PUBLIZIERBARER ENDBERICHT

A) Projektdaten

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B) Projektübersicht

1 Kurzfassung / Abstract

Der anhaltende Klimawandel wird die Verbreitung von Arten stark verändern. Es ist jedoch unklar, ob Arten auch durch rasche Anpassung an die neuen Umgebungsbedingungen reagieren können, was schließlich zur Entstehung neuer Arten führen würde. Da die Änderung der genetischen Sequenz durch Mutation zu langsam ist, um mit dem Klimawandel Schritt zu halten, konzentrierten wir uns auf epigenetische Veränderungen (strukturelle Modifikationen der DNA und Wechselwirkungen mit small RNAs) als Möglichkeit einer raschen und erblichen Veränderung des Genoms. Die untersuchten Modellorganismen sind zwei nah verwandte Arten von Gebirgspflanzen, *Heliosperma pusillum*, das auf die alpine Zone beschränkt ist, und *H. veselskyi*, das unter überhängenden Felswänden der montanen Zone verbreitet ist. Beide Arten sind trotz des Fehlens genomweiter genetischer Differenzierung morphologisch und ökologisch unterschiedlich was darauf hindeutet, dass epigenetische Mechanismen an ihrer Divergenz beteiligt waren.

Hauptziel des Projektes war es, Licht auf die komplexen Wechselwirkungen zwischen Genotyp, Epigenotyp und Umwelt zu werfen, mit dem Ziel die Möglichkeit rascher Evolution als Reaktion auf den Klimawandel besser beurteilen zu können. Wir konzentrierten uns auf den Vergleich räumlich naher (innerhalb weniger Kilometer horizontaler Distanz) Paare von Populationen, die jeweils zusammengesetzt waren aus *H. veselskyi* aus tieferen Lagen und dem 200-500m höher vorkommenden *H. pusillum.* Die durchschnittlichen Temperaturunterschiede an den Wuchsorten innerhalb der Populationspaare entsprechen damit in etwa der für das 21. Jahrhundert vorhergesagten Temperaturerhöhung. Diese Populationspaare untersuchten wir auf genetische und epigenetische Differenzierung sowie deren Rolle bei der Anpassung an divergierende Umweltbedingungen. Darüber hinaus sammelten wir wichtige Daten über biotische und abiotische (z. B. Temperatur) Habitatbedingungen, testeten die Kreuzbarkeit zwischen den beiden Spezies und untersuchten morphologische und ultrastrukturelle sowie ökophysiologische Divergenzen zwischen beiden Arten.

Wir untersuchten Ausmaß und Struktur der genetischen und epigenetischen Variation sowohl innerhalb und zwischen wilden Populationen von *H. veselskyi* und *H. pusillum* als auch in reziprok transplantierten Individuen und in einem „common garden“ herangezogenen Pedigrees, mit dem Ziel, die evolutionäre Bedeutung dieser Variation zu ergründen. Als ersten Schritt in unseren molekularen Analysen untersuchten wir genetische Daten – von RADseq generierte, genomweite Single Nucleotide Polymorphisms (SNPs) – um die Populationsstruktur und die Evolutionsgeschichte der untersuchten Populationspaare zu verstehen und die Häufigkeit sowie das Ausmaß des Genflusses zwischen diesen Populationen abzuschätzen. Zusätzlich wurde die genomweite DNA-Methylierung unter Verwendung einer im Rahmen dieses Projekts neu entwickelten Methode (bsRADseq), die Bisulfit- und Next-Generation-Sequenzing kombiniert und eine effiziente bioinformatische Analyse-Pipeline beinhaltet, bestimmt. Unsere zu testende Hypothese war, dass angesichts der erwarteten Umgebungsempfindlichkeit von epigenetischen Signalen genomweite Unterschiede in der DNA-

Methylierung zwischen den beiden Spezies bestehen. Unter Verwendung statistischer Ansätze durchsuchten wir die allgemeinen Differenzierungsmuster in den generierten Datensätzen hinsichtlich Hinweise auf Selektion einzelner (Epi)Loci. Dies beruht auf der Annahme, dass Loci unter positiver Selektion eine signifikant höhere Differenzierung als der Großteil der genomweiten Loci aufweisen sollten, während Loci unter reinigender Selektion eine niedrigere Differenzierung zeigen sollten. Ziel der Untersuchung war auch die evolutionäre Bedeutung von Epi-Loci für einzelne Individuen aus den sechs Populationspaaren im Detail zu hinterfragen. Weitere Annotationsanalysen wurden durchgeführt, um die biologische Bedeutung der gewonnenen Daten zu erhöhen. Weiters untersuchten wir die Umgebungsempfindlichkeit der DNA-Methylierung indem wir alle Epi-Loci bei reziproken Transplantationen untersuchten. Darüber hinaus wurde der vererbbare Teil der epigenetischen Variabilität über drei Generationen quantifiziert. Abschließend verschnitten wir die verschiedenen Ebenen molekularer Informationen mit komplementären phänotypischen Daten, um die Verbindungen zwischen Genotyp, Epigenotyp und Phänotyp zu definieren und dadurch zusätzliche Informationen über die Muster von Selektion und deren Ziele zu gewinnen.

Unsere Ergebnisse widersprachen den meisten unserer anfänglichen Hypothesen, die auf dem Stand der Kenntnisse zum Zeitpunkt des Projektbeginns basierten. **A.** Zum Beispiel haben wir innerhalb der sechs Populationspaare bis zu fünf unabhängige Entstehungen von *H. veselskyi* aus *H. pusillum* gefunden, was darauf hindeutet, dass eine Anpassung an montane Wuchsorte häufig und relativ leicht erreichbar ist. Diese Divergenzereignisse passierten verteilt über einen Zeitraum zwischen 15.000 und 500 Jahren und trotz des Wirkens von Genfluss; die genomische Divergenz erscheint aber weitgehend durch Drift geformt zu sein. **B.** Die entdeckten Methylierungsmuster sind in verschiedenen Sequenzkontexten stark verschieden (d.h. CpG, CHH, CHG), sie sind jedoch überraschend stabil zwischen *H. veselskyi* und *H. pusillum*, die nur begrenzte und punktuelle epigenetische Divergenz aufweisen. Dies kann auf eine unerwartet starke, reinigende Selektion hinweisen, die auf DNA-Methylierung innerhalb eines Genom-Abschnittes, hauptsächlich in oder um Gene herum, wirkt. **C.** Obwohl wir eine signifikante Umgebungsempfindlichkeit der DNA-Methylierung festgestellt haben, die etwa 20% der CpG-Positionen (in der Nähe von Genen) beeinflusst, übertrifft dies nicht die Labilität der DNA-Methylierung in einer stabilen Umgebung. Daher erscheint bei *Heliosperma* das umweltbedingte „Reset“ der DNA-Methylierung nicht als eine auf eine Generation beschränkte, groß angelegte Reaktion auf Umweltveränderungen. Allerdings könnten örtliche Reaktionen, die außerhalb der untersuchten genomischen Regionen liegen, oder Änderungen, die über mehrere Generationen vonstattengehen, eine wichtige Rolle bei der Anpassung an unterschiedliche Bedingungen spielen. Außerdem lassen unsere Analysen auch besondere episodische Selektionsdrücke wie etwa Fraßdruck unberücksichtigt. **D.** Innerhalb der Populationspaare wurden nur begrenzte DNA-Methylierungsunterschiede zwischen den beiden Spezies beobachtet, von denen nur wenige über alle Populationspaare hinweg konsistent sind. Dies deutet darauf hin, dass die lokale Selektion eine Rolle bei der Gestaltung von DNA-Methylierungsmustern und genetischen Loci spielen kann, von denen epigenetischen Signale abhängen könnten.

Insgesamt zeigen unsere Ergebnisse, dass die wahrscheinlich klimatisch bedingte Evolution von *H. veselskyi* – einem morphologisch, anatomisch, ökophysiologisch und ökologisch abweichendem, polytopisch entstandenem Nachkommen des weitverbreiteten *H. pusillum* – rasch

verlief und im Holozän stattfand. Auf der anderen Seite liefern unsere Daten nur begrenzte Hinweise für adaptive Evolution über epigenetische Mechanismen – in unserem Fall Cytosin-Methylierung – als Reaktion auf veränderte Umweltbedingungen. Basierend auf unseren Ergebnissen zeigt sich wiederum, dass epigenetische Anpassung von Pflanzen an den anthropogenen Klimawandel unwahrscheinlich ist, was den "konventionellen" Klimaschutz notwendiger denn je macht.

2 Executive Summary

The ongoing climate change will strongly reshape the distribution of species. It remains unclear, however, if species will be able to react also by rapid adaptation to the new environmental conditions eventually leading to the emergence of new species. As alteration of the genetic sequence by mutation is too slow to keep pace with climate change, we focused on epigenetic change (structural modifications of the DNA and interactions with small RNAs) as a rapid and heritable alteration of the genome. Our model organisms were two closely related species of mountain plants, *Heliosperma pusillum* restricted to the alpine zone and *H. veselskyi*, which inhabits cliff overhangs in the montane zone. Both species are morphologically and ecologically distinct despite the absence of genome-wide genetic differentiation, suggesting that epigenetic mechanisms were involved in their divergence.

The main aim of the project was to shed light on the complex interactions between genotype, epigenotype and environment with the final goal of evaluating the possibility of rapid evolution as a reaction to climate change. We focused on the comparison of geographically close (within a few kilometres horizontal distance) population pairs of the lower-elevation *H. veselskyi* and the higher-elevation *H. pusillum* separated by 200–500 m altitudinal difference. The average temperature differences within the population pairs thus roughly match the temperature increase projected for the 21st century. Using these pairs of populations, we evaluate genetic and epigenetic differentiation and their role in adaptation to divergent environments. In addition, we gathered crucial data describing the plants' biotic and abiotic (e.g., temperature) growing environment, tested the crossability between the two species and explored morphological and ultrastructural as well as ecophysiological divergence between the two species.

We investigated the extent and structure of genetic and epigenetic diversity within and among wild populations of *H. veselskyi* and *H. pusillum*, as well as of reciprocally transplanted individuals and pedigrees in a common garden, with the final aim of assessing the evolutionary implications of this variation. As the first step in our molecular analyses we screened genetic data – genome-wide SNPs derived from RADseq to understand the population structure and evolutionary history of the studied population pairs, in order to estimate the frequency and extent of gene flow between these populations. In addition, genome-wide DNA methylation was profiled using a method newly developed in the framework of this project (bsRADseq), combining bisulfite and next generation sequencing and establishing an efficient bioinformatic methodology for analysis. Our hypothesis to evaluate here was that given the expected environmental sensitivity of epigenetic signals, large-scale, genome-wide differences in DNA methylation will exist between the two species. By using statistical approaches, we searched the general patterns of differentiation in the obtained datasets for signatures of selection on individual (epi)loci with the rationale that loci under positive selection should present a significantly higher differentiation than the bulk of genome-wide loci, while loci under purifying selection will show lower differentiation. We also aimed to question in detail the evolutionary significance of epi/loci across individuals from the six population pairs. Further annotation analyses aimed to increase the biological meaning of the obtained data. We also interrogated the environmental sensitivity of DNA methylation, again by investigating all epi-loci in reciprocal transplantations. In addition, the heritable portion of the

epigenetic variability was quantified across three generations. We finally aimed to integrate the various levels of molecular information, and complementary phenotypic data in order to define the links between genotype, epigenotype and phenotype, together with providing additional information on the patterns of selection and their targets.

Our results contradicted most of our initial hypotheses, which were based on the state of knowledge at the time the project application was conceived. **A.** For instance, within the six population pairs we uncovered as much as five independent origins of *H. veselskyi* from *H. pusillum*, suggesting that adaptation to the montane localities is frequent and relatively easily achievable. These divergence events are scattered between 15,000 and 500 years ago and happened in the presence of some gene flow; however the genomic divergence appears largely shaped by drift. **B.** The uncovered methylation patterns are strongly divergent in different sequence contexts (i.e., CpG, CHH, CHG), but they are surprisingly stable between *H. veselskyi* and *H. pusillum*, with only limited and punctual epigenetic divergence between them. This may indicate unexpectedly strong purifying selection acting on DNA methylation in a genomic portion mostly in or around genes. **C.** Whereas we identified a significant environmental sensitivity of DNA methylation affecting ca. 20% of CpG positions (again around genes), this does not exceed the lability of DNA methylation in a stable environment. Therefore, in the *Heliosperma* system environmental reset of DNA methylation does not appear as a one-generation, large-scale response to environmental change. However, localized responses that may reside outside the targeted genomic region or multi-generation alterations may still play an important role towards adaptation to different conditions, whereas our analyses may have also missed particularly episodic selection pressures, such as herbivory. **D.** Only limited DNA methylation differences between the two species have been observed in each population pair, of which few are consistent across population pairs. This indicates that local selection may play a role in shaping DNA methylation patterns or the genetic loci on which such epigenetic signals may depend.

Altogether, our results indicate that the likely climate-driven evolution of *H. veselskyi* – a morphologically, anatomically, ecophysiologically and ecologically divergent, polytopically evolved descendent of widespread *H. pusillum* – was rapid and took place in the Holocene. On the other hand, our data provide limited evidence for adaptive evolution via epigenetic mechanisms – in our case cytosine methylation – as a response to changed environmental conditions. This in turn indicates that based on our results plants are unlikely to adapt epigenetically to ongoing anthropogenic climate change, rendering “conventional” climate protection more necessary than ever.

3 Hintergrund und Zielsetzung / Background and aims

Initial situation / motivation for the project

Apart from extinction or displacement, climate change may drive adaptation, especially in sessile organisms such as plants. However, our understanding of frequency, extent and molecular mechanisms involved in rapid adaptive responses is still in its infancy. This project aimed to investigate the genetic and epigenetic basis of differentiation into higher and lower elevation species within the *Heliosperma pusillum* (Caryophyllaceae) species complex. This is a monophyletic group comprising perennial caespitose herbs that inhabit rocky habitats mostly on calcareous substrates in mountain ranges of southern Europe from the Sierra Cantábrica in the West to the Carpathians in the East (Frajman & Oxelman 2007). We focused here on geographically close (within a few kilometres horizontal distance) population pairs of the lower-elevation (i.e., below the timber line) *H. veselskyi* and the higher-elevation (i.e., alpine) *H. pusillum* separated by 200–500 m altitudinal difference in the SE Alps. The average temperature difference within these population pairs thus roughly matches the temperature increase projected for the 21st century (Intergovernmental Panel on Climate Change 2011). The higher elevation taxon is characterized by glabrous or sparsely hairy leaves and occasional presence of unicellular glands (Neumayer 1923; Frajman & Oxelman 2007), whereas plants of lower elevations share a dense indumentum with long, multicellular, sticky glandular trichomes. Dense indumentum has been explained as adaptation offering protection against drought, herbivores, and/or UV radiation (e.g., Levin 1973; Kärkkäinen et al. 2004; Hanley et al. 2007) and previous genetic analyses in other systems indicate that the inheritance of this trait can be simple (e.g., Kärkkäinen & Ågren 2002). *Heliosperma veselskyi* and *H. pusillum* are generally treated as different species in national Floras (Poldini 2002; Fischer et al. 2008), but – based on preliminary weakly-resolving sequenced-based phylogenies – they were previously suggested to have probably originated several times independently (Frajman & Oxelman 2007).

Objectives of the project

The main aim of the project was to shed light on the complex interactions between genotype, epigenotype and environment in shaping phenotypes as response to warmer and drier habitats with the final goal of evaluating the possibility of rapid evolution in response to climate change. Using six pairs of populations of *Heliosperma veselskyi* and *H. pusillum*, we focused on the pattern of reciprocal genetic and epigenetic differentiation as well as on the potential roles of genetic and epigenetic differentiation in plant adaptation to divergent environments. Complementary environmental, ecological, phenotypic (morphometric and anatomical) and crossing-experiment data were initially planned to be obtained with other funding. Due to unfortunate conditions (see section 2.2.5 below) and as these complementary data were of vital importance for the interpretation of the genetic and epigenetic data, we had to produce much of these data within the framework of the present project (within *Work Package [WP] 4*, see below).

We investigated the extent and structure of genetic and epigenetic diversity within and among wild populations of *H. veselskyi* and *H. pusillum*, as well as of reciprocally transplanted individuals and

pedigrees in a common garden, with the final aim of assessing the evolutionary implications of this variation. **A)** (*WP1*) As the first step in our molecular analyses we screened genetic data – genome-wide SNPs derived from RADseq (Baird & al. 2008) to understand the population structure and evolutionary history of the studied population pairs, in order to estimate the frequency and extent of gene flow between these populations. **B)** (*WP2*) In addition, genome-wide DNA methylation was profiled using a method newly developed in the framework of this project (bsRADseq, Trucchi et al. 2016a), combining bisulfite and next generation sequencing and establishing an efficient bioinformatic methodology for analysis. Our hypothesis to evaluate here was that given the expected environmental sensitivity of epigenetic signals, large-scale, genome-wide differences in DNA methylation will exist between the two species. **C)** (*WP1* and *WP2*) By using statistical approaches, we searched the general patterns of differentiation in the obtained datasets for signatures of selection on individual (epi)loci with the rationale that loci under positive selection should present a significantly higher differentiation than the bulk of genome-wide loci, while loci under purifying selection will show lower differentiation. **D)** (*WP3*) We also aimed to question in detail the evolutionary significance of selected candidate (epi-)loci in triggering ecological divergence by analysing them with locus-by-locus approaches across up to six populations and up to 120 individuals to confirm the results obtained. This work package was extended significantly by investigating the patterns of all (and not only the candidate) epi/loci across individuals from the six population pairs (i.e., altogether 120 individuals). This change in our research plan ensured a higher throughput within the same budget, while freeing some of the working time of the personnel involved that could be dedicated to complementary phenotypic analyses. Further annotation analyses aimed to increase the biological meaning of the obtained data. **E)** (*WP3*) We also interrogated the environmental sensitivity of DNA methylation, again by investigating all epi-loci (and not only the candidate epi-loci as originally planned) in reciprocal transplantations. **F)** (*WP3*) In addition, the heritable portion of the epigenetic variability was quantified across three generations. **G)** (*WP4*) We finally aimed to integrate the various levels of molecular information, and complementary phenotypic data in order to define the links between genotype, epigenotype and phenotype, together with providing additional information on the patterns of selection and their targets. Unforeseen turns of events (see below) impaired the planned achievement of complementary phenotypic data, but extensive efforts of our team finally enabled us to obtain those data within an extended project period.

4 Projektinhalt und Ergebnisse / Project content and results

Genetic patterns of differentiation – RADseq (WP1)

Milestones

1. Signals of either purifying or divergent selection are identified using outlier approaches
2. Population structure is unravelled
3. The frequency of gene flow within and among the six pairs of populations is determined

The average number of raw RADseq pairs of reads for each of the 120 samples from natural populations after quality filtering was 2.1 million. A very small portion of these reads blasted to a Viridiplantae transposable elements database (0.5–4.1% depending on individual). In turn, 41% of the RADseq loci mapped to available transcriptomes of related species, indicating that our data is enriched for euchromatic regions. Altogether, 1,097 high-quality, polymorphic RADseq loci, containing 3,401 SNPs, have been retained for further analyses. Our metagenomic analyses on the non-plant RADseq loci (i.e., distinguished on the base of coverage depth) demonstrate significantly divergent biotic conditions, in particular with regard to bacteria and fungi on the leaves of plants in the localities of *H. pusillum* and *H. veselskyi* (Trucchi et al. 2016b).

Genetic relatedness was governed by geographic proximity, but not by environment, as populations clustered by population pair rather than by species (Fig. 1). There was no support for a grouping according to the two species (e.g., Mantel test ecological vs. genetic distance, $p = 0.89$), and isolation-by-distance models were confirmed for each species separately, as well as for the global sampling. This indicates that the two taxa should be best treated as ecotypes, a result confirmed by the lack of reproductive isolation uncovered by crossing experiments (Fig. 10). For the sake of simplicity, however, we treat them as species throughout this report. Several phylogenomic and population genetic analyses gave evidence of at least five independent events of divergence between *H. pusillum* and *H. veselskyi* (e.g., Fig. 1; Malinsky *et al.* 2016; Trucchi *et al.* 2016b) that happened in different time horizons during the last 15,000 years. At the global level, the differentiation among *H. veselskyi* populations was slightly stronger than among those of *H. pusillum*. Our demographic inference was able to reject a model of divergence with no migration in all population. For two population pairs (1 and 5) the isolation with migration scenario obtained the highest goodness-of-fit, whereas in the rest of population pairs distinguishing between isolation with migration and secondary contact was impossible. No evidence of admixture was detected between the two species at localities 5 and 6, whereas weak signals of gene flow were uncovered in the four other localities. However, in all populations pairs the migration rates estimated were lower than one individual per generation (Trucchi *et al.* 2016b), with the exception of locality 4, where the migration rate from *H. veselskyi* to *H. pusillum* was 1.6.

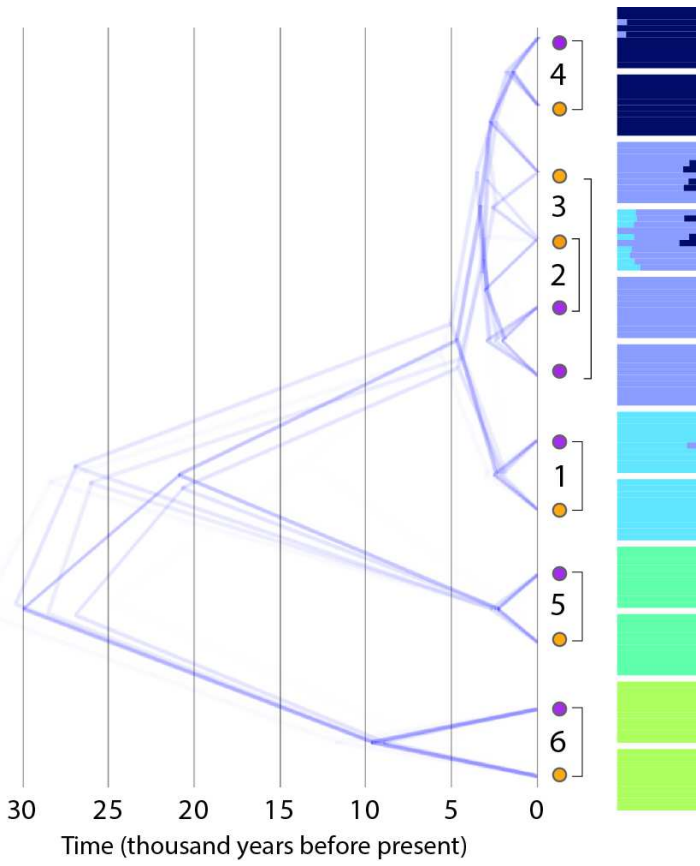

Figure 1. Time-calibrated SNAPP coalescent tree and Bayesian *k*-means results. All alternative topologies for the population tree are shown. Bar plots show inferred proportion of ancestry relative to five clusters as obtained with fastStructure. Colours identifying each cluster are randomly assigned. *Heliosperma pusillum* and *H. veselskyi* populations are shown as orange and purple filled circles, respectively. Modified after Trucchi et al. (2016b).

Analysing the six population pairs separately, we found between 79 and 116 loci with an extreme level of differentiation (expressed by the fixation index, F_{ST}). With a few exceptions, the number of highly divergent loci shared among different population pairs, however, was not different from random expectations (Fig. 2). The lack of sharing of outliers between different localities indicates that drift and/or local adaptation strongly shape the structure of populations in this group. A haplotype-based Bayesian search for loci under selection suggested one candidate RAD locus (Fig. 13), albeit with a probability slightly exceeding the significance threshold. This locus was annotated as a putative E3 ubiquitin ligase.

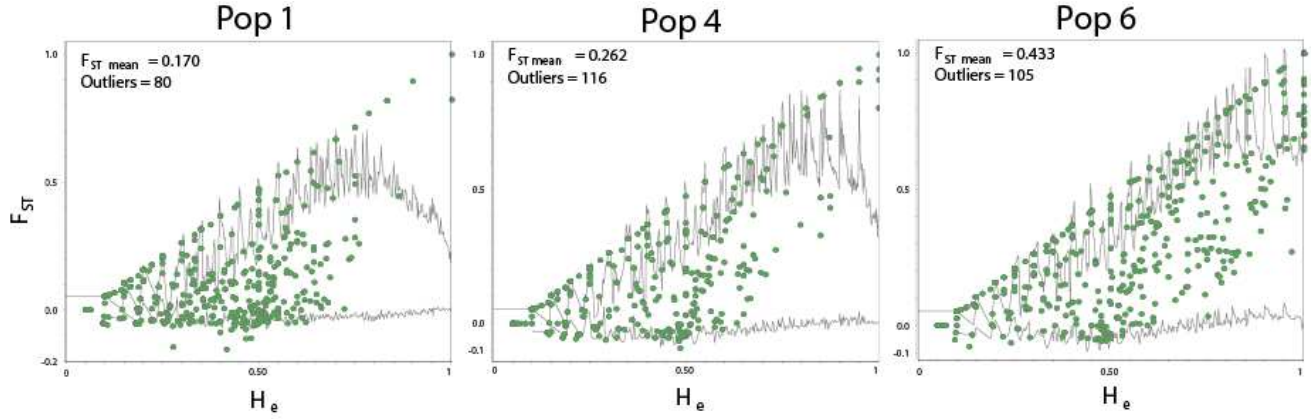

Figure 2. Example of highly divergent loci across three pairs of populations of *H. pusillum* and *H. veselskyi.* Joint distribution of genetic differentiation (F_{ST}) and expected heterozygosity (H_e) across all loci (i.e. haplotypes) in each population pair. The average F_{ST} and the number of highly divergent loci in each species pair are indicated. Green dots: observed loci; gray lines: upper and lower bounds of the joint distribution of F_{ST} and H_e estimated by 500,000 coalescence-based simulations. Modified after Trucchi *et al.* submitted.

Exploration of DNA methylation – bsRADseq (WP2)

Milestones
1. The extent of cytosine methylation, which is one of the most frequent and important epigenetic modifications, is determined
2. The frequency/diversity of epialleles is determined

All our sequenced bsRADseq libraries appeared consistently converted by the sodium bisulfite treatment. Using technical replicates we were able to assess a high level of reproducibility of the methylation assay, ranging from 93 to 95% (Trucchi et al. 2016a). The mean efficiency of mapping to the reduced reference genome built by concatenating ~1,710 RADseq loci was 21.5% with an average number of mapping reads per sample of ~617,000. The relatively low mapping efficiency is a consequence of the selection of loci applied when assembling the reduced-representation genome.

Across all samples, the average methylation of the screened cytosines in CpG, CHG and CHH context was 74%, 1.8% and 1.1% respectively (Trucchi *et al.* 2016a). After filtering for minimum coverage and excluding all positions, where a sequence context change occurred in at least one individual, we compared the average methylation level by position between *H. pusillum* and *H. veselskyi* in 2,855 CpG, 5,555 CHG and 18,427 CHH positions (Fig. 3). Surprisingly, we found a generally conserved pattern of methylation across all samples, independent of the native habitat type. A slightly higher variance in the methylation level was found in CpG methylation, probably due to a much higher methylation level in this context.

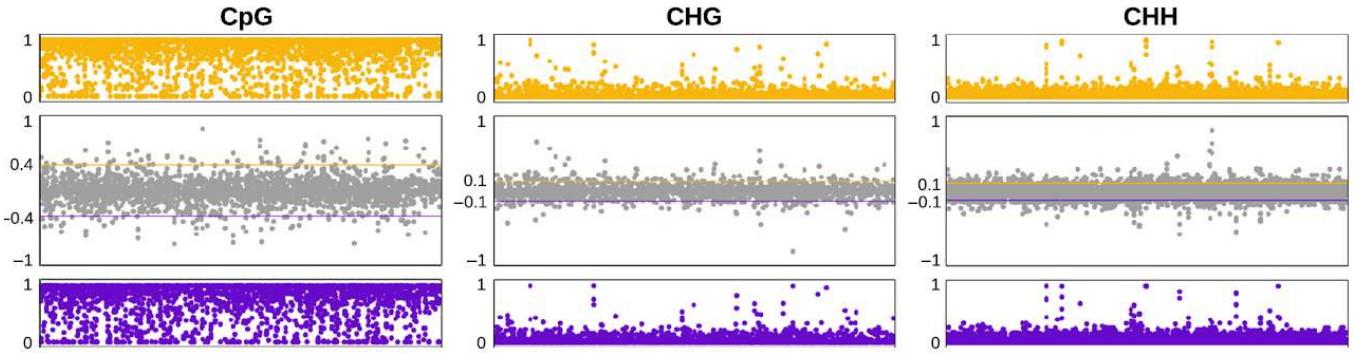

Figure 3. Differential methylation between the two *Heliosperma* species, given separately for each methylation context. 2,855, 5,555 and 18,427 cytosine positions were assessed in the CpG, CHG and CHH contexts respectively. The top panels (orange) show the average methylation of *H. pusillum*, the bottom panels (blue) show the average methylation of *H. veselskyi*. The middle panels (grey) show the methylation difference between the two species, where a value of 1 indicates complete methylation in *H. pusillum* and complete lack of methylation in *H. veselskyi*, while a value of -1 indicates the opposite. The lines in the middle panels indicate the 95% quantile. Modified after Trucchi *et al.* 2016a.

Several outlier positions showing a signal of differential methylation between the two species could be identified in this context. We compared the distributions of the methylation as recorded across individuals in each population pair by a two-sample Kolmogorov-Smirnov test. A total of 212 cytosine positions occurring on 156 RAD loci, across all contexts, showed a *p*-value < 0.05 and 17 positions,

occurring in 15 different RAD loci, showed a *q*-value < 0.05. Several cytosine positions co-occurring on the same locus showed a similar significant signal of divergence in different population pairs (Fig. 4). Blasting each locus sequence to the NCBI nt database and to the *Silene vulgaris* transcriptome, it was possible to annotate 72 of the 156 loci including cytosine positions with significant *p*-value and six of the 15 loci including positions with significant *q*-values. Considering the latter case, among the suggested genes we found a galactose-oxidase and a few proteins with binding or catalytic activity. The high proportion of positions mapping to genes might be due to the general enrichment in coding regions by the RADseq restriction enzyme.

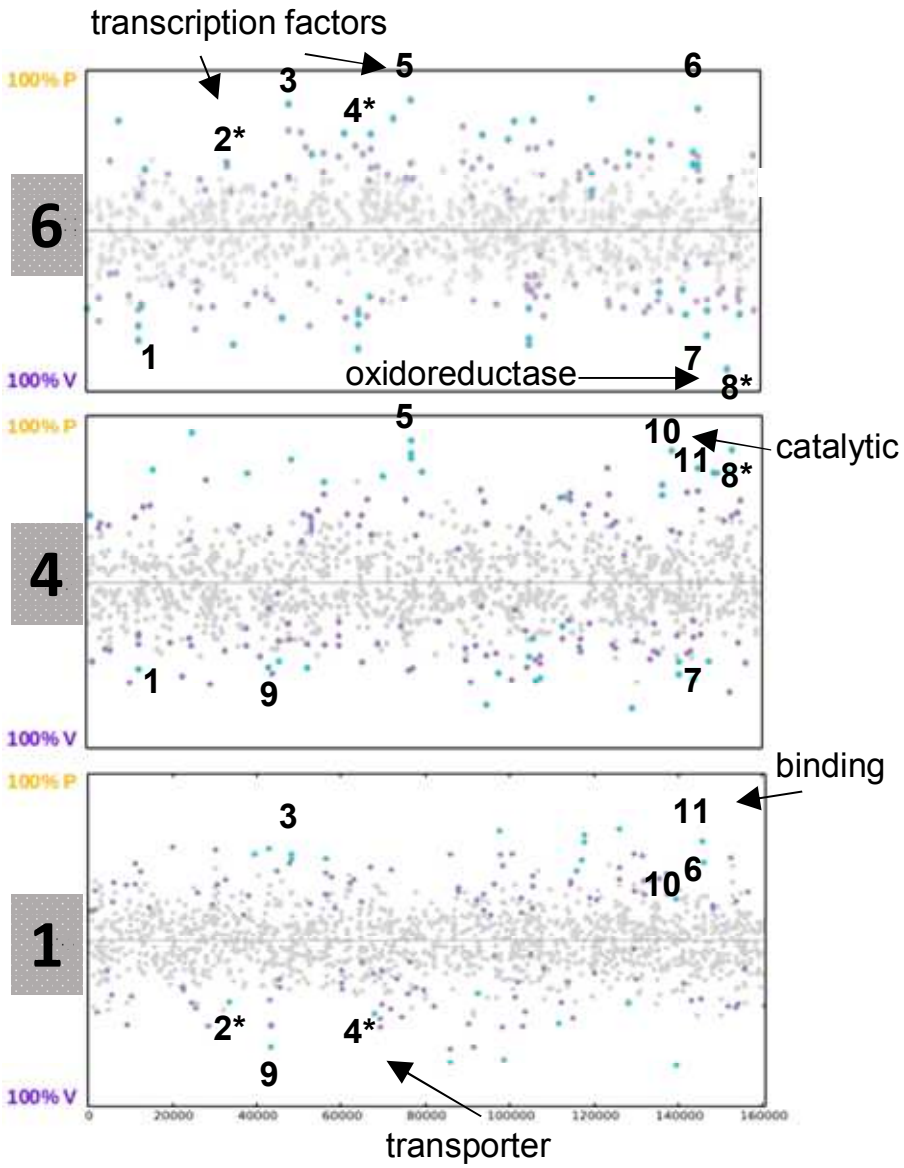

Figure 4. Examples of differential CpG methylation between the two *Heliosperma* species at three different localities. The purple dots indicate outliers with $p < 0.05$, the turquois dots outliers with $q < 0.05$. The numbers from 1 to 11 indicate shared outliers in different population pairs. Successful annotations are also indicated.

***Detailed analyses of relevant (epi)loci* (*WP3*)**

Milestones
1. Variation and traces of selection in candidate epi-/genetic loci identified in *WP1* (RADseq) and *WP2* (bsRADseq) are established
2. The stability of eventual epigenetic variation in different environments as well as its heritability is determined

The only identified genetic outlier locus (Fig. 13) featured five SNPs, but only one non-synonymous substitution with a non-random segregation among populations and species (Fig. 5). This SNP is in a first codon-position changing the amino acid from Glutamic acid in most individuals of *H. pusillum* to Aspartic acid in most individuals of *H. veselskyi*. The locus was retrieved in the RADseq data from only 43 individuals out of 120 which were in total analysed. Sanger-sequencing of this locus using primers designed out of the coding regions was used to confirm the unique amplification of this locus in 30 randomly selected individuals (Fig. 5). Primers designed within the exonic region, in contrast, produced double amplifications: sequences of this locus were found together with a set of sequences of a putative paralog (Fig. 5). The paralog locus was sequenced in 30 individuals and had 12 substitutions in the 94 base pairs of the single-end RAD locus plus a 3-base pairs insertion. One substitution was in the SbfI restriction site thus preventing this paralog to be present in our RAD dataset. Nevertheless, both alleles found at this paralog locus seemed functional (no stop codon in the amplified region in the same reading frame) and mapped to the same region of the *S. vulgaris* transcriptome. Orthologous alleles of our target locus were grouped in three major clusters: one including only *H. veselskyi* from population pairs 1, 2, 3; one including only *H. pusillum* from population pairs 2, 4 and 6; one including *H. pusillum* from population pairs 1, 2, 3, and 4, and *H. veselskyi* from population pairs 1 and 2.

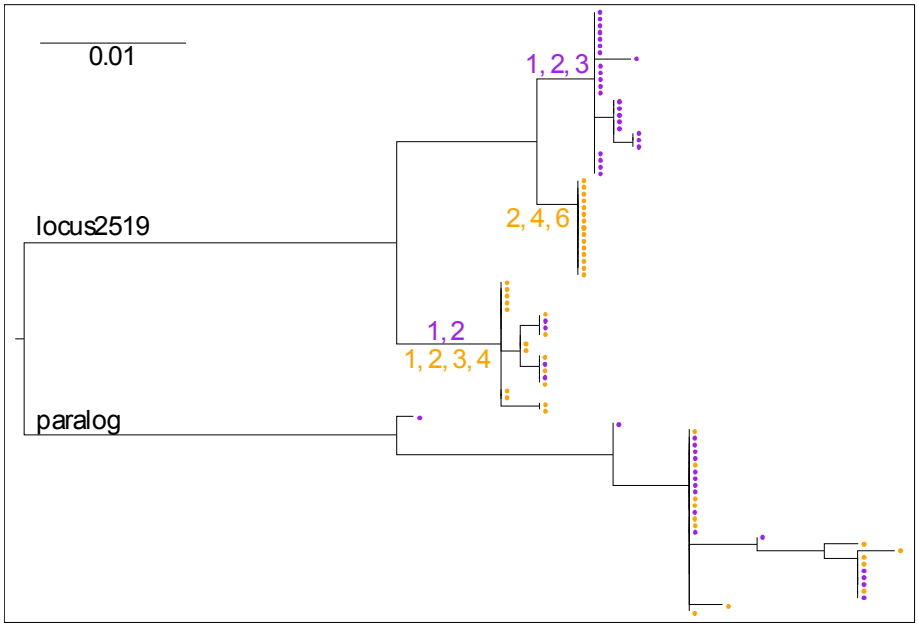

Figure 5. Maximum-likelihood tree of all Sanger sequences obtained from *Heliosperma pusillum* (orange) and *H. veselskyi* (purple) using different primer pairs of the putative outlier locus 2519 identified by Bayescan (a U3-Ubiquitin ligase enzyme) and its paralog. Modified after Trucchi *et al.* (2016b).

For the DNA methylation data, all loci have been analysed across the 120 individuals and several outliers have been identified in the comparisons of each population pair of the two species. Several consistent patterns across two such pairs of populations have been identified and annotated (Fig. 4), but none in more than two such pairs, indicating that most epigenetic divergence is driven either by drift or by adaptation to local conditions, specific to the respective locality. The heritability of DNA methylation patterns have been followed across three generations of selfed plants (see Fig. 6 for two examples), starting from three lines for each of the species. Selfing (i.e., uniparental reproduction) was performed to enable an easier estimation of heritability. The heritability level uncovered was relatively high, but was differentiated according to context and to starting DNA methylation status (Fig. 6). Non-methylation was faithfully inherited in CHH and CHG context (greater than 99% heritability), followed by methylation at CpG positions (75–80% heritability), and non-methylation at CpG sites (55–65% heritability). Methylation at CHH and CHG contexts was highly instable, with more than 50% of the sites in this category losing completely their methylation. Our data, however, is enriched for genes, hence it is representative for a particular genomic portion. In addition, it is unclear how significant the contribution of selfing (i.e., increased homozygosity and unmasking of deleterious alleles) to these patterns is.

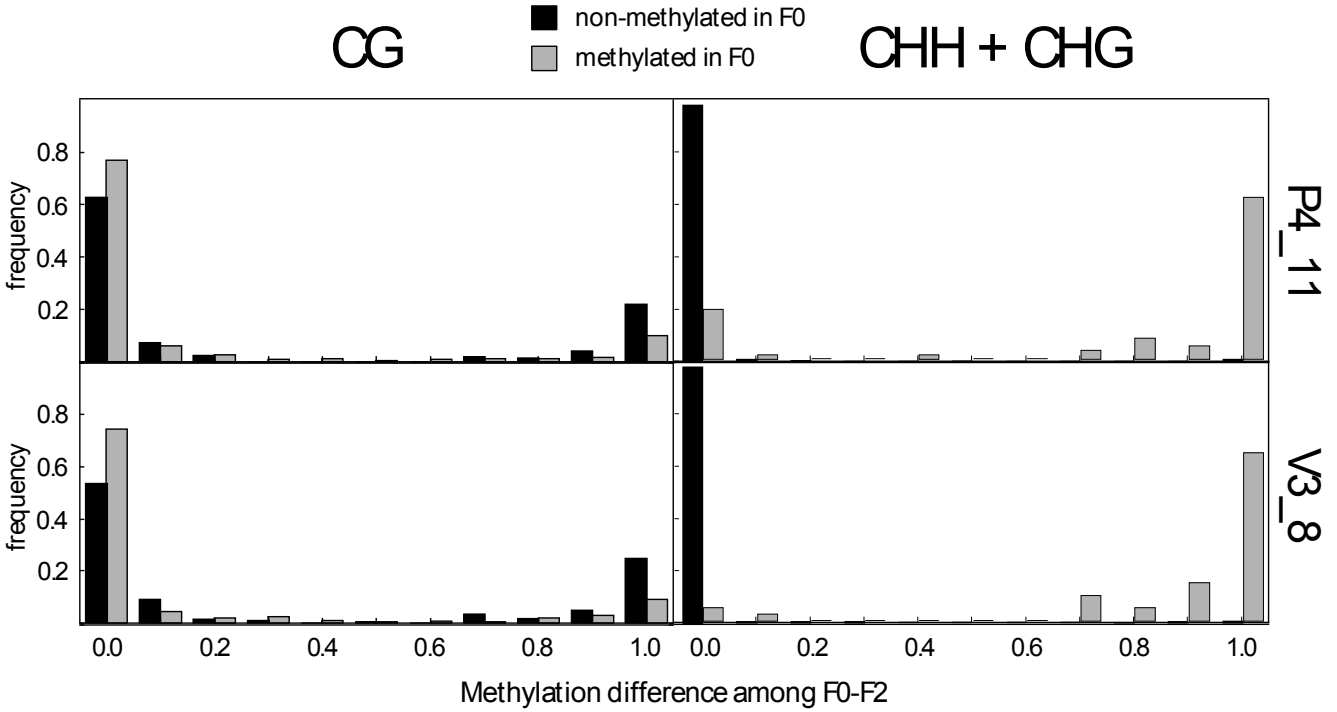

Figure 6. Level of methylation across CpG and CHH/CHG positions in F2 progeny, compared to the respective F0. Here one example each is given from the three F0 lines of *H. pusillum* (upper panels) and *H. veselskyi* (lower panels), respectively. We first selected only the cytosine positions which were covered by bisulfite-converted reads in the F0 individual (50 < coverage < 300). We then filtered these positions to be covered in at least one of its F2 individuals and we estimated the range of differentiation in the methylation level across all F0 and F2 individuals. We analysed the methylated (% of reads supporting the methylated state > 0.65) and the unmethylated (% of reads supporting the methylated state < 0.35) F0 positions separately. The proportion of cytosines across the whole range of differentiation (between 0 and 1) is reported (Trucchi *et al*. in preparation).

Finally, we investigated the environmental sensitivity of DNA methylation with reciprocal transplantations. We observed that *H. pusillum* plants increased (by more than 0.5) their methylation level at a typical *H. veselskyi* locality at 10% of the CpG sites, whereas it demethylated 8.2% of the sites (altogether 18.2% methylation changes). *H. veselskyi* methylated 8.8% of its sites at the *H. pusillum* locality, whereas it demethylated 8.9% of its CpG sites (altogether 17.6% methylation changes). Altogether, we observed a high impact of the environment in altering DNA methylation, but the magnitude of alterations observed does not exceed the general lability of DNA methylation observed when estimating its heritability in a 'constant' environment.

Synthesis and publication (WP4)

Milestones

1* *[added milestone, for details see 2.2.5 below]* Detailed ecological and phenotypic (morphological, anatomical and ecophysiological) as well as cross-compatibility data allow to solidly interpret the genetic data in a climatic context.

1. A synthesis of the various data describing extent, stability and heritability of epigenetic variation is reached. The ability of plants to react to climate change with rapid, epigenetic modification is unravelled.
2. Peer-reviewed publications and publications intended to inform conservation managers are written.

In the following the ecological and phenotypic (morphological, anatomical and ecophysiological) as well as cross-compatibility data sets are shortly summarised and major results are highlighted.

Ecology: We observed a discrete delimitation into two main environmental regimes following the species boundary across all population pairs as well as within population pairs (Fig. 7ab) – which corroborates the hypothesis that divergent selection pressure, acting in the same direction at several locations drives the parallel differentiation of both species. Major environmental conditions underlying the separation are soil moisture, irradiance, and temperature, varying uniformly among all population pairs, and thus representing possible selective forces. In all regions, populations of *H. veselskyi* are characterised by a higher thermal input, a longer vegetation period, much lower soil water availability and reduced irradiance (evidenced also by both temperature measurements and indicator values).

Morphology: We observed discrete morphological differentiation accompanying environmental divergence, which separated the populations following the species boundary consistently in all regions (Fig. 7cd). This indicates that the multiple differentiation is directional; it is most likely caused by a similar divergence in selection regimes. In contrast, differentiation of populations by neutral processes in isolated populations without environmental selection pressure is expected to lead to stochastic variation between populations evolved in parallel; a pattern not seen in our data. We observed greater morphological variance within *H. veselskyi*, most likely mirroring the parallel, independent evolution of its populations. Finally, the environmental differentiation correlated significantly with morphological, but not with the genetic differentiation seen in the RADseq data, supporting the hypothesis that natural selection influences population differentiation to a greater extent than genetic drift.

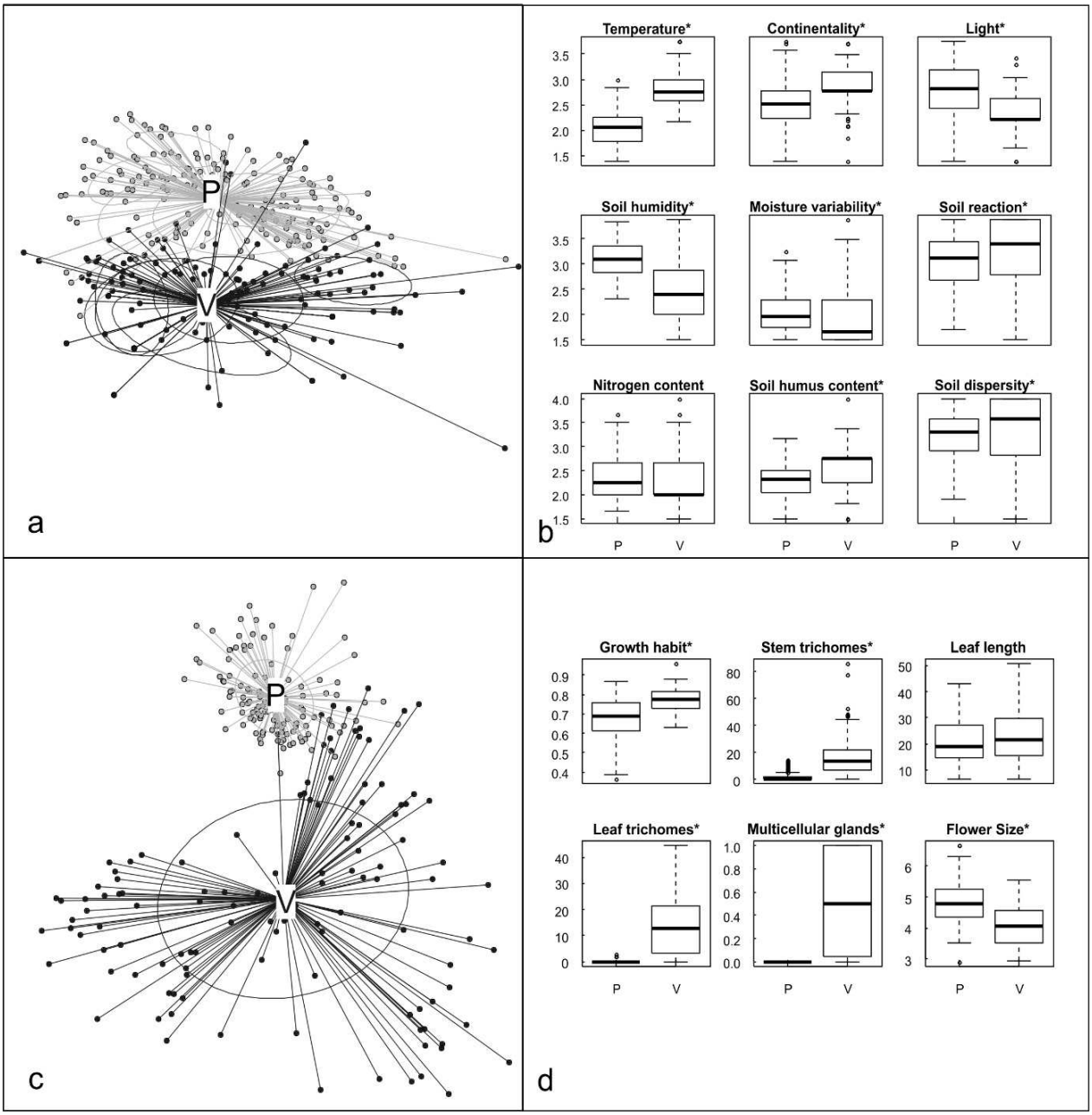

Figure 7. Ecological (a-b) and morphological (c-d) differentiation of *Heliosperma pusillum* (grey; 180 and 121 individuals) and *H. veselskyi* (black; 173 and 120 individuals) as derived from two-dimensional non-metric multidimensional scaling (nMDS, a, c). The nMDSs were based on dissimilarity matrices of mean Landolt indicator values of plant species growing within a circular area 0.2 m radius centred on *Heliosperma* individuals (a, b) and 38 morphological characters (c, d). using Bray-Curtis distances and applying 9999 permutations. Kruskal stress value was 0.140 and 0.163 for the NMDS of Landolt indicator values and morphological characters, respectively. Confidence ellipses are defined by the centroid and the standard deviation of individuals pooled for each species (a, c). Lines connect individuals to species centroids and centroids of populations within population pairs (indicated by numbers) in the left and right panels, respectively. Differences in single mean Landolt indicator (b) and aggregated morphological traits (d). Significant differences (α=0.05) are indicated by asterisks. The box plots show the medians well as the 25 and 75 percentiles. Values outside 1.5 × interquartile ranges are illustrated as outliers. Modified from Bertel et al. (unpubl.).

Adaptation: Establishment during two years revealed strong evidence of adaptation for both species, as the local species had a higher establishment than the foreign species at both growing sites (Fig. 8). Since we did not find any sign of adaptation in traits characterising germination, size or growth velocity, we conclude that other phenotypic traits are altered in both species – probably as a result of natural selection – and allow successfully establishment at the home site. Experiments further suggested that water availability, annual variation of environmental conditions and herbivore pressure likely differ between habitats and differentially affect the establishment of individuals of both species.

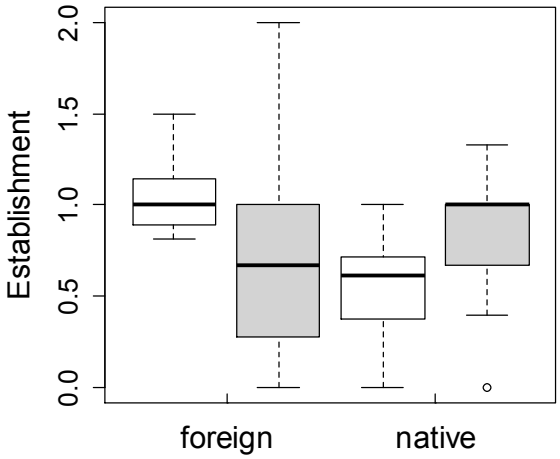

Figure 8. Establishment of *Heliosperma pusillum* (white boxes) and *H. veselskyi* (grey boxes) under foreign and native habitat conditions derived from reciprocal transplantations in natural populations (population pair 4) Box plots show median as well as 25 and 75 percentiles. Values outside 1.5 × interquartile ranges are illustrated as outliers. Modified from Bertel et al. (unpubl.).

Divergence in ecophysiology and leaf anatomy (Fig. 9): Investigations were conducted with plants from natural growing sites and plants grown from seeds in a common garden to disentangle plastic and heritable components of trait expression. Whereas trait values in natural populations are shaped by both environmental influences during development and an individual's (epi-)genetic constitution, traits measured in the common garden allow for estimating the (epi-)genetically based differentiation of species under uniform environmental conditions. The anatomical and eco-physiological traits mirrored the environmental divergence between natural habitats suggesting that different anatomical and functional traits are favoured at the particular growing sites: In leaf anatomy higher irradiance of the alpine growing site of *H. pusillum* were accompanied by higher thickness of leaves and palisade layers. The higher stomatal area index reflected the better water availability of the alpine sites (Bertel et al. in press). Similarly, photosynthetic light and temperature responses mirrored different irradiance and daily minimum temperatures. In the common garden, both species adjusted their anatomical and functional traits plastically in response to the shift in environmental conditions. However, traits differed between both species, suggesting that the differentiation between the two species is not solely based on phenotypic plasticity but also has an (epi-)genetic basis. In summary, early-stage speciation between *H. pusillum* and *H. veselskyi* is probably environmentally induced, as the close connection between trait values and microclimatic conditions suggests that functional differentiation is adaptive.

Reproductive isolation: There was no evidence of intrinsic reproductive barriers since fitness parameters measured under uniform conditions were not lower in inter- than in intraspecific crosses

(Fig. 10). Moreover, morphometric analyses of the offspring clearly showed that the differentiation of the parental species is heritable, likely adaptive, and the intermediate morphology of hybrids may lead to reduced hybrid fitness in parental habitats. Reproductive isolation is thus caused by extrinsic factors, most likely distance-dependent reduction in gene flow resulting from genetically fixed ecological differences and adaptation to distinct habitat conditions via pre- and postzygozic fitness disadvantages of immigrants and hybrids, respectively. As reproductive isolation is solely based on environmentally, extrinsic barriers, it may be sensitive to environmental changes and subsequent vegetation shifts; only completion of reproductive isolation by endogenous mechanisms may on the long term ensure the stability of the diverging species.

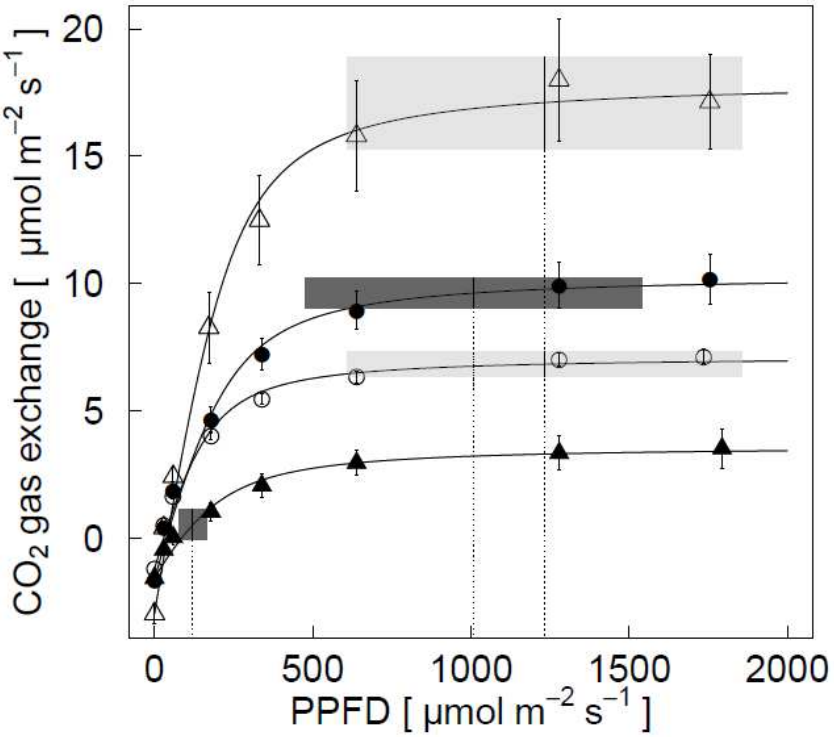

Figure 9. Net photosynthetic light response obtained on leaves of *Heliosperma pusillum* (circles) and *H. veselskyi* (triangles), grown at the natural growing sites (open symbols, PN and VN) or in a common garden (filled symbols, PG and VG). Vertical lines inside boxes represent mean values of daily maximum PPFD of the season, the box length (natural growing sites, light grey; common garden, dark grey) indicates the standard deviation of mean maximum PPFD, whereas the vertical position of the box indicates photosynthetic rates at mean daily maximum PPFD and its height the respective standard errors. Modified from Bertel et al. (2016a).

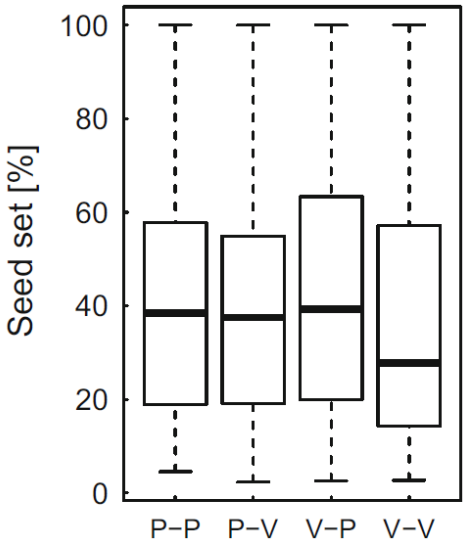

Figure 10. Fitness parameters of offspring derived from intra- and interspecific cross-pollinations of *Heliosperma pusillum* (P) und *H. veselskyi* (V). The first letter indicates the pollen recipient. Seed set represents the proportion of fully developed seeds per capsule. Boxplots show the median and the 25/75 percentiles. Whiskers are 1.5 times interquartile ranges, values outside are indicated as outliers. Modified from Bertel et al. (2016b).

Synthesis

Climate models suggest that global change will occur mainly through changes in temperature and precipitation regimes. The divergent growing sites of our study plants can serve as a proxy for forthcoming changes. Specifically, the montane *H. veselskyi* grows in habitats, which on average exhibit a 3.73 ± 0.11 °C higher daily mean air temperature (Bertel et al. unpubl.) and which are much drier than those of the alpine *H. pusillum*. Our study was thus well conceived to unravel the impact of global change in mountain areas on the evolutionary potential of species via epigenetic change, with a particular focus on adaptation to enhanced temperatures and decreased precipitation. The availability of ecological data allowed to place the patterns of cytosine methylation modification into the context of a warming climate.

Our project was highly exploratory, including developing methodologies and analytical pipelines for population level investigations of DNA methylation levels across wild populations in a non-model system. **Initial expectations at the start of the project** (following also the knowledge in the available literature at the start of the project) were that **A.** the populations of montane *H. veselskyi* have formed 2–3 times from populations of alpine *H. pusillum*; **B.** due to significantly divergent environment parameters at the localities of *H. pusillum* and *H. veselskyi*, large-scale differences in DNA methylation patterns will be observed between the two species; **C.** some of these methylation patterns have formed as a rapid, but stochastic response to climatic stress (i.e., warmer, drier habitats with temperature in a similar range as the predicted climatic changes in the next decades) and have been selected by the environment; and **D.** due to similar selection pressures, similar DNA methylation alterations will be observed in independently diverged populations of *H. veselskyi*.

Our results, however, contradicted most of these hypotheses. **A.** Within the six population pairs we uncovered as much as five independent origins of *H. veselskyi* from *H. pusillum*, suggesting that adaptation to the montane localities is frequent and relatively easily achievable. These divergence events are scattered between 15,000 and 500 years ago and happened in the presence of some gene flow; however the genomic divergence appears largely shaped by drift. **B.** The uncovered methylation patterns are as expected strongly divergent in different sequence contexts (i.e., between CpG, CHH and CHG), but they are surprisingly stable between *H. veselskyi* and *H. pusillum*, with only limited and punctual epigenetic divergence between them. This may indicate unexpectedly strong purifying selection acting on DNA methylation in a genomic portion mostly in or around genes (see also Neri *et al*. 2017). **C.** Whereas we identified a significant environmental sensitivity of DNA methylation affecting ca. 20% of CpG positions (again around genes), this does not exceed the lability of DNA methylation in a stable environment. Therefore, **in the *Heliosperma* system environmental reset of DNA methylation does not appear as a one-generation, large-scale response to environmental change**. However, localized responses that may reside outside the targeted genomic region or multi-generation alterations may still play an important role towards adaptation to different conditions, whereas our analyses may have also missed particularly episodic selection pressures, such as herbivory. **D.** Only limited DNA methylation differences between the two species have been observed in each population pair, and very few of those are consistent when analysing different population pairs. This indicates that local selection may play a role in shaping DNA methylation patterns or the genetic loci on which such epigenetic signals may depend.

Publications: On the outreach side, **37 contributions to international and national conferences** have been presented and **nine publications intended for publication in peer-reviewed scientific journals** have been drafted within this work package (**four already published**).

5 Schlussfolgerungen und Empfehlungen / 2.3 Conclusions to be drawn

Taken together and viewed from the perspective of climatic change, our results indicate that the likely climate-driven evolution of the disjunctly distributed mountain plant *H. veselskyi* – a morphologically, anatomically, ecophysiologically and ecologically divergent, polytopically evolved montane descendent of widespread alpine *H. pusillum* – was rapid and took place in the Holocene. On the other hand, our data provide limited evidence for genome-wide epigenetic alterations – in our case cytosine methylation – as a response to changed environmental conditions. In the *Heliosperma* system and in our reduced representation – but still based on genome-wide data – DNA methylation appears to have more a constitutive role, being generally conserved between different localities and environments. This in turn indicates that based on our results plants may have only limited means to adapt epigenetically to ongoing anthropogenic climate change, rendering "conventional" climate protection more necessary than ever.

More specifically, our results contradicted many of our initial hypotheses, which were based on the state of knowledge at the time the project application was conceived. **A.** For instance, within the six population pairs we uncovered as much as five independent origins of *H. veselskyi* from *H. pusillum*, suggesting that adaptation to the montane localities is frequent and relatively easily achievable. These divergence events are scattered between 15,000 and 500 years ago and happened in the presence of limited gene flow; however the genomic divergence appears largely shaped by drift. **B.** The uncovered methylation patterns are as expected strongly divergent in different sequence contexts (i.e., between CpG, CHH and CHG), but they are surprisingly stable between *H. veselskyi* and *H. pusillum*, with only limited and punctual epigenetic divergence between them. This may indicate unexpectedly strong purifying selection acting on DNA methylation in a genomic portion mostly in or around genes. **C.** Whereas we identified a significant environmental sensitivity of DNA methylation affecting ca. 20% of CpG positions (again around genic regions which represented our targeted genome representation), this does not exceed the lability of DNA methylation in a stable environment. Therefore, in the *Heliosperma* system environmental reset of DNA methylation does not appear as a one-generation, large-scale response to environmental change. However, localized responses that may reside outside the targeted genomic region or multi-generation alterations may still play an important role towards adaptation to different conditions, whereas our analyses may have also missed particularly episodic selection pressures, such as herbivory. **D.** Only limited DNA methylation differences between the two species have been observed in each population pair, of which few are consistent across population pairs. This indicates that local selection may play a role in shaping DNA methylation patterns or the genetic loci on which such epigenetic signals may depend.

The time of local divergence estimated at the investigated localities is consistently postdating the last glaciation. Ecological divergence could have been triggered by the rapid spread of forests induced by Holocene warming, separating montane stands below overhanging cliffs from alpine populations. Globally, the structure among the populations of the two species reflects a clear isolation-by-distance pattern. This may be related to the limited time elapsed since the colonization of recently de-glaciated mountains or to gene flow maintaining genetic contiguity. Indeed, whereas some traces of admixture or

incomplete lineage sorting between the *H. pusillum* populations are visible across some localities, the *H. veselskyi* populations seem more isolated from each other. This can be a consequence of the insular, disjunct habitat preferred by the montane *H. veselskyi*, whereas the populations of *H. pusillum* are more continuously distributed.

Our approach uncovered only limited shared molecular differentiation at different population pairs of the two species, that could be explained by independent, different genetic and/or epigenetic mutations occurring in or around the same gene, or polygenic traits underlying ecological adaptations. The short divergence time estimated in each *Heliosperma* population pair renders a significant contribution of novel genetic mutations unlikely. Hence, repetitive divergence from standing molecular variation appears more probable (i.e. collateral evolution by shared ancestry). However, as adaptive traits are likely polygenic, the signature of selection may be difficult to detect unless more advanced analytical approaches, not applicable on the data type at hand, are used. For example, the short RADseq loci, which formed our reference for epigenetic investigations, allowed us to work only with single methylation polymorphism (SMPs) and did not allow calling any differentially methylated regions (DMRs) likely important for trait divergence.

More importantly, the reduced representation of our RADseq dataset screened only a limited portion of the genome (0.1%) and of the genes (estimated 3.5%). It is thus very likely that any adaptive locus, or neutral loci linked to it, was not covered in our genome scan. In addition, if selection is acting on standing variation present in the ancestral population, the signature of divergence around loci under selection is expected to be minimal. These aspects could then exacerbate the difficulties in finding adaptive loci.

Different demographic processes such as multiple divergence events and/or independent secondary contacts, likely characterize the evolutionary history of sister species pairs adapted to divergent conditions at medium to large geographical scales. At the local scale and if effective population size is small, drift can largely overwhelm the marks left by selection. In addition, different instances of local divergence could be affected by a diverse range of biotic (e.g. pathogen load) and abiotic (e.g. microclimatic conditions) factors of local selection.

Recent genomic investigations suggested parallel ecological divergence as common to several organisms. The results of our project confirm with solid data that the alpine-mountain group of *Heliosperma pusillum* s.l. is a highly relevant system for detailed investigations of the adaptive and convergent processes underpinning rapid phenotypic responses to new environments. In particular the repeated nature of adaptation to warmer and drier habitats in *Heliosperma*, which also happened at different time points in the Holocene, offers natural replicates to study in detail the mechanisms enabling organisms to rapidly cope with environmental stressors. Our molecular, environmental, morphological and eco-physiological data brought about important background information, but the genomic coverage was still insufficient to pinpoint the exact molecular targets of natural selection in this system. The obtained results form the basis of several publications and of the PhD thesis of Clara Bertel, the PhD student financed by the ACRP project (thesis to be submitted for review in due course – anticipated in May 2017).

Based on the results obtained in the ACRP project, a follow-up project (for the PhD student Aglaia Szukala, who started in Oct 2016) is already funded through the Vienna doctoral school of population genetics (www.popgen-vienna.at) and will investigate whole transcriptomes of the two species in a common garden and across transplantations. The transcriptomic data will provide information on both regulatory changes and any potential coding sequence variation that may be important for the recurrent evolution of montane plants from alpine populations. In a future project to be submitted to FWF, we will further complement the transcriptomic data with whole genome resequencing and whole genome bisulfite sequencing, taking advantage of the technological developments in throughput of next generation sequencing. For this aim efforts are currently under way to assemble a reference genome for *Heliosperma pusillum* based on a combination of Illumina HiSeq and PacBio reads.

Altogether, this plan of research will make *Heliosperma pusillum* a model system for evolutionary biology and will specifically pinpoint the molecular components underlying this iterative ecological divergence and to fully understand the evolutionary forces driving it. This will ultimately allow for a comprehensive understanding of the interplay between environmentally-sensitive molecular components, such as epigenetic regulation and transposable elements, and the more stable genomic components, such as coding DNA sequences.

C) Projektdetails

6 Methodik / Material and methods

Plant material. *N.B. The original plan was to investigate only three population pairs in WP1 and WP2 and to use locus-by-locus approaches (WP3) to cover the remaining population pairs up to a total of six. Deviating from this plan (and outlined in the First Interim Report) we subjected all six population pairs (120 individuals) to the NGS methodology, therefore making much more diffuse the distinction of WP1–WP3. The initial plan was impaired by external events and, given the rapid developments in the field, appeared also to compromise the possibility of publishing all results in high-ranking journals.*

Our sampling included six pairs of geographically close populations of the higher elevation *H. pusillum* and the lower elevation *H. veselskyi* in the south-eastern Alps (Fig. 11): **1**. Grödental/Val Gardena (Dolomiten/Dolomiti, South Tyrol, Italy); **2**. Höhlensteintal (Dolomiten/Dolomiti, South Tyrol, Italy); **3**. Anetwände (Lienzer Dolomiten, Eastern Tyrol, Austria); **4**. Val Cimoliana (Alpi Carniche, Friuli, Italy); **5**. Sella Nevea (Alpi Giulie, Friuli, Italy); **6**. Idrijca valley (Primorska, Slovenia). In all populations data logger (TidbiT) were installed that monitor the temperature in order to provide the background abiotic divergence between the habitats of *H. pusillum* and *H. veselskyi*. In each population, leaf material from ten individuals of each species dried in silica gel was used for molecular analyses of the genetic and epigenetic variation (*WP1–3*); we aimed to collect leaves at the same developmental stage to avoid any developmentally related variation in DNA methylation patterns. Representative parts of each of these 120 individuals have been conserved as herbarium specimens.

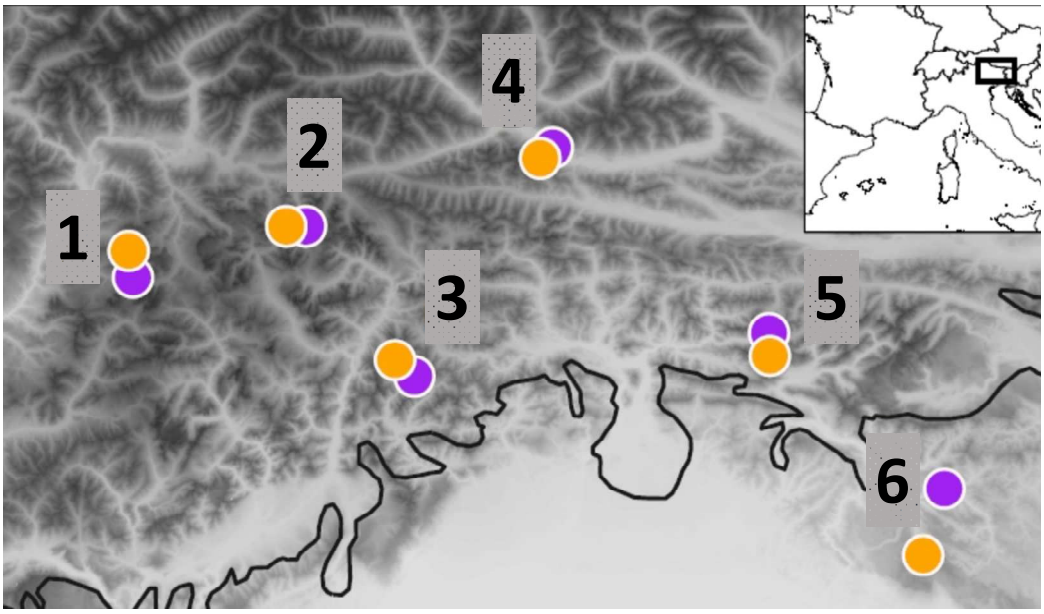

Figure 11. Map of the investigated localities (*H. pusillum*: orange; *H. veselskyi*: purple) in the south-eastern Alps. The extension of the ice sheet during the Last Glacial Maximum is shown with a solid black line. The insert shows the position of the sampling area in Europe. Modified after Trucchi *et al.* (2016b).

Genetic patterns of differentiation – RADseq (WP1). Using genome-wide SNPs produced with restriction site associated DNA sequencing (RADseq), we investigated the patterns of genomic divergence across the six pairs of neighbouring populations of *H. pusillum* and *H. veselskyi*. Single-digest RAD libraries were prepared using the restriction enzyme SbfI and a protocol adapted from Baird *et al.* (2008) with modifications. The libraries of individually barcoded samples were finally sequenced with Illumina HiSeq at VBCF (http://www.vbcf.ac.at/facilities/next-generation-sequencing/) as 100 bp paired-end reads. The raw reads were processed with Stacks to build genomic loci and call SNPs. The genomic loci were also used to create a 'synthetic' genomic reference for the epigenomic analyses (i.e., *WP2*). A metagenomic approach (using MEGAN) was applied to non-plant RADseq loci to characterize the native biotic environment of populations of *H. veselskyi* and *H. pusillum* (i.e., the so called 'phyllosphere'; Trucchi *et al.* 2016b).

We further used the SNP dataset derived from the plant RADseq loci for a combination of phylogenomic investigations (with RAxML, Treemix and SNAPP), analyses of population structure (e.g., fastStructure, fineStructure and summary statistics), demographic inferences (with fastsimcoal2) and outlier searches (e.g., Lositan and BayeScan) to investigate the number of divergence events between the two lineages, to test for parallel adaptation and to understand the processes that shaped their recent evolutionary history. We first asked if the observed genomic patterns support the hypothesis of multiple origins of the ecological divergence between *H. pusillum* and *H. veselskyi*. We then evaluated the strength of isolation by distance versus isolation by environment among localities and we investigated the extent and direction of gene flow between and within the two species. We further assessed the relative probability of three demographic models representing different historical processes behind the observed pattern of divergence: strict isolation, isolation-with-migration and secondary contact following a period of allopatric separation. We also asked if the observed phenotypic divergence resulted from a similar genomic response to similar selective pressures by searching for adaptive loci, diverging in the same direction in different population pairs. To detect signatures of selection, outlier approaches have been used, estimating the probability of each locus to be under divergent or purifying selection. Finally, annotation analyses have been performed, making use of an available transcriptome of related *Silene vulgaris* (http://silenegenomics.biology.virginia.edu/; Sloan et al. 2011).

Exploration of DNA methylation – bsRADseq (WP2). We developed bsRADseq (Trucchi *et al.* 2016a), a high-resolution method for DNA methylation quantification across hundreds of individuals. This NGS-based technique is suitable for non-model organisms and results in a reduced representation of the genome that is consistent across multiple individuals, revealing the patterns of DNA methylation at base-pair resolution across the genomic fragments represented. Sequencing in the presence of sodium bisulfite chemically converts unmethylated cytosine to uracil; thus, by comparing the sequence of treated to untreated DNA, it is possible to identify the methylation status of cytosines in the original sample (Fig. 12). BsRADseq is a reduced-representation approach, but it has the same flexibility as RADseq, where the choice of the restriction enzyme determines the proportion of the genome that will be genotyped. In addition, bsRADseq features all standard characteristics of bisulfite sequencing, including accuracy and base-pair resolution when detecting DNA methylation. This technique opens up

the opportunity for genome wide epigenetic investigations of evolutionary and ecological relevance, especially for questions that require information from a large number of individuals in species with a medium to large genome size. If a reference genome is available for the study group, the sequences can be mapped back to known positions. However, for non-model organisms without a published genome, such as *Heliosperma*, we developed a pipeline (Fig. 12) for the construction of synthetic references for mapping bisulfite converted RADseq reads using loci built from standard RADseq data (here available from *WP1*). We demonstrated the flexibility of this approach across a range of model and non-model organisms (Trucchi *et al.* 2016a), investigating the extent of epigenetic divergence between individuals that thrive in different ecological conditions. An advantage inherent to the approach we developed is that it automatically incorporates, and takes into account, the genetic differences among analysed individuals. As we mapped the bisulfite-converted reads to the actual sequences of each individual sample, we could clearly identify methylated Cs without the risk of misidentifying C → T SNPs as SMPs (single methylation polymorphism; Schmitz et al. 2013) (see Fig. 12c).

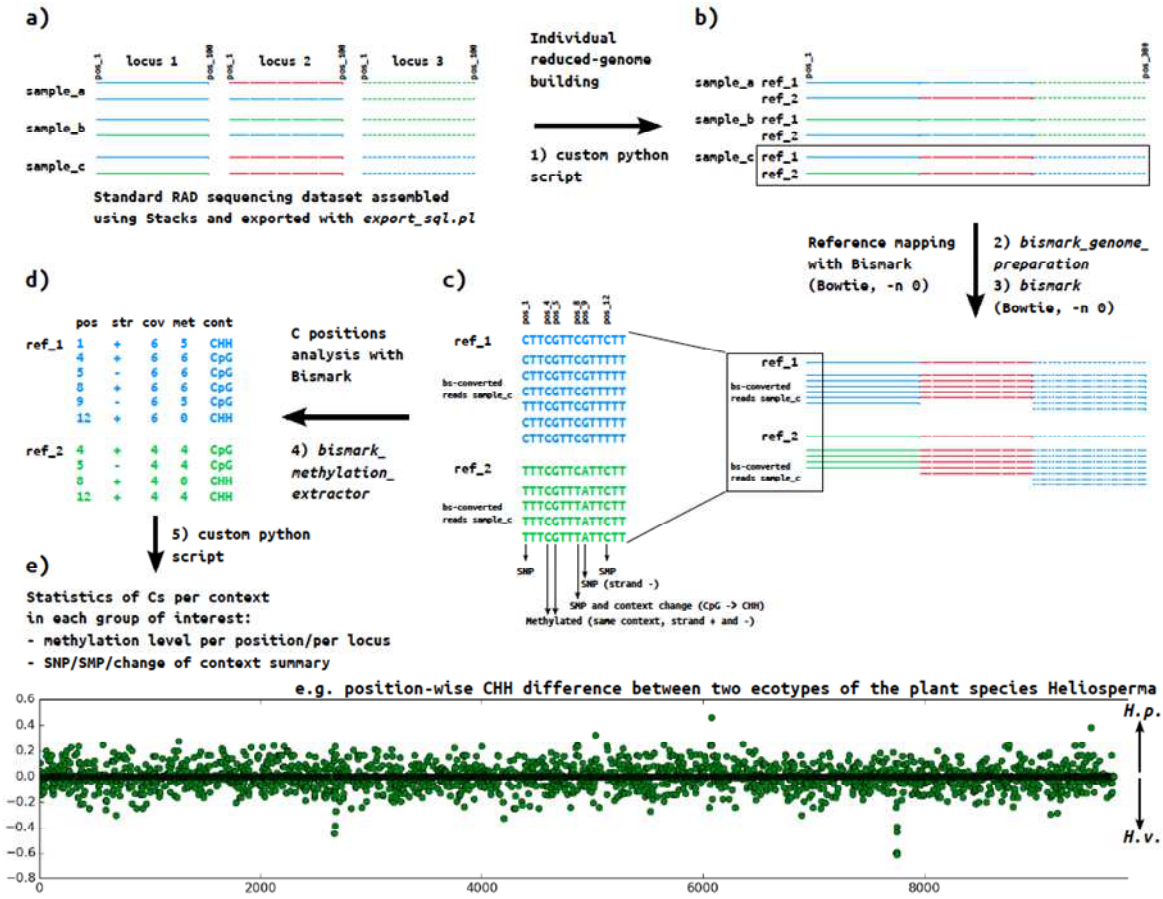

Figure 12. Workflow of the bsRADseq analysis when no reference genome is available. (a) Standard RADseq data is first assembled, and further (b) an individual-specific reduced reference genome is built. (c) Mapping the bsRADseq reads of each individual sample to its own reduced reference genome and analysis of the methylation level at each cytosine position separately in each individual sample. (d) Example of Bismark output. Pos: position; str: strand; cov: number of reads supporting that position; met: number of reads supporting methylation at that position; cont: context of the cytosine position according to the reference. (e) Results across individual samples are summarized and statistical tests assessing significance of methylation differentiation between groups of samples can be performed. Here, the average methylation difference between the two *Heliosperma* species across all CHH positions screened is shown. Modified after Trucchi *et al.* (2016a).

We further compared individuals and identified methylation differences (i.e. a cytosine present in the reference sequences of all genotyped individuals) and nucleotide differences (i.e. cytosine position present only in some of the genotyped individuals). In addition, the context in which a position was found in each individual was also compared to identify changes of context due to neighbouring SNPs (Fig. 12c). This is an important information as distinct molecular mechanisms are responsible for maintaining the methylation state through DNA replication according to different sequence context (CpG, CHG and CHH), making the context critical for the maintenance of the methylation. Thus, when a base substitution occurs in the flanking region of a methylated cytosine, the sequence context can change and the cytosine is expected to lose its methylation status as a result. In such a case, the difference in methylation has a clear genetic determinant in the neighbouring sequence. Statistical tests (i.e., two-sample Kolmogorov-Smirnov) of methylation differentiation between groups were performed obtaining a *p*-value for each position, which was transformed into *q*-values associated with each comparison. We have questioned in detail the possibility that rapid adaptation to divergent habitats is driven from the epigenetic level, by searching for signatures of divergent selection in epigenetic markers in each population pair (similar to *WP1*) (Fig. 12e). By looking at sets of disjunctly distributed, recurrently formed species that converged to similar phenotypes (and adapted to similar ecologies) we searched for loci that show parallel frequency shifts in response to similar environmental pressures.

Detailed analyses of relevant (epi)loci (WP3). N.B. Our original plan was to base this WP on traditional Sanger DNA sequencing and locus-by-locus bisulfite sequencing in order to extend our sampling from 90 to 120 individuals for selected, candidate (epi)loci. However, in the same line as outlined above under “Plant Material”, we chose to switch methodology to NGS-based analyses, which were also employed in WP1 and WP2. This switch was approved by DI Müller on October 1st, 2015. The change maximized the gained information, investigating at once all genomic loci (candidate and non-candidate), across natural populations, transplantations and progenies, rather than focusing only on a subset of candidate loci. In addition, comparability of data across work packages was optimized. The obtained information from the non-candidate loci was important to understand background epigenetic processes at the population level and fine tune our analyses (e.g., define an appropriate level of significance for our inferences).

The single emergent candidate genetic locus (Fig. 13) was Sanger-sequenced in order to test its validity. A mini-contig was built using the paired-end reads of all individuals at that locus. The mini-contig was blasted to the published *Silene vulgaris* transcriptome and annotated. Intronic and exonic regions were identified and several sets of primers were designed within and outside the coding region. We compared the results of amplification using only primers matching the non-coding part with amplifications obtained with primers matching only the coding region. A maximum-likelihood tree of all of the sequences amplified was built with RAxML.

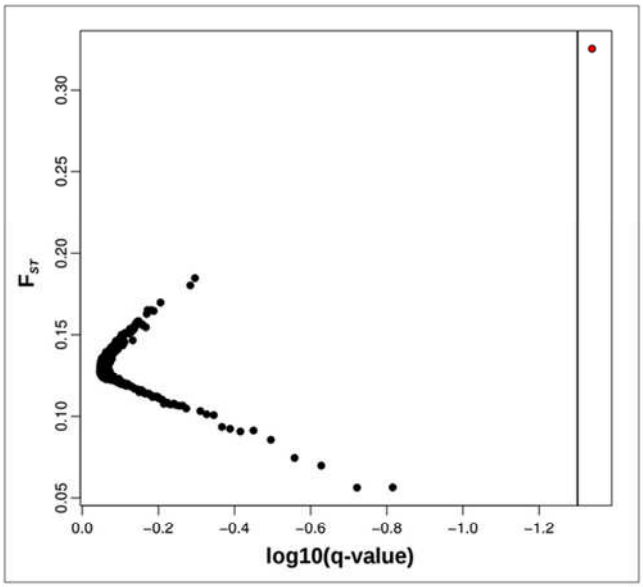

Figure 13. Bayesian inference of loci under selection. Plot of genetic differentiation (F_{ST}) between *H. pusillum* and *H. veselskyi* as a function of *q*-values for each of the 1,097 loci. A false discovery rate threshold of 0.05 is shown as vertical solid line. The candidate outlier locus (*locus2519*) is coloured in red. This is an E3-ubiquitin ligase that features a non-synonymous SNP. Figure modified after Trucchi *et al.* (2016b).

We further applied RADseq (to build individual references as explained above) and bsRADseq to tackle the following questions with specific sets of individuals. *i)* In order to investigate how stable the phenotypic divergence between the two species is and to evaluate the extent of environmental sensitivity of epigenetic signals we interrogated the effect of the environment on phenotypes by investigating bsRADseq data obtained from reciprocally transplanted individuals (i.e., seedlings of *H. pusillum* were transplanted to a natural *H. veselskyi* locality and vice versa; controls were also performed and analysed). *ii)* In addition, the heritable portion of the epigenetic variability within populations was quantified across three generations of *H. pusillum* and *H. veselskyi* cultivated under uniform conditions in the Botanical Garden of the University of Innsbruck. To simplify the assessment of inheritance, pollinators were excluded and the plants were selfed with hand-pollination to produce the next generation. Three F0 plants of each species were used to start the experiment and up to ten F2 individuals per F0 line were analysed with bsRADseq.

Synthesis and publication (WP4). The aim of this work package was to integrate the information obtained from the various levels of molecular information – i.e. (1) the RADseq data targeting genetic patterns obtained in *WP1*, (2) the bsRADseq data targeting epigenetic patterns in *WP*2, and (3) the outlier search, heritability and environmental stability test conducted in *WP3* – with patterns of phenotypic divergence. This approach, which significantly broadened the originally proposed research, allowed us to reach a comprehensive conclusion on the links between genotype, epigenotype and phenotype. In addition, this synthesis provided additional information on the patterns of selection and their targets with the final goal of establishing the ability of plants to quickly react to climate change.

In addition to the tasks outlined in the proposal, we conducted detailed ecological, anatomical, morphological and ecophysiological investigations in order to solidly interpret the genetic data in a climatic context. To this end, we investigated all population pairs and evaluated the degree of environmental and morphological divergence between the two species in a six-fold replicated system. **First**, we determined if habitats of the two species consistently differ in their environmental conditions; we measured air and soil temperatures with data loggers and conducted vegetation inventories to

characterise the habitats by indicator values of the accompanying plant species. **Second**, we conducted morphometric analyses based on traits measured from herbarium vouchers of plants from their natural growing sites. **Third**, in order to prove the influence of natural selection, we tested if individuals of both species are adapted to their habitat conditions by conducting reciprocal transplantation experiments and reciprocal fitness experiments in climate chambers adjusted to the conditions of both habitats. We assessed their fitness, by quantification of germination rate, establishment success, size, and growth velocity. **Forth**, we studied environmentally induced and (epi-)genetically based leaf anatomical and functional (ecophysiological) divergence in population pair 3 in detail. As characterisation of natural habitats indicated pronounced environmental differences in temperature, irradiance and water availability, we focused on traits potentially reflecting the adaptation to differential temperature, irradiance and moisture supply. Specifically, we measured photosynthetic light and temperature response by a gas exchange measurement system (GFS-3000, Walz), studied cellular characteristics of leaves reflecting adaptation and adjustment to water availability, i.e. osmotic, pre-dawn, actual and saturation water potential by a dew point hygrometer (PSYPRO, Wescor), assessed various leaf anatomical traits by light microscopy and investigated chloroplast ultrastructure by transmission electron microscopy. To compare it with microclimatic divergence, we measured photosynthetically active photon flux density by a quantum sensor and leaf temperatures at a 30 min interval by climate stations in parallel. **Finally**, as in *WP1* we found little evidence for gene flow between geographically close populations of *H. pusillum* and *H. veselskyi*, we explored the mechanisms causing the observed reproductive isolation in population pairs 3 and 4. We tested if the lack of gene flow is a result of intrinsic reproductive isolation by cross-pollinations and fitness experiments of offspring. Further, by applying morphometric analyses to offspring of intra- and interspecific crosses we investigated whether phenotypic differentiation is heritable – indicating the onset of speciation – and whether hybrids exhibit morphologically intermediate phenotypes. Breakdown of phenotypic differentiation in intraspecific crosses grown in a common garden would suggest that the morphological differentiation of phenotypes is caused by phenotypic plasticity. Further, intermediate morphology of hybrids may confer maladaptation to parental growing sites and thus allows insights into the fitness reduction at natural growing sites.

7 Arbeits- und Zeitplan / Work and time plan

Due to the official project start on 1.4.2013 instead of the 1.2.2013 as scheduled in the application and the *de facto* start on the 1.7.2013 (begin of the employment of the PhD student) the project was roughly 5 months delayed from the very beginning as compared to the calendar months schedule in the application. Further, it proved difficult to find a suitable, highly qualified Postdoc, who started only in May 2014. Therefore, we asked in two steps for a cost-neutral prolongation of the project from 36 months to 45 months, which was granted by the project officer. The following table gives an overview of the actual duration of work packages and tasks along with the approximate allocation of personnel (project funded and university funded).

Work package and Task		2013									2014										2015										2016											
	calender month	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
	project month	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
WP 1 RADseq																																												
Task 1: library construction																																												
Task 2: data analyses																																												
	Emiliano Trucchi (50%)																																											
	Maria T. Lorenzo (50%)																																											
	Ovidiu Paun																																											
WP 2 BS-RADseq																																												
Task 1: library construction																																												
Task 2: data analyses																																												
	Emiliano Trucchi (50%)																																											
	Maria T. Lorenzo (50%)																																											
	Ovidiu Paun																																											
WP 3 Locus-by-locus																																												
Task 1: validation of candidate loci																																												
Task 2: environmental sensitivity																																												
Task 3: heritability																																												
	Emiliano Trucchi (50% & 97.5%)																																											
	Clara Bertel (75%, 62.5%)																																											
	CTA Innsbruck																																											
	Peter Schönswetter																																											
	Ovidiu Paun																																											
WP 4 Synthesis and publication																																												
Additional Task: Phenotypic differentiation and climatic adaptation																																												
	Emiliano Trucchi (50% & 97.5%)																																											
	Clara Bertel (75%, 62.5%)																																											
	Peter Schönswetter																																											
	Božo Frajman																																												
	Ovidiu Paun																																												
WP 5 Management																																												
	Peter Schönswetter																																												

duration of Work Package
duration of task
enrolement of a project-funded person
enrolement of a University-funded person

8 Publikationen und Disseminierungsaktivitätenw / Publications

J, publication in international, peer-reviewed journal: **X**, published; **(X)**, submitted or manuscript in advanced state; **D**, Dissemination; **P**, Presentation (lecture or poster presentation)

	J	D	P
Bertel, C., Schönswetter, P., Frajman, B., Holzinger, A., Neuner, G. (in press) Leaf anatomy of two reciprocally non-monophyletic mountain plants (*Heliosperma* sp.): Does heritable adaptation to divergent growing sites accompany the onset of speciation? **Protoplasma**, doi:10.1007/s00709-016-1032-5.	X		
Bertel, C., Buchner, O., Schönswetter, P., Frajman, B., Neuner, G. (2016). Environmentally induced and (epi-)genetically based physiological trait differentiation between *Heliosperma pusillum* and its polytopically evolved ecologically divergent descendent, *H. veselskyi* (Caryophyllaceae: Sileneae). **Botanical Journal of the Linnean Society** 182: 658–669.	X		
Bertel, C., Hülber, K., Frajman, B., Schönswetter, P. (2016). No evidence of intrinsic reproductive isolation between two reciprocally non-monophyletic, ecologically differentiated mountain plants at an early stage of speciation. **Evolutionary Ecology** 30: 1031–1042.	X		
Trucchi, E., Mazzarella, A., Gilfillan, G. D., Lorenzo Romero, M., Schönswetter, P., & Paun, O. (2016). BsRADseq: screening DNA methylation in nonmodel species. **Molecular Ecology** 25: 1697–1713.	X		
Malinsky, M., Trucchi, E., Lawson, D.J., Falush, D. (2016) RADpainter and fineRADstructure: population inference from RADseq data. *bioRxiv* doi: http://dx.doi.org/10.1101/057711		X	
Trucchi, E., Frajman, B., Haverkamp, T.H.A., Schönswetter, P., Paun, O. (2016) Genomic and metagenomics analyses reveal parallel ecological divergence in *Heliosperma pusillum* (Caryophyllaceae). *bioRxiv* doi: http://dx.doi.org/10.1101/044354		X	
Trucchi, E., Frajman, B., Haverkamp, T.H.A., Schönswetter, P., Paun, O. (submitted) Genomic analyses reveal parallel ecological divergence in *Heliosperma pusillum* (Caryophyllaceae). New Phytologist in review.	X		
Bertel C, Hülber K, Frajman B, Schönswetter P. (manuscript in advanced state) Consistent ecological and morphological differentiation of ecotypes within the mountain plant *Heliosperma pusillum* and a higher survival at their native site suggest that natural selection is involved in their multiple parallel evolution. Planned for New Phytologist.	(X)		
Trucchi, E., Frajman, B., Baar, J., Schönswetter, P., Paun, O. (in preparation) Epigenomics of parallel adaptation during ecotype formation in *Heliosperma* (Caryophyllaceae). Planned for *Molecular Biology and Evolution*.	(X)		
Bertel, C. (2013) Can environmentally-induced phenotypic variation lead to recurrent speciation? Insights from the mountain plant *Heliosperma pusillum*? Lecture at the **Bad Feilnbach Summer School in Plant Evolution and Systematics**, Bad Feilnbach, Germany, 04.10.2013–06.10.2013			X

Flatscher, R., Frajman, B., Schönswetter, P., Paun, O. (2013) Stay high or get low: exploring epigenetic correlates of altitudinal variants in *Heliosperma pusillum* (Caryophyllaceae). Lecture at the 2nd **BioSyst EU 2013** conference, Vienna, 18.02.2013–22.02.2013			X
Paun, O. (2013) Comparative genomics in environmental context to understand evolution. Invited lecture at the **University of Salzburg**, Dec 2013.			X
Paun, O. (2013) Ecological genomics in non-models. Invited lecture at the **Vienna Graduate School of Population Genetics**, University of Veterinary Medicine, Vienna, Nov 2013.			X
Paun, O. (2013) Plant evolution from beyond genetics. Invited lecture at the **University of Zürich**, Switzerland, Nov 2013.			X
Bertel, C., Frajman, B., Hülber, K., Paun, O., Flatscher, R., Schönswetter, P. (2014) Can environmentally induced phenotypic variation lead to recurrent speciation? – Insights from the mountain plant *Heliosperma pusillum* (Caryophyllaceae)? Lecture at the conference **16. Treffen der Österreichischen Botanikerinnen und Botaniker**, Graz, Austria, 25.–27.9.2014.			X
Frajman, B., Bertel, C., Flatscher, R., Hülber, K., Trucchi, E., Paun, O., Schönswetter, P. (2014) Did environmentally induced phenotypic variation led to recurrent speciation in the *Heliosperma pusillum* group (Caryophyllaceae)? Lecture at the conference **Gothenburg Systematikdagarna**, Gothenburg, Sweden, Nov 2014.			X
Paun, O. (2014) Genomic approaches to evolution in non-models. Invited lecture at the **Natural History Museum, Oslo**, Norway, 25.11.2014.			X
Paun, O., Flatscher, R., Lorenzo, M.T., Trucchi, E., Frajman, B., Schönswetter, P. (2014) Stay high or get low: can epigenetic variation lead to recurrent speciation? Lecture at the conference **Evolution 2014**, Raleigh, North Carolina, USA, Jun 2014.			X
Paun, O., Flatscher, R., Lorenzo, M.T., Trucchi, E., Frajman, B., Schönswetter, P. (2014) Stay high or get low: can epigenetic variation lead to recurrent speciation? Lecture at the conference **EuroEvoDevo 2014** – 5th Meeting of the European Society for Evolutionary Developmental Biology, Vienna, Austria, Jul 2014.			X
Trucchi, E., Flatscher, R., Lorenzo, M.T., Frajman, B., Schönswetter, P., Paun, O. (2014) Stay high or get low: can epigenetic variation lead to recurrent speciation? Lecture at the conference **GfOE 2014**, Hildesheim, Germany, Sept 2014.			X
Bertel, C., Trucchi, E., Paun, O., Frajman, B., Hülber, K. & **Schönswetter, P**. (2015) Können epigenetische Änderungen eine rasche Anpassung an den Klimawandel ermöglichen? Lecture at the conference **16. Österreichischer Klimatag 2015**, Wien, Austria, 28.4.–30.4.2015.			X
Paun, O. (2015) Convergent and adaptive processes during evolution. Invited lecture at the conference **Systematikdagarna 2015**. Lund, Sweden, Nov 2015.			X
Paun, O. (2015) Evolution *from beyond genetics*. Invited Evening Lecture at the Linnean Society of London, Oct 2015.			X
Paun, O. (2015) *Genomic approaches to evolution in non-models*. Invited seminar at the CSIC Sevilla, Spain, Nov 2015.			X
Paun, O. (2015) Genomic approaches to variation and adaptation. Invited seminar at the Institute for Biodiversity and Environmental			X

Research, University Brunei Darussalam, Brunei, May 2015.			
Schönswetter, P., Bertel, C., Hülber, K., Trucchi, E., Paun, O., **Frajman, B.** (2015) Environmentally induced recurrent speciation – a driver of diversification also on the Balkans? Lessons from Eastern Alpine *Heliosperma pusillum* and *H. veselskyi* (Caryophyllaceae). Lecture at the conference **6th Balkan Botanical Congress**, Rijeka, Croatia, 14.–18.9.2015.			X
Trucchi, E. (2015) (Epi)genomics of parallel adaptation in an alpine plant. Invited lecture at the **Centre for Ecological and Evolutionary Synthesis**, University of Oslo, Norway, Apr 2015.			X
Trucchi, E., Flatscher, R., Romero, M.L., Frajman, B., Schönswetter, P., Paun, O. (2015) Epigenetic divergence and parallel evolution in *Heliosperma pusillum* (Caryophyllaceae). Lecture at the conference **ESEB2015, Congress of the European Society for Evolutionary Biology**, Lausanne, Switzerland, Aug 2015.			X
Bertel, C., Frajman, B., Hülber, K., Neuner, G., Schönswetter, P. (2016) Can environmentally induced phenotypic variation lead to recurrent speciation? – Insights from the mountain plant *Heliosperma pusillum*. Lecture at the conference **29th Conference of the Plant Population Biology Section of the Ecological Society of Germany, Austria and Switzerland**, Třeboň, Czech Republik, 06.05.2016			X
Bertel, C., Frajman, B., Neuner, G., Hülber, K., Schönswetter, P. (2016) Can environmentally induced phenotypic variation lead to recurrent speciation? – Insights from the mountain plant *Heliosperma pusillum*. Lecture at the conference **22nd European annual Meeting for PhD Students in Evolutionary Biology**. Burgsvik, Gotland, Sweden, 15.9.2016.			X
Bertel, C., Frajman, F., Neuner, G., Hülber, K., Schönswetter, P. (2016) Wie können ökologische und phänotypische Differenzierung zur Entstehung neuer Arten beitragen – Einblicke von multidisziplinären Untersuchungen an der Alpenpflanze *Heliosperma pusillum* sensu lato (Caryophyllaceae). Lecture at the conference **17. Treffen der Österreichischen Botanikerinnen und Botanike**r. Universität für Bodenkultur (BOKU), Vienna, September 2016.			X
Paun, O. (2016) Convergent and adaptive processes during evolution. Invited seminar at the **University of Cluj**, Romania, Nov 2016.			X
Paun, O. (2016) Ecological & evolutionary genomics in non-model organisms using RADseq. Invited seminar at the **Slovak Academy of Sciences**, Bratislava, Slovakia, Dec 2016.			X
Paun, O. (2016) *Genomic approaches to evolution in non-models*. Invited seminar at the **CeMM Research Center**, Austria, Jun 2016.			X
Trucchi, E. (2016) More than SNPs, other things RADseq can be useful for. Invited lecture at the **NGS Workshop, BIOM Spring Camp, Research Facility Studenec**, Institute of Vertebrate Biology, Czech Academy of Sciences. April 2016.			X
Trucchi, E. 2017 (forthcoming). Epigenomics of parallel adaptation during ecotype formation. Invited lecture at the symposium **Plant epigenetics: from mechanisms to ecological relevance** (40th New Phytologist symposium), Vienna, Sep 2017.			X
Flatscher, R., Schönswetter, P., Paun, O., Frajman, B. (2013) Can epigenetic differentiation cause the formation of ecotypes? Insights			X

from stable altitudinal variants in the mountain plant *Heliosperma pusillum* (Caryophyllaceae). Poster presentation at the **Congress of the European Society for Evolutionary Biology 2013**, Lisbon, Portugal, 19.08.2013–24.08.2013.			
Bertel, C., Frajman, B., Neuner, G., Hülber, K., Schönswetter, P. (2014) Exploring phenotypic divergence in the mountain plant Heliosperma pusillum (Caryophyllaceae). Poster presentation at the conference **20. Tagung der Austrian Society of Plant Biology (ATSPB)**, Lunz, 20.06.2014.			X
Bertel, C., Neuner, G., Schönswetter, P., Hülber, K. (2014) Exploring phenotypic divergence in the mountain plant *Heliosperma pusillum* (Caryophyllaceae). Poster presentation at the conference **20. Meeting of the Austrian Society of Plant Biology (ATSPB)**, Lunz, Austria, 20.06.2014.			X
Geisler, J., Frajman, B., Hülber, K., Schönswetter, P. (2014) Performance of Heliosperma *veselskyi* and *Heliosperma pusillum* across different environmental conditions in common garden cultures. Poster presentation at the conference **16. Treffen der Österreichischen Botanikerinnen und Botaniker**, Graz, Austria, 25.–27.9.2014.			X
Paun, O., Trucchi, E., Lorenzo, M.T., Flatscher, R., Frajman, B., Schönswetter, P. (2014) Stay high or get low: can epigenetic variation lead to recurrent speciation? Poster presentation at the conference **PopBio 2014**, 27th Plant Population Biology Conference, Book of abstracts, Konstanz, Germany, May 2014.			X
Sansone, T., Flatscher, R., Bertel, C., Hülber, K., **Erschbamer, B. (**2014) Does local adaptation govern seedling recruitment in closely related species? Poster presentation at the conference **PopBio 2014**, 27th Plant Population Biology Conference, Konstanz, Germany, May 2014.			X
Trucchi, E., Flatscher, R., Lorenzo, M.T., Frajman, B., Schönswetter, P., Paun, O. (2014) Unexpected genome-wide DNA methylation stability across plant populations adapted to divergent habitats. **FISV 2014**, Pisa, Italy, Sept 2014.			X
Bertel, C., Frajman, B., Hülber, K., Buchner, O., Schönswetter, P., Neuner, G. (2015) Did environmental heterogeneity lead to functional differentiation in the mountain plant *Heliosperma pusillum*? Poster presentation at the conference **From Molecules to the Field, Botanikertagung München**, München, Germany, 30.08.-03.09.2015.			X
Bertel, C., Frajman, B., Hülber, K., Buchner, O., Schönswetter, P., Neuner, G. (2015) Can environmentally induced phenotypic variation lead to recurrent speciation? - Insights from ecophysiological investigations of the mountain plant *Heliosperma pusillum* (Caryophyllaceae). Poster presentation at the conference **26. Congress of the Scandinavian Plant Physiology Society (SPPS)**, Stockholm, 10.08.2015.			X
Frajman, B., Bertel, C., Hülber, C., Trucchi, E., Paun, O., Schönswetter, P. (2015) Did environmentally induced phenotypic variation led to recurrent speciation in the *Heliosperma pusillum* group (Caryophyllaceae)? Poster presentation at the conference **Caryophyllales 2015**, Berlin, Germany, 13.–18.9.2015.			X
Trucchi, E. Instructor at 2015 Workshop on Molecular Evolution, Cesky Krumlov, Czech Republic, January-February 2015.		X	

Trucchi, E. Instructor at the RADseq workshop of the University of Milan, Unimont and University of Romse La Sapienza. Edolo, Italy, June 2015.		X	
Trucchi, E. Instructor at 2016 Workshop on Popuation and Speciation Genomics, Cesky Krumlov, Czech Republic, January-February 2015.		X	
Trucchi, E and **Paun, O.** contributed to the workshop "*sEpiDiv: Towards understanding the causes and consequences of epigenetic diversity*" in Leipzig, Germany. Trucchi E. introduced to the audience the newly developed bsRADseq method and the bioinformatic analyses.		X	
Symposium "Speciation Genomics" at the meeting of the Society for Molecular Biology and Evolution, Vienna, Austria, July 2015. Organised by **O. Paun** with P. Novikoka		X	
Symposium "Evolutionary Epigenetics: Switching from Models to the Field" at the ESEB meeting, Lausanne, Switzerland, Aug 2015. Organised by **O. Paun** with C. Alonso.		X	
Symposium "Evolutionary epigenetics" at the BioSystEU2013 Congress, Vienna, Austria, Feb 2013. Organised by **O. Paun**.		X	
(forthcoming) Symposium "Plant epigenetics: from mechanisms to ecological relevance" (40th *New Phytologist* symposium), Vienna, Austria, Sep 2017. Local organiser **O. Paun**.		X	