3.75	3.75
T2	2016	55_3_1	3.28	3.23	3.23
T2	2016	58_1_3	2.85	3.9	3.9
T2	2016	66_2_2	2.6	3.73	3.73
T2	2016	79_3_3	2.25	4.12	4.12
T2	2016	82_3_3	3.09	3.77	3.77
T2	2016	83_3_1	2.65	4.19	4.19
T2	2016	84_4_2	2.33	4.3	4.3
T2	2016	90_2_3	3.28	3.8	3.8
T2	2016	96_2_3	2.47	4.19	4.19

Chapter 3

Phenotypic responses, reproduction mode and epigenetic patterns under temperature treatments in the alpine plant species *Ranunculus kuepferi* (Ranunculaceae)

Eleni Syngelaki, Mareike Daubert, Simone Klatt and Elvira Hörandl

Abstract

Plant life in alpine habitats is shaped by harsh abiotic conditions and cold climates. Phenotypic variation of morphological characters and reproduction can be influenced by temperature stress. Nevertheless, little is known about the performance of different cytotypes under cold stress and how epigenetic patterns could relate to phenotypic variation. *Ranunculus kuepferi*, a perennial alpine plant, served as a model system for testing the effect of cold stress on phenotypic plasticity, reproduction mode, and epigenetic variation.

Diploid and autotetraploid individuals were placed in climate growth cabinets under warm and cold conditions. Morphological traits (height, leaves and flowers) and the proportion of well-developed seeds were measured as fitness indicators, while flow cytometric seed screening (FCSS) was utilized to determine the reproduction mode. Subsequently, comparisons with patterns of methylation-sensitive amplified fragment-length polymorphisms (AFLPs) were conducted.

Diploids grew better under warm conditions, while tetraploids performed better in cold treatments. Epigenetic patterns were correlated with the expressed morphological traits. Cold stress reduced the reproduction fitness but did not induce apomixis in diploids.

Overall, our study underlines the potential of phenotypic plasticity for acclimation under environmental conditions and confirms the different niche preferences of cytotypes in natural populations. Results help to understand the pattern of geographical parthenogenesis in the species.

3.1 Introduction

Environment is well known to be a major factor, together with genotype, to influence the expression of a phenotype in living organisms (West-Eberhard, 2008). Alpine habitats are characterized by short growth periods and cold spells, eventually with nocturnal frost during flowering time (spring or summer), which can influence several developmental processes in plants and activate adaptive traits (Körner, 2003; Nagy and Grabherr, 2009; Hedhly, 2011). In that context, phenotypic plasticity is defined as the ability of an organism, as a single genotype, to differentially respond to environmental stimuli (Brandshaw, 1965; Schlichting, 1986). The alterations of the phenotype can be depicted in morphology, in physiology, in gene expression, as single changes, or as a combination of these characteristics of organisms (West-Eberhard, 2008; Price *et al.*, 2003; Whitman and Agrawal, 2009; Munns, 2011; Laland *et al.*, 2015). The effect of phenotypic plasticity on plants, as sessile organisms, seems to be of great importance, regarding the acclimation to environmental conditions (West-Eberhard, 2008; Nicotra *et al.*, 2011; Richards *et al.*, 2017). The adaptive value of phenotypic plasticity should not be considered eminent *per se*, as adaptation is a complex procedure that is implied by plastic responses that are beneficial and can be transmitted to next generations (Schlichting, 1986; Kopp and Matuszewski, 2013; Chevin and Hoffmann, 2017).

As an immediate response to a change inbiotic or abiotic environmental conditions, individuals often show the capacity of phenotypic accommodation (West-Eberhard, 2003; Whitman and Agrawal, 2009), as well as acclimation to the new conditions, which are established in time (Chevin and Hoffmann, 2017). The latter term is often understood as environmental tolerance, estimated through the phenotypic plasticity of several fitness traits (Merilä and Hendry, 2013; Chevin and Hoffmann, 2017).

In the past few decades, a growing numberof studies approached phenotypic plasticity and its evolutionary aspects on organisms, as well as population level (Via and Lande, 1985; Schlichting, 1986; Sterns, 1989; Pigliucci, 2001; Franks *et al.*, 2013; Laland *et al.*, 2015; Kelly, 2019). A reliable body of them highlighted the triggering role of environmental conditions and changes, which often challenge an organism's survival and reproduction (Angers *et al.*, 2010; Kooke *et al.*, 2015). Studies on the genetic and epigenetic background of phenotypic plasticity (Richards *et al.*, 2017) and its correlation with transcriptional differentiation (Donelson *et al.*, 2017; Kelly, 2019) posed various interesting research questions on plasticity under extreme environmental conditions.

Plastic responses are the way of organisms potentially coping with extreme conditions and, thus, contributing positively to the colonization of novel habitats (Yeh and Price, 2004; Sol *et al.*, 2005) via selection of fitting phenotypes (Schlichting and Pigliucci, 1998; West-Eberhard, 2003; Lande, 2009; Whitman and Agrawal, 2009; Chevin and Lande,

2010). Several traits could be potentially involved in such procedures (Kopp and Matuszewski, 2013; Kelly, 2019). Nicotra *et al.* (2010) reviewed several studies on phenotypic plasticity and climate change, trying to highlight the fitness traits that seem to be up- or down regulated as part of the phenotypic response. Such phenotypic traits in plants can be stem height at maturity, flowering time and size at reproduction stage, leaf size and further morphological characteristics, and number/size of seeds (Westoby, 1998; Cornelissen *et al.*, 2003; Metcalf *et al.*, 2003). For alpine plants, a reduction in stem height and dense, cushion-like growth is regarded as acclimation or even adaptation to short vegetation periods, lower temperature, and exposure to freezing (Körner, 2003), while polyploidization and mode of reproduction can be affected by cold stress conditions (Ramsey and Schemske, 1998). Mirouze and Paszkowski (2011) proposed that this plasticity of plants to new environmental and possibly unfavorable conditions is linked to DNA methylation variation, which could regulate growth and reproduction to fit the changes (Lacaze *et al.*, 2009) and may lead to microevolutionary events in plants (Zhang *et al.*, 2013).

Phenotypic plasticity can also occur in the mode of reproduction. Apomixis is defined as asexual reproduction via seeds, *i.e.* agamospermy (Nogler, 1984). It is a heritable trait (Nogler, 1984; Ozias-Akins and van Dijk, 2007) and occurs in 78 families and more than 290 genera of angiosperms (Hojsgaard *et al.*, 2014). Apomixis is usually facultative, which means that both sexual and apomictic seeds can be produced by the same plant, in variable proportions (Nogler, 1984; Hojsgaard *et al.*, 2014; Schmidt, 2020). Apomictic plants are mostly perennials, and an individual plant may express variation in sexual/apomictic seed formation in different years (Klatt *et al.*, 2018). Apomixis appears to be a consequence of a temporal or spatial deregulation of genes regulating the sexual pathway (Koltunow and Grossniklaus, 2003; Grimanelli, 2012), but its genetic and epigenetic background and the environmental influence on the expression of that trait are rather complicated and remain enigmatic (Schmidt, 2020).

In flowering plants, apomicts are commonly polyploids. Polyploidy is thought to have many effects on vigor, physiology, morphology, and other adaptive traits (te Beest *et al.*, 2012), and genomic changes associated with the formation of polyploid cytotypes were thought to lead to the induction of apomixis (Carman, 1997; Koltunow and Grossniklaus, 2003; Comai, 2005). However, growing evidence suggests that apomixis can originate spontaneously in diploid wild populations in low frequencies (Schinkel *et al.*, 2016) and in diploid populations under cold temperature stress (Klatt *et al.*, 2018). A successful establishment of polyploids is often connected to a niche differentiation of cytotypes (Levin, 1975; Levin, 2003). Apomictic polyploid taxa tend to have environmental tolerances and colonization abilities at high latitudes and altitudes, which are not observed among their diploid and sexual progenitors ("geographical parthenogenesis") (Vandel, 1928; Bierzychudek, 1985; Hörandl, 2006).

Experimental research suggests that abiotic conditions like temperature and light (Hörandl and Hadacek, 2013; Klat *et al.*, 2016; Klatt *et al.*, 2018; Ulum *et al.*, 2020) can directly alter the mode of reproduction in plants. Specifically, low temperature may trigger apomictic seed formation, albeit at low frequencies (Klatt *et al.*, 2018). Case studies on apomictic clonal dandelions demonstrated the immediate response of plants to abiotic stress conditions, which are linked to methylation patterns and heritability of traits (Verhoeven *et al.*, 2010a, b; Verhoeven and Preite, 2013) and could contribute to differentiation of populations according to latitudinal gradients (Preite *et al.*, 2015).

We hypothesized that cold exposure would influence morphological and reproductive traits; therefore, we used *Ranunculus kuepferi* Greuter & Burdet, a high-mountain perennial herb with diploids and autotetraploids as main cytotypes (Cosendai and Hörandl, 2010; Cosendai *et al.*, 2011), as a suitable system to test our hypothesis. The reproduction mode of these cytotypes is predominantly sexual for the diploid plants and facultative apomictic for the autotetraploid plants, with varying proportions of sexual and asexual seeds (Burnier *et al.*, 2009; Schinkel *et al.*, 2016). The species is primarily distributed across the European Alps (as well as the northern Apennines and Corsica) and at altitudes between 1300 and 2800 m (Burnier *et al.*, 2009; Cosendai and Hörandl, 2010; Cosendai *et al.*, 2011; Kirchheimer *et al.*, 2016; Schinkel *et al.*, 2016). This distribution pattern indicates a pronounced geographical parthenogenesis pattern in the European Alps (Cosendai *et al.*, 2013), where diploid populations are restricted to the southwestern Alps and tetraploid populations have colonized previously glaciated areas, i.e., northern, central, and eastern Alps (Burnier *et al.*, 2009; Cosendai and Hörandl, 2010; Küpfer, 1974). Tetraploids exhibit a pronounced niche shift toward higher elevations and colder temperatures (Kirchheimer *et al.*, 2016; Schinkel *et al.*, 2016), which are associated with their reproduction mode and, in that regard, seem to provide asexual taxa with a distributional advantage (Kirchheimer *et al.*, 2018).

Previous studies on wild populations showed that genetic differentiation between cytotypes is very low, and that within cytotypes is on a similar level (F_{st}s are around 0.3 for both cytotypes), independent of their reproduction mode (Cosendai *et al.*, 2013). A molecular dating revealed that the tetraploid cytotype originated only 10–80 thousand years ago (Kirchheimer *et al.*, 2018), probably via multiple and recurrent polyploidization events (Cosendai *et al.*, 2011; Schinkel *et al.*, 2017). Epigenetic studies using methylation-sensitive amplified fragment-length polymorphisms (MS-AFLPs) on the species suggested differential profiles in the cytotypes and a connection to abiotic environmental conditions of the epigenetic variation in natural populations and experimental treatments (Schinkel *et al.*, 2020; Syngelaki *et al.*, 2020). This epigenetic variation was further correlated with an elevation in natural populations (Schinkel *et al.*, 2020) and showed higher persistence under cold treatment in experimental conditions (Syngelaki *et al.*, 2020). Thus, we assume a putative epigenetic background of the niche

shift of tetraploids in the Alps, which helps to understand the geographical parthenogenesis scenario for the species. Additionally, concerning several epigenetic mechanisms, it is indicated that methylation patterns also configure, together with genotype, phenotypic plasticity, under changing environmental and developmental conditions (Bossdorf *et al.*, 2010; Kooke *et al.*, 2015).

The aim of the current study was to investigate the putative temperature sensitivity of morphological responses under the different ploidy levels of individuals, in order to decipher how the two cytotypes cope with different temperature conditions. For that purpose, we exposed diploid and tetraploid plants of *R. kuepferi* to different controlled temperature treatments, to quantify plasticity and their phenotypic response. Furthermore, we explored the effect of treatment on the reproduction mode of the species and more specifically the probability of a positive correlation between cold temperature and induction of apomixis, as previously reported by Klatt *et al.* (2018).

By assessing methylation variation data of the same year of treatment (Syngelaki *et al.*, 2020), we focused on possible correlations of phenotypic patterns with MS-AFLP profiles. We investigated whether the methylation profiles of vegetative parts differentiate for phenotypic traits according to ploidy and treatment conditions. The results provided us with insights into the potential of *R. kuepferi* to acclimate to cold conditions during the postglacial establishment of the species in the European Alps.

3.2 Materials and Methods

Plant Material and Experimental Design

Diploid and tetraploid individuals of the species *Ranunculus kuepferi* were collected at 102 sampling sites throughout the distribution range of the species in the European Alps (Schinkel *et al.*, 2016) during the flowering seasons of 2013 and 2014. Consequently, they were transferred to the Old Botanical Garden of Göttingen University, where they were repotted in garden soil and overwintered outdoors. Their ploidy level was determined via flow cytometry measurements on silica-gel-dried leaf material collected in the field (Schinkel *et al.*, 2016).

During the early spring of 2014 (beginning of sprouting and flowering period), a subset of these individuals (see Table S1, Supplementary Materials) was placed in two climate chambers MC1000E (Snijders Scientific, Tilburg, the Netherlands), which implemented different temperature conditions but all other environmental variables were kept equal (Klatt *et al.*, 2018). The conditions of the cold and warm temperature treatments are presented in Table 1. The current experimental design was favored for the purpose of investigating the temperature preferences of the two cytotypes implied by Schinkel *et al.*

(2016) and Kirchheimer *et al.* (2016), with cold temperature treatments simulating the harsh high alpine temperature conditions of the tetraploid cytotype's typical habitats. The treatment of Klatt *et al.* (2018) was kept until 2016 and rotated in 2017 before the beginning of the present study (Syngelaki *et al.*, 2020). All measures were taken from 2017 under the conditions specified in Table 1.

Table 1. Temperature treatment conditions during plant growth and seed formation.

Plant Ploidy	Cold Treatment		Warm Treatment	
	Diploid	Tetraploid	Diploid	Tetraploid
No.plants	164		189	
	74	90	92	97
Light regime (μmol·m^{-2}·s^{-1}, PAR)	ca. 700*			
Photoperiod	16 h; 10h of full light and 3 + 3 of twilight			
Temperature during the light/dark period (°C)	+7 °C day/+2 °C night; frost treatment; −1 °C cold shocks for three nights per week		+15 °C day/+10 °C night	

PAR: Photosynthetically active radiation in 400-700nm.

*Measured with a Quantum light meter (Spectrum Technologies Inc., Aurora, IL, USA) during the full light period (100% intensity) at level of early leaf tips and first buds. Plants were rotated weekly in the cabinet to avoid effects of light and temperature gradients.

In the early spring of 2017, 353 individuals from 63 populations were sampled, targeting as precisely as possible the distribution range of the species in the Alps (see Table S1, Supplementary Materials). The individuals were categorized into four groups regarding their treatment and ploidy: cold diploids, cold tetraploids, warm diploids, and warm tetraploids (CD, CT, WD, and WT, respectively). During the flowering period of 2017, leaf material was collected from all the plants, which was stored in silica gel for further molecular analysis.

Morphological Growth Variables/Seed Set/Reproductive Fitness

Flowers of the diploid individuals are self-incompatible, and they have fewer carpels and more well-developed stamens than the ones of tetraploids, which are also to some degree self-compatible (Cosendai *et al.*, 2013). Although tetraploids are mainly apomictic, pollen is still needed for endosperm formation, as it fertilizes the polar nuclei (pseudogamy) (Küpfer, 1974; Burnier *et al.*, 2009; Cosendai *et al.*, 2013; Ladinig *et al.*, 2013). Hence, we placed the two cytotypes in separate space sections of the climate chambers and pollinated the individuals manually at least thrice with the pollen of plants belonging to the same ploidy level and treatment. To prevent any unwanted cross-pollination event, flowers were covered with small perforated plastic bags as long as they were blooming.

At the peak of the flowering time, measurements of the vegetative parts of the individuals (153 individuals from both growth chambers, belonging to 50 populations) were conducted during the peak of the flowering period of each chamber, regarding the height of the stem (including flower), the number and length of the leaves, and the number of flowers per individual. These morphological growth variables are most informative according to Schinkel *et al.* (2016). All measurements took place the same day for each chamber and were handled further in order to investigate any potential ploidy and treatment effect between the groups.

At the late stage of pollinated flowers/early stage of achene formation, stems were sealed tightly with tape in small perforated plastic bags until the harvesting of ripe fruits (single-seeded achenes). Well-developed achenes were separated from the undeveloped achenes manually by using forceps, with the former resisting the pressure because of their properly formatted endosperm and the latter shattering as they were empty (Schinkel *et al.*, 2016; Klatt *et al.*, 2018). Thereupon, the seed set, *i.e.* the proportion of well-developed achenes of the total number of achenes per flower per individual, was calculated, as a measure of reproductive fitness of both cytotypes under both treatments. Well-developed achenes, after being kept for at least a week at room temperature, were placed into paper bags and stored on silica gel at 4 °C, prior to further analyses.

Methylation-Sensitive Amplified Fragment-Length Polymorphisms (MS-AFLPs)

A subset of 100 randomly selected individuals (25 per group; Table S1, Supplementary Materials), originating from 57 populations, was selected to elucidate the effects of temperature treatments on the DNA methylation. DNA was isolated from the dried leaf material collected in 2017, using the Qiagen DNeasy Plant Mini Kit, and was processed further with a slightly modified protocol of Paun *et al.* (2012), to investigate the patterns of epigenetic variation through the methylation-sensitive amplified fragment-length polymorphisms (MS-AFLPs). Regarding fragment scoring, the resulted

electropherograms from the ABI Prism 3700/3730 (Applied Biosystems, Waltham, MA, USA) capillary sequencer went through the following scoring pipeline: Peakscanner v.2 (Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA), RawGeno 2.0-1 (Arrigo *et al.*, 2009), and MSAP_calc script (Schulz *et al.*, 2013). A detailed wet lab and fragment scoring methodology can be found in Methods S1 (Supplementary Materials), and the DNA methylation study results on the species *Ranunculus kuepferi* are presented elsewhere (Syngelaki *et al.*, 2020).

Flow Cytometric Seed Screening (FCSS)

The flow cytometric seed screening (FCSS) method was employed to determine the reproduction mode of each seed (achene) and investigate whether temperature, especially cold stress, affects it, by quantifying the proportion of sexual versus asexual seed formation. FCSS can distinguish the ploidy levels of both endosperm and embryo per single seed and allows the reconstruction of reproductive pathways, by calculating the ratios of endosperm to embryo ploidy levels, which differs between sexual and apomictic seeds (Matzk *et al.*, 2000).

Up to 10 well-developed seeds (when applicable) per individual were analyzed with a slightly modified FCSS protocol (Matzk *et al.*, 2000), initially introduced by Schinkel *et al.* (2016). Seeds were prepared in separate 2 mL Eppendorf tubes with two steel beads (Qiagen, Hilden, Germany) (Ø 4 mm) for each tube and were ground with Tissue Lyzer II (Qiagen, Hilden, Germany) (stroke rate: 30 Hz for 7s). Subsequently, nucleus isolation and staining were performed in two steps using the Otto buffers (Otto, 1990; Dolezel and Bartos, 2005; Dolezel *et al.*, 2007). First, 200 µL of Otto I buffer wasadded to the ground seed material for a minimum of 30s, to extract the nuclei from the cells. Then, 30 µm mesh filters (CellTrics, Partec GmbH, Münster, Germany) were used to filtrate the mixture into 3.5 mL plastic tubes (55 × 12 mm, Sarstedt, Nümbrecht, Germany) and the plastic tubes containing the filtrate were placed in a dark chamber to proceed with the staining of nuclei. Second, 800 µL of Otto II buffer, containing the stain 4′,6-diamidino-2-phenylindole (DAPI), in a concentration of 300 µg mL^{-1}, wasadded to the filtrate and, after an incubation of minimum 5 min in the dark, the measurement of the final solution was performed on a CyFlow Space flow Cytometer (Sigma-Aldrich, Partec GmbH, Münster, Germany) in the blue fluorescence channel [ultraviolet (UV) light-emitting diode (LED), wavelength 365 nm]. A diploid and a tetraploid *R. kuepferi* plant were used as an external ploidy reference standard to adjust the gain of the UV LED lamp, and parameters were kept equal. Resulting histograms presenting the Gaussian means were obtained and analyzed with the FloMax software, version 2.81 (Quantum Analysis GmbH, Münster, Germany).

The Gaussian means of the peaks refer to the mean values of the DNA content for every tissue, uncovering the ploidy levels of the embryo and the endosperm of the seed. The peak index of each seed, i.e., the ratio of the mean peak value of the endosperm to the mean peak value of the embryo, was calculated, as it is required for the interpretation of plausible reproductive pathways (see Figure S1, Supplementary Materials). To link peak ratios to reproductive pathways, the studies of Cosendai and Hörandl (2010), Schinkel *et al.* (2016), and Klatt *et al.* (2018) on *R. kuepferi* were utilized (see Table S2, Supplementary Materials). A peak index threshold of 1.65 was set to classify all seeds with lower peak indices as sexual. Seeds with peak indices values of minimum 2.0 were classified as asexual (apomictic) (see Figure S1, Supplementary Materials). The proportions of different reproduction modes of the seeds were quantified for each individual and, thereafter, were pooled for each of the four groups, in order to pairwise compare their reproductive modes.

Statistical Analyses

Regarding the pairwise comparisons of the Groups (WD, CD, WT, CT), multiway ANOVAs and the nonparametric Wilcoxon and Kruskal–Wallis tests were computed on morphological growth, seed set, and reproduction mode datasets. All datasets mentioned above were handled as table formats in Excel 2016, and percentages of seed set and reproduction mode (sexuality, apomixis, and BIII hybrids) were arcsine transformed to match the normal distribution of the data. Analyses were performed in R (R Core Team, 2020) under R Studio environment (RStudio Team, 2016). The visualization of descriptive statistics was carried out with ggplot2 R package (Wickham, 2009). All data used for descriptive statistics can be found in Table S3 (Supplementary Materials).

To compare the DNA methylation data produced previously (Syngelaki *et al.*, 2020) with the current morphological growth dataset, non-Euclidean, Jaccard distances of DNA methylation data were calculated under vegan 2.5-6 R package (Oksanen *et al.*, 2019) and visualized with ggplot2, with color referring to the morphological growth data and shape to the predefined groups. Linear models (LMs) and generalized linear models (GLMs), produced under R, were also employed to investigate these relationships further and check the hypothesis of morphological growth data being predicted by the different types of epiloci. Group was selected as an extra predictor, as it indicated the ploidy level and the treatment conditions for each individual.

We conducted linear models for morphological traits (stem height and leaf length) which are continuous numerical variables, while for the traits that refer to count data (number of flowers and number of leaves), generalized linear models, assuming Poisson distributions, were employed (Bolker *et al.*, 2009; Zuur *et al.*, 2009). The normal

distribution of the continuous variables was tested by Shapiro–Wilk's test, prior to linear modeling. For stem height, only the observations that differed from zero were used. As the resulting *p*-values of Shapiro–Wilk's test were not significant, the null hypothesis, *i.e.* that data follow a normal distribution, could not be rejected, and we didnot need to further transform those variables. Furthermore, we set the intercept, *i.e.* the expected mean value of *y* when all *x*=0, to zero, regarding both types of statistical models. Without this step, the coefficients estimated the mean in each group but the difference from a reference group.

3.3 Results

Morphological Growth Data

Overall, the growth of tetraploid plants was affected under warm treatment, as they produced significantly lower numbers of flowers and leaves, as well as significantly shorter leaves and shorter stems in the warm treatment than in the cold one (Figure 1). Paralleling the two ploidy levels under warm treatment, we observed that diploid plants attained significantly higher values for all the morphometric measurements than the tetraploids (Figure 1). Moreover, diploid individuals developed significantly more leaves under the cold treatment than under the warm one. Herein, differences were regarded as highly significant at the 5% level of probability and slightly/marginal significant at the 10% level of probability.
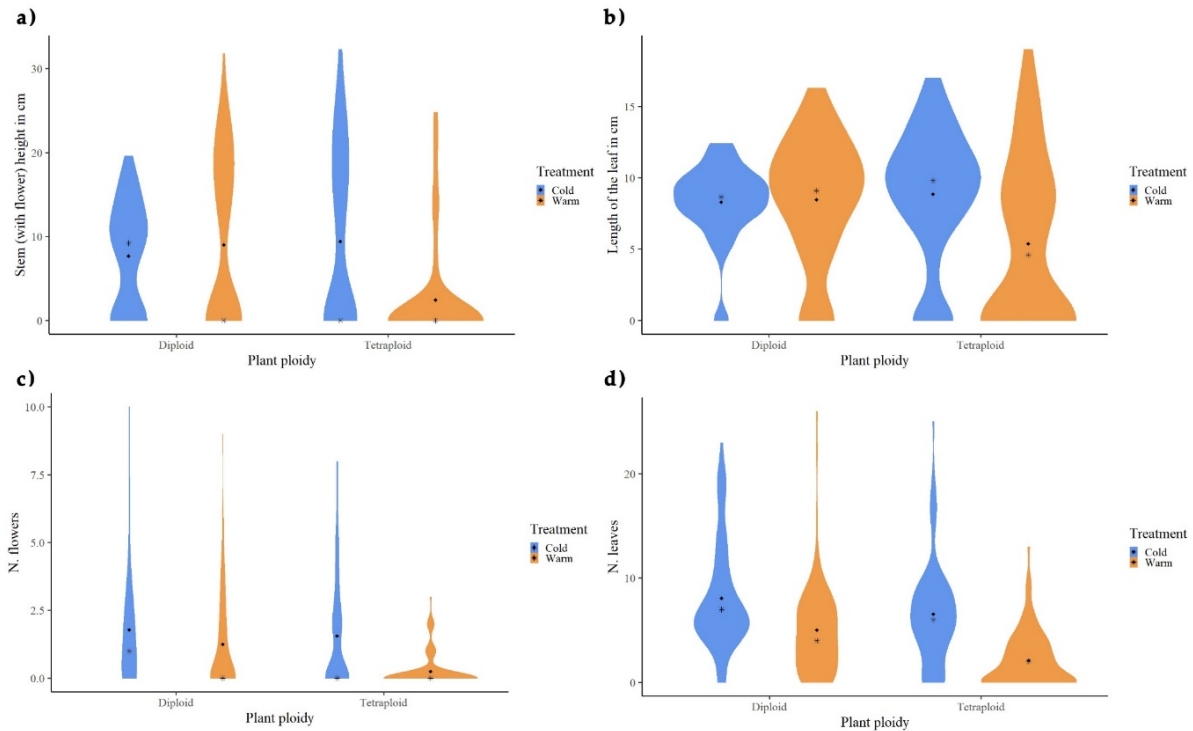
Figure 1. Violin plots of morphological traits of diploid and tetraploid *Ranunculus kuepferi* individuals under cold (blue) and warm (orange) temperature treatments: (a) stem height (with flower) in cm, (b) length of the longest leaf per plant in cm, (c) total number of flowers per plant, and (d) total number of leaves per plant.

Seed Set (Reproductive Fitness) and Reproduction Mode

A total number of 14,404 seeds were harvested from 143 individuals (49 populations) in both treatment conditions. On average, 20.25% of them were well-developed (Figure 2), classified among CD, CT, WD, and WT with percentages of 10.97%, 4.7%, 84.13%, and 0.2%, respectively. Statistical analyses on the seed set data showed that diploids have a significantly higher seed set than tetraploids under both treatments, while diploids produced significantly more well-developed seeds under the warm treatment (Figure 2).

From the 701 FCSS measurements, 688 seeds, collected from 87 individuals, were clearly interpretable, while 13 were excluded from further analyses as there were extreme irregularities in embryo and endosperm development (histograms in Figure S1; reproductive pathways in Table S2; FCSS data in Table S3, Supplementary Materials). Flow cytometric seed screening results confirmed that the sexual mode of reproduction is dominant for the diploid individuals, whereas apomixis is higher expressed in the tetraploid cytotype (see Figure S2, Supplementary Materials). Except for sexual and apomictic seeds, in which the ploidy of the embryo is equal to that of the mother plant, there were cases detected with a ploidy shift in the embryo compared to the mother plant. In such cases, which are defined as BIII hybrids (Nogler, 1984) (Figure S1b,

Supplementary Materials), an unreduced egg cell was fertilized by reduced pollen, resulting in a higher-ploid embryo. BIII hybrids are considered to perform partial apomixis (i.e., apomeiosis only) and be a potential pathway to polyploidization (Schinkel *et al.*, 2017). Interestingly, a relatively high number of individuals (16) in the WD group displayedpartially apomictic reproduction as they were classified as BIII hybrids (Figure S2).
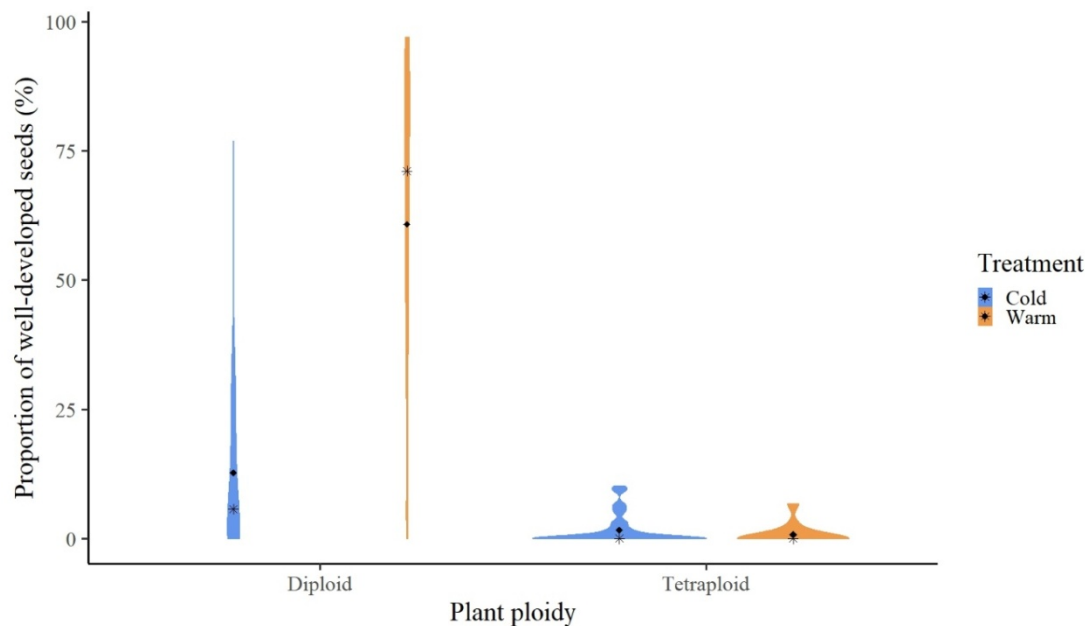

Figure 2. Violin plots of reproduction fitness of diploid and tetraploid *Ranunculus kuepferi* individuals under cold (blue) and warm (orange) temperature treatments.

Comparison of Morphological Growth Data with MS-AFLP Data

In a previous study, a ploidy effect was confirmed for all three types of epiloci (internally, externally, and nonmethylated) under cold conditions and for externally and internally methylated under warm conditions. Moreover, a treatment effect was observed in diploids regarding the internallymethylated epiloci (Syngelaki *et al.*, 2020). The Jaccard distance matrices of the respective MS-AFLP data for all types of epiloci, calculated separately for each cytotype, were visualized as multidimensional scaling diagrams and present a pronounced variation of the groups and a "relaxed" correlation of the morphological traits with the epigenetic data. Treatments are more sharply separated in the diploid cytotype than in the tetraploid one, as cold diploids are gathered mainly in two clusters, whereas the epigenetic patterns of the tetraploid groups seem to overlap a lot (Figures 3 and 4).

Concerning the number of flowers for each cytotype, diploids did not seem to form an epigenetic cluster by increasing or decreasing the number of flowers per individual. As already shown by Figure 1c, treatment affects this morphological trait, as the highest values were found in warm diploids, while cold diploids had mostly individuals with zero to one flower (Figure 3a). For the tetraploids, the highest numbers of flowers were found under cold conditions, and these individuals tended to cluster in MS-AFLP patterns toward the *y*-axis (Figure 3b).
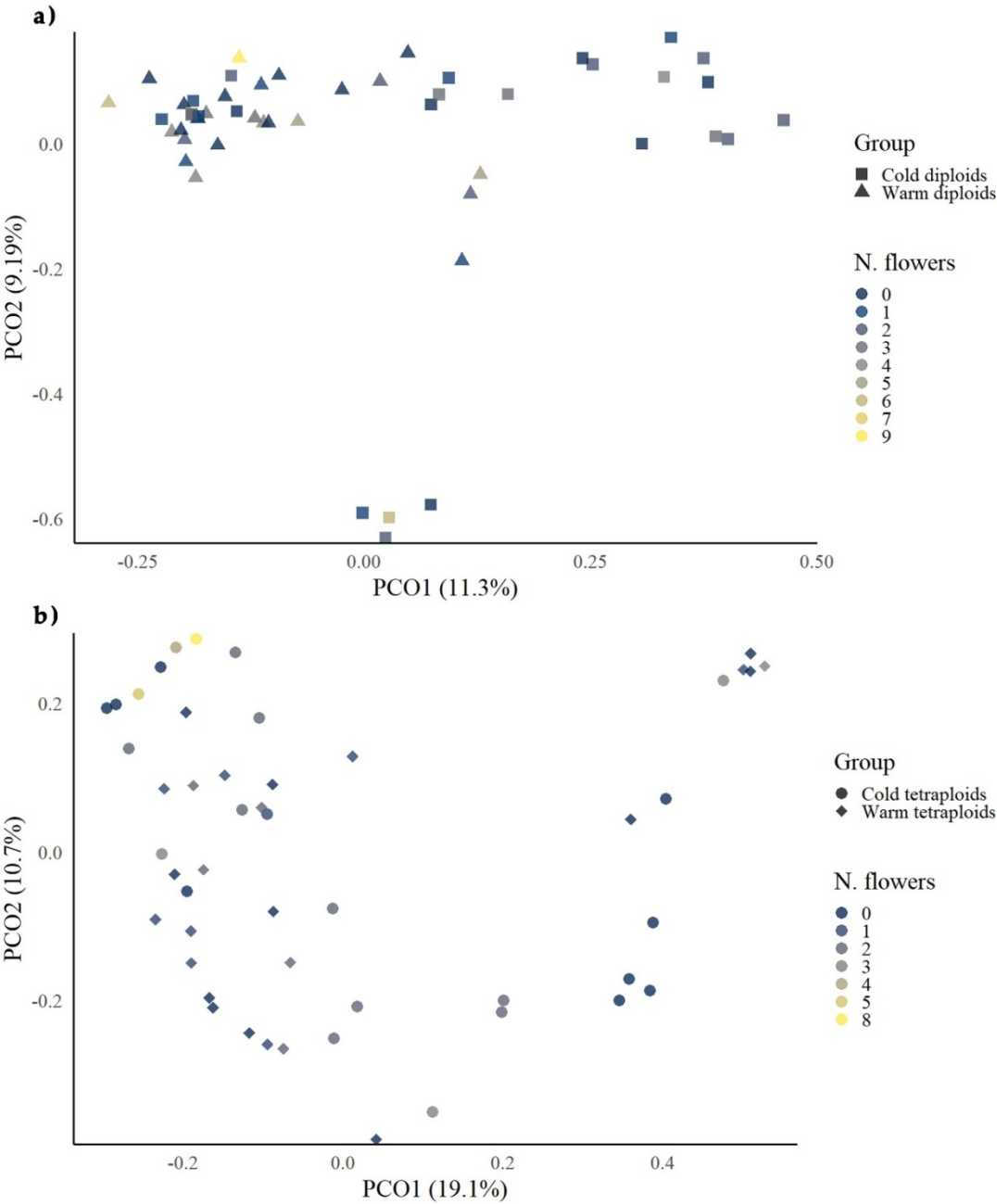
Figure 3. PCoAs of DNA methylation-sensitive amplified fragment-length polymorphisms (MS-AFLPs) against the number of flowers on treatment level of *Ranunculus kuepferi* diploid and tetraploid individuals: (a) diploid plants; (b) tetraploid plants.

The correlation of epigenetic patterns with the number of leaves was slightly stronger. MSAP patterns, regarding both treatments, tended to gather on "relaxed" clusters along the *x*-axis (toward *y*-axis) for diploid individuals with a higher number of leaves in the warm treatment (Figure 4a), while, in tetraploids, there wasa slight differentiation of leaf number and epigenetic patterns along the *x*-axis, with the highest numbers of leaves in the cold treatment (Figure 4b).
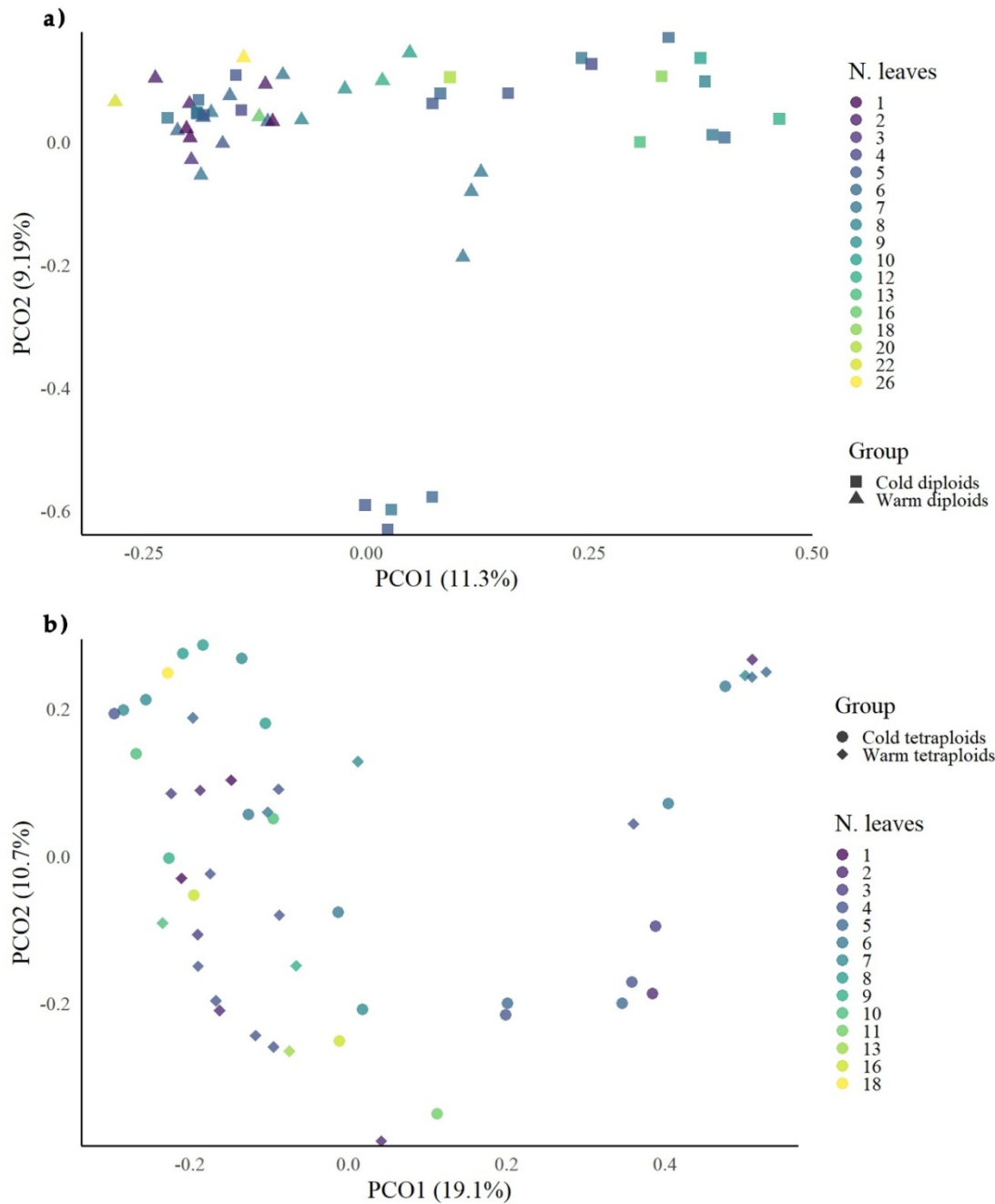
Figure 4. PCoAs of DNA methylation patterns (MS-AFLPs) against the number of leaves on treatment level of *Ranunculus kuepferi* diploid and tetraploid individuals: (a) diploid plants; (b) tetraploid plants.

Further comparisons of the two datasets via linear models (LMs) and generalized linear models (GLMs) unveiled that non-methylated epiloci (predictor) have a significant and a slightly significant negative correlation with leaf length and number of leaves (responses), respectively (Table 2). Furthermore, modeling results showed that groups were, mostly, highly significant and positively correlated with all different

morphological traits, except for the case of WT regarding the number of flowers and the externally methylated epiloci (Table 2b). All linear models presented here (Table 2a) had highly significant p-values.

Table 2. Linear model and generalized linear model results, investigating the relationship of morphological traits with DNA methylation (MS-AFLP) patterns and predefined groups. MS-AFLP patterns are reported separately for different types of epiloci (Syngelaki *et al.*, 2020): (a) linear models (model *p*-values < 0.0001); (b) generalized linear models.

	Nonmethylated Epiloci				Internally Methylated Epiloci				Externally Methylated Epiloci			
	EST	SE	t	p	EST	SE	t	p	EST	SE	t	p
					(a) Linear Models (LMs)							
Stem height												
Epiloci's arcsin	0.3109	0.9664	0.322	0.749	0.317	0.7598	0.417	0.678	−0.5181	0.7724	−0.671	0.505
Group WD	21.0011	2.4582	8.543	<0.0001	20.9473	2.2096	9.48	<0.0001	22.7812	2.2576	10.091	<0.0001
Group WT	14.9239	2.4573	6.073	<0.0001	15.14	1.8074	8.376	<0.0001	16.9496	2.5926	6.538	<0.0001
Group CD	11.3751	2.6498	4.293	<0.0001	11.3147	2.3252	4.866	<0.0001	13.3097	2.209	6.025	<0.0001
Group CT	18.653	2.3273	8.015	<0.0001	18.836	1.7315	10.879	<0.0001	20.5472	2.4101	8.525	<0.0001
Leaf length												
Epiloci's arcsin	0.7476	0.3653	2.047	0.0435	−0.0095	0.3222	−0.029	0.977	−0.2165	0.3482	−0.622	0.535
Group WD	8.1467	0.9546	8.534	<0.0001	9.7367	0.9122	10.674	<0.0001	10.1492	0.9062	11.199	<0.0001
Group WT	8.9927	0.9476	9.49	<0.0001	10.5581	0.7544	13.996	<0.0001	11.1192	1.0916	10.186	<0.0001
Group CD	7.5579	0.9898	7.636	<0.0001	9.2389	0.9716	9.509	<0.0001	9.7112	0.9849	9.86	<0.0001
Group CT	9.4605	0.8641	10.948	<0.0001	10.8027	0.6849	15.772	<0.0001	11.371	1.0967	10.369	<0.0001

		(b) Generalized Linear Models (GLMs)										
No. of flowers												
Epiloci's arcsin	0.0019	0.1519	0.012	0.9901	−0.0077	0.1327	−0.058	0.9536	0.0973	0.1514	0.643	0.5202
Group WD	2.0764	0.4299	4.83	<0.0001	2.0971	0.4089	5.128	<0.0001	1.8705	0.4126	4.533	<0.0001
Group WT	0.836	0.3644	2.294	<0.05	0.8495	0.2705	3.14	<0.01	0.5777	0.4362	1.324	0.1854
Group CD	1.8357	0.4325	4.245	<0.0001	1.8601	0.4206	4.423	<0.0001	1.6387	0.4363	3.756	<0.001
Group CT	1.7965	0.381	4.715	<0.0001	1.8091	0.3075	5.884	<0.0001	1.5369	0.4789	3.21	<0.01
No. of leaves												
Epiloci's arcsin	−0.5111	0.3085	−1.657	0.0975	−0.3444	0.287	−1.2	0.23	0.0663	0.3181	0.208	0.835
Group WD	8.4683	0.8596	9.852	<0.0001	8.1792	0.8334	9.814	<0.0001	7.2984	0.8359	8.731	<0.0001
Group WT	5.7235	0.7948	7.201	<0.0001	5.1557	0.6189	8.331	<0.0001	4.4241	0.9462	4.676	<0.0001
Group CD	8.8534	0.8903	9.945	<0.0001	8.4953	0.896	9.482	<0.0001	7.5825	0.9142	8.294	<0.0001
Group CT	8.6126	0.7854	10.966	<0.0001	8.1359	0.6513	12.493	<0.0001	7.5378	1.0138	7.436	<0.0001

EST: estimate of each coefficient; SE: standard error of the estimate; t: t-value, *i.e.* the coefficient divided by its standard error; p: p-value for the coefficient.

3.4 Discussion

In the current study, the variability of several morphological traits together with shifts of the reproduction mode in two cytotypes of *R. kuepferi* along cold (stress) and warm (control) temperature treatments was explored, simulating natural conditions in the Alps. The results confirmed the correlation of phenotypic responses with the ploidy level and treatment conditions, while, for reproduction mode, only the ploidy effect was confirmed as reported previously (Klatt *et al.*, 2018), as there were no significant differences in treatments.

Furthermore, tetraploids had significantly improved growth under the cold treatment and seemed to suffer under the warm conditions. Comparing the growth response of the two cytotypes under warm conditions, diploids did better than tetraploids. This prominent reaction of the tetraploid cytotype to the cold treatment, in addition to the slightly better performance of the diploid cytotype under warm treatment, strengthens the hypothesis of the ecological background of the geographical parthenogenesis pattern of the species (Schinkel *et al.*, 2016; Kirchheimer *et al.*, 2018). This hypothesis proposes the occurrence of the diploid cytotype in the warmer climate of the southwestern Alps and of the tetraploid cytotype in colder conditions at higher elevations inthe rest of the Alps.

The interaction between morphological traits and DNA methylation patterns, evaluated for different cytotypes, could indicate an aspect of the molecular, more specifically, epigenetic, background of environmental response and/or phenotypic plasticity.

Overall, the treatment shift did affect the phenotypic plasticity of the two cytotypes. A repeated exposure to a condition for several flowering periods may induce acclimation and eventually put adaptive procedures in motion. Below, we attempt to disentangle and interpret all results.

Phenotypic Plasticity and Morphological Traits

Regarding the response of the four selected morphological traits to the treatments, our findings support the hypothesis of a niche preference depending on the cytotype documented by Kirchheimer *et al.* (2016; 2018). The observed ploidy effect under warm treatment and the treatment effect for the tetraploids under cold treatment imply a potential of phenotypic plasticity, which could sustain fitness under shifted environmental conditions (Bateson, 2015). In natural environments, plants show phenotypic variation under a wider range of conditions. If such a variation is inherited by next generations, it is a trait, which could be associated with adaptation and evolution (van Kleunen and Fisher, 2003; Bossdorf and Pigliucci, 2009; Miryeganeh and Saze, 2019). This ability could give an advantage to an organism to colonize freshly

available ecological niches. Under this process, the evolution of several adaptive traits could be expected, in favor of a successful establishment to the new niche (Wyles, 1983; Pfenning and McGee, 2010; Bateson, 2015). Moreover, cytotypes with low genetic divergence (Cosendai *et al.*, 2011; Cosendai *et al.*, 2013) rely on phenotypic variation to manage to colonize different environments (Vrijenhoek and Parker, 2009). Thus, we suggest that the post-glacial colonization of the Alps by tetraploid populations of *R. kuepferi* and the distribution of the two cytotypes was fostered by their phenotypic response to climatic conditions.

Previous studies on morphological traits in natural populations of *R. kuepferi* (Schinkel *et al.*, 2016) showed that tetraploid plants in the Alps often exhibit "alpine dwarfism" as an adaptation to higher elevations and harsh conditions (Körner, 2003). Our results suggest that tetraploids grow best under a cold treatment, which approximates the optimal alpine conditions of the natural habitats for this cytotype, as they do significantly better than those under warm treatment. Our experiments did not include the extreme cold conditions that occur at the highest elevations of the Alps (Schinkel *et al.*, 2016), which explains why we did not observe dwarf growth. Although temperature,as an environmental factor, plays the most decisive role in the ecology of each cytotype (Kirchheimer *et al.*, 2016), the phenotypic response of the tetraploid cytotype under cold conditions may entail a spatiotemporal heterogeneity of several environmental variables, *e.g.* moisture, pH, and nutrients (Kelly, 2019). Kirchheimer *et al.* (2016) found a niche shift of tetraploids toward more acidic soils. At the highest elevations in the Alps, nutrient-poor soils may also reduce growth performance (Körner, 2003). Furthermore, we hypothesized that the adaptive effects of polyploidy, *e.g.* increasing cell size (te Beest *et al.*, 2012) may be expressed in a stronger way in the climate growth chambers, where only temperature varies and the other proxies are kept equal for the scope of current experimental design. For the diploids, our results are consistent with the study on natural populations (Schinkel *et al.*, 2016), as diploids grew better and had more flowers than tetraploids under warm conditions. Our warm treatments appear to match their natural optimal conditions. The higher number of flowers, together with better seed set (Schinkel *et al.*, 2016), resulted in a higher seed yield for diploids and, hence, a fitness advantage in their optimal, warmer climatic niche.

Epigenetic Patterns and Morphological Traits

The epigenetic background of phenotypic plasticity was noteworthily discussed (Pigliucci, 2001; West-Eberhard, 2003; Mirouze and Paskzowski, 2011; Noble *et al.*, 2014; Bateson, 2015), while the potential of epigenetic inheritance of plastic phenotypic traits in plants was debated by several authors (Johannes *et al.*, 2009; Cortijo *et al.*, 2014; Miryeganeh and Saze, 2019), with cytosine (DNA) methylation being quite important in such procedures (Paskowski and Grossniklaus, 2011). DNA methylation is an epigenetic

mechanism, which seems to be strongly correlated with phenotypic plasticity to internal and external stimuli (Wyles *et al.*, 1983), which could be advantageous to occupy a wider distribution niche (Pal and Milkos, 1999; Angers *et al.*, 2010). Such stress-induced epigenetic patterns provide rapid responses to fluctuating environmental conditions and could have an impact on individual fitness (Crews *et al.*, 2007; Bossdorf and Pigliucci, 2009; Angers *et al.*, 2010; Castonguay and Angers, 2012; Herrera *et al.*, 2013; Nicotra *et al.*, 2015; Rubenstein *et al.*, 2016: Verhoeven *et al.*, 2016).. In case this phenotypic variation is heritable, DNA methylation could provide a mechanism of adaptive microevolution in plants (Herman and Sultan, 2011; Zhang *et al.*, 2013), which is faster and independent from traditional genetic evolution (Jablonka and Lamb, 1995; Richards, 2006; Bossdorf and Pigliucci, 2009). Epigenetic variation differed in *R. kuepferi* between cytotypes, and also varied under climatic conditions, both in natural populations (Schinkel *et al.*, 2020) and under controlled conditions (Syngelaki *et al.*, 2020).

Here, we compared the DNA methylation patterns with all the measured morphological traits per individual, keeping in mind their ploidy level and treatment conditions. Furthermore, we focused on the number of leaves and number of flowers, as they were important for our hypotheses regarding the survival and the fitness of *R. kuepferi*. Concerning the leaves as a morphological trait, it wasintriguing to inspect their interaction with the epigenetic patterns, as they are the main photosynthetic organ of plants (Maugarny-Cales and Laufs, 2018). Tholen *et al.* (2012) suggested that biomass production of leaves is the main factor influencing photosynthesis, while Yamori et al. (Yamori *et al.*, 2014) appraised the temperature acclimation of photosynthesis, its underlying mechanisms, and their heritable potential. Moreover, photosynthetic plasticity is triggered by stress environmental conditions in cotton cultivars (Aspinwall *et al.*, 2015). In *Arabidopsis thaliana*, the phenotypic plasticity of leaves has an epigenetic basis and is associated with DNA hypomethylation (Kooke *et al.*, 2015).

The correlation of some morphological traits with the epigenetic patterns, which wasindicated in our study, may refer to the invoked reaction of the individuals toward the shifted conditions after three years (Syngelaki *et al.*, 2020) of being acclimated in previous experimental treatments (Klatt *et al.*, 2018). In particular, our findings showed a stronger correlation of phenotypic plasticity in leaves than in flowers with epigenetic variation, while, for both cytotypes, leaves exhibited higher values under the cold treatment.

Hence, the stress response of individuals was expressed as phenotypic plasticity, which may be controlled by DNA methylation variation,as well as by the genetic background. Such a hypothesis of epigenetic control on the phenotypic plasticity of the species wasfurther supported by the negative correlation of the leaf length and the number of

leaves with the patterns of non-methylated epiloci for all the predefined groups. The significant relationship of nonmethylated epiloci withleaf length and the number of leaves, in comparison to the non-significant effect of the other types of epiloci, may highlight the importance of this epilocus in the mechanisms of phenotypic response, *e.g.* the gene expression toward the new temperature conditions. Non-methylated epiloci are often linked with DNA demethylation, which is responsible for variations in phenotypic plasticity, by extending its environmental sensitivity (Kooke *et al.*, 2015), while global demethylation of genomic DNA in response to abiotic environmental stress could regulate gene expression (Steward *et al.*, 2002; Shan *et al.*, 2013). However, the observed non-methylation patterns could also reflect an underlying genetic variation and should rather be regarded as indicative of a high methylation dynamics under stress conditions (Zhang *et al.*, 2018).

The observed variation in some of the morphological traits and the epigenetic patterns seemed to affect the fitness of individuals under the new environmental conditions, thus proposing a Jack-and-master scenario (Richards *et al.*, 2006) for the species. In that scenario, changes in traits can contribute to a higher fitness and/or be opportunistic, *e.g.* because of epigenetic asymmetry, profiting in such a way the establishment of a species in a new environment. Epigenetic asymmetry is often observed under changing environmental conditions, as stochastic epigenetic changes may result in high levels of plasticity, "weird" phenotypes, and even developmental disturbances (Palmer and Strobeck, 1986; Palmer, 1996; Moller and Swaddle, 1997; Dongen, 2006; Angers *et al.*, 2010; Angers *et al.*, 2020).

Nevertheless, the degree of phenotypic and epigenetic response of the species in fluctuating biotic and abiotic environmental conditions is a rather complicated process, where exposure time may also play a role (Angers *et al.*, 2010). Thus, further studies, which would investigate the mechanisms of phenotypic response and factors that prompt it, *e.g.* exploring harsher environmental conditions, will help us to address our hypotheses more efficiently.

Reproduction Mode under Temperature Treatments

Differences in seed set and reproduction mode among the cytotypes confirmed the results of previous studies on the species in natural populations (Cosendai and Hörandl, 2010; Schinkel *et al.*, 2016) and under experimental conditions (Klatt *et al.*, 2018). Seed set is negatively affected by cold conditions, with diploids having significantly lower abortion rates under both temperature conditions, while tetraploids produced only a handful of well-developed seeds under warm conditions. Cold and frost conditions decrease seed set and injure the reproductive tissues of alpine plants, *e.g.* in *Saxifraga bryoides* (Ladinig and Wagner, 2007) and *Ranunculus hirtellus* (Kumar and Singhal, 2011).

Moreover, Ladinig and Wagner (2007) suggested that the repeated moderate frost treatment, applied also here, mimics temperature conditions occurring in high mountains and provokes frost injury in reproductive shoots, which could also result in full fruit loss. Such damaging effects were observed by Klatt *et al.* (2018) regarding the present experimental design.

Results of the current study did not imply a significant cold-induced production of apomictic seeds in the diploid cytotype as observed by Klatt *et al.* (2018), but rather suggest a phenotypic plasticity on reproduction mode from one year to the other in these perennial plants, as observed in the earlier study. In accordance with earlier findings (Syngelaki *et al.*, 2020), we speculate that these results may be correlated with the shift of treatment for the plants, which activated the complex stress responses in plants (Dewitt *et al.*, 1998) such as the plastic development of flowers in *R. kuepferi*. More specifically, not all individuals produce flowers every year, as they can rest for one or more years. It is also hypothesized that female development takes place before sprouting, asin various alpine plants (Körner, 2003). The severity of stress conditions also plays a role in the plants' response and can underline the cost of plasticity in extreme environmental conditions (Dewitt *et al.*, 1998; Suzuki *et al.*, 2014; Chevin and Hoffmann, 2017). Finally, the occurrence of BIII hybrids, which were detected in the group of WD, confirmed the hypothesis of a "female triploid bridge" as the first step to polyploidization and apomictic mode of reproduction in natural populations (Schinkel *et al.*, 2017).

To summarize, temperature stress does affect phenotypic plasticity of morphological traits in *R. kuepferi*, with responses linked to DNA methylation patterns. In addition, the phenotypic plasticity of *R. kuepferi* most likely helps to acclimate the cytotypes to their respective climatic niches. If traits were heritable, then they would have an adaptive value and explain the geographical parthenogenesis pattern of the species in the Alps. The putative epigenetic background of phenotypic plasticity suggests that DNA methylation, in comparison to DNA mutations, provides rapid reactions of an organism to variable environmental conditions but does not necessarily ensure the stability of a phenotype. Thus, the high phenotypic variability of asexual organisms could allow for a higher or equal niche dynamic as for sexual plants. Regarding these interactions of DNA methylation and gene regulation, the next step ininvestigating the stress response of *R. kuepferi* would be to identify the gene expression profiles of both cytotypes under stress and controlled conditions.

3.5 References

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3.6 Supporting Information

Table S1. List of individuals placed in the climate chambers. Listed are the sampling ID, ploidy level, treatment, country, province and altitude. The individuals, which were used for the MSAP laboratory work, are highlighted with green (Schinkel *et al.*, 2016; Klatt *et al.*, 2018; Syngelaki *et al.*, 2020).

SampleID	Leaf ploidy	Temperature treatment 2017	Country	Region	Altitude	MSAPs
3_2_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	2291	Y
3_4_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	2291	N
3_4_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	2291	N
23_1_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1616	N
23_2_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1616	N
23_2_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1616	N
23_4_1	diploid	warm	France	Provence-Alpes-Côte d'Azur	1616	Y
23_4_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1616	N
24_2_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1925	N
24_2_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1925	N
24_3_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1925	N
24_4_1	diploid	warm	France	Provence-Alpes-Côte d'Azur	1925	N
24_4_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1925	Y
25_1_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1435	N
25_2_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1435	N
25_2_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1435	N
25_3_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1435	Y
25_3_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1435	N
25_4_1	diploid	warm	France	Provence-Alpes-Côte d'Azur	1435	N
26_1_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1456	N
26_2_1	diploid	warm	France	Provence-Alpes-Côte d'Azur	1456	N
26_2_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1456	N
26_3_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1456	N
26_3_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1456	Y
26_4_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1456	N
27_1_1	diploid	warm	France	Rhônes-Alpes	1449	N
27_1_2	diploid	warm	France	Rhônes-Alpes	1449	N
27_2_1	diploid	warm	France	Rhônes-Alpes	1449	Y
27_2_2	diploid	warm	France	Rhônes-Alpes	1449	N
27_2_3	diploid	warm	France	Rhônes-Alpes	1449	N
27_3_1	diploid	warm	France	Rhônes-Alpes	1449	N
27_4_3	diploid	warm	France	Rhônes-Alpes	1449	N
28_2_2	diploid	warm	Italy	Piemonte	1685	N
28_3_1	diploid	warm	Italy	Piemonte	1685	Y

SampleID	Leaf ploidy	Temperature treatment 2017	Country	Region	Altitude	MSAPs
28_3_2	diploid	warm	Italy	Piemonte	1685	Y
28_4_2	diploid	warm	Italy	Piemonte	1685	N
28_4_3	diploid	warm	Italy	Piemonte	1685	N
29_1_1	diploid	warm	Italy	Piemonte	2020	N
29_1_2	diploid	warm	Italy	Piemonte	2020	Y
29_2_2	diploid	warm	Italy	Piemonte	2020	N
29_4_1	diploid	warm	Italy	Piemonte	2020	N
30_1_1	diploid	warm	Italy	Piemonte	1743	N
30_1_3	diploid	warm	Italy	Piemonte	1743	Y
31_3_3	diploid	warm	Italy	Piemonte	1937	Y
31_4_1	diploid	warm	Italy	Piemonte	1937	N
31_4_2	diploid	warm	Italy	Piemonte	1937	N
32_1_3	diploid	warm	Italy	Piemonte	2320	N
32_4_1	diploid	warm	Italy	Piemonte	2320	N
32_4_2	diploid	warm	Italy	Piemonte	2320	Y
33_3_2	diploid	warm	Italy	Piemonte	2328	N
33_3_3	diploid	warm	Italy	Piemonte	2328	Y
33_4_3	diploid	warm	Italy	Piemonte	2328	N
112_1_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1626	N
112_2_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1626	N
112_2_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1626	N
112_3_1	diploid	warm	France	Provence-Alpes-Côte d'Azur	1626	Y
112_3_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1626	Y
112_3_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1626	N
115_1_1	diploid	warm	France	Provence-Alpes-Côte d'Azur	1891	N
115_1_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1891	N
115_2_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1891	N
115_3_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1891	Y
115_4_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1891	N
116_1_1	diploid	warm	France	Provence-Alpes-Côte d'Azur	1953	Y
116_1_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1953	N
116_2_1	diploid	warm	France	Provence-Alpes-Côte d'Azur	1953	N
116_3_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1953	Y
117_1_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1632	N
117_1_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1632	N
117_2_1	diploid	warm	France	Provence-Alpes-Côte d'Azur	1632	N
117_3_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1632	Y
117_4_1	diploid	warm	France	Provence-Alpes-Côte d'Azur	1632	N
117_4_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1632	N
202_1_1	diploid	warm	France	Provence-Alpes-Côte d'Azur	1829	N
202_1_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1829	N

SampleID	Leaf ploidy	Temperature treatment 2017	Country	Region	Altitude	MSAPs
202_2_1	diploid	warm	France	Provence-Alpes-Côte d'Azur	1829	N
202_4_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1829	N
203_2_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1840	N
203_4_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	1840	N
203_4_3	diploid	warm	France	Provence-Alpes-Côte d'Azur	1840	Y
233_1_1	diploid	warm	France	Provence-Alpes-Côte d'Azur	2185	N
233_2_1	diploid	warm	France	Provence-Alpes-Côte d'Azur	2185	Y
233_2_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	2185	N
233_3_2	diploid	warm	France	Provence-Alpes-Côte d'Azur	2185	N
28B_2_1	diploid	warm	Italy	Piemonte	1685	N
28B_3_1	diploid	warm	Italy	Piemonte	1685	Y
28B_4_3	diploid	warm	Italy	Piemonte	1685	N
31B_1_1	diploid	warm	Italy	Piemonte	1937	N
31B_1_3	diploid	warm	Italy	Piemonte	1937	Y
31B/32B_2_2	diploid	warm	Italy	Piemonte	1937	Y
32B_1_2	diploid	warm	Italy	Piemonte	2320	Y
32B/31B_4_3	diploid	warm	Italy	Piemonte	1937	N
16_4_1	tetraploid	warm	France	Provence-Alpes-Côte d'Azur	2078	N
17_1_1	tetraploid	warm	France	Provence-Alpes-Côte d'Azur	2357	N
17_2_1	tetraploid	warm	France	Provence-Alpes-Côte d'Azur	2357	N
17_3_3	tetraploid	warm	France	Provence-Alpes-Côte d'Azur	2357	N
17_4_3	tetraploid	warm	France	Provence-Alpes-Côte d'Azur	2357	Y
34_2_1	tetraploid	warm	France	Rhônes-Alpes	2120	Y
34_2_3	tetraploid	warm	France	Rhônes-Alpes	2120	N
34_4_1	tetraploid	warm	France	Rhônes-Alpes	2120	N
36_1_1	tetraploid	warm	France	Rhônes-Alpes	2152	N
36_2_3	tetraploid	warm	France	Rhônes-Alpes	2152	N
36_4_1	tetraploid	warm	France	Rhônes-Alpes	2152	Y
36_4_2	tetraploid	warm	France	Rhônes-Alpes	2152	N
37_1_2	tetraploid	warm	Italy	Val d'Aosta	2115	Y
37_2_3	tetraploid	warm	Italy	Val d'Aosta	2115	N
37_3_1	tetraploid	warm	Italy	Val d'Aosta	2115	N
38_1_2	tetraploid	warm	France	Rhônes-Alpes	2182	Y
40_1_1	tetraploid	warm	Switzerland	Wallis	1860	N
40_2_1	tetraploid	warm	Switzerland	Wallis	1860	N
40_3_3	tetraploid	warm	Switzerland	Wallis	1860	N
40_4_3	tetraploid	warm	Switzerland	Wallis	1860	N

SampleID	Leaf ploidy	Temperature treatment 2017	Country	Region	Altitude	MSAPs
41_4_3	tetraploid	warm	Italy	Val d'Aosta	2174	Y
42_1_2	tetraploid	warm	Switzerland	Wallis	1789	Y
42_1_3	tetraploid	warm	Switzerland	Wallis	1789	N
42_2_2	tetraploid	warm	Switzerland	Wallis	1789	N
42_3_3	tetraploid	warm	Switzerland	Wallis	1789	N
43_4_1	tetraploid	warm	Switzerland	Wallis	2012	N
43_4_3	tetraploid	warm	Switzerland	Wallis	2012	N
45_1_3	tetraploid	warm	Switzerland	Wallis	2400	N
45_2_2	tetraploid	warm	Switzerland	Wallis	2400	N
45_2_3	tetraploid	warm	Switzerland	Wallis	2400	N
45_3_2	tetraploid	warm	Switzerland	Wallis	2400	N
45_3_3	tetraploid	warm	Switzerland	Wallis	2400	N
47_1_1	tetraploid	warm	Switzerland	Graubünden	2211	N
47_2_1	tetraploid	warm	Switzerland	Graubünden	2211	N
47_3_2	tetraploid	warm	Switzerland	Graubünden	2211	N
47_3_3	tetraploid	warm	Switzerland	Graubünden	2211	Y
48_1_1	tetraploid	warm	Switzerland	Graubünden	2262	N
48_2_2	tetraploid	warm	Switzerland	Graubünden	2262	N
50_1_2	tetraploid	warm	Switzerland	Graubünden	2322	N
50_1_3	tetraploid	warm	Switzerland	Graubünden	2322	N
50_3_2	tetraploid	warm	Switzerland	Graubünden	2322	N
50_3_3	tetraploid	warm	Switzerland	Graubünden	2322	N
51_3_1	tetraploid	warm	Austria	Tirol	2286	Y
51_3_3	tetraploid	warm	Austria	Tirol	2286	N
53_1_2	tetraploid	warm	Switzerland	Graubünden	2456	N
53_1_3	tetraploid	warm	Switzerland	Graubünden	2456	Y

SampleID	Leaf ploidy	Temperature treatment 2017	Country	Region	Altitude	MSAPs
53_2_1	tetraploid	warm	Switzerland	Graubünden	2456	N
53_3_2	tetraploid	warm	Switzerland	Graubünden	2456	N
53_4_3	tetraploid	warm	Switzerland	Graubünden	2456	N
54_1_1	tetraploid	warm	Italy	Lombardia	2303	N
54_2_3	tetraploid	warm	Italy	Lombardia	2303	Y
54_3_1	tetraploid	warm	Italy	Lombardia	2303	N
54_4_1	tetraploid	warm	Italy	Lombardia	2303	N
58_1_1	tetraploid	warm	Italy	Trentino Alto Adige/ Südtirol	2117	Y
58_3_3	tetraploid	warm	Italy	Trentino Alto Adige/ Südtirol	2117	Y
58_4_1	tetraploid	warm	Italy	Trentino Alto Adige/ Südtirol	2117	N
66_1_3	tetraploid	warm	Italy	Trentino Alto Adige/ Südtirol	2101	N
66_2_1	tetraploid	warm	Italy	Trentino Alto Adige/ Südtirol	2101	N
66_3_2	tetraploid	warm	Italy	Trentino Alto Adige/ Südtirol	2101	N
66_3_3	tetraploid	warm	Italy	Trentino Alto Adige/ Südtirol	2101	N
66_4_2	tetraploid	warm	Italy	Trentino Alto Adige/ Südtirol	2101	N
74_1_1	tetraploid	warm	Austria	Osttirol	2117	N
74_1_2	tetraploid	warm	Austria	Osttirol	2117	Y
74_2_1	tetraploid	warm	Austria	Osttirol	2117	N
75_3_1	tetraploid	warm	Switzerland	Graubünden	2678	N
78_3_2	tetraploid	warm	Switzerland	Wallis	2000	Y
79_1_3	tetraploid	warm	Switzerland	Graubünden	2280	N
79_2_1	tetraploid	warm	Switzerland	Graubünden	2280	N
79_2_3	tetraploid	warm	Switzerland	Graubünden	2280	Y
79_3_2	tetraploid	warm	Switzerland	Graubünden	2280	N
79_4_1	tetraploid	warm	Switzerland	Graubünden	2280	N
85_2_1	tetraploid	warm	Austria	Kärnten	2184	N
85_4_1	tetraploid	warm	Austria	Kärnten	2184	Y
88_3_1	tetraploid	warm	Switzerland	Graubünden	2300	Y

SampleID	Leaf ploidy	Temperature treatment 2017	Country	Region	Altitude	MSAPs
89_1_2	tetraploid	warm	Switzerland	Graubünden	2265	N
89_2_1	tetraploid	warm	Switzerland	Graubünden	2265	N
92_2_2	tetraploid	warm	Switzerland	Graubünden	2260	N
92_3_2	tetraploid	warm	Switzerland	Graubünden	2260	N
92_4_2	tetraploid	warm	Switzerland	Graubünden	2260	N
93_2_2	tetraploid	warm	Switzerland	Wallis	2405	N
96_1_1	tetraploid	warm	France	Provence-Alpes-Côte d'Azur	2300	N
96_3_1	tetraploid	warm	France	Provence-Alpes-Côte d'Azur	2300	N
96_3_3	tetraploid	warm	France	Provence-Alpes-Côte d'Azur	2300	N
103_4_1	tetraploid	warm	Italy	Lombardia	2290	N
103_4_2	tetraploid	warm	Italy	Lombardia	2290	Y
106_1_2	tetraploid	warm	Italy	Trentino Alto Adige/ Südtirol	2142	N
106_3_2	tetraploid	warm	Italy	Trentino Alto Adige/ Südtirol	2142	N
106_3_3	tetraploid	warm	Italy	Trentino Alto Adige/ Südtirol	2142	N
106_4_1	tetraploid	warm	Italy	Trentino Alto Adige/ Südtirol	2142	Y
108_4_3	tetraploid	warm	Switzerland	Graubünden	2171	N
111_1_3	tetraploid	warm	France	Provence-Alpes-Côte d'Azur	2243	N
111_3_2	tetraploid	warm	France	Provence-Alpes-Côte d'Azur	2243	Y
111_3_3	tetraploid	warm	France	Provence-Alpes-Côte d'Azur	2243	Y
116_4_1	tetraploid	warm	France	Provence-Alpes-Côte d'Azur	1953	Y
116_4_2	tetraploid	warm	France	Provence-Alpes-Côte d'Azur	1953	N
208_2_1	tetraploid	warm	France	Provence-Alpes-Côte d'Azur	1924	Y
3B_1_2	tetraploid	warm	France	Provence-Alpes-Côte d'Azur	2291	Y
3_1_2	diploid	cold	France	Provence-Alpes-Côte d'Azur	2291	N
3_1_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	2291	N
3_2_2	diploid	cold	France	Provence-Alpes-Côte d'Azur	2291	Y
3_3_2	diploid	cold	France	Provence-Alpes-Côte d'Azur	2291	Y
3_4_1	diploid	cold	France	Provence-Alpes-Côte d'Azur	2291	N
23_3_2	diploid	cold	France	Provence-Alpes-Côte d'Azur	1616	N
23_3_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	1616	Y
23_4_2	diploid	cold	France	Provence-Alpes-Côte d'Azur	1616	N
24_1_2	diploid	cold	France	Provence-Alpes-Côte d'Azur	1925	Y
24_3_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	1925	N

SampleID	Leaf ploidy	Temperature treatment 2017	Country	Region	Altitude	MSAPs
24_4_2	diploid	cold	France	Provence-Alpes-Côte d'Azur	1925	N
25_1_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	1435	Y
25_2_1	diploid	cold	France	Provence-Alpes-Côte d'Azur	1435	N
25_3_1	diploid	cold	France	Provence-Alpes-Côte d'Azur	1435	N
25_4_2	diploid	cold	France	Provence-Alpes-Côte d'Azur	1435	N
25_4_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	1435	N
26_1_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	1456	N
26_4_1	diploid	cold	France	Provence-Alpes-Côte d'Azur	1456	Y
27_2_2	diploid	cold	France	Rhônes-Alpes	1449	Y
27_3_2	diploid	cold	France	Rhônes-Alpes	1449	N
27_3_3	diploid	cold	France	Rhônes-Alpes	1449	N
27_4_1	diploid	cold	France	Rhônes-Alpes	1449	N
28_1_2	diploid	cold	Italy	Piemonte	1685	N
28_2_1	diploid	cold	Italy	Piemonte	1685	N
28_2_3	diploid	cold	Italy	Piemonte	1685	Y
28_3_3	diploid	cold	Italy	Piemonte	1685	N
29_1_3	diploid	cold	Italy	Piemonte	2020	N
29_2_3	diploid	cold	Italy	Piemonte	2020	N
29_3_1	diploid	cold	Italy	Piemonte	2020	N
29_4_2	diploid	cold	Italy	Piemonte	2020	Y
30_1_2	diploid	cold	Italy	Piemonte	1743	Y
31_2_3	diploid	cold	Italy	Piemonte	1937	Y
32_1_2	diploid	cold	Italy	Piemonte	2320	N
32_3_2	diploid	cold	Italy	Piemonte	2320	N
32_3_3	diploid	cold	Italy	Piemonte	2320	Y
32_4_3	diploid	cold	Italy	Piemonte	2320	N
33_1_1	diploid	cold	Italy	Piemonte	2328	N
33_3_1	diploid	cold	Italy	Piemonte	2328	N
33_4_1	diploid	cold	Italy	Piemonte	2328	N
33_4_2	diploid	cold	Italy	Piemonte	2328	Y
112_1_1	diploid	cold	France	Provence-Alpes-Côte d'Azur	1626	N
112_1_2	diploid	cold	France	Provence-Alpes-Côte d'Azur	1626	Y
112_2_1	diploid	cold	France	Provence-Alpes-Côte d'Azur	1626	N
112_4_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	1626	N
115_1_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	1891	N
115_2_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	1891	N
115_3_1	diploid	cold	France	Provence-Alpes-Côte d'Azur	1891	N
115_4_1	diploid	cold	France	Provence-Alpes-Côte d'Azur	1891	N
115_4_2	diploid	cold	France	Provence-Alpes-Côte d'Azur	1891	Y
116_1_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	1953	Y
116_2_2	diploid	cold	France	Provence-Alpes-Côte d'Azur	1953	N

SampleID	Leaf ploidy	Temperature treatment 2017	Country	Region	Altitude	MSAPs
116_2_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	1953	N
116_3_1	diploid	cold	France	Provence-Alpes-Côte d'Azur	1953	Y
116_3_2	diploid	cold	France	Provence-Alpes-Côte d'Azur	1953	N
117_1_1	diploid	cold	France	Provence-Alpes-Côte d'Azur	1632	N
117_2_2	diploid	cold	France	Provence-Alpes-Côte d'Azur	1632	N
117_2_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	1632	N
117_3_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	1632	Y
202_1_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	1829	N
202_3_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	1829	Y
203_2_2	diploid	cold	France	Provence-Alpes-Côte d'Azur	1840	Y
203_4_1	diploid	cold	France	Provence-Alpes-Côte d'Azur	1840	N
233_2_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	2185	Y
233_3_1	diploid	cold	France	Provence-Alpes-Côte d'Azur	2185	N
233_3_3	diploid	cold	France	Provence-Alpes-Côte d'Azur	2185	N
28B_1_1	diploid	cold	Italy	Piemonte	1685	N
28B_1_2	diploid	cold	Italy	Piemonte	1685	Y
28B_1_3	diploid	cold	Italy	Piemonte	1685	N
28B_2_2	diploid	cold	Italy	Piemonte	1685	N
31B_3_3	diploid	cold	Italy	Piemonte	1937	Y
32B_1_1	diploid	cold	Italy	Piemonte	2320	Y
32B/31B_2_1	diploid	cold	Italy	Piemonte	2320	Y
32B_2_3	diploid	cold	Italy	Piemonte	2320	N
32B_3_2	diploid	cold	Italy	Piemonte	2320	N
17_2_3	tetraploid	cold	France	Provence-Alpes-Côte d'Azur	2357	N
17_3_1	tetraploid	cold	France	Provence-Alpes-Côte d'Azur	2357	N
17_3_2	tetraploid	cold	France	Provence-Alpes-Côte d'Azur	2357	N
17_4_1	tetraploid	cold	France	Provence-Alpes-Côte d'Azur	2357	N
17_4_2	tetraploid	cold	France	Provence-Alpes-Côte d'Azur	2357	Y
34_1_1	tetraploid	cold	France	Rhônes-Alpes	2120	Y
34_2_2	tetraploid	cold	France	Rhônes-Alpes	2120	N
36_1_2	tetraploid	cold	France	Rhônes-Alpes	2152	N
36_1_3	tetraploid	cold	France	Rhônes-Alpes	2152	N
36_2_1	tetraploid	cold	France	Rhônes-Alpes	2152	Y
36_2_2	tetraploid	cold	France	Rhônes-Alpes	2152	N
37_2_2	tetraploid	cold	Italy	Val d'Aosta	2115	N
37_3_2	tetraploid	cold	Italy	Val d'Aosta	2115	N
37_4_1	tetraploid	cold	Italy	Val d'Aosta	2115	Y
40_1_2	tetraploid	cold	Switzerland	Wallis	1860	N
40_2_2	tetraploid	cold	Switzerland	Wallis	1860	N

SampleID	Leaf ploidy	Temperature treatment 2017	Country	Region	Altitude	MSAPs
40_3_1	tetraploid	cold	Switzerland	Wallis	1860	Y
40_4_2	tetraploid	cold	Switzerland	Wallis	1860	N
41_2_2	tetraploid	cold	Italy	Val d'Aosta	2174	Y
42_1_1	tetraploid	cold	Switzerland	Wallis	1789	N
42_2_3	tetraploid	cold	Switzerland	Wallis	1789	N
42_3_2	tetraploid	cold	Switzerland	Wallis	1789	Y
42_4_2	tetraploid	cold	Switzerland	Wallis	1789	N
42_4_3	tetraploid	cold	Switzerland	Wallis	1789	N
45_1_1	tetraploid	cold	Switzerland	Wallis	2400	N
45_1_2	tetraploid	cold	Switzerland	Wallis	2400	Y
45_2_1	tetraploid	cold	Switzerland	Wallis	2400	N
45_4_1	tetraploid	cold	Switzerland	Wallis	2400	N
47_1_2	tetraploid	cold	Switzerland	Graubünden	2211	N
47_1_3	tetraploid	cold	Switzerland	Graubünden	2211	N
47_2_2	tetraploid	cold	Switzerland	Graubünden	2211	Y
47_2_3	tetraploid	cold	Switzerland	Graubünden	2211	N
47_4_1	tetraploid	cold	Switzerland	Graubünden	2211	N
48_3_2	tetraploid	cold	Switzerland	Graubünden	2262	N
48_4_2	tetraploid	cold	Switzerland	Graubünden	2262	Y
50_2_1	tetraploid	cold	Switzerland	Graubünden	2322	Y
50_2_2	tetraploid	cold	Switzerland	Graubünden	2322	N
50_3_3	tetraploid	cold	Switzerland	Graubünden	2322	N
50_4_1	tetraploid	cold	Switzerland	Graubünden	2322	N
50_4_3	tetraploid	cold	Switzerland	Graubünden	2322	N
53_2_2	tetraploid	cold	Switzerland	Graubünden	2456	N

SampleID	Leaf ploidy	Temperature treatment 2017	Country	Region	Altitude	MSAPs
53_2_3	tetraploid	cold	Switzerland	Graubünden	2456	N
53_3_1	tetraploid	cold	Switzerland	Graubünden	2456	Y
53_3_3	tetraploid	cold	Switzerland	Graubünden	2456	N
54_1_2	tetraploid	cold	Italy	Lombardia	2303	N
54_2_2	tetraploid	cold	Italy	Lombardia	2303	Y
54_3_3	tetraploid	cold	Italy	Lombardia	2303	N
54_4_2	tetraploid	cold	Italy	Lombardia	2303	N
54_4_3	tetraploid	cold	Italy	Lombardia	2303	N
55_3_1	tetraploid	cold	Austria	Tirol	2557	Y
58_1_2	tetraploid	cold	Italy	Trentino Alto Adige/ Südtirol	2117	N
58_1_3	tetraploid	cold	Italy	Trentino Alto Adige/ Südtirol	2117	Y
58_4_3	tetraploid	cold	Italy	Trentino Alto Adige/ Südtirol	2117	N
66_1_1	tetraploid	cold	Italy	Trentino Alto Adige/ Südtirol	2101	N
66_2_2	tetraploid	cold	Italy	Trentino Alto Adige/ Südtirol	2101	Y
66_2_3	tetraploid	cold	Italy	Trentino Alto Adige/ Südtirol	2101	N
74_2_2	tetraploid	cold	Austria	Osttirol	2117	N
74_4_2	tetraploid	cold	Austria	Osttirol	2117	N
79_3_1	tetraploid	cold	Switzerland	Graubünden	2280	N
79_3_3	tetraploid	cold	Switzerland	Graubünden	2280	Y
82_3_3	tetraploid	cold	Italy	Lombardia	2500	Y
83_3_1	tetraploid	cold	Austria	Osttirol	2271	Y
84_1_2	tetraploid	cold	Austria	Kärnten	2236	N
84_4_2	tetraploid	cold	Austria	Kärnten	2236	Y
85_3_2	tetraploid	cold	Austria	Kärnten	2184	N
85_3_3	tetraploid	cold	Austria	Kärnten	2184	N
88_1_1	tetraploid	cold	Switzerland	Graubünden	2300	N
88_1_2	tetraploid	cold	Switzerland	Graubünden	2300	N
88_2_3	tetraploid	cold	Switzerland	Graubünden	2300	N
90_2_3	tetraploid	cold	Switzerland	Wallis	2477	Y
92_2_1	tetraploid	cold	Switzerland	Graubünden	2260	N

SampleID	Leaf ploidy	Temperature treatment 2017	Country	Region	Altitude	MSAPs
92_4_1	tetraploid	cold	Switzerland	Graubünden	2260	N
93_2_1	tetraploid	cold	Switzerland	Wallis	2405	N
93_2_3	tetraploid	cold	Switzerland	Wallis	2405	N
93_3_3	tetraploid	cold	Switzerland	Wallis	2405	N
96_1_3	tetraploid	cold	France	Provence-Alpes-Côte d'Azur	2300	N
96_2_3	tetraploid	cold	France	Provence-Alpes-Côte d'Azur	2300	Y
96_3_2	tetraploid	cold	France	Provence-Alpes-Côte d'Azur	2300	N
96_4_2	tetraploid	cold	France	Provence-Alpes-Côte d'Azur	2300	N
96_4_3	tetraploid	cold	France	Provence-Alpes-Côte d'Azur	2300	N
103_1_2	tetraploid	cold	Italy	Lombardia	2290	N
106_2_1	tetraploid	cold	Italy	Trentino Alto Adige/ Südtirol	2142	N
106_2_2	tetraploid	cold	Italy	Trentino Alto Adige/ Südtirol	2142	N
106_2_3	tetraploid	cold	Italy	Trentino Alto Adige/ Südtirol	2142	Y
106_4_3	tetraploid	cold	Italy	Trentino Alto Adige/ Südtirol	2142	N
108_3_2	tetraploid	cold	Switzerland	Graubünden	2171	N
108_3_3	tetraploid	cold	Switzerland	Graubünden	2171	Y
111_1_1	tetraploid	cold	France	Provence-Alpes-Côte d'Azur	2243	N
111_1_2	tetraploid	cold	France	Provence-Alpes-Côte d'Azur	2243	N
111_2_3	tetraploid	cold	France	Provence-Alpes-Côte d'Azur	2243	Y

Table S2. Reproductive modes (a) and special cases of assumed reproduction modes (b) for diploid and tetraploid *Ranunculus kuepferi* plants under temperature treatments. The FCSS data for embryo and endosperm ploidy in single seeds presented in (b) were excluded from further analysis.

	Genome contribution to embryo/endosperm	Embryo: endosperm	Peak index	Path	Number of observations (seeds, ssFCSS)		
	Egg cell + sperm nucleus/fused polar nuclei + sperm nucleus (nuclei)				Cold	Warm	Total
a) Reproduction mode						
Diploid plants						
Sexual	1Cx(m) + 1Cx(p)/2Cx(m) + 1Cx(p)	2:3	1.5	A	206	359	565
Apomictic	2Cx(m) + 0Cx(p)/4Cx(m) + 0Cx(p)	2:4	2	B	1	6	7
	2Cx(m) + 0Cx(p)/4Cx(m) + 1Cx(p)	2:5	2.5	C	0	2	2
	2Cx(m) + 0Cx(p)/4Cx(m) + 2Cx(p)	2:6	3	D	2	0	2
B$_{III}$ hybrid	2Cx(m) + 1Cx(p)/4Cx(m) + 1Cx(p)	3:5	1.67	E	0	7	7
	2Cx(m) + 2Cx(p)/4Cx(m) + 2Cx(p)	4:6	1.5	F	0	1	1
	2Cx(m) + 2Cx(p)/(4Cx(m))*2 + 2Cx(p)	4:10	2.5	G	0	8	8
Tetraploid plants						
Sexual	2Cx(m) + 2Cx(p)/4Cx(m) + 2Cx(p)	4:6	1.5	H	6	0	6
Apomictic	4Cx(m) + 0Cx(p)/8Cx(m) + 0Cx(p)	4:8	2	I	2	0	2
	4Cx(m) + 0Cx(p)/8Cx(m) + 1Cx(p)	4:9	2.25	J	3	5	8
	4Cx(m) + 0Cx(p)/8Cx(m) + 2Cx(p)	4:10	2.5	K	52	1	53
	4Cx(m) + 0Cx(p)/8Cx(m) + 3Cx(p)	4:11	2.75	L	8	0	8
	4Cx(m) + 0Cx(p)/8Cx(m) + 4Cx(p)	4:12	3	M	11	0	11
	4Cx(m) + 0Cx(p)/8Cx(m) + 6Cx(p)	4:14	3.5	N	8	0	8

b) Assumed reproduction mode						
Diploid plants						
Apospory with polyspermy	2Cx(m) + 0Cx(p)/4Cx(m) + 3Cx(p)	2:7	3.5	2	1	3
Apospory with polyspermy	2Cx(m) + 1Cx(p)/4Cx(m) + 3Cx(p)	3:7	2.33	1	1	2
Apospory with polyspermy	2Cx(m) + 1Cx(p)/4Cx(m) + 4Cx(p)	3:8	2.67	0	2	2
Apospory with polyspermy	2Cx(m) +2Cx(p)/4Cx(m) + 5Cx(p)	4:9	2.25	0	2	2
Apospory with endosperm endopolyploidy& polyspermy	2Cx(m) + 2Cx(p)/(4Cx(m))*2 + 8Cx(p)	4:16	4	0	1	1
Tetraploid plants						
Apospory with endopolyploidy	4Cx(m) + 0Cx(p)/(8Cx(m))*2 + 2Cx(p)	4:18	1:4.5	2	0	2
Hypertetraploid aneuploidy with autonomous endosperm	5Cx(m) + 0Cx(p)/10Cx(m) + 0Cx(p)	5:10	2	1	0	1

Cx :ploidy based on DNA content; m: maternal genome contribution; p: paternal genome contribution.

Table S3. Vegetative growth data for the measured morphological traits, seed yield data and reproduction mode of the seeds in diploid and tetraploid *Ranunculus kuepferi* plants under temperature treatments (Supplementary excel file; can be found under the Supplementary data of the published article).

a)

b)

c)
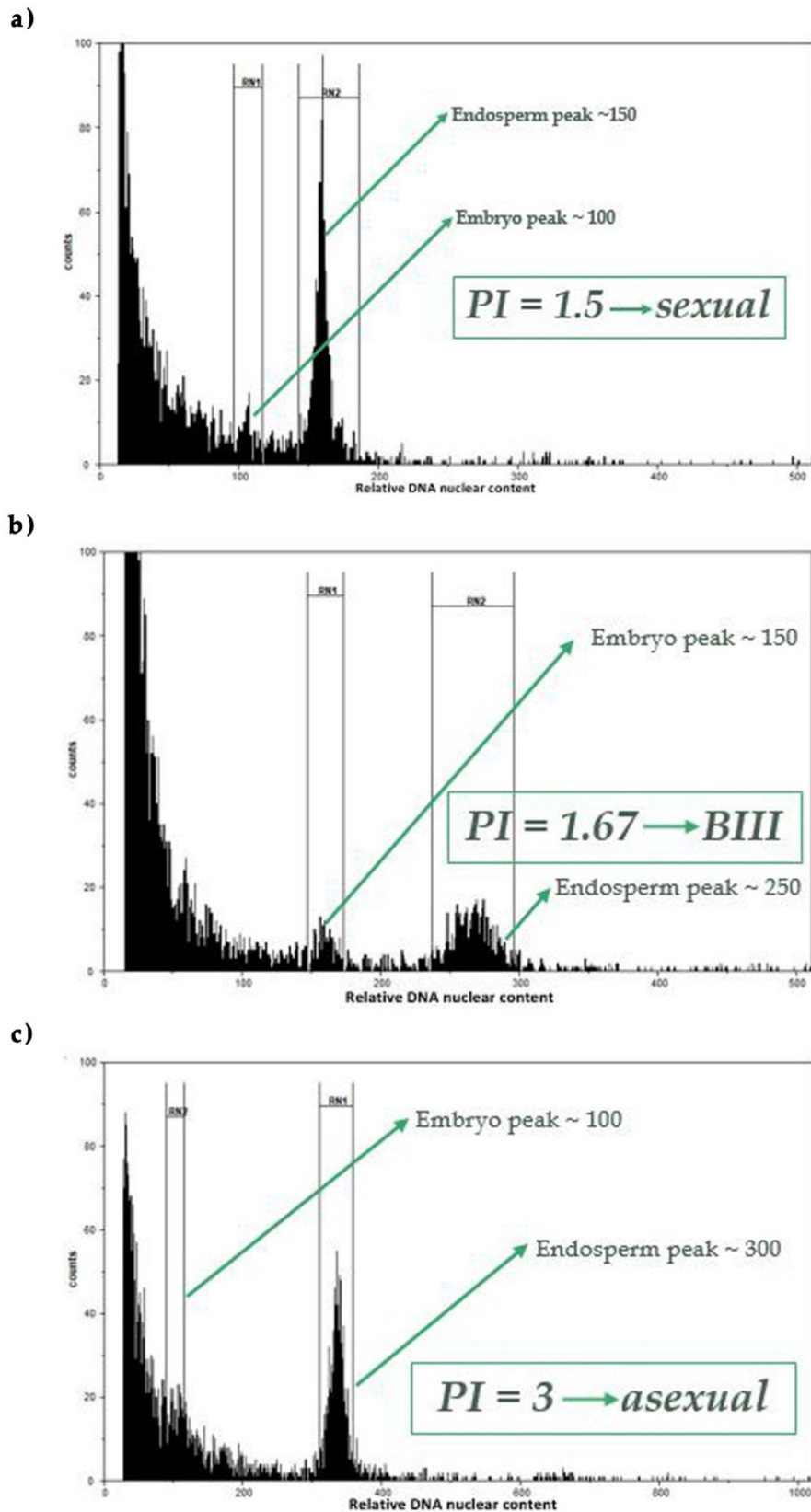
Figure S1. Representative histograms of sexual (a), BIII hybrid (b) and apomictic seeds (c) of *Ranunculus kuepferi*. All histograms refer to seeds of diploid mother plants.
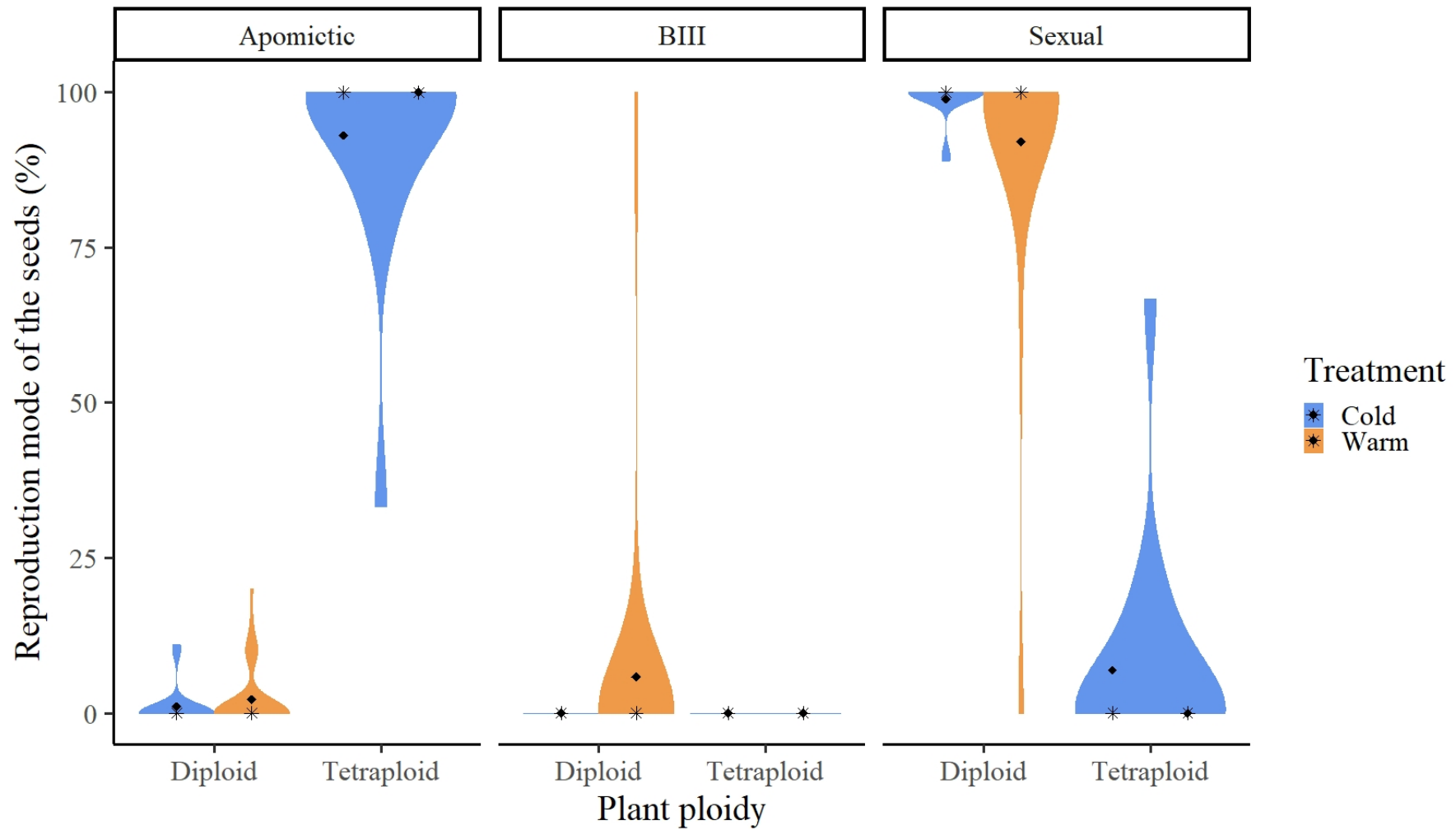
Figure S2. Influence of the temperature treatments on the mode of reproduction in diploid and tetraploid *Ranunculus kuepferi* plants. Violin plots show the percentages of apomictic seeds, BIII hybrids (partially apomictic seeds) and sexual seeds produced by plants under the cold and warm treatment.

Methods S1. Methylation-sensitive amplified fragment length polymorphisms (MS-AFLPs or MSAPs): Lab protocol and fragments scoring pipeline (Syngelaki *et al.*, 2020).

DNA from the leaf material was isolated using the QiagenDNeasy Plant Mini Kit, with a slightly modified protocol. At the second step, 360 µl AP1 Buffer and 40 µl PVP 2.6% were added and incubation time for the elution was prolonged to 30 min. The extracted samples of 100 individuals for each treatment year were screened according to a slightly modified protocol of Paun *et al.* (2012). Restriction and ligation were carried out in two parallel reactions, each one with a different restriction enzyme. The restriction enzymes, which were used, are MspI & HpaII. They are methylation sensitive restriction enzymes, i.e. isoschizomers, that recognize the same DNA sequence (CCGG), but differ in the sensitivity regarding the methylation state of C, and used as the frequent cutters, while EcoRI is used as the rare cutter. Ligation products were subjected to pre-selective amplification, whereupon selective amplification was performed with a set of three primer combinations with three selective nucleotides to each primer, used before for an AFLP analysis on the species (Cosendai *et al.*, 2011). Ligation, pre-selective and selective amplification products went through a quality and quantity check on a 1.5% agarose gel and diluted 10-fold dilution prior to pre-selective, selective amplification and fragment analyses, respectively. The final selective-PCR products were prepared with GeneScan ROX 500 (Thermo Fisher Scientific, Waltham, MA, USA) as the internal size standard and fragment analyzed on the ABI Prism 3700/3730 (Applied Biosystems, Waltham, MA, USA) capillary sequencer.

The technical reproducibility of resulting electropherograms was checked by replicating 100% of accessions, i.e. duplicates were produced for every sample used throughout the MSAP protocol steps, to minimize the false positive fragment peaks. We transformed electropherograms of raw data into a binary dominant-marker matrix. Peak Scanner2 was used to determine the height, width & the area of all peaks. The output of the Peak Scanner2 was then imported to RawGeno 2.0-1 (Arrigo *et al.*, 2009) to proceed with the binning of detected peaks, the analysis of replication and the filtering of samples of low quality. RawGeno handles a single dye color at a time, so presence/absence of fragments binary matrices were obtained for each of the three dyes (Blue; FAM, Green; HEX, Yellow; NED) and

then they were merged in a final binary matrix. Fragments between 50 and 600 base pairs were scored.In order to optimize the fragment detection and minimize the risk of false positives, a run of RawGeno with an R script (Arrigo *et al.*, 2009) was performed, which checked stepwise (~5760 steps) the binning and filtering parameters. Going through the resulting table, the optimal combination of the parameters was chosen for each dye and the respective binary matrices were produced. The selection of parameters represent a balance between quality measures, *e.g.* the error rate and bin reproducibility, and informativity, measured with the data polymorphism.

The merged binary matrix of optimized dataset for each treatment year was dealt with MSAP_calc script in R (Schulz *et al.*, 2013), to distinguish the four possible methylation conditions, using the 'Mixed Scoring 2' approach for scoring the following conditions: I) no methylation (both MspI and HpaII cut the restriction site), II) holo- or hemi-methylation of internal cytosine (^{HMe}CG or ^{Me}CG; MspI cuts the restriction site), III) hemimethylation of external cytosine (^{HMe}CCG; HpaII cuts the restriction site) and IV) holomethylation of external cytosine or of both cytosines or hemi-methylation of both cytosines or mutations (none of them cuts the restriction site). In 'Mixed Scoring 2' condition I was scored as 100 (non-methylated), condition II as 010 (internally-methylated), condition III as 001 (externally methylated), and condition IV as '000' and refers to a non-distinguishable situation, *e.g.* an ambiguous methylation or a mutation status. Condition IV was, therefore, excluded from further statistical analyses.

Chapter 4

Gene expression profiles suggest a better cold acclimation of polyploids in the alpine species *Ranunculus kuepferi* (Ranunculaceae)

Eleni Syngelaki, Claudia Paetzold and Elvira Hörandl

Abstract

Alpine habitats are shaped by harsh abiotic conditions and cold climates. Temperature stress can affect phenotypic plasticity, reproduction and epigenetic profiles, which may affect acclimation and adaptation. Distribution patterns suggest that polyploidy seems to be advantageous under cold conditions. Nevertheless, whether temperature stress can induce gene expression changes in different cytotypes, and how the response is initialized through gene set pathways and epigenetic control remain vague for non-model plants.

The perennial alpine plant *Ranunculus kuepferi* was utilized to investigate the effect of cold stress on gene expression profiles. Diploid and autotetraploid individuals were exposed to cold and warm conditions in climate growth chambers and analyzed via transcriptome sequencing and qRT-PCR.

Overall, cold stress changed gene expression profiles of both cytotypes and induced cold acclimation. Diploids changed more gene set pathways than tetraploids, and suppressed pathways involved in ion/cation homeostasis. Tetraploids mostly activated gene set pathways related to cell wall and plasma membrane. An epigenetic background for gene regulation in response to temperature conditions is indicated.

 Results suggest that perennial alpine plants can respond to temperature extremes via altered gene expression. Tetraploids are better acclimated to cold conditions, enabling them to colonize colder climatic areas in the Alps.

4.1 Introduction

Temperature stress is affecting several developmental processes in the life cycle of flowering plants (Hedhly, 2011) and is considered a key constraint to the geographical distribution of species. Aside from distributional ranges, changes in average temperature can affect the phenology, defense capacity, growth and development of plants e.g., (Quint *et al.*, 2016; Gangappa *et al.*, 2017; Lau et *al.*, 2018; Casal & Balasubramanian, 2019; Fiorucci *et al.*, 2020).

Cold temperature stress is defined as chilling (0–20°C) or freezing (< 0°C) and represents a major abiotic stress, threatening growth and development e.g., (Chinnusamy *et al.*, 2007; Liu *et al.*, 2017). Most notably, it induces biochemical, physiological, structural and morphological modifications e.g., (Nagy & Grabherr, 2009; Wani *et al.*, 2013; Wani *et al.*, 2016; Banerjee *et al.*, 2017; Körner, 2021) such as changes in light utilization, ROS production, carbon assimilation, photosynthesis rate, membrane permeability, fluidity and cell wall architecture e.g., (Garsed *et al.*, 1987; Chinnusamy *et al.*, 2007; Sanghera *et al.*, 2011; Theocharis *et al.*, 2012; Longo *et al.*, 2017; Kazemi–Shahandashti & Maali-Amiri, 2018; Megha *et al.*, 2018).

Flowering plants evolved various adaptation strategies in order to survive and reproduce under adverse temperature conditions, such as plastic responses, which are observed to be positively correlated to colonization of novel habitats e.g., (Yeh & Prince, 2004; Sol *et al.*, 2005) and subsequent selection of fitting phenotypes over many generations (Schlichting & Pigliucci, 1998; West-Eberhard, 2003; Lande, 2009; Whitman & Agrawal, 2009; Chevin & Lande, 2010). In the past decades, a growing amount of studies have been focused on phenotypic plasticity, one component of which is thought to be changes in gene expression patterns, and its evolutionary aspects e.g., (Via & Lande, 1985; Schlichting, 1986; Sterns, 1989; Pigliucci, 2001; Franks *et al.*, 2013; Laland *et al.*, 2015; Kelly, 2019). A reliable subset of them focuses on the triggering role of environmental conditions (Angers *et al.*, 2010; Kooke *et al.*, 2015) and the capacity of individuals for phenotypic accommodation (West-Eberhard, 2003; Whitman & Agrawal, 2009) as well as acclimation to the new conditions (Chevin & Hoffmann, 2017).

The molecular response of plants towards environmental conditions is dynamic and extremely complex, as a typical plant cell possesses more than 30,000 genes (Cramer *et al.*, 2011). Alterations on a phenotype can be depicted in morphology, physiology and gene expression, as single changes or a combination of these (Price *et al.*, 2003; West-Eberhard, 2008; Whitman & Agrawal, 2009; Munns, 2011; Laland *et al.*, 2015). The genes involved in these procedures can be induced by cold *per se* or by the relative state of dehydration following cold stress (Griffith & Yaish, 2004). As plants are sessile organisms, the effects of cold stress seem to be of great importance regarding the acclimation to environmental conditions e.g., (West-

Eberhard, 2008; Nicotra *et al.*, 2010; Richards *et al.*, 2017), with timing, combination and intensity of the stress parameters presumably playing an important role e.g., (Suzuki *et al.*, 2014).

Cold acclimation, *i.e.* the acquisition of increased freezing tolerance upon prior exposure to non-lethal low temperatures (Guy, 1990; Thomashow, 1999; Ding *et al.*, 2019), is a sophisticated mechanism plants evolved to endure cold stress. It is moderated via structural and functional remodeling. Profound changes in gene expression profiles affect the composition of the transcriptome, proteome and metabolome e.g., (Chinnusamy *et al.*, 2007; Jammohammadi *et al.*, 2015). Gene expression depicts the way phenotypes are determined under particular environmental conditions (Auld *et al.*, 2010). The combination of environment and genotype influences the expression of a phenotype in a world of continuously changing conditions (West-Eberhard, 2008).

Polyploidy has several effects on vigor, physiology, morphology and other adaptive traits and can result in increased survival fitness in harsher environments (Li *et al.*, 2011; te Beest *et al.*, 2012; Van de Peer *et al.*, 2017; Wei *et al.*, 2019). Polyploids are specifically more abundant in high latitudes and regions with colder climates (Rice *et al.*, 2019). It is hypothesized that under cold temperature conditions polyploid plants are reducing cell numbers and increasing cell size (te Beest *et al.*, 2012), thus adjusting their growth and exposure of reproductive tissues towards the putative adaptive morphology of alpine dwarfism (Körner, 2021). The shift to asexual reproduction modes in some polyploids and epigenetic modifications are suggested to further improve their adaptation to stress conditions (Osborn *et al.*, 2003; Soltis *et al.*, 2014; Körner, 2021).

Epigenetic modifications, such as DNA methylation, histone modifications and chromatin rearrangement can directly or obliquely regulate gene expression e.g., (Bird 2002; Yan *et al.*, 2010; Law & Jacobsen, 2010; Harris *et al.*, 2018; Xiao *et al.*, 2019). In plants, DNA methylation is a documented epigenetic mechanism, which could mediate phenotypic plasticity within a single generation (Bossdorf *et al.*, 2010) and between generations (Boyko *et al.*, 2010). DNA methylation changes can be induced by environmental stimuli, either biotic or abiotic e.g., (Sherman & Talbert, 2002; Hu *et al.*, 2011; Dowen *et al.*, 2012), while DNA methylation profiles could be somewhat fixed *e.g.* for transgenerational inheritance e.g., (Vaughn *et al.*, 2007; Johannes *et al.*, 2009; Finnegan, 2010; Hirsch *et al.*, 2013). Furthermore, DNA methylation changes, *e.g.* gene silencing, can also be triggered by genomic stresses, such as polyploidization and hybridization, which could also result in genome-wide transcriptional rewiring e.g., (Adams & Wendel, 2005; Grant-Downton & Dickinson, 2005; Dong *et al.*, 2006; Baubec *et al.*, 2010; Li *et al.*, 2011; Jones, 2012). Thus, DNA methylation can be beneficial in the procedures following

polyploidization events concerning the re-establishment of genomic balance and structural and functional remodeling (Soltis *et al.,* 2010; Hegarty *et al.,* 2011; Madlung & Wendel, 2013; Alonso *et al.,* 2016).

Phenotypic plasticity, here defined as the ability of a single genotype to differentially respond to environmental stimuli (Brandshaw, 1965; Schlichting, 1986), is thought to be under genetic and epigenetic control e.g., (Richards *et al.,* 2017) and often correlated with transcriptional differentiation (Donelson *et al.,* 2017; Kelly, 2019). An epigenetic background of phenotypic plasticity suggests that DNA methylation provides a plant with a more rapid reaction to variable environmental conditions compared to DNA mutation. However, the resulting phenotype is not necessarily stable (Angers *et al.,* 2010).

Most studies on gene expression under temperature stress were so far conducted on annual model organisms like *Arabidopsis thaliana* or on crop plants e.g., (Miura & Furumoto, 2013; Ding *et al.,* 2019; Zhang *et al.,* 2019). Little is known on the plasticity of perennial plants growing under natural, extreme conditions. *Ranunculus kuepferi* Greuter & Burdet is an alpine perennial herb appropriate for studying acclimation to cold conditions. The species is primarily distributed across the European Alps, in altitudes between 1300 – 2800 m (Burnier *et al.,* 2009; Cosendai & Hörandl, 2010; Cosendai *et al.,* 2011; Kirchheimer *et al.,* 2016; Schinkel *et al.*, 2016) along a pronounced geographical parthenogenesis scenario (Cosendai *et al.,* 2013). Geographical Parthenogenesis in general describes related sexual and asexual organisms having different geographical distributions (Hörandl, 2006). In *R. kuepferi*, diploid plants are predominantly sexual and restricted to the warmer Southwestern Alps, whereas autotetraploid plants are facultative apomicts (aposporous), with varying proportions of sexual and asexual seeds, and colonize previously glaciated areas, *i.e.* the Northern, Central and Eastern Alps as well as the northern Apennines and Corsica (Küpfer, 1974; Burnier *et al.,* 2009; Cosendai & Hörandl, 2010; Schinkel *et al.,* 2016) (Syngelaki *et al.*, 2020a). Tetraploid populations occur at higher elevations in the European Alps than diploids and exhibit a pronounced niche shift towards colder temperatures (Kirchheimer *et al.,* 2016; Schinkel *et al.,* 2016) (Syngelaki *et al.*, 2020a). High alpine habitats are characterized by short growth periods and cold spells, eventually with nocturnal frost during flowering time (spring or summer). It was suggested that the niche differentiation between the cytotypes is associated with a combination of climatic conditions and reproduction mode, with the asexual taxa having a distributional advantage towards cooler conditions (Kirchheimer *et al.,* 2018).

Previous studies revealed that the tetraploid cytotype originated 10 - 80 kyears ago (Kirchheimer *et al.,* 2018), presumably by multiple and recurrent autopolyploidization events (Cosendai *et al.,* 2011; Schinkel *et al.,* 2017). The genetic

differentiation between cytotypes, regardless of reproduction mode, is very low and on a similar level within cytotypes (F_{st}values ~ 0.3 for both cytotypes) (Cosendai *et al.,* 2013). The epigenetic background of the species proposes a differentiation of the cytotypes, but also a correlation of abiotic environmental conditions with the epigenetic variation in natural populations and in experimental conditions (Schinkel *et al.,* 2020; Syngelaki *et al.,* 2020a). Thus, a putative epigenetic background of the niche shift of the tetraploids in the Alps is indicated, with epigenetic variation being associated with elevation in natural populations and higher persistence under cold treatments (Schinkel *et al.,* 2020; Syngelaki *et al.,* 2020a). Syngelaki *et al.* (2020b) highlighted the potential of phenotypic plasticity, with changes of growth parameters linked to DNA methylation patterns, for acclimation to environmental conditions. These experiments confirmed the different niche preferences of cytotypes in natural populations, as diploids grow better under warm conditions, while tetraploids perform better in cold treatments.

Herein, we employed diploid and tetraploid plants already used by Syngelaki *et al.* (2020a, b) and exposed them to different controlled temperature treatments, to assess the gene expression profiles of individuals. We aim to investigate whether cold temperature stress has an effect on gene expression and try to get further insights into the expression dynamics. A temperature-sensitivity of gene expression profiles is speculated, which could act as a rapid response towards stressful environments. We also investigated the differentiation of the gene expression profiles according to the ploidy level of the individuals. We hypothesize that the observed niche shift of the tetraploid cytotype has the physiological background of a better cold acclimation. Finally, we associated the gene expression results with DNA methylation, on a transcriptomic level, as a correlation between them would explain the potential of tetraploid *R. kuepferi* to adapt to cold conditions at higher altitudes during the postglacial recolonization of the European Alps.

4.2 Materials and Methods

Plant material and experimental design

Diploid and tetraploid plants of *R. kuepferi* utilized in the present study were part of a long-term temperature stress experiment, as described in Klatt *et al.* (2018) and Syngelaki *et al.* (2020a, b). Plants were collected throughout the distribution range of the species in the European Alps (Kirchheimer *et al.,* 2016) during the growing seasons of 2013 & 2014. Subsequently, the plants were re-potted in garden soil at the Old Botanical Garden of Göttingen University, where their ploidy level was defined via Flow Cytometry of silica gel dried leaf material collected in the field (Schinkel *et al.,* 2016). For the scope of the current experimental design, which was

conducted from 2014 onwards, the plants were exposed to different temperature conditions during the sprouting and flowering period, while the rest of the parameters were kept equal. The settings for the transcriptome study were as in Syngelaki *et al.* (2020a, b) (Table 1). The temperature conditions were simulating the natural environment of the species in the Alps, including freezing during some nights.

Table 1. Temperature treatment conditions during plant growth and sampling collection. Light regime was measured with a Quantum light meter (Spectrum Technologies Inc., Aurora, IL, USA) during the full light period (100% intensity) at the level of early leaf tips and first buds. Plants were rotated weekly in the cabinet to avoid effects of light and temperature gradients.

No.Plants	Cold Treatment 164		Warm Treatment 189	
	Diploid 74	Tetraploid 90	Diploid 92	Tetraploid 97
Light regime (µmol m⁻² s⁻¹, SAR)	~ 700			
Photoperiod (h)	16; 10 of full light and 3 + 3 of twilight			
Temperature during the light/dark period(°C)				
Daytime	7		15	
	2			
Night	−1(cold shocks for three nights per week)		10	

Altogether 262 individuals were categorized into four groups corresponding to their ploidy level and treatment: cold diploids (CD, 63 plants), warm diploids (WD, 79 plants), cold tetraploids (CT, 71 plants), and warm tetraploids (WT, 49 plants). At the peak of the flowering season in beginning of summer 2019, leaf tissue was collected simultaneously from three individuals per group, each originating from different natural populations, immediately frozen in liquid nitrogen and stored in −80°C. Sample collection localities can be found in Supplementary Table S1.

RNA extraction and sequencing

Frozen leaf tissue was pulverized in liquid nitrogen and a maximum of 100 mg of powder was used for RNA isolation with the RNAeasy Plant® Mini Kit (QIAGEN, Hilden, Germany) following the provided protocol. RNA quantity and quality were determined with Nanodrop, a Qubit™ with the RNA HS Assay Kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Library preparation and sequencing with HiSeq 4000 (Illumina, San Diego, CA, USA) was conducted at the Transcriptome and Genome Analysis Laboratory of the Microarray & Deep-Sequencing Core Facility (UMG, Georg-August-Universität, Göttingen, Germany) producing 50bp single end reads.

Bioinformatics

The quality of reads was assessed using FastQC v.0.11.4 (Andrews, 2010). Raw reads were trimmed with CutAdapt v.2.3 (Martin, 2011), removing adapters and bases with a phred score below 30 and removing reads shorter than 30bp after trimming. As there is no available genomic reference for *R.kuepferi*, the transcriptomes from all diploid individuals were pooled for a *de novo* assembly with Trinity v.2.8.6 and default parameters, except for max. memory was set to 50Gb (Grabherr *et al.* 2011, 2013) to produce a pseudoreference. The quality of the resulting assembly was verified with BUSCO v.3.0.2 (Manni *et al.*, 2021; Supplementary Figure S1). TransDecoder v.5.5.0 (Haas et *al.*, 2013) was used to identify the longest Open Reading Frame per assembled contig. Coding sequences were annotated using the blastp algorithm under NCBI-BLAST v.2.10.0 (Johnson *et al.*, 2008) and the December 2020 release of Uniprot as reference. Annotation reports were produced using Trinotate v.3.2.1 (Bryant *et al.*, 2017). Trimmed reads of each sample were mapped against the annotated pseudoreference individually using Bowtie2 v.2.3.5.1 with default parameters (Longread & Salzberg, 2012).

Raw counts of mapped reads were calculated employing the Rsubread/Subread package v.2.4.0 (Liao *et al.,* 2019) in R/Bioconductor (v.4.0.3 and v.4.1.0/v.3.12 and v.3.13, respectively) (R Core Team, 2021) in R Studio (R Studio Team, 2016). Resulting matrices were further analyzed with DESeq2 v.1.30.0 (Love *et al.*, 2014) with a false discovery rate (FDR) threshold of < 0.05 and the Benjamini-Hochberg

p-value normalization adjustment (Jafari & Ansari-Pour, 2019 Loci were identified as differentially expressed regarding the comparison of interest (cytotypes, treatments), with the group of Warm Diploids (WD) used as reference, and were visualized in a heatmap with ggplot2 v.3.3.5 (Wickham, 2009).

ClusterProfiler v.4.0.2 (Yu *et al.*, 2012) was used for Gene Set Enrichment Analysis. This package currently only accepts single organism references via AnnotationDbi v.1.55.1 (Pages *et al.*, 2021). Of the currently accepted reference genomes, *A. thaliana* is most closely related to *R. kuepferi* (Bell *et al.*, 2010). Consequently, the pseudoreference was annotated again as described above using the updated TAIR 10 release [The Arabidopsis Information Resource (TAIR), http://www.arabidopsis.org; accessed on 11 March 2021] and the resulting annotations were employed in ClusterProfiler for a separate run for each cytotype, checking all subontologies and with a number of 1 Mio. permutations. For these analyses the warm treatment was set as the control condition. Dot plots were generated with enrichplot v.1.13.1 (Yu, 2021).

qRT-PCR

Quantitative real time RT-PCR was conducted to validate the differential gene expression revealed by bioinformatic analyses. The annotation reports of the pseudoreference were screened for possible Genes of Interest (GOIs), which are stated to be related, directly or indirectly, to DNA methylation and gene regulation (Finnegan & Kovac, 2000; Miura & Furumoto, 2013; Matzke & Mosher, 2014; Banerjee *et al.*, 2017; Bewick *et al.*, 2017; Lanciano & Mirouze, 2017; Bräutigam & Cronk, 2018; Harris *et al.*, 2018; Xiao *et al.*, 2019; Chang *et al.*, 2020; Ritonga & Chen, 2020) as well as housekeeping genes (Joseph *et al.*, 2018; Yu *et al.*, 2019). Primers were designed for two methyltransferases (CAMT3, PMT2), two demethylases (JM706, JMJ25), the AGO4B gene, which is participating in the RNA-directed DNA methylation (RdDM) pathway in *Arabidopsis* and rice (Lanciano & Mirouze, 2017) and the housekeeping gene *Actin* (Supplementary Table S2). Primers specificity was tested with a touch-down PCR; products were sequenced and compared to the respective gene in the pseudoreference.

Complementary DNA synthesis and qRT-PCR was performed using QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) and the Rotor-Gene SYBR Green PCR kit (QIAGEN, Hilden, Germany) in QIAGEN Rotor-Gene Q cycler, equipped with Q-Rex Software and following the instructions of the manufacturer for two cycling steps and a 45 cycles PCR program for three technical replicates per sample. In order to evaluate the differential gene expression, the amplification performance as the log of fold change was calculated with the ΔΔCt method (Livak & Schmittgen, 2001) in Excel 2016, using the housekeeping gene *Actin* as endogenous control for normalization and warm diploid individuals were considered the reference.

4.3 Results

Pseudoreference and mapping

In the current study, gene expression profiles of six diploid and six tetraploid individuals of *R. kuepferi,* under cold (stress) and warm (control) temperature treatments were explored. Sequencing of the samples resulted in a mean of 31,918,319 raw reads per sample with a mean of 31,707,039 reads retained after trimming. The assembly of the pseudoreference resulted in 71,444 transcripts, with 15,224 of them functionally annotated. Through Bowtie2 mapping, we obtained an average mapping rate of 93.08% per sample (Table 2).

Table 2. Summary of sequencing and mapping data for the *R. kuepferi* transcriptomes, against the pseudoreference resulting from Trinity *de novo* assembly of all reads from diploid samples.

Group†	Sample ID	Untrimmed Reads	Total Trimmed Reads	Mapped Reads	Mapped Reads (%)
CD	30_1_2	1,335,657	1,326,485	1,214,557	91.56
CD	115_4_2	39,260,122	39,044,345	37,240,848	95.38
CD	116_3_1	46,805,434	46,545,107	43,938,918	94.4
CT	41_2_2	37,813,775	37,625,107	34,877,792	92.7
CT	90_2_3	35,989,875	35,824,317	33,353,051	93.1
CT	108_3_3	36,888,834	36,687,214	33,980,212	92.62
WD	24_4_3	29,197,541	29,058,835	27,467,724	94.52
WD	29_1_2	34,122,764	33,708,095	31,629,139	93.83
WD	33_3_3	29,530,425	29,378,666	27,521,885	93.68
WT	42_1_2	29,277,225	29,108,345	26,919,488	92.48
WT	74_1_2	31,858,591	31,626,671	28,839,855	91.19
WT	106_4_1	30,939,580	30,551,281	27,988,255	91.61

†WD: warm diploid, CD: cold diploid, WT: warm tetraploid, CT: cold tetraploid

Differential gene expression

A total of 2617 significantly differentially expressed genes were identified between the four predefined groups. Among all groups, more genes were found to be down-regulated compared to up-regulated (Table 3).

Table 3. Differentially expressed genes of *R. kuepferi* transcriptomes regarding the comparison of interest (cytotypes, treatments).

	Gene Count	Percentage (%)
Expressed genes	19,033	
Differentially expressed genes	2,617	13.75
Up-regulated	1,055	5.5
Down-regulated	1,562	8.2
Outliers	469	2.5

Gene expression was correlated with temperature, while the ploidy level of the plants under the same environmental conditions did not affect the gene expression strongly (Figure 1).
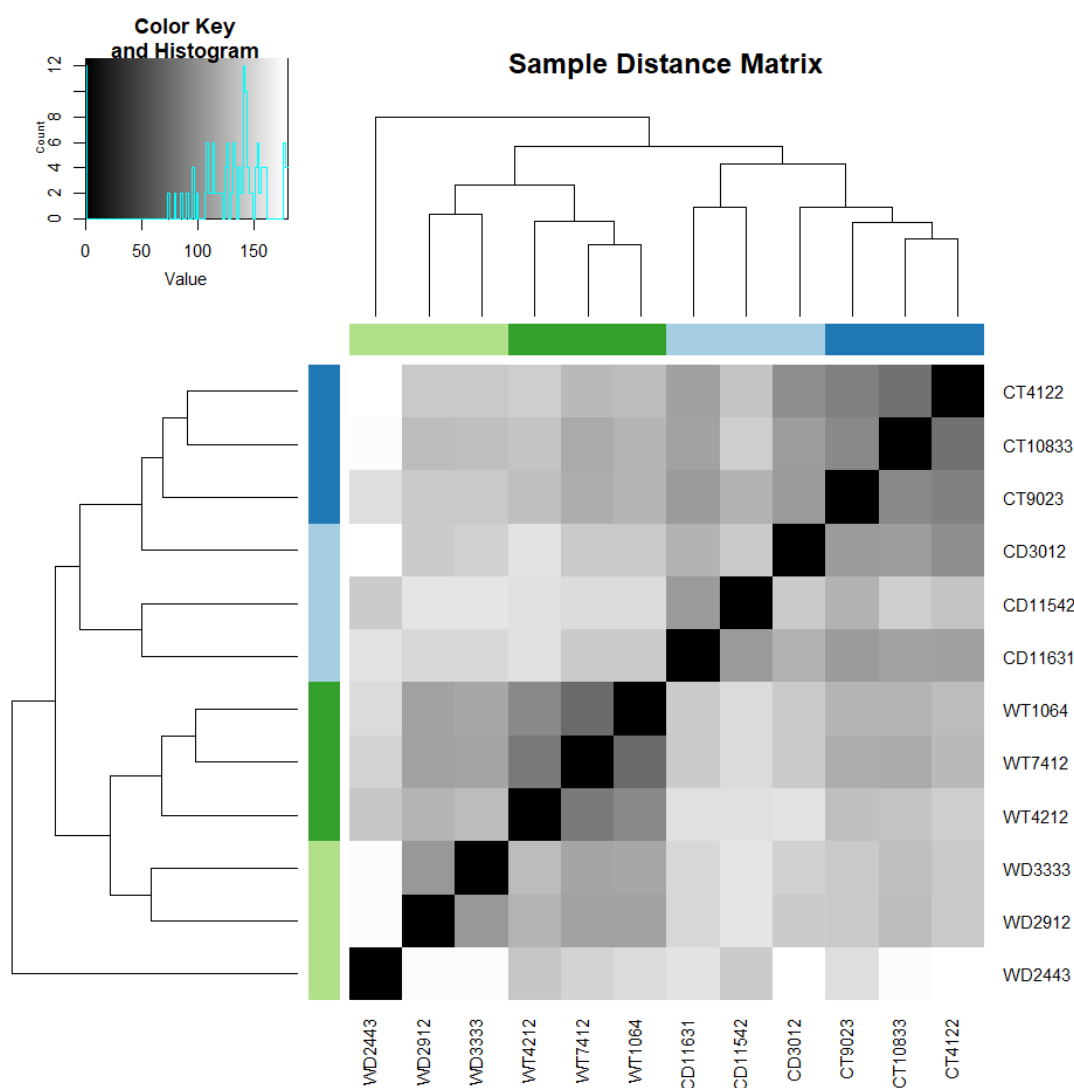
Figure 1. Heat map showing the expression of transcriptomes of diploid and tetraploid *R. kuepferi* plants under warm (control) and cold (stress) conditions. Regarding the sample IDs the capital letters stand for the predefined groups (WD: warm diploid, CD: cold diploid, WT: warm tetraploid, CT: cold tetraploid) and the numbers stand for the populations in the wild (Supplementary Table S1).

One WD individual (WD2443) seems to present gene expression patterns different from all the other samples. The same individual seems to be an outlier also in the qRT-PCR analysis.

Gene Set Enrichment Analyses

Gene set enrichment was successfully assigned and statistically significant for 59 pathways in the diploids and 20 pathways in the tetraploids. Enriched gene sets with higher GeneRatios, which in ClusterProfiler are defined as 'count/setSize,' where 'count' is the number of genes that belong to a given gene set, while 'setSize' is the total number of genes in the gene set, are presented in Figures 2 and 3.

Figure 2. Significantly enriched gene set pathways resulting from the analysis of differentially expressed genes in diploid individuals of *R. kuepferi*.

Regarding Figure 2, an extract of all resulted enriched pathways is shown due to graphical purposes and a dotplot of all pathways is provided in Supplementary Data (Supplementary Figure S2). Overall, 25 and 13 pathways have been activated in diploid and tetraploid individuals, respectively. Pathways which are linked to the plasma membrane e.g., 'anchored component of plasma membrane', '(cation) transmembrane transporter activity' and the cell wall e.g., 'cell wall organization or biogenesis', 'plant-type cell wall', as well as the 'cold acclimation' and 'hydrolase activity' pathways, are activated in both cytotypes (Figures 2 and 3).
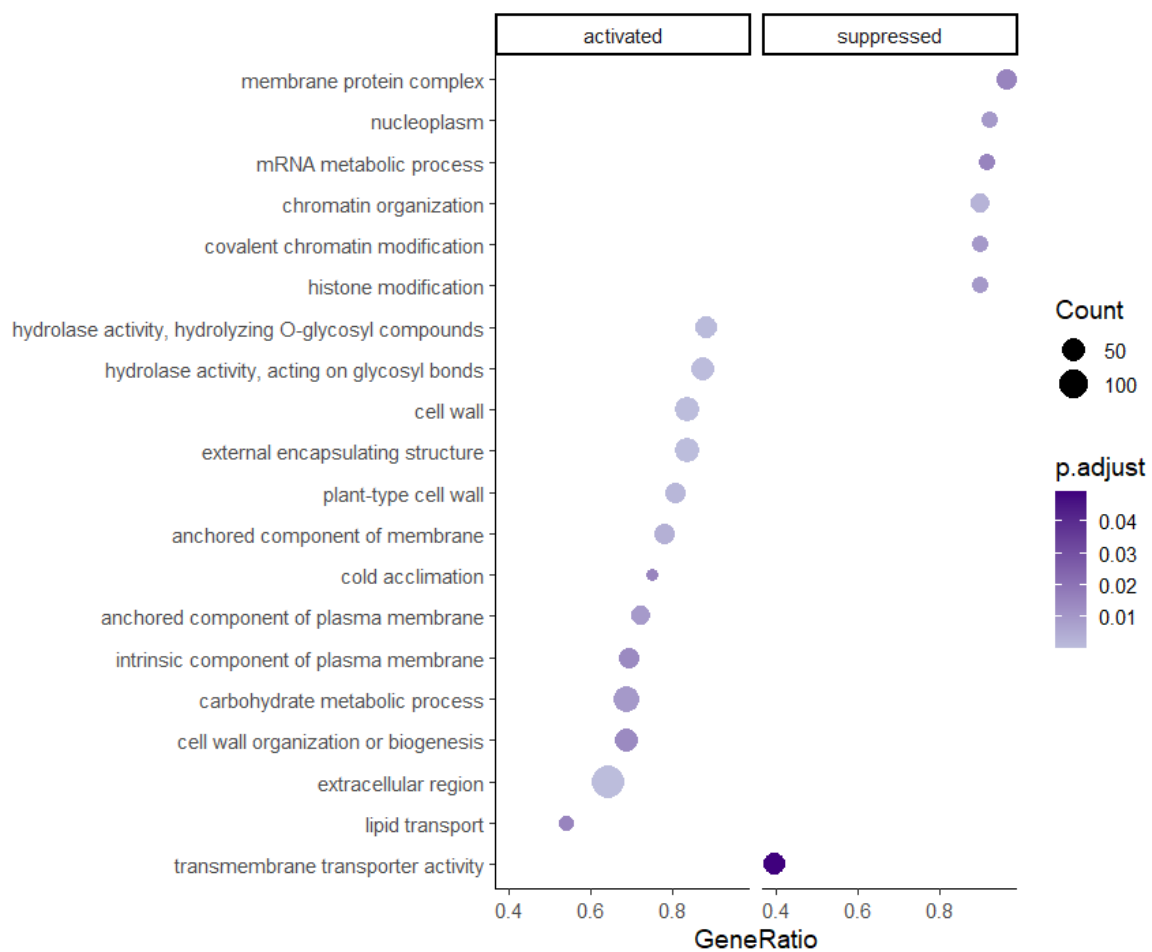
Figure 3. Significantly enriched gene set pathways resulting from the analysis of differentially expressed genes in tetraploid individuals of *R. kuepferi*.

However, pathways related to ion/cation homeostasis and enzymic activity, such as '(cellular) metal ion homeostasis' and 'protein serine/threonine kinase activity' are enriched only in diploids, while pathways such 'histone/chromatin modification' and 'lipid transport' are enriched only in tetraploid individuals.

Genes of Interest and qRT-PCR

A total of 38 genes of interest, identified by their involvement in DNA methylation, were significantly differentially expressed among the four predefined groups (Table 4).

Table 4. List of differentially expressed genes related to DNA methylation in *R. kuepferi* leaves. Annotation is according to UniProt. The genes which are highlighted are employed as genes of interest (GOIs) for the real-time qRT-PCR.

GeneID	Organism‡	Function	Regulation
AGL15	BRANA	Agamous-like MADS-box protein	Down
AGL62	ARATH	Agamous-like MADS-box protein	Down
AGO1A	ORYSJ	Protein argonaute 1A	Down
AGO4B	ORYSJ	Protein argonaute 4B	Down
ALKB2	ARATH	DNA oxidative demethylase	Down
AP1	VITVI	Agamous-like MADS-box protein	Down
ATX4	ARATH	Histone-lysine N-methyltransferase	Up
CAMT3	PETHY	Caffeoyl-CoA O-methyltransferase 3	Down
CMTA3	ARATH	Calmodulin-binding transcription activator	Down
COMT1	POPKI	Caffeic acid 3-O-methyltransferase	Down
DRM1L	ARATH	DNA (cytosine-5)-methyltransferase	Down
EPFL2	ARATH	EPIDERMAL PATTERNING FACTOR-like protein 2	Down
EPFL6	ARATH	EPIDERMAL PATTERNING FACTOR-like protein 6	Down
EPFL9	ARATH	EPIDERMAL PATTERNING FACTOR-like protein 9	Down
FDM1	ARATH	Factor of DNA methylation 1	Up
JM706	ORYSJ	Lysine-specific demethylase	Down
JMJ25	ARATH	Lysine-specific demethylase	Down
LAMT	CATRO	Loganic acid O-methyltransferase	Down
MADS1	VITVI	Agamous-like MADS-box protein	Up
MBD2	ARATH	Methyl-CpG-binding domain-containing protein 2	Up
MBD6	ARATH	Methyl-CpG-binding domain-containing protein 6	Down
METE	CATRO	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	Down
METE2	ORYSJ	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase 2	Down
PEAM1	ARATH	Phosphoethanolamine N-methyltransferase 1	Down
PMT1	ARATH	Probable methyltransferase PMT1	Down
PMT2	ARATH	Probable methyltransferase	Down
PMT4	ARATH	Probable methyltransferase	Down
PMT7	ARATH	Probable methyltransferase	Down
PMT8	ARATH	Probable methyltransferase	Down
PMTB	ARATH	Probable methyltransferase	Down
PMTD	ARATH	Probable pectin methyltransferase	Down
PMTI	ARATH	Probable methyltransferase	Down
PMTQ	ARATH	Probable methyltransferase	Down
PMTT	ARATH	Probable pectin methyltransferase	Down
RP6L1	ARATH	Protein RRP6-like 1	Up
RP6L2	ARATH	Protein RRP6-like 2	Up
RRP8	ARATH	Ribosomal RNA-processing protein 8	Up
SUVR1	ARATH	Probable inactive histone-lysine N-methyltransferase	Down

‡Abbreviations: ARATH = Arabidopsis thaliana, BRANA = Brassica napus, CATRO = Catharanthus roseus, PETHY = Petunia hybrida, POPKI = Populus kitakamiensis, ORYSJ = Oryza sativa ssp. japonica, VITVI = Vitis vinifera.

The correlation of differential gene expression analysis with epigenetics was validated via qRT-PCR. For almost all individuals the expression of all five of the selected genes was down-regulated (Supplementary Table S3), corresponding to the differential expression analysis results of DESeq2. Only for one individual, an up-regulation of all the genes was detected. This individual is the same that separates from the rest of the samples under cold treatment (Figure 1).

4.4 Discussion

Plant acclimation to cold stress induces various cellular processes through a cascade of change in gene expression and protein synthesis e.g., (Smallwood & Bowles, 2002; Zhu *et al.*, 2007). It is estimated that between 4% and 12% of the transcriptome of *A. thaliana* changes after a respective period of several hours, days or weeks of exposure to chilling temperatures (Hannah *et al.*, 2005; Knight & Knight, 2012). This differentiation in gene expression combined with the observation of different gene clusters being up-regulated during different times of the stress exposure indicates a hierarchy in the functional response, with signaling of harmful conditions or increasing freezing tolerance comes first (Fowler & Thomashow, 2002; Hannah *et al.*, 2005; Robinson & Parkin, 2008), while circadian clocks being hypothesized to play an important role in general regulation (Nohales & Kay, 2016; Ritonga & Chen, 2020). Polyploidy may affect cold acclimation as polyploids are thought to perform better under cold conditions e.g., (Rice *et al.*, 2019). We analyzed here for the first time gene expression of a perennial alpine plant under different temperature conditions, and evaluated effects of different ploidy levels on the response to cold stress.

Ploidy effects on gene expression and the distribution pattern

The ploidy level of the individuals *per se* does not appear to be a significant contributor to the observed differential gene expression (Figure 1). This differs from previous studies on DNA methylation patterns, mode of reproduction, and morphological traits of *R. kuepferi* which revealed significant ploidy effects (Klatt *et al.*, 2018; Syngelaki *et al.*, 2002a, b). The congeneric species of the *Ranunculus auricomus* complex also showed strong ploidy effects in gene expression profiles of ovules (Pellino *et al.*, 2020). In our study, the main effects in gene expression changes are due to treatments, not to ploidy. In autopolyploids, transcription profiles can be influenced by a multitude of factors, which are caused by genome duplication e.g., dosage effects due to the presence of additional copies of genes (Madlung & Wendel, 2013). However, autotetraploid rice does seemingly not show a genome-wide dosage effect on gene expression; likely because subfunctionalization maintains the functional pleonasm of duplicated genes and avoids any energy waste (Wagner, 2005; Drummond & Wilke, 2008; Qian *et al.*, 2010; Zhang *et al.*, 2015). Regarding the WD individual, which is an outlier for both

the gene expression profiles and qRT-PCR analysis, there was no indication of lower RNA quality during wet and dry lab manipulation of the specimen. Probably, the conditional plasticity and the gene-environment interaction (West-Eberhard, 2008; Bateson, 2015) of the individual, as well as the micro site of origin of the natural population, which belongs to the sympatric zone of the two cytotypes (Schinkel *et al.*, 2016), could play the major role for its observed phenotype.To investigate further how the two cytotypes cope with stress conditions, gene set enrichment for each ploidy level was assessed. Several key regulatory pathways and their interactions have been documented previously e.g., (Takahashi *et al.*, 2004; Cramer *et al.*, 2011; Puijalon *et al.*, 2011; Jutsz & Gnida, 2015; Ritonga & Chen, 2020).

In *R. kuepferi*, diploids respond more intensively to temperature treatments compared to the tetraploids, as a higher number of gene sets is significantly differentially expressed (Figures 2 and 3). Hence, diploids appear to be more stressed by cold conditions than tetraploids. The cold stress treatments of our experiments are quite similar to the natural habitat conditions of the tetraploid *R. kuepferi* plants in the Alps (Syngelaki *et al.*, 2020a). As the cold adapted genotypes are thought to have a distinct advantage over non-adapted ones in frost-prone environments, such as high mountain areas (Nagy & Grabherr, 2009), we hypothesize that the postglacial colonization of the Alps by autotetraploid populations of *R. kuepferi* (Schinkel *et al.*, 2016) did rely to a large extent on the phenotypic variation towards the climatic conditions, pronounced also as differential gene expression (Vrijenhoek & Parker, 2009). Thus, the geographical parthenogenesis scenario, which interrelates the cytotypes with different ecological backgrounds (Schinkel *et al.*, 2016; Kirchheimer *et al.*, 2018), is further supported. Results support the simulation study of postglacial recolonization in the Alps, which identified the acclimation/adaptation of tetraploids to a colder climatic niche in higher and more northern parts of the Alps as one decisive character of the geographical parthenogenesis pattern (Kirchheimer *et al.*, 2018). Our results here suggest that this niche shift has a direct physiological background of cold tolerance and is less likely due other characteristics of alpine habitats (like a lower pathogen pressure, among others).

Functional aspects of gene expression related to cold acclimation

Although some of the differentially expressed gene sets overlap between ploidy levels, there are some characteristics for either group. In diploids, most pathways are linked to ion/cation homeostasis and activity and are suppressed. Additional pathways are connected to the plasma membrane, cell wall, and hydrolase activity. Similar pathways are present in tetraploid individuals, with a greater focus on membranes and cell wall. In contrast to diploids, the tetraploids suppressed only six pathways, mostly related to chromatin and histone modification. In both cytotypes the cold acclimation pathway is activated.

As access point of the cell, membranes are injured by adverse environmental conditions, yet their stability contributes to cell behavior and activity maintenance (Theocharis *et al.*, 2012; Chen *et al.*, 2018; Ritonga & Chen, 2020). Pathways related to cell lipid composition, such as the ones activated in the tetraploids, play an important role in the maintenance of plasma membrane functionality e.g., (de Palma *et al.*, 2008; Janska *et al.*, 2010) and are affecting the downstream expression of genes linked to resilience to lower temperature (Dutilleul *et al.*, 2012). Maintenance of plasma membrane functionality is especially important for freezing tolerance in alpine plants, as a fluid membrane allows transfer of water from the protoplast into the intercellular space, where extracellular ice nucleation takes place, leaving the protoplast unfrozen (Körner, 2021). We suppose that our short freezing treatments (−1°C during three nights per week) induced these expression changes. However, we observed no apparent damage of leaves in cold treatments (Syngelaki *et al.*, 2020b), and hence both cytotypes are tolerant to short-term freezing and thawing during the day.

Decreasing membrane fluidity, coupled with its interaction with the cell wall, is considered to be one of the first cold sensors (Murata & Los, 1997; Martiniere *et al.*, 2011; Ma & Liu, 2019). The connection of membrane rigidification to cytoskeletal rearrangements, calcium influxes and kinases, acts as trigger for the subsequent low-temperature response e.g., (Orvar *et al.*, 2000; Sangwan *et al.*, 2002).

Changes in the composition of the cell wall can strongly affect plant stress resistance (Zhu, 2016) as stress can up-regulate the expression of e.g., expansins and xyloglycan-modifying enzymes, which are acting on cell wall remodeling (Tenhaken, 2014). The hydrolase activity pathways activated in both ploidy levels in *R. kuepferi* under cold stress may indicate their unique roles in cell wall modification (Sulova *et al.*, 2003; Eklof & Brumer, 2010). The cytoskeleton is also affected by cold stress (Pokorna *et al.*, 2004; Ma & Liu, 2019) and its interactions with membranes and the cell wall play a distinctive role in cold stress tolerance (Abdrakhamanova *et al.*, 2003; Kasemi-Shahandashti & Maali-Amiri, 2018; Ding *et al.*, 2019). The 'cell wall organization or biogenesis' pathway, which is activated in both diploid and tetraploid *R. kuepferi* individuals (Figures 2 and 3) is relevant to the cytoskeleton and its modifications under cold stress.

The pathways of ion/cation homeostasis and activity, as well as the serine/threonine protein kinase activity are thought to be temporal and spatial events downstream of Ca^{2+} signaling e.g., (Monroy *et al.*, 1993; Lewis *et al.*, 1997). A change in intracellular calcium ion concentration is one of the earliest signaling events triggering the response of plants to cold stress (Sanders *et al.*, 2002; Knight & Knight, 2012; Shi *et al.*, 2015) with Ca^{2+} dynamics being detected within 40s after cold stress exposure (Zhu *et al.*, 2013). Presumably Ca^{2+} channels are primary sensors for cooling rate and Ca^{2+} efflux transporters are absolute temperature sensors (Plieth, 1999; White, 2009), while oscillations of Ca^{2+} are linked to stomatal closure in *Arabidopsis thaliana*, as a response to cold stress (Allen *et al.*, 2000).

Furthermore, the serine/threonine protein kinases are plasma membrane receptor-like kinases (RLKs), several of which are calcium-moderated and promote the expression of cold-responsive genes (COR) through the activation of a mitogen-activated protein kinase (MAPK) cascade (Cheng *et al.*, 2013; Zhu *et al.*, 2016; Ye *et al.*, 2017; Liu *et al.*, 2018).

The intriguing suppression of the latter pathways, especially in diploid *R. kuepferi*, corroborates the hypotheses of tangled information encoded through Ca^{2+} kinetics, which are constantly changing as a complex mechanism of stress response and are also influenced by 'cold memory', i.e., former exposure to cold stress conditions (Knight *et al.*, 1996; Knight & Knight, 2012). We hypothesize that suppressed pathways of ion/cation homeostasis in diploids are probably linked to stomatal closure and consequently reduction of CO_2 uptake/carbon gain (Taiz & Zeiger, 2010). This would explain the lower growth performance of diploids under cold conditions, as observed in the experiments of Syngelaki *et al.* (2020b).

Gene expression related to DNA methylation

Deciphering the epigenetic background of plants, which are exposed to abiotic stress, is a constantly developing field e.g., (Richards *et al.*, 2017). DNA methylation is correlated with histone proteins and their post-transcriptional modifications, as the conversion of these modifications to DNA methylation profiles is often thought to be more stable e.g., (Zhu, 2008; Jones, 2012). These interactions are associated with gene expression profiles and gene transcription in general in response to cold stress (Chinnusamy & Zhu, 2009; Kim *et al.*, 2015) and could encompass changes in chromatin structure and accessibility (Strahl & Allis, 2000; Lang-Mladek *et al.*, 2010). Chromatin remodeling has a putative function as a plant thermometer, representing a relatively direct connection between cold and gene expression (Kumar & Wigge, 2010; Kumar *et al.*, 2012). In the present study, the pathways of histone and chromatin modifications are suppressed in the tetraploid individuals (Figure 3). Additionally, several genes, directly or indirectly related to DNA methylation, are significantly differentially expressed in both ploidy levels (Table 4). This result corroborates previous results of methylation-sensitive AFLP screenings that the cytotypes exhibit different methylation profiles (Schinkel *et al.*, 2020; Syngelaki *et al.*, 2020a). The differential expression of genes correlated with DNA methylation is further validated by the qRT-PCR results, where the two methyltransferases (CAMT3, PMT2), two demethylases (JM706, JMJ25) and the argonaute protein AGO4B were all found to be down-regulated, as expected from the bioinformatical analyses of the transcriptomes. This fits the overall pattern of loss of methylated MS-AFLP fragments after dramatic temperature changes (Syngelaki *et al.*, 2020a). Methylation patterns may conserve the transgenerational epigenetic memory of response to cold treatments and hence differential

acclimation and adaptation of cytotypes (Schinkel *et al.*, 2020, Syngelaki *et al.* 2020a).

To summarize, the present study demonstrates the responses of diploid and tetraploid *R. kuepferi* plants towards cold stress in their gene expression patterns. Although both ploidy levels activate genes related to cold acclimation, the gene set pathways differ between cytotypes, suggesting a better cold acclimation of tetraploids than diploids. Consequently, our results strongly support the hypothesis of a physiological background of the observed ecological and geographical differentiation patterns between cytotypes. Altogether, cold stress induces differentially expressed gene profiles and several gene set pathways are involved in the response, either being activated or suppressed. Seemingly, these parallel mechanisms invoke energy conservation and help individuals to survive in novel and/or extreme environments. Lastly, DNA methylation is indicated to contribute to the regulation of gene expression and may preserve a different epigenetic memory for the two cytotypes.

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4.6 Supporting Information

Table S1. List of individuals used for the laboratory work. Listed are the sample and group IDs, ploidy level, treatment as well as country, province and altitude of origin (Schinkel *et al.*, 2016; Klatt *et al.*, 2018).

Sample ID	Ploidy	Temperature Treatment	Group	Country	Region	Altitude (m)
30_1_2	diploid	cold	CD	Italy	Piemonte	1743
115_4_2	diploid	cold	CD	France	Provence-Alpes-Côte d'Azur	1891
116_3_1	diploid	cold	CD	France	Provence-Alpes-Côte d'Azur	1953
41_2_2	tetraploid	cold	CT	Italy	Val d'Aosta	2174
90_2_3	tetraploid	cold	CT	Switzerland	Valais	2477
108_3_3	tetraploid	cold	CT	Switzerland	Graubunden	2171
24_4_3	diploid	warm	WD	France	Provence-Alpes-Côte d'Azur	1925
29_1_2	diploid	warm	WD	Italy	Piemonte	2020
33_3_3	diploid	warm	WD	Italy	Piemonte	2328
42_1_2	tetraploid	warm	WT	Switzerland	Valais	1789
74_1_2	tetraploid	warm	WT	Austria	Osttirol	2117
106_4_1	tetraploid	warm	WT	Italy	Trentino Alto Adige/ Südtirol	2142

† WD: warm diploid, CD: cold diploid, WT: warm tetraploid, CT: cold tetraploid

Table S2. List of primers used for qRT-PCR validation.

Gene ID	Sequence	
	Primer 5´- 3´	Primer 3´-5´
ACT	TCTCCTTGCTCATTCTGTCG	TTCCCTTTACGCCAGTGGTC
AGO4B	GTTGCTCTGAAACTAGAATG	CCACCACTTCCCTTCAAC
CAMT3	CTTTCACGATTCAACACTCC	GAACTCAGAGAAATAACAGC
JM706	AGTATGTTTGCATGGCATGTG	TCGGAGTTTGATGGGTAGTG
JMJ25	TTCATCACTGCGCTTCCATTCC	CTTGATGTGGGCACCCTGC
PMT2	GCAGTGATTGGTGTTCTCGG	GTCAGACTCATCCCTACCAGG

Table S3. Results of qRT-PCR and Delta-delta analyses. The ΔΔCt is calculated as following: ΔCt of the average Cp for the three runs of each inidividual minus the average ΔCt of all 3 samples of the control group, i.e. the WD group (same setting was used in DESeq2 analysis).

		Houskeeping gene	Genes of interest(GOIs)														
		ACTb	AGO4B			CAMT3			JM706			JMJ25			PMT2		
Sample name	Biological groups	Average Cp	Average Cp	$2^{-(\Delta\Delta Ct)}$	$\log[2^{-(\Delta\Delta Ct)}]$	Average Cp	$2^{-(\Delta\Delta Ct)}$	$\log[2^{-(\Delta\Delta Ct)}]$	Average Cp	$2^{-(\Delta\Delta Ct)}$	$\log[2^{-(\Delta\Delta Ct)}]$	Average Cp	$2^{-(\Delta\Delta Ct)}$	$\log[2^{-(\Delta\Delta Ct)}]$	Average Cp	$2^{-(\Delta\Delta Ct)}$	$\log[2^{-(\Delta\Delta Ct)}]$
30_1_2	CD	26.8	21.52	0.63	-0.2	35.47	0	-7.73	31.83	0.13	-0.9	29.1	0.23	-0.63	19.39	1.71	0.23
115_4_2	CD	29.1	19.96	9.13	0.96	38.67	0	-8	32.83	0.31	-0.51	29.52	0.85	-0.07	21.25	2.32	0.37
116_3_1	CD	26.76	21.72	0.53	-0.28	34.88	0	-7.56	32.2	0.09	-1.02	31.77	0.04	-1.45	20.65	0.69	-0.16
41_2_2	CT	26.75	22.54	0.3	-0.52	33.63	0	-7.19	35.29	0.01	-1.95	31.13	0.05	-1.26	20.29	0.88	-0.06
90_2_3	CT	27.43	22.77	0.41	-0.39	35.04	0	-7.41	31.73	0.21	-0.68	30.3	0.16	-0.81	20.78	1.01	0
108_3_3	CT	25.63	22.93	0.11	-0.98	34.94	0	-7.92	32.33	0.04	-1.4	30.89	0.03	-1.52	20.3	0.41	-0.39
24_4_3	WD	34.18	23.17	33.16	1.52	0	148764	5.17	33.6	6.18	0.79	31.31	8.37	0.92	26.01	2.9	0.46
29_1_2	WD	30.96	26.57	0.34	-0.47	0	15920	4.2	32.98	1.02	0.01	31.31	0.89	-0.05	24.92	0.66	-0.18
33_3_3	WD	29.47	27	0.09	-1.05	43.61	0	-9.37	34.18	0.16	-0.8	32.56	0.13	-0.87	23.76	0.52	-0.28
42_1_2	WT	34.14	27.65	1.44	0.16	33.26	0	-4.85	32.99	9.21	0.96	32.39	3.85	0.59	28.71	0.43	-0.36
74_1_2	WT	29.89	25.36	0.37	-0.43	37.66	0	-7.46	34.21	0.21	-0.68	32.54	0.18	-0.74	23.3	0.97	-0.01
106_4_1	WT	29.03	23.41	0.79	-0.1	37.96	0	-7.8	30.94	1.1	0.04	31.11	0.27	-0.57	22.53	0.91	-0.04
NTC	--	0	29.51	0	-10.68	0	0	-5.12	30.76	0	-8.64	31.14	0	-9.32	39.08	0	-13.76
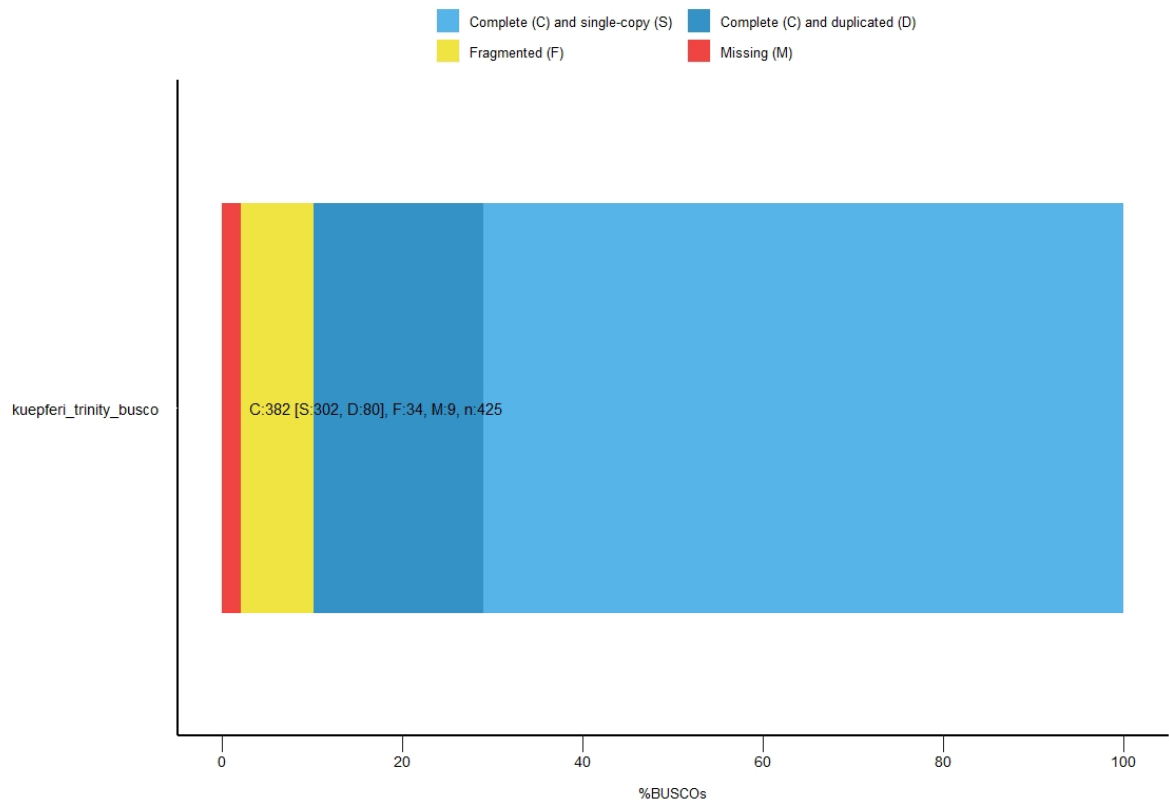
Figure S1. BUSCO (Benchmarking Universal Single-Copy Orthologs) plot of the *R. kuepferi* transcriptome pseudoreference.

Figure S2. All Gene set pathways significantly enriched from the analysis of differentially expressed genes in diploid individuals of *R. kuepferi*.

Raw Data Availability

The raw sequences files are stored and available for download in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA756988.

5. Discussion

5.1 Effects of polyploidy

In the current session I will discuss how the two cytotypes of *R. kuepferi* react under temperature treatment, and try to entangle any advantageous effects of polyploidy in phenotypic plasticity and acclimation. Such effects will be examined regarding reproductive fitness, mode of reproduction, morphological traits, epigentic and gene expression patterns, as well as gene set pathways. Previous bibliography documented indications of polyploidy could contribute in long-term evolutionary flexibility (*e.g.* Comai, 2005; Paterson, 2005; van de Peer and Meyer, 2005).

Reproductive fitness of the species, as defined from the seed set measurements, does differentiate among the cytotypes under each temperature treatment, and follows a temperature pattern, *i.e.* diploids presented significantly lower abortion rates in comparison to tetraploids under both temperature treatments, while tetraploids are doing much better under cold conditions.

Furthermore, it is documented that several alpine species *e.g. Saxifraga bryoides* and *Ranunculus hirtellus* are affected by cold and frost conditions, with a decrease in seed sets and injuries of the reproductive tissues (Kumar and Singhal, 2011; Suzuki *et al.*, 2014). These differences in seed set among the cytotypes confirm results of previous studies on the species in natural populations (Cosendai and Hörandl, 2010; Schinkel *et al.*, 2017) and under experimental conditions (Klatt *et al.*, 2018).

The latter study also empowers the hypothesis of a cold-induced production of apomictic seeds in diploid *R. kuepferi* (Klatt *et al.*, 2018), but this is not supported by my findings. As *R. kuepferi* is a perennial plant, I would suggest that results from my project reflect the phenotypic plasticity of flower development and reproduction mode from one year to the other and also correlated with the tangled responses of stress (*e.g.* Dewitt *et al.*, 1998), which were induced from the shift of treatments for the plants, which was performed the same year (2017) of seed collection (Syngelaki *et al.*, 2020a; Chapter 2). Trying to decipher these results regarding the mode of reproduction, I will point to previous studies and observations.

Firstly, in natural populations, a female triploid bridge via unreduced egg cells was detected as the major pathway towards polyploidization and shift to apomixis in *R.kuepferi* (Schinkel *et al.*, 2017). Hence, the occurrence of BIII hybrids, which were detected in the warm diploids after the treatment shift, follows such a scenario, as the first step to polyploidization and apomictic mode of reproduction.

Based on my personal observations, some individuals may rest for one or more years and not develop any parts, or grow only a couple leaves and no flowers, while the lifespan of the plants is unclear. Moreover, embryological analysis on ovules via clearing techniques and DIC microscopy, following the methods of Hojsgaard *et al.* (2014), implied female development before sprouting, as described for various alpine plants (Körner, 2021). Finally, the molecular mechanisms behind apomixis are rather complicated and remain enigmatic (*e.g.* Pellino *et al.*, 2013), so further studies are needed to understand their genetic and epigenetic backgrounds, which is proposed by Grimanelli (2012).

The differentiation of the cytotypes is also documented for the measured morphological traits (Syngelaki *et al.*, 2020b; Chapter 3), which implies a potential of phenotypic plasticity and could sustain fitness (van Kleunen and Fisher, 2001) under the shifted temperature conditions. Overall, the hypothesis of niche preference depending on the cytotype is further supported (Kirchheimer *et al.*, 2016; Kirchheimer *et al.*, 2018). Previous studies on these morphological traits in natural populations of *R. kuepferi* showed a phenotypic variation under a wider range of conditions (Schinkel *et al.*, 2017). Results on diploid individuals are consistent with the study on natural populations (Syngelaki *et al.*, 2020b; Chapter 3), as they appear to have a fitness advantage under warm temperature contiditions, which match their natural optimal climatic niche (Kirchheimer *et al.*, 2016; Schinkel *et al.*, 2017; Kirchheimer *et al.*, 2018).

Regarding tetraploid individuals in the Alps, they exhibit "alpine dwarfism" (Schinkel *et al.*, 2017), as an adaptation to higher elevations and harsh conditions (Körner, 2021). However, tetraploids under cold temperature treatment, which simulates the optimal conditions of their natural alpine habitats, do significantly better than under warm temperature treatment. The absence of dwarf growth phenotype in tetraploids is correlated with the absence of the extreme cold conditions at highest elevations of the Alps (Schinkel *et al.*, 2017). Although temperature is stated to be the most decisive environmental factor on the ecology of *R. kuepferi* cytotypes (Kirchheimer *et al.*, 2016), other environmental variables like pH, nutrients and moisture (Kelly, 2019), may also be important on the phenotypic response towards cold temperature stress. More specifically, growth performance at higher elevations in the Alps is known to be influenced by poor nutrient soils, while tetraploid plants of *R. kuepferi* are showing a niche shift towards more acidic soils. For the scope of the current experimental design, in climate growth chambers only temperature varies and the rest of the proxies are kept equal, allowing the adaptive effects of polyploidy *e.g.* increasing cell size (te Beest *et al.*, 2012) to be expressed in a stronger way.

Hypothesizing that such a phenotypic variation of the cytotypes under different temperature conditions could be inherited by the next generations, it is an adaptive trait (*e.g.* Wyles *et al.*, 1983; van Kleunen and Fisher, 2003; Bossdorf and Pigliucci, 2009; Miryeganeh and Saze, 2019), which could be associated with a successful colonization of different environments (Noble *et al.*, 2014) and an establishment to freshly available ecological niches (van Kleunen and Fisher, 2001; Vrijenhoek and Parker, 2009; Pfenning and McGee, 2010; Miryeganeh and Saze, 2019). Thus, a post-glacial colonization of the Alps by tetraploid populations of *R. kuepferi* is suggested, with the phenotypic plasticity of the two cytotypes towards the climatic conditions shaping their distribution.

The epigenetic background of the two cytotypes under the different temperature treatments and the treatment shift of the plants is detected via the methylation approach of MS-AFLPs (Paun *et al.*, 2012). This method is preferred for non-model plants, as their genome has not been sequenced yet, in order to highlight the genome-wide patterns of epigenetic variation (Schrey *et al.*, 2013). Firstly, results showed an epigenetic differentiation between the two cytotypes, for both years of analysis (Syngelaki *et al.*, 2020a; Chapter2), which is in contrast to their very low genetic differentiation in wild populations of the AFLP study of Cosendai *et al.* (2011, 2013). Such an epigenetic differentiation of cytotypes was also observed in methylation patterns studied in wild populations (Schinkel *et al.*, 2020). Moreover, DNA methylation patterns in the wild populations rather followed a temperature gradient than the biogeographical pattern, while they seem to correlate with reproduction mode of each cytotype (Schinkel *et al.*, 2020).

The lack of genetic structure in tetraploids is thought to be a concequence of their recent origin and rapid postglacial colonization of the Alps (Kirchheimer *et al.*, 2018), while the observed variation in DNA methylation patterns over short timescales (Syngelaki *et al.*, 2020a; Chapter 2) could be spontaneous and independent from genetic background (Vidalis *et al.*, 2016). Since tetraploid *R.kuepferi* is autopolyploid, side-effects of hybridity on methylation patterns can be ruled out (*e.g.* Chen, 2007).

The epigenetic consequences of polyploidy are well documented prior to the current project, as epigenetic mechanisms such as DNA methylation in allo- and autopolyploids can lead to gene expression alterations (*e.g.* gene silencing) and genome-wide transcriptional rewiring (*e.g.* Baubec *et al.*, 2010; Li *et al.*, 2011; Madlung and Wendel, 2013; Song and Chen, 2016). Results on *R. kuepferi* methylation patterns and their consistency (Syngelaki *et al.*, 2020a; Chapter 2) suggest that polyploidization affects methylation variation independently from genetic background variation and environment, while they could reflect a heritable

pattern, as ploidy levels are highly stable within natural populations (Cosendai *et al.*, 2010; Schinkel *et al.*, 2016).

Other studies on autopolyploid plants, such as *Taraxacum officinale* and *Dianthus broteri*, an endemic species of the Iberian Peninsula, demonstrated that *de novo* methylation can be triggered (Verhoeven *et al.*, 2010; Alonso *et al.*, 2016), while an increase of the global cytosine methylation in six species of the grass species complex *Cymbopogon* was observed (Lavania *et al.*, 2012). Autotetraploid cytotypes of *R. kuepferi* in the Alps have an extended biogeographical and altitudinal distribution (Cosendai and Hörandl, 2010; Schinkel *et al.*, 2016; Kirchheimer *et al.*, 2016), but neither geographical structure nor isolation-by-distance appeared in their gene pool (Cosendai *et al.*, 2013) while the cold treatment conditions, which were applied in the experimental design of the climate growth chambers, are quite similar to the natural habitat conditions of the cytotypes in the Alps (Syngelaki *et al.*, 2020a; Chapter 2).

Kirchheimer *et al.* (2016) suggested that the niche optimum of autotetraploids is placed in the direction of cooler conditions than the one for diploids, presumably due to the change in the reproductive system of an originally warm-adapted species during postglacial re-colonization of higher regions in the Alps (Kearney, 2005; Hörandl, 2006). Thus, tetraploids managed to surmount high elevation barriers and establish new populations throughout a greater distribution range (Kirchheimer *et al.*, 2018), with the shift to facultative apomixis probably being advantageous to rapid colonization (Kirchheimer *et al.*, 2018) (Syngelaki *et al.*, 2020a; Chapter 2).

DNA methylation changes, which are controlled by *e.g.* hormone signals, may occur at specific genes (Kim *et al.*, 2009) and functional responses may be restricted to this limited number of loci. Thus, the functional interpretation of the data of anonymous MS-AFLP marker loci is unclear. Hence, to capture the stress-induced phenotypic variation of the two cytotypes of *R. kuepferi*, as well as to try to draw functional interpretation of DNA methylation patterns, a gene expression study under temperature treatment conditions was conducted (Chapter 4).

The results of the latter study, do not advocate *per se* for polyploidy being a significant contributor to the stress responses, as they were observed by differentiated gene expression patterns, but rather differentiating on the number and characteristics of the Gene Set Enrichment pathways, which are expressed for each group. These findings may indicate that changes in gene expression connected with polyploidy may contribute to heterosis, indicating a variation that may allow adaptive advantages towards novel conditions (Comai, 2005). In general, diploid individuals of *R. kuepferi* appear to respond more intensively to

cold temperature treatments than tetraploid ones, as a higher number of Gene Sets Enrichment pathways is significantly differentially expressed in them.

More specifically, most pathways expressed differentially in diploid individuals are linked to ion/cation homeostasis and activity and they were suppressed, while Gene Set Enrichment pathways differentially expressed in tetraploid individuals are mainly focusing on membranes and cell wall, as well as chromatin and histone modification.

The intriguing suppression of the pathways related to ion/cation homeostasis and activity, as well as the serine/threonine protein kinase activity, in diploid individuals of *R. kuepferi*, corroborates the hypotheses of tangled information encoded through Ca^{2+} kinetics under cold stress conditions (Sanders *et al.*, 2002; Knight & Knight, 2012; Shi *et al.*, 2015), which could also be influenced by 'cold memory', *i.e.* former exposure to cold stress conditions (Knight *et al.*, 1996; Knight & Knight, 2012). I further assume that such pathways in diploids, linked to oscillations of Ca^{2+} could explain the lower growth performance of diploids under cold conditions, regarding the morphological traits which were measured, as they could provoke the stomatal closure and consequently the reduction of CO_2 uptake/carbon gain (Taiz & Zeiger, 2010). However, temperature treatments seem to be the main factor influencing the gene expression patterns. Previous studies on autotetraploid rice do not seemingly show a genome-wide dosage effect on gene expression patterns (Zhang *et al.*, 2015), empowering the role of subfunctionalization in maintaining the functional pleonasm of duplicated genes and avoiding any energy waste (Wagner, 2005; Drummond & Wilke, 2008; Qian *et al.*, 2010; Madlung & Wendel, 2013).

Overall, the post-glacial colonization of the Alps by autotetraploid populations of *R. kuepferi* (Schinkel *et al.*, 2016) seems to rely, among other factors, on the phenotypic variation, pronounced also as differential gene expression, towards the climatic conditions, with the cold adapted genotypes having a distinct advantage over non-adapted ones in frost-prone environments, such as high mountain areas (Nagy & Grabherr, 2009; Vrijenhoek & Parker, 2009). Finally, results of the gene expression study further support the geographical parthenogenesis scenario of the species, towards the ecological niche shift of the two cytotypes (Schinkel *et al.*, 2016; Kirchheimer *et al.*, 2018) and more specifically, the decisive role of acclimation/adaptation of tetraploids to a colder climatic niche in higher and more northern parts of the Alps (Kirchheimer *et al.*, 2018), under a scope of direct physiological background of cold tolerance.

5.2 Effects of temperature treatments

In this session I will discuss how the temperature treatments affect the phenotypic plasticity and acclimation of the cytotypes, regarding the epigenetic and gene expression background. Some of the treatment effects were already discussed in the previous session, as it is thought to be heavily influenced by the ploidy background of the individuals. Altogether, DNA methylation profiles of perennial plants, such as *R. kuepferi*, do change over their lifetime, independently from environmental influence. However, it is quite unlikely that such a drastic shift from one year to another would just be an effect of ageing, as it occurred synchronously in plants from different origins.

Several studies show that the exposure to biotic or abiotic environmental stresses can trigger epigenetic changes, which seem to persist even after the stress is relieved, resulting, thus, in a stress memory that can be stable throughout the lifetime of an organism or even across generations, especially in plants (*e.g.* Verhoeven *et al.*, 2010; Dowen *et al.*, 2012; Richards *et al.*, 2012; Verhoeven & Preite, 2013; Nicotra *et al.*, 2015; Foust *et al.*, 2016). Furthermore, the evolutionary aspects of stress-induced epigenetic variation and putative epigenetic inheritance have been noteworthy discussed (*e.g.* Wendel and Rapp, 2005; Richards, 2006; Bossdorf *et al.*, 2008; Johannes *et al.*, 2008; Richards *et al.*, 2017).

Regarding the treatment effect on *R. kuepferi* individuals, only after the reciprocal change of treatments we observed significant changes of the methylation profiles for the same individuals. Hereby, the temperature treatment shift happened from 2016 to 2017, concerning the comparison of same individuals for these two years, resulted in less fragments (epiloci) after the shift, *i.e.* from 754 (2016) to 493 (2017) (Syngelaki *et al.*, 2020a; Chapter 2). Additionally, this epigenetic variation related to the treatment shift, regardless of the treatment's direction, may depict the dynamics of DNA methylation under stress conditions (Bartels *et al.*, 2018; Zhang *et al.*, 2018) and the epigenetic control on the phenotypic plasticity of the species (Zhang *et al.*, 2012; Richards *et al.*, 2017) (Syngelaki *et al.*, 2020a; Chapter 2).

The 2017 results for *R. kuepferi* (Syngelaki *et al.*, 2020a; Chapter 2) may empower the argument of cold-induced DNA methylation changes, as described by *e.g.* Song *et al.* (2015) regarding the alpine to subnivale species *Chorispora bungeana*, while forest trees set off several epigenetic mechanisms, including DNA methylation, to elicit rapid phenotypic variations in response to environmental changes (Mamadou *et al.*, 2018). Hence, I suggest that the findings of 2016, which represent the 3rd year (2014 – 2016) of cultivation in growth chambers under the same conditions, still reflect to a great extent the background methylation profiles from the natural habitats, potentially related to differential cold acclimation of diploids and tetraploids and the dynamics of DNA methylation during cold acclimation

(Liu *et al.,* 2017) (Syngelaki *et al.,* 2020a; Chapter 2). Moreover, I assume that an extreme change of temperature is needed to alter methylation patterns independently from ploidy variation, such as the extreme temperature shifts occuring under natural weather conditions in the Alps, where temperature fluctuates strongly between day, night and seasons and where extremely low temperatures down to -24 °C occur in higher altitudes (Körner, 2021). Thus, the high phenotypic plasticity of *R. kuepferi* regarding methylation patterns, may be responsive to the fluctuating climatic conditions (Syngelaki *et al.,* 2020a; Chapter 2).

Concerning the gene expression profiles of the individuals, they differentiate under treatments (Chapter 4), indicating a cascade of key regulatory pathways provoked by stress conditions (*e.g.* Takahashi *et al.,* 2004; Cramer *et al.,* 2011; Puijalon *et al.,* 2011; Jutsz & Gnida, 2015; Ritonga & Chen, 2020), whereas both cytotypes express gene sets related to cold acclimation, plasma membrane, cell wall and hydrolase activity. I interpret such gene expression changes as a consequence of the short freezing treatments (-1° C during three nights per week) taking place in the climate growth chambers. As no apparent damage of leaves in cold treatments was observed (Syngelaki *et al.,* 2020b; Chapter 3), I would further suggest that both cytotypes are tolerant to short-term freezing and thawing during the day.

As access points of the cell, membranes are injured by adverse environmental conditions (*e.g.* Theocharis *et al.,* 2012). Thus, in order to maintain cell activity and behavior under environmental stress, membrane stability is vital (Chen *et al.,* 2018; Ritonga & Chen, 2020). Decreasing membrane fluidity, coupled with its interaction with the cell wall, contributes to maintenance of plasma membrane functionality and is considered as one of the first cold sensors (Murata & Los, 1997; Martiniere *et al.,* 2011; Ma & Liu, 2019). A fluid membrane in alpine plants, which have to encounter freezing conditions, allows transfer of water from the protoplast into the intercellular space, where extracellular ice nucleation takes place, leaving the protoplast unfrozen (Körner, 2021).

5.3. Correlations of epigenetic patterns with morphological traits and gene expression patterns

The epigenetic background of phenotypic plasticity, together with cytosine DNA methylation, as an epigenetic mechanism, which seems to be strongly correlated with phenotypic plasticity to internal and external stimuli (Miryeganeh and Saze, 2019), has been notably discussed (*e.g.* Pigliucci, 2001; van Kleunen and Fisher, 2001; West-Eberhard, 2003; Mirouze and Paskzowski, 2011; Johannes *et al.*, 2009). Stress-induced epigenetic patterns could have an impact on an individual's fitness (*e.g.* Crews *et al.*, 2007; Angers *et al.*, 2010; Castonguay and Angers, 2012; Herrera and Bazaga, 2013; Nicotra *et al.*, 2015; Rubenstein *et al.*, 2016; Verhoeven *et al.*, 2016; Miryeganeh and Saze, 2019), while it could also be advantageous towards a wider distribution niche (*e.g.* Pal and Milkos, 1999; Angers *et al.*, 2010).

The potential of epigenetic inheritance of plastic phenotypic traits in plants is argued for by several studies (*e.g.* Johannes *et al.*, 2009; Paskowski and Grossniklaus, 2011; Cortijo *et al.,* 2014; Miryeganeh and Saze, 2019). In case such phenotypic variation is heritable, DNA methylation could provide a mechanism of adaptive microevolution in plants (*e.g.* Herman and Sultan, 2011; Zhang *et al.*, 2012), which is faster and independent from traditional genetic evolution (Jablonka and Lamb, 1995; Richards, 2006; Miryeganeh and Saze, 2019).

By comparing the DNA methylation patterns with the morphological traits of leaves and flowers per individual (Syngelaki *et al.*, 2020b; Chapter 3), the hypotheses regarding the survival and the fitness of *R. kuepferi* towards the shifted conditions after three years being acclimated in previous experimental treatments (Klatt *et al.*, 2018; Syngelaki *et al.*, 2020a), are further investigated. A stronger correlation of phenotypic plasticity in leaves than in flowers with epigenetic variation, as it is pronounced by DNA methylation variation, is observed, while for both cytotypes leaves are exhibiting higher values under the cold treatment (Syngelaki *et al.*, 2020b; Chapter 3). The hypothesis of epigenetic control on the phenotypic plasticity of the species is further supported by the negative correlation of the leaf length and the number of leaves with the patterns of non-methylated epiloci for both cytotypes and under both treatments (Syngelaki *et al.*, 2020b; Chapter 3).

These findings indicate an effect on the fitness of individuals under the new environmental conditions, proposing, thus, a Jack-and-master scenario (Richards *et al.*, 2006) for the species. The epigenetic asymmetry, which is proposed from such a scenario, is often observed under changing environmental conditions, as stochastic epigenetic changes may result in high levels of plasticity, "weird" phenotypes and even developmental disturbances (*e.g.* Palmer and Strobeck, 1986; Palmer, 1996; Moller and Swaddle, 1997; Dongen, 2006; Angers *et al.*, 2010; Angers *et al.*, 2020).

Such changes may promote the establishment of a species towards freshly available environments, contributing further to the post-glacial colonization of the species in the Alps. However, the correlations of phenotypic plasticity with epigenetic variation of the species in fluctuating biotic and abiotic environmental conditions is a rather complicated process, with time of exposure also playing a role (Angers *et al.*, 2010).

Trying to further explore the mechanisms of phenotypic plasticity and factors that prompt it, I will address the correlations of DNA methylation with the gene expression profiles (Chapter 4). Kooke *et al.* (2015) proposed that DNA methylation dynamics could extend the environmental sensitivity of the phenotypes and consequently, could regulate gene expression (*e.g.* Steward *et al.*, 2002; Shan *et al.*, 2013; Rakei *et al.*, 2016), by playing a role in the maintenance of cell stability under stress (Song *et al.*, 2015).

It is further known that DNA methylation profiles under cold stress conditions, together with histone modifications and non-histone proteins, are associated with gene expression profiles (Chinnusamy & Zhu, 2009; Kim *et al.*, 2015) and could encompass chromatin structure and accessibility (*e.g.* Strahl & Allis, 2000; Lang-Mladek *et al.*, 2010; Jones, 2012; Zhang *et al.*, 2018). Moreover, it is proposed that a prolonged cold stress treatment decreases the level of DNA methylation and gene expression is increased after the occurrence of demethylation (Shan *et al.*, 2013), while chromatin remodeling has a putative function as a plant thermometer, indicating, thus, a relatively direct connection between cold and gene expression (Kumar & Wigge, 2010; Kumar *et al.*, 2012).

Regarding the results of my gene expression study of *R. kuepferi* individuals (Chapter 4), several genes, directly or indirectly related to DNA methylation, are significantly differentially expressed in the tetraploid individuals, while the Gene Set pathways of histone and chromatin modifications are suppressed under the same cytotype. The differential expression of genes correlated to DNA methylation is further validated by the qRT-PCR results. These findings fit the overall pattern of loss of methylated MS-AFLP fragments after dramatic temperature changes (Syngelaki *et al.*, 2020a; Chapter 2) and indicate that DNA methylation patterns could conserve the transgenerational epigenetic memory of cold stress responses towards a differential acclimation cytotypes (Schinkel *et al.*, 2020; Syngelaki *et al.*, 2020a).

5.4 Conclusion and Prospects

To summarize, my thesis demonstrates the temperature and polyploidy effects on the DNA methylation profiles, fitness and mode of reproduction, phenotypic plasticity of several morphological traits and gene expression profiles, as well as their interactions, in *R. kuepferi*.

More specifically, the DNA methylation differentiation among the two cytotypes and the high epimutational dynamics of the different types of epiloci attest that phenotypic plasticity of epigenetic mechanisms catalyzes the response towards cold stress conditions. Additionally, cold temperature stress induces differentially expressed gene profiles, with several gene set pathways, either activated or suppressed, being involved in the response, with the differentiation of the gene set pathways between cytotypes suggesting a better cold acclimation of tetraploids than of diploids.

This hypothesis is further supported by the correlations of DNA methylation with the morphological traits and the differential gene expression, drawing, thus, an extended concept of rapid reaction to shifted environments, acclimation to cold temperature conditions and a putative epigenetic memory for each cytotype. Interestingly, this acclimation further correlates the cytotypes with their respective climatic niches in the wild, as they were described by Kirchheimer *et al.* (2016).

Furthermore, if the putative epigenetic background of phenotypic plasticity would be a heritable trait, which could ensure a relative stability of the phenotypes (Angers *et al.*, 2010), it could have an adaptive value and advocate further on the geographical parthenogenesis pattern proposed for the species in the Alps (Kirchheimer *et al.*, 2016; Kirchheimer *et al.*, 2018). Thus, higher DNA methylation differentiation and phenotypic plasticity of asexual organisms could allow for a higher or equal niche dynamics compared to sexual plants. To empower this scenario, a physiological background of the observed ecological and geographical differentiation patterns between cytotypes is suggested by the results of the differential gene expression study.

5.5 References

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List of Publications

Syngelaki, E., Schinkel, C.C-F., Klatt, S. and Hörandl, E. (2020a). Effects of temperature treatments on cytosine-methylation profiles of diploid and autotetraploid plants of the alpine species *Ranunculus kuepferi* (Ranunculaceae). *Front. Plant Sci.*, 11, 435.

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Syngelaki, E., Paetzold, C., and Hörandl, E. (*submitted*). Gene expression profiles of diploid and tetraploid plants of the alpine species *Ranunculus kuepferi* (Ranunculaceae) under cold stress conditions. Submitted to *The Plant Journal*

Thesis Declarations

Chapter 2 - **Effects of temperature treatments on cytosine-methylation profiles of diploid and autotetraploid plants of the alpine species *Ranunculus kuepferi* (Ranunculaceae).**

ES, EH designed research; CS, SK, EH collected plant materials; ES, CS, SK performed experiments; ES collected and analyzed data; ES wrote the paper with contributions from EH. All authors commented on and discussed previous versions of the paper.

Chapter 3 - **Phenotypic responses, reproduction mode and epigenetic patterns under temperature treatments in the alpine plant species *Ranunculus kuepferi* (Ranunculaceae).**

ES, EH designed research; SK, EH collected plant materials; ES, MD performed experiments; ES collected and analyzed data; ES wrote the paper with contributions from EH. All authors commented on and discussed previous versions of the paper.

Chapter 4 - **Gene expression profiles of diploid and tetraploid plants of the alpine species *Ranunculus kuepferi* (Ranunculaceae) under cold stress conditions.**

ES, EH designed research; ES performed experiments; ES, CP collected and analyzed data; ES wrote the paper with contributions from EH. All authors commented on and discussed previous versions of the paper.

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Curriculum Vitae

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