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Erigeron glabratus* subsp. *candidus* (Asteraceae): an overestimated taxon or an endemic subspecies of the Koralpe? Phylogeny and Taxonomy of the endemic *Erigeron glabratus* subsp. *candidus

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Zusammenfassung

Lebensräume mit einem Reichtum an Endemiten nehmen im Naturschutz eine besondere Stellung ein und gelten als besonders schützenswert. An der Grenze der Steiermark und Kärntens befindet sich die Koralpe. Eine Bergkette, welche einst während der letzten Eiszeit als Refugium für viele subalpine und alpine Arten galt und aufgrund ihrer hohen Bodenvielfalt heute ein Habitat für mehrere Endemiten darstellt. Marmorbänder ziehen sich durch die Landschaft der Koralpe und bilden Mikrohabitate für kalkliebende Pflanzenarten. Ein Lokalendemit dieser Bergkette ist die Unterart *Erigeron glabratus* subsp. *candidus* (Asteraceae), welche sich besonders durch ihre einheitlich weiße Zungenblütenfarbe und die Einköpfigkeit der Blüten pro Individuum morphologisch von ihrer verwandten und stark morphologisch variablen Unterart *E. glabratus* subsp. *glabratus* unterscheidet. Der Lokalendemit *E. glabratus* subsp. *candidus* ist ausschließlich auf der Koralpe verbreitet, wohingegen die nah-verwandte Unterart vom Balkan, über die gesamten Alpen bis in die Pyrenäen Habitate besiedelt. Aufgrund der hohen Variabilität der Morphologie in den alpinen *Erigeron* Arten und besonders in der Unterart *E. glabratus* subsp. *glabratus*, ist die Taxonomie in dieser Gattung schwer zu bestimmen. Besonders die beiden Unterarten von *E. glabratus* führten in der Vergangenheit zu wissenschaftlichen Diskussionen. So wurde die Unterart der Koralpe als eigene Art kategorisiert, bevor sie als Subspezies eingeteilt wurde. Jedoch ist der Verwandtschaftsgrad zwischen beiden Unterarten immer noch stark umstritten und auch die Morphologie stellte sich in vorherigen Studien als kein klares Unterscheidungsmerkmal heraus. Ziel dieser Studie ist es den genauen Verwandtschaftsgrad der beiden Unterarten zu ermitteln, um so Rückschlüsse auf die Taxonomie des Lokalendemiten ziehen zu können. Um die Populationsstrukturen innerhalb dieser Art in den zentralen Ostalpen zu identifizieren, wurden zehn Populationen der Unterart *E. glabratus* subsp. *glabratus* und eine Population von *E. glabratus* subsp. *candidus* mittels Restriction site associated (RAD) Sequenzierung analysiert. Die Ergebnisse zeigen keine klare Differenzierung des Koralpe-Lokalendemiten. Die sehr geringe genetische Differenzierung von *E. glabratus* subsp. *candidus* schließt eine genetische Kategorisierung als Unterart aus. Die Fixierung der einheitlichen Merkmale der Koralpe-Populationen könnten durch die geringen Populationsgrößen begründet liegen und eine Anpassung an die ökologischen Standortbedingungen der Koralpe sein.

Abstract

Endemic rich habitats possess a high interest for conservation biology and the protection of them is a key element for national nature conservation. The Koralpe is a mountain range with a high endemism rate and during the last ice age it was a refugium for many subalpine and alpine species. The landscape of this mountain range is defined by marble bands, which are forming microhabitats for calcicole biota. One of the local endemics is *Erigeron glabratus* subsp. *candidus*, which is restricted to the marble bands of the Koralpe. The alpine species of the genus *Erigeron* are known for their morphological variability, which leads to taxonomical uncertainties. Ranking taxa as subspecies is not clear without ambiguity. Especially the boundaries between the two subspecies *E. glabratus* subsp. *candidus* and *E. glabratus* subsp. *glabratus* led to several controversial discussions in the past and the relation between the two subspecies remains still as a question. Former studies revealed a weak morphological and ecological differentiation between them. The aim of this study is the uncovering of the genetic relation between the two subspecies by using Restriction site associated (RAD) Sequencing. Ten populations of *E. glabratus* subsp. *glabratus* were sampled all over the Central Eastern Alps and one population of *E. glabratus* subsp. *candidus* was collected at the Koralpe. The results of the population genetic analyses reveal no genetic differentiation of *E. glabratus* subsp. *candidus* from its related subspecies. The morphological characteristics of the *candidus* subspecies, such as the white colour of the petals, might be due to the adaptation to the environmental conditions of the Koralpe or might be explained by the small population sizes. Nevertheless, the identified low genetic variability and differentiation between the two subspecies rejects the categorisation of them as two separate subspecies. Still, the protection of the Koralpe, especially of the marble bands and their vegetation, should be considered as a main object of local nature conservation.

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Introduction

Endemism describes the restricted distribution of species to specific geographical regions, which reveals an exceptional contribution of local areas with high endemism rates to global biodiversity. Endemic species often form small populations, which can be highly adapted to habitats and climate conditions and can be vulnerable to environmental changes and anthropogenic impacts (Essl *et al.*, 2011; Komposch, 2017). Therefore, prioritising endemic rich regions for protection interests is a key element for national nature conservation. Hence, it is essential to understand the biogeographical distribution patterns, as well as the ecology, sociology and phylogeny of endemic species. The geographical distribution of species can be driven by several factors, such as the dependency of climatic or ecological niches, interspecific competition or it can be caused by regional processes, defined by the geological and climatic history of an area.

Climatic fluctuations during Quaternary cold periods heavily influenced the intraspecific genetic structure and distribution of species in Europe (Taberlet *et al.*, 1998; Tribsch and Schönswetter, 2003). The decrease of the temperature, as well as the average depression of the climatic snow line to 1200 m strongly affected the Eastern Alps and large regions were covered by an ice sheet during the Pleistocene glaciation (Tribsch, 2004). As a result, organisms were forced to migrate to unglaciated refugial habitats, which were located especially at peripheral parts of the Alps. The caused range restriction can be seen as a main driver for vicariant speciation (Major, 1988). Patterns in intraspecific genetic variation were influenced by the survival of populations during and after glacial periods, as well as by fragmentation, isolation and hybridisation after secondary contact with the possible result of the evolution of genetically distinct lineages (Comes and Kadereit, 1998; Taberlet *et al.*, 1998; Dynesius and Jansson, 2000; Tribsch, 2004). The provided opportunities for speciation in refugial areas results in species rich regions, thus Tribsch (2004) observed a significant correlation between high endemism and low glacial ice cover in the Eastern Alps. In addition, especially calcareous regions at the southern and the eastern border of the Eastern Central Alps are showing an increased number of endemics, which can be explained by the predominance of calcareous refugia (Tribsch and Schönswetter, 2003).

Located at the south-eastern edge of the Alps with a north-south oriented crest lies one of the Pleistocene refugia, called the Koralpe. During the last ice age, six isolated small glaciers

covered the highest alpine parts of the Koralpe (Berg and Drescher, 2018). All the other parts served as a refugial area for alpine and subalpine species. The main rocks are gneisses and mica-schists, which are nutrient-rich with a low saturation of bases. All over the mountain range, bands and spots of marble and amphibolites are shaping the landscape and are providing the surrounding area with bases and calcium. These restricted islands of calcareous microhabitats are increasing the local soil- and biodiversity by permitting the colonisation of calcicole biota. The importance of the Koralpe as being a habitat with high biodiversity can be manifested by the abundance of many endemic species. For example, 18 endemic vascular plant species can be found all over the mountain range including three local endemics, *Alchemilla philonotis* FRÖHN, *Doronicum cataractanum* Widder and *Erigeron glabratus* Hoppe & Hornsch. subsp. *candidus* (Widder) Huber (Komposch *et al.*, 2016).

Ranking taxa as subspecies remains still as a long-running controversy due to the fact that boundaries between subspecies are not clear without ambiguity and can be contradicted by molecular phylogenetic data (Phillimore and Owens, 2006). The boundaries between the two subspecies of *Erigeron glabratus* were also controversially discussed during the last decades, which led to several changes inside the taxonomy of this taxon. The genus *Erigeron* harbours around 200 species of which Huber (1993) distinguished between nine alpine *Erigeron* species, including the two subspecies *E. glabratus* subsp. *glabratus* and *E. glabratus* subsp. *candidus*. The alpine *Erigeron* species are showing a high variability in natural populations. Especially in contact zones, intermediates can be observed as a result of introgression and hybridisation (Huber and Zhang, 1991; Huber, 1993; Utelli *et al.*, 1995). The similarities among the alpine species, as well as the variability inside the species and populations, complicate the coding of the taxonomy.

The endemic *E. glabratus* subsp. *candidus* is one of the taxa, which has evolved a constant morphology within its populations. In comparison to *E. glabratus* subsp. *glabratus*, the most significant traits of *E. glabratus* subsp. *candidus* are the white colour of the ligulate florets, as well as the development of exclusively single flowering heads (Widder, 1932; Huber and Leuchtman, 1992; Wilfing, 2013). The subspecies *E. glabratus* subsp. *glabratus* is developing a broader pattern of morphological traits. For example, the colour of the ligulate florets can vary between white and deep purple. Furthermore, it can express more than one flowering head, and diversify in its heights. In addition, the two subspecies are geographically

separated from each other. The contribution of *E. glabratus* subsp. *candidus* is restricted to the marble bands of the Koralpe, whereas *E. glabratus* subsp. *glabratus* is growing on limestone widespread from the Balkan Peninsula, over the Alps to the Pyrenees (**Figure 1**). Due to the morphological unity of the subspecies *candidus*, Widder (1932) ranked it as an own species: *Erigeron candidus*. In contrast, Huber (1993) disagreed with Widder and categorised the taxon as a subspecies due to the weak boundaries to *Erigeron glabratus*. He justified the new ranking based on the similarities in their chemotaxonomy (Huber and Leuchtman, 1992; Oberhänsli and Huber, 1993), sociological behaviour and ecological requirements. In a continuative study, Katrin Wilfing (2013) analysed the same characteristics, as Huber observed in his studies, and revealed that *E. glabratus* subsp. *candidus* is only weakly differentiated from *E. glabratus* subsp. *glabratus*. Additionally, she sequenced several populations of both subspecies and closely related taxa by using the Internal transcribed Spacer (ITS) as a nuclear marker for revealing a phylogenetic relationship. It turned out that ITS was an uninformative marker for this kind of study and therefore a new approach will be tested in this project.

The aim of this study is to decode the genetic relationship between the two subspecies by using multiple polymorphous sites of the genome. The usage of restriction site associated DNA Sequencing (RAD-seq) provides the opportunity to detect thousands of single nucleotide polymorphisms (SNPs) in a cost-effective way (Rowe *et al.*, 2011). Furthermore, the revealing of the genetic diversity among the two subspecies shall be used to identify whether *Erigeron glabratus* subsp. *candidus* can be ranked as a subspecies or whether the two subspecies are genetically not distinguishable and should be seen as one taxon.

Material and Methods

Sampling

For this survey we studied a total of 70 specimens of *Erigeron glabratus* collected in 12 localities across its distributional range in the Eastern Central Alps. Sampling localities are with a few exceptions the same reported by Wilfing (2013) but were newly sampled during the summer of 2018. The dataset includes populations of two subspecies. Eleven of them belong to *E. glabratus* subsp. *glabratus* and one to the endemic *E. glabratus* subsp. *candidus*. The number of sampled specimens ranged between 12 and 4 depending on the encountered population sizes, except from the single specimen of subsp. *glabratus* collected from Tyrnauer Alm. Information about the sampling localities of the populations are listed in the following table (**Table 1**). A list with information about all single individuals is represented in the supplementary part (Appendix: **Table S.1**)

Table 1: Sampling localities of the populations including number of sampled individuals, average latitude [decimal degree] and longitude values [decimal degree], as well as the average altitude [m].

Population	Species	Sampled Individuals	Location	Average Altitude	Average Latitude	Average Longitude
EcanKor	<i>Erigeron glabratus</i> subsp. <i>candidus</i>	12	Koralpe (Bärentalkar)	1752,92	46.81384	11.39221
EglaBue	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	5	Hochschwab (Bürgeralm)	1683,40	47.58249	15.23453
EglaDob	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	5	Dobratsch	1769,40	46.59530	13.70500
EglaEis	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	7	Nockberge (Eisentalhöhe)	2107,14	46.93778	13.76130
EglaGab	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	6	Gleinalpe (Gaberl)	1699,00	47.07981	14.92528
EglaML	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	4	Monte Lussari	1742,50	46.48048	13.52326
EglaPet	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	7	Petzen (Kniepstattel)	2032,57	40.52547	14.76663
EglaSchi	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	4	Nockberge (Schiestelnock)	2013,25	46.88954	13.79904
EglaSchne	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	5	Schneetalpe	1876,00	47.70248	15.59528
EglaSchoe	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	7	Schöckl	1438,00	47.19786	15.45986
EglaSpei	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	7	Großbeck (Speiereck)	1983,71	47.12916	13.63969

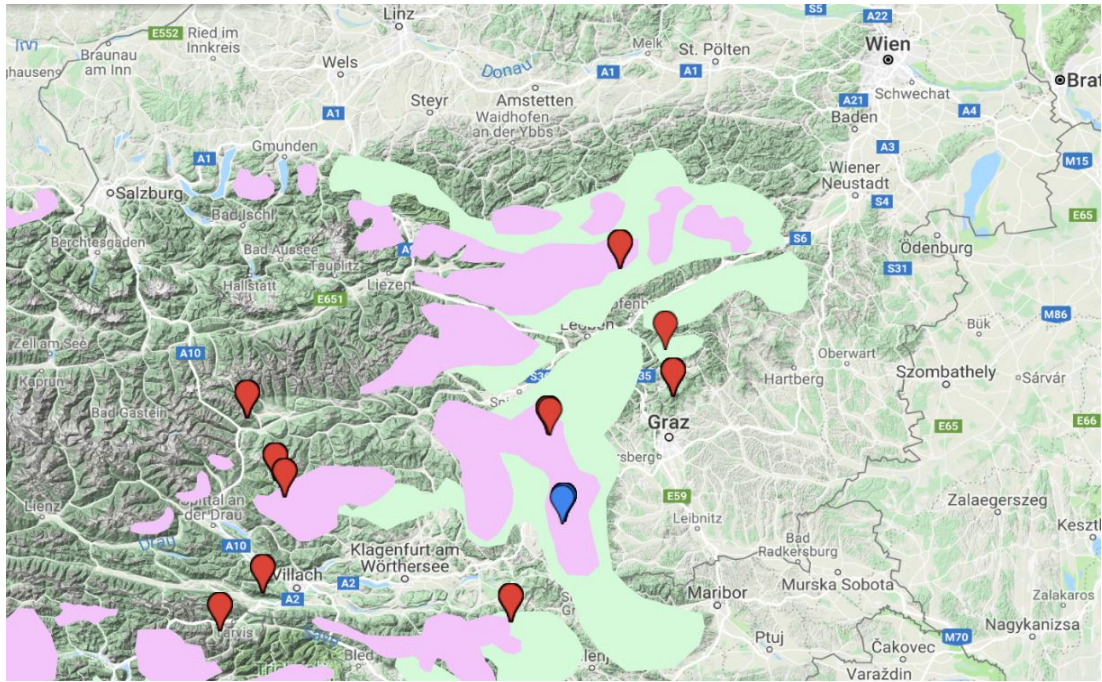


Figure 1: Sampling locality map showing all 11 populations of the Central Eastern Alps. The pink patterns are representing hypothetical alpine refugia and the green patterns are identifying hypothetical subalpine refugia of the Eastern Alps, mapped based on the last glacial maximum from van Husen (1997), visualised in Google Maps (2019).

DNA Extraction

For DNA extraction, two to three leaves were excised per individual sample. Before starting the extraction, between 5 to 25 mg of the dried leaf material were taken into a 2 ml Eppendorf cup with three glass beads and homogenised in a grinder. Genomic DNA was extracted from silica-dried leaf tissues using the Invisorb® Spin Plant Mini Kit (STRATEC Molecular GmbH) with minor modifications. After the homogenisation, 1.5 ml of cold Sorbitol buffer was added to the samples. Before use, the buffer was prepared with 1% 2-mercaptoethanol and 1% Polyvinylpyrrolidone (PVP). The samples were incubated at 4°C for 15 minutes. The cups were centrifuged at 4°C at 1000 rpm for 30 minutes and afterwards the supernatant was discarded and the total DNA of the samples was extracted following the manufacturer's instructions. The quality of the DNA extractions was visually assessed by means of agarose gel electrophoresis (1% TRIS-Borate-EDTA (TBE) agarose gel, run for 30 minutes at 90 V). Further quantification of the quality of each extraction was carried out

using a NanoDrop system (Thermo Fisher Scientific). DNA content was further quantified on 1 µl per extraction using the dsDNA HS Assay Kit (Thermo Fisher Scientific) in the Invitrogen Qubit 3.0 Fluorometer.

RAD library preparation and sequencing

The measured DNA quantities [ng/µl] were used to calculate the sub libraries for the RAD sequencing. A table including all information about the sub libraries is listed in the supplementary part (Appendix: **Table S.3**). In total, six sub-libraries were established, each with 12 individuals. RAD-Seq libraries were prepared following a protocol adapted from Baird et al. (2008) of the Department of Botany and Biodiversity of the University of Vienna (Prof. Dr. Ovidiu Paun and Prof. Dr. Gerald Schneeweiss). To ensure homogeneous sequencing intensities across samples, all isolates were diluted to contain a sample volume of 40 µl. Two Samples, Eglaschne1 and Eglabue4, had lower DNA concentrations than targeted (Appendix: **Table S.3**). They were not diluted but were duplicated to compensate for possible underrepresentation. The diluted isolates were first digested using the restriction enzyme 15U *PstI* (New England Biolabs, Appendix: **Figure S.1**), at 37°C and two hours of incubation. Digestion was terminated by inactivating the enzyme at 80°C for 20 minutes. Then, specific P1 adapters were ligated to the open ends of the digested templates using a T4 DNA ligase (New England Biolabs). Adapters contain unique hexameric inline barcodes, which allow the sequencing of multiplexed libraries (Baird *et al.*, 2008). Digested isolates were incubated overnight at 16°C. Ligase activity was afterwards inactivated, incubating at 65°C for 10 minutes.

After ligation, barcoded libraries were pooled in six batches of 12 individuals for further processing. Pooling was done at random to avoid introducing systematic bias. Afterwards, sonication was used to shear the DNA in fragments of 400 bp of average size. Sonication was carried out using a Bioruptor® Pico (Diagenode) using two cycles of 30 seconds “on” and 60 seconds “off” and a temperature of 4 °C.

After shearing of DNA, each batch was cleaned using the MinElute Reaction Cleanup Kit (Qiagen). Then, adequate fragment sizes were selected, using a right to a left double selection, using the SPRIselect beads in the Biomek FX Liquid Handler (Beckman Coulter) following the manufacturer’s technical application note (Bélisle *et al.*, 2016). For the right

size selection x0.55 volume of SPRI reagent was added to the samples while for the left size selection x0.25 volume was used. Next, the Quick Blunting™ Kit (New England Biolabs) was used with an incubation time of 30 minutes at room temperature to convert incompatible 5' or 3' overhangs to blunt-ended, 5' phosphorylated DNA. To finalize the first stage of library preparation, each batch was first purified using the MinElute PCR Cleanup Kit (Qiagen), then it was supplemented with 1µl of 100mM dATP, and was finally purified using the same kit as before. At this stage, DNA content was quantified using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) to maintain homogeneous DNA contents across library batches. The DNA content was readjusted to that of the second batch, since the DNA content of the first sub-library was too low (Appendix: **Table S.4**).

Before carrying the final library amplification, each batch was supplemented with P2 adapters which were ligated using a T4 ligase. To prevent the amplification of DNA fragments which do not possess the P1 adapter, the P2 adapter has divergent ends and contains reverse complement sites of the reverse amplification primer site. This way, only fragments ligated to the P1 adapter were selected and amplified.

Finally, all six batches were pooled equimolarly and amplified. Before PCR amplification, pooled batches were left-size selected using x0.8 volumes of SPRI reagent. An 18 cycle PCR amplification was run using the Phusion® High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs), P1-PCR and P2-PCR primers. The PCR product, as well as a negative control, including 5 µl of the PCR Master Mix and a positive control, consisting of 2 µl DNA sample which had not been amplified, were checked on a 1% TBE gel and the DNA concentration was measured, again (Appendix: **Table S.5**).

Finally, to overcome the inefficient size selection obtained with SPRI beads, the final size selection was done using Pippin Prep (Sage Science) with a 1,5% dye-free cassette and a target fragment size between 300 and 850 bp. Size ranges were checked on a Bioanalyzer using a High-Sensitivity DNA Kit (Agilent Technologies). Multiplexed libraries were sequenced at the Vienna Biocenter Sequencing facility (VBCF, Vienna, Austria), where it was sequenced on an Illumina HiSeq 2500 platform using a single-end 100 bp cycle run.

Identification of RAD loci and Filtering of SNPs

The main processing of RAD-seq data was carried out using a *de novo* approach using Stacks v2.3 (Catchen *et al.*, 2011). First the quality-fasta file retrieved from the single-end Illumina run was demultiplexed using the `process_radtags.pl` script. The following parameters were used: *Pst*I as the restriction enzyme, discarding reads with low quality scores, cleaning data by removing any reads with uncalled bases and allowing one mismatch when rescuing Rad tags and single-end barcodes for maintaining adapter-free reads.

Read assembly was done using the stacks pipeline implemented in the `denovo_map.pl` script, which automates the use of the different stacks components: `ustacks`, which builds loci; `cstacks`, which creates a catalogue of the loci and `sstacks`, which matches the reads against the catalogue (Catchen *et al.*, 2013). Although alternative parametrizations were also tried, *de novo* mapping was run using a value of two for the number of allowed nucleotide mismatches between two stacks to merge them into a locus (M) and a value of three for the number of mismatches found between stacks between individuals (n). While these settings already reduce the interference of paralogous loci, the dataset was further filtered whitelisting loci containing at most 10 SNPs.

To output SNPs for further analysis, the Stacks program population was used with a maximum observed heterozygosity of 0.65 to filter out pooled paralogs. Loci that were presented in less than 20 % of the populations were removed from the data set. For the alignment of the reads, a pseudo reference was necessary and created with a python script written by Dennis Larsson. The pseudo reference was used to align the reads with the `ref_map.pl` program of Stacks, as well as to generate a SNP calling, by running the default settings with a minimum number of three identical reads for maintaining a stack. The created bam files were used for a second population run in Stacks. The SNPs were filtered based on the established whitelist. The final filtering was performed using `vcftools v0.1.16` (Danecek *et al.*, 2011) by removing alleles with uninformative SNPs using the `maf`-function, by determining the percentage of missing data of 20% using the `max-missing`-function and by excluding two individuals from the data set, which were considered to possess poor data: *EglaEis5* and *EglaSchi1*. For further analysis, the final vcf files were converted with `PGDSpider v.2.1.1.5` into phylip and nexus files (Lischer and Excoffier, 2012).

Population genetics

To gain an overview of the molecular diversity within and among the populations, Arlequin ver. 3.5 (Excoffier and Lischer, 2010) was used to calculate the mean number of pairwise differences (π) and the molecular genetic variation (Θ_s). In addition, the number of private alleles in each population, as well as the type of substitutions, transversion or transition, were identified. The values of the *E. glabratus* subsp. *glabratus* from the Grazer Bergland were excluded due to the fact that only one individual was collected and this is why the sample was not useful for measuring population based genetic variation parameters.

To investigate the population structure, the on variational Bayesian framework for posterior inference based FastSTRUCTURE 1.0 (Raj *et al.*, 2014) was used with a hypothetical number of populations of $k=10$. With the usage of the web-based software StructureSelector (Li and Liu, 2018), the visualisation of the population structure and the identification of the best-fitting number of genetic clusters (K) were performed. Another approach to identify a certain structure within the data, was conducted by using FineRADStructure (Malinsky *et al.*, 2018).

The population differentiation was measured with the fixation index F_{ST} using vcftools v0.1.16 (Danecek *et al.*, 2011). Further statistical analyses were computed in RStudio ver. 1.2.1335 (RStudio, 2012). The R package *adegenet* ver. 1.3-1 (Jombart and Ahmed, 2011) was used for the following approaches. To investigate the relations among the sampled individuals, the on genetic distances based Neighbour-Joining method was used and trees were visualised with the web-based software iTOL (Letunic and Bork, 2019). Furthermore, a Principle Component Analysis (PCA) was conducted to summarise the genetic diversity among the individuals. In addition, the data was tested for spatial structures using a Mantel test. The Mantel test gives an opportunity to identify a correlation between genetic and geographic distances. For this kind of analysis, pairwise Euclidean distances were calculated and a histogram, as well as a scatterplot were computed to visualise Isolation by Distance patterns.

In addition to the F_{ST} calculations, other population differentiation statistics were performed using the R package *mmod* ver. 1.3.3 (Winter, 2012). The pairwise Nei's G_{ST} (Nei, 1973), as well as the pairwise Jost's D (Jost, 2008) statistics were computed to identify the genetic differentiation among the two subspecies.

Results

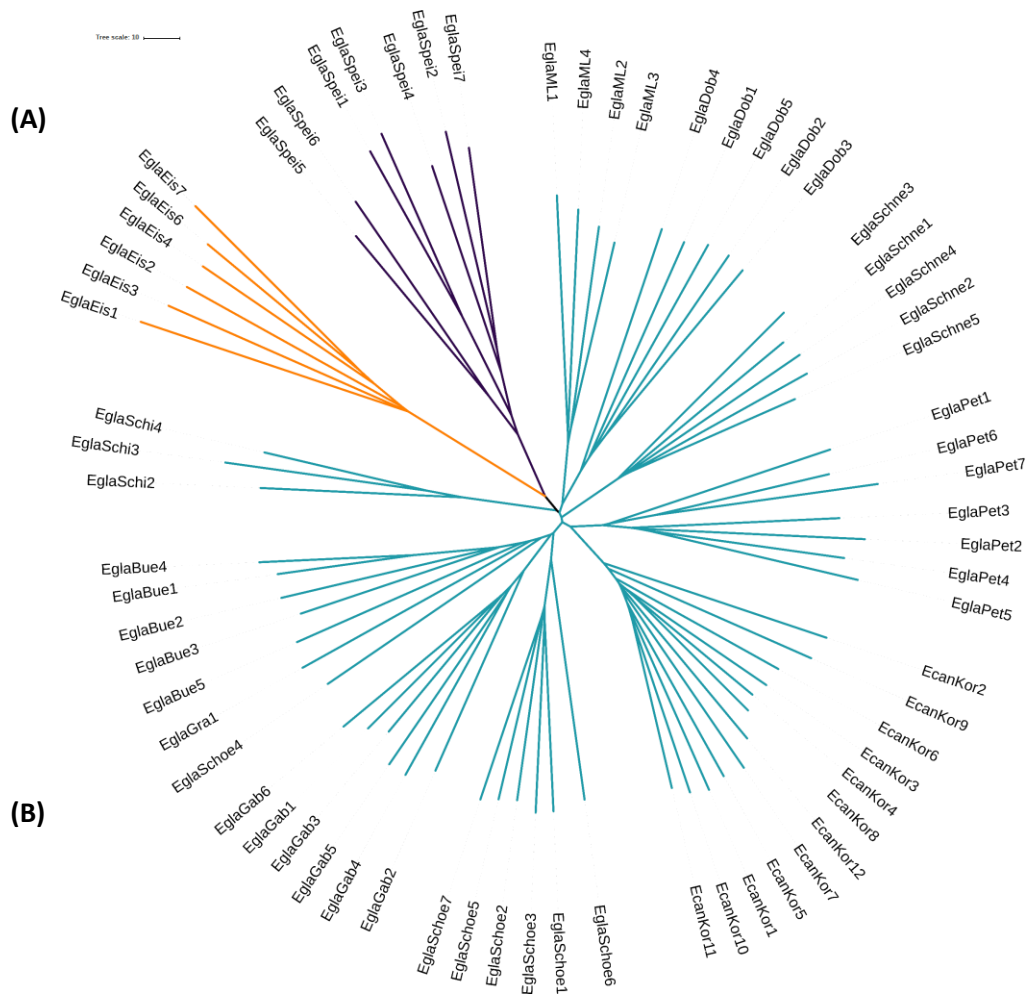
Substitution Sites

After demultiplexing and running the Stacks pipeline with all the whitelist filtering steps, 32,608 polymorphic sites were generated. Filtering loci with a maximum of 20% of missing data, produced 17,404 loci for further analysis.

The genetic variances between populations explained by the number of occurring substitution sites, as well as the types of substitution within each population is shown in the supplementary part (Appendix: **Table S.6**). The molecular genetic variation given by Theta.S (Θ_s) based on the number of segregating sites revealed the lowest genetic variation within the *E. glabratus* subsp. *candidus* (can: 983.587) population. The highest value of Θ_s was exhibit by the Italian population (ml: 1579.339). Furthermore, the mean numbers of pairwise differences measured as π were identifying *E. glabratus* subsp. *candidus* as the population with the lowest pairwise differences (can: 668.413). The more Central Alps populations, such as from Eisentalhöhe (eis: 2095.678) and from Speiereck (spei: 2191,697), showed the highest values of π . Similar results were observed by comparing the numbers of private alleles of all populations. The sampled population of *E. glabratus* subsp. *candidus* possessed 32 private substitution sites and the highest number was determined inside the Speiereck individuals (spei: 565 private alleles).

Population Structure

The analysis of the inferring population structure by using FastSTRUCTURE revealed a pattern of weak population structuring (**Figure 1.B**). The best number of genetic clusters was calculated as K=3. The three clusters showed a separation between the two more Central Alps populations, from the Eisentalhöhe and from Speiereck, from all the other sampled populations. *E. glabratus* subsp. *candidus* indicated the same genetic structure as all the other Eastern Alps populations. The same results were observed by using different numbers of genetic clusters (Appendix: **Figure S.2**)



K=3

Figure 2: Population structure of the sampled *Erigeron glaberratus* populations. **(A)** Neighbour-Joining Tree of all sampled individuals. The different colours represent the observed genetic structure within the populations, computed by FastSTRUCTURE **(B)**. The best fitting K was visualised using StructureSelector.

The same clustering was noticed by calculating a PCA based on the genetic differences of all the individuals (**Figure 2**). The sampled individuals of the *E. glaberratus* subsp. *candidus* did not show a genetic divergence from all the other Eastern Alps populations of the other subspecies, with the exception of the two more Central Alps populations. The Eigenvalues of the PCA revealed low genetic differences due to the flat slope of the Eigenvalues between

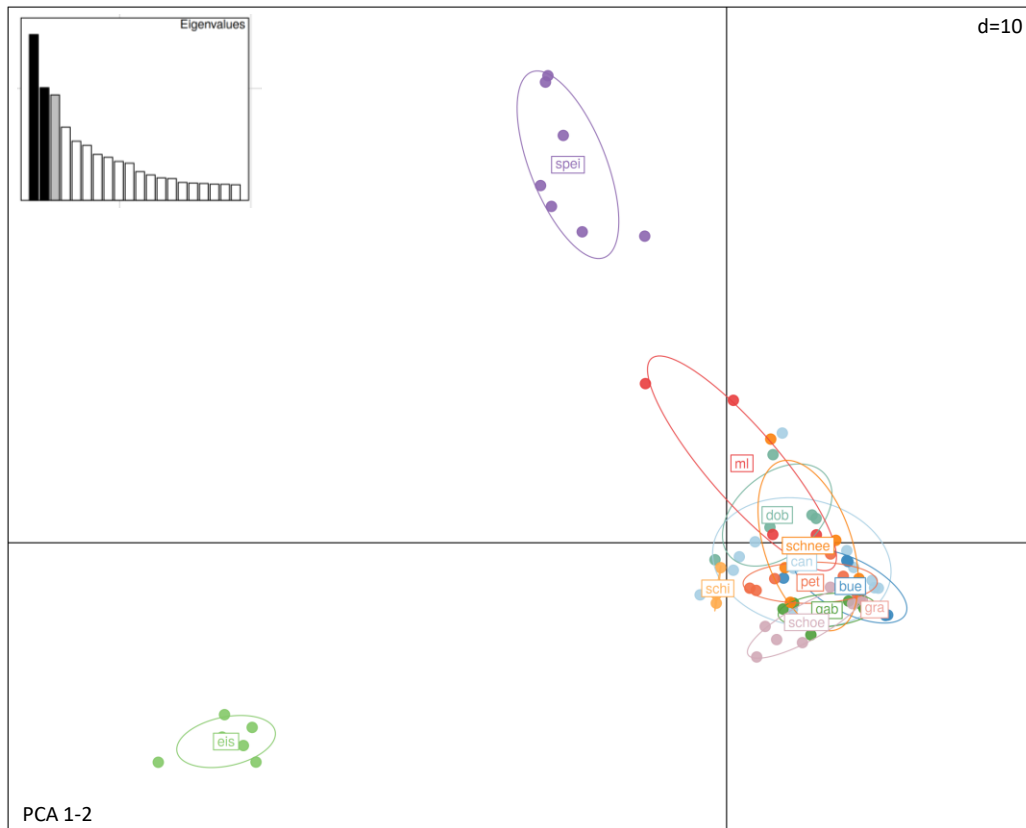


Figure 3: Results of the PCA (10 dimensions, $d=10$) based on genetic distances. PC 1 is representing 11.75% of the variance and PC 2 is indicating 7.98 % of the amount of the variance. Each colour highlights individuals of the same population.

all principle components. Only 11 % of the variances of the individuals can be explained by the first principle component.

Another way to understand the genetic structure among populations was revealed by using FineRADStructure. In general, FineRADStructure utilises haplotype linkage information to cluster common ancestry patterns. The co-ancestry patterns for all sampled individuals of the subspecies indicated only a clustering by locality and no shared ancestors between the different populations (Appendix: **Figure S.6**). A clustering by locality was also observed in the Neighbour-Joining tree (**Figure 1.A**). The unrooted Neighbour-Joining tree represented a star-like orientation with no clear clustering. Only the individuals sampled at the Eisentalhöhe and at Speiereck showed a weak

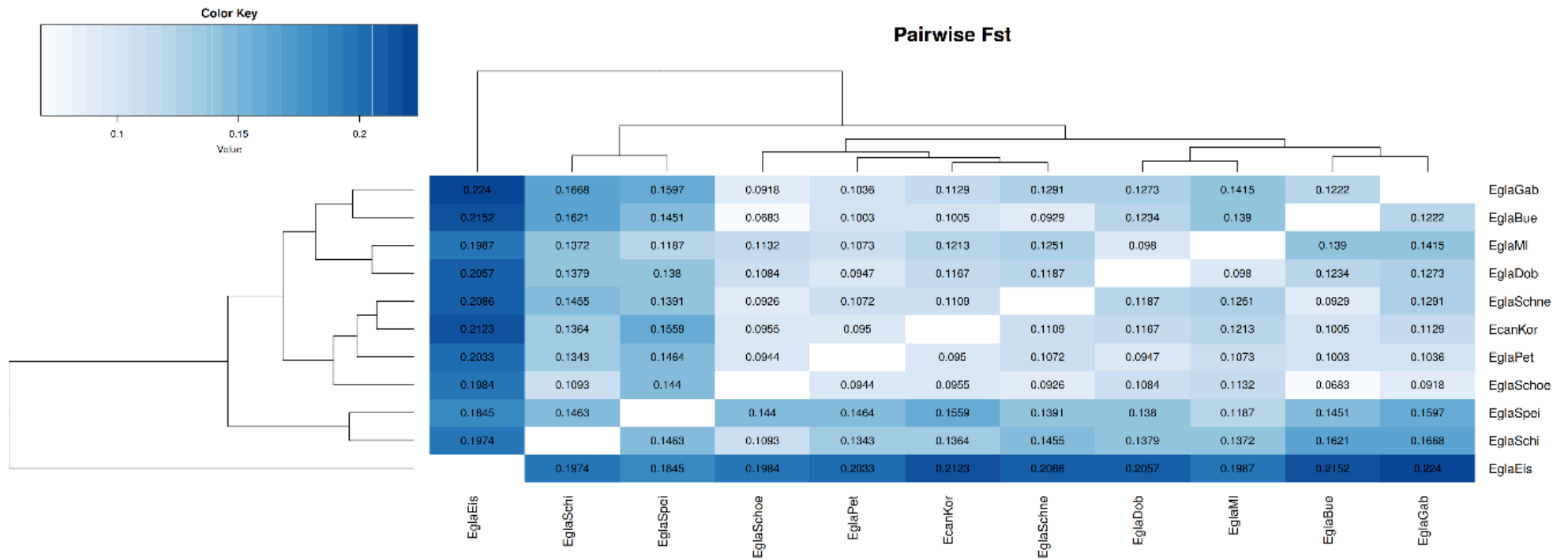


Figure 4: Results of the Pairwise F_{ST} statistics represented as a heatmap. Numbers of F_{ST} can range between zero and one, with one as the highest genetic diversity.

distance from the other populations. The subspecies of interest, *E. glabratus* subsp. *candidus*, did not show any strong divergence from *E. glabratus* subsp. *glabratus*.

Genetic Diversity

By calculating the pairwise F_{ST} , the genetic diversity within and among the sampled populations indicated again the genetic divergence of *E. glabratus* subsp. *glabratus* from the Eisentalhöhe from all the other populations. F_{ST} values can be ranked from zero to one, with zero indicating no differentiation between two populations. The highest difference was observed between the individuals of the Eisentalhöhe and individuals from the Bürgeralm (**Figure 3**). The other two populations from the more Central Alps sites, Speiereck and Schiestelnock, were also showing a stronger signal for genetic differentiation. The weakest genetic differentiation can be observed inside the population of the *E. glabratus* subsp. *glabratus* from the Schöckl mountain. Individuals of *Erigeron glabratus* subsp. *candidus* revealed no significant differentiation pattern inside the data. Similar results were observed by using other genetic diversity statistics, such as pairwise G_{ST} (Appendix: **Figure S.5**) and pairwise Jost's D (Appendix: **Figure S.4**).

Isolation by Distance

The results of the Mantel-Test, as shown in **Figure 4**, manifested a correlation between the geographical and genetic distances. By running the Mantel-Test on all individuals, strong outliers got visible (**Figure 4, A and C**). The outliers were created by the two more western populations from the Eisentalhöhe and Speiereck. Excluding these two populations, had a positive impact on the isolation by distance by increasing the significance of the correlation (**Figure 4, B and D**).

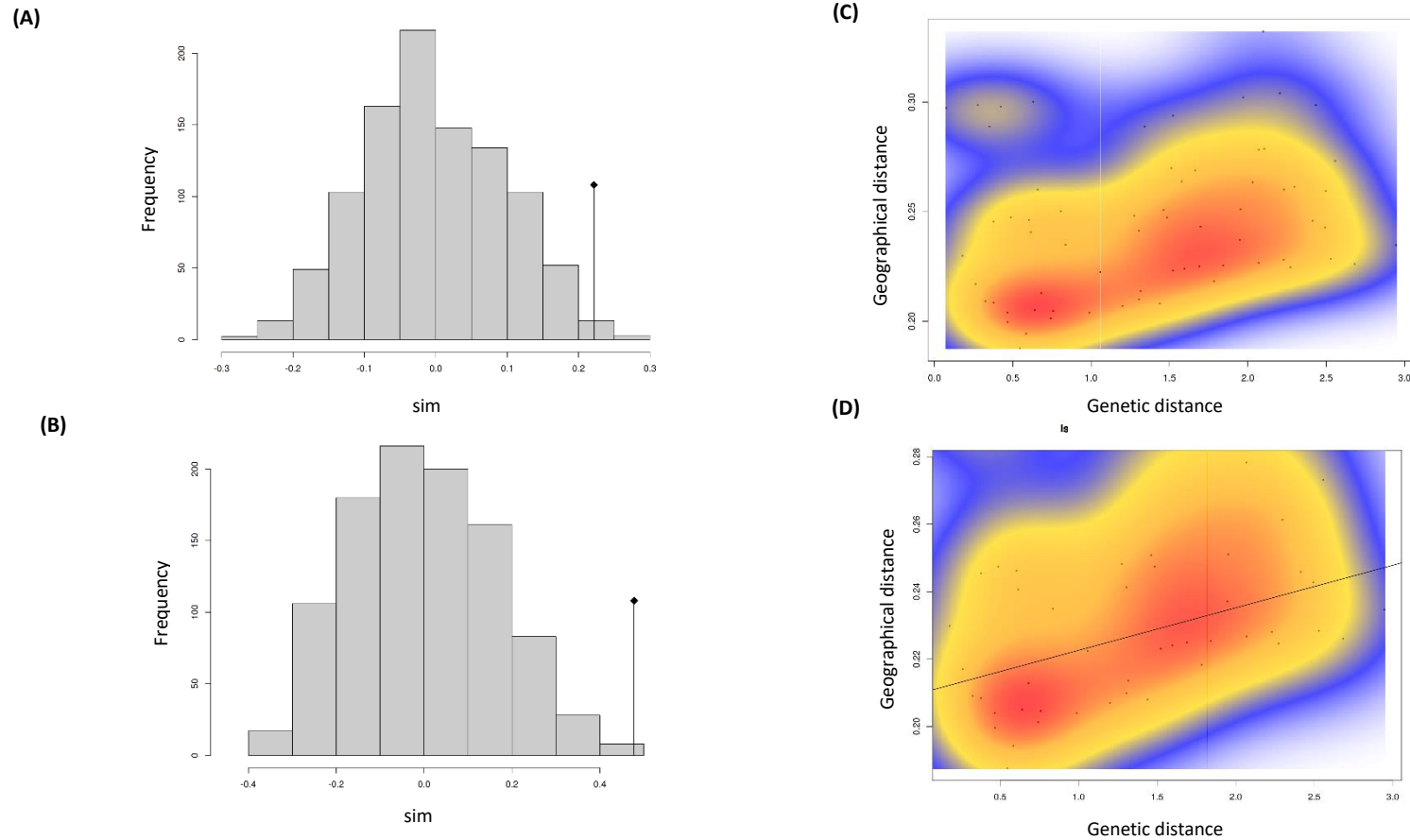


Figure 5: Results of the Mantel-Test: Histogram of simulated values (sim) of measured correlation between geographical distance and genetic distance (diamonds) of **(A)** all populations (observed $r = 0.222$, $p\text{-value} = 0.016$) and **(B)** without outlier (Eisentalhöhe and Speiereck were excluded, observed $r = 0.477$, $p\text{-value} = 0.003$). Coloured scatterplots (warmer colours indicating higher densities of points) of the genetic and geographical distances representing the relationship between both distances of all sampled individuals **(C)** and of the samples without outlying populations of Eisentalhöhe and Speiereck **(D)**.

Discussion

The alpine *Erigeron* species are known for their morphological variability within their populations. The subspecies *E. glabratus* subsp. *glabratus* is one of these taxa, which can develop a range of morphological traits, for example the colour of the petals can vary from dark purple to white in one population. Therefore, the taxon *E. glabratus* was once called *E. polymorphus* (Huber, 1993). In comparison, the subspecies *E. glabratus* subsp. *candidus* is quite unitary in its morphology, developing only single flowering heads with a white colour per individual. Especially the number of flowering heads was revealed as a significant difference between the two subspecies (Huber, 1993; Wilfing, 2013). A fixation of morphological traits could assume that genetic differences also appear between these two subspecies. The analysis of the ITS-marker, conducted by Katrin Wilfing (2013), identified no genetic differences between these two subspecies at this nuclear site of the genome. The investigation of multiple loci, which were randomly attached by the RAD-sequencing, provided an informative technique to achieve an overview of the population structure within the Eastern Central Alps populations.

The genetic structure of the two subspecies is giving no evidence for a genetic divergence between these two taxa (**Figure 2 & 3**). In addition, the Neighbour-Joining tree represents a clustering by locality with only small differentiation patterns and a high genetic similarity of the populations (**Figure 2.A**). The only observed differentiation occurs in populations, which are located in more central parts of the Alps, such as the individuals sampled at Speiereck (Salzburg) and individuals from the Eisentalhöhe (Nockberge, Carinthia). These two populations are showing more genetic diversity, for example as it can be revealed from the counts of the number of private alleles and from the high Θ_S and π values (Appendix: **Table S.6**). Comes & Kadereit (1998) assumed a reduction in genetic diversity with increasing the distance from a refugium. They explained this phenomenon with repeated population bottleneck events that appeared during postglacial expansion. Additionally, they suggested higher levels of genetic diversity in refugial areas. In this study, the theory of Comes & Kadereit (1998) cannot be confirmed. Not only the populations of Eisentalhöhe and Speiereck are showing a greater genetic diversity, also other populations from non-refugial areas, which were covered by glaciers during the last ice age, are representing higher genetic variability within their populations, for example individuals from Monte Lussari (**Figure 1**,

Appendix: **Table S.6**), whereas, populations from hypothetical refugial areas, such as the Koralpe (*E. glaberratus* subsp. *candidus*), from Gaberl (Styria) or Bürgeralm (Styria), have lower numbers of substitutional sites or private alleles. The low Θ_S and π values of *E. glaberratus* subsp. *candidus* could have been influenced by the sample size of the population, but the low diversity can also be observed in the reduced number of private substitution sites of the *candidus* population, which is not depending on the sample size. Higher genetic diversity in non-refugial areas might occur due to introgression with other alpine *Erigeron* species or the localities might have been a contact zone for several expanding refugial populations. Which means that *E. glaberratus* species from different refugial zones, such as from the southern or western edges of the Alps, might have recolonised the Central Alps and increase the genetic diversity in these contact zones. But to test this assumption, populations from all over the distribution sites of *E. glaberratus* would be necessary to be analysed. Even though the populations of Eisentalhöhe and Speiereck are identified to be more differentiated from the other populations, the F_{ST} values are still quite low and around zero, which indicates only a weak signal for divergence.

Nevertheless, bottleneck populations in the history of *E. glaberratus* might have led to the fixation of the *E. glaberratus* subsp. *candidus* characteristics, such as the single flowering heads. The Koralpe offers only a small habitat restricted to the marble bands, which are surrounded by more acidic soils. On the one hand, this may lead to smaller populations, which are limited in their ability to perform gene flow with other populations and on the other hand it may have led to adaptation to the local conditions. Selection against mating between individuals specialised to different local environments can lead to higher specialisation and to a gradual speciation, which can be subdividing species (Dynesius and Jansson, 2000). A gradual speciation cannot be confirmed for *E. glaberratus* subsp. *candidus* due to the high genetic similarity to *E. glaberratus* subsp. *glaberratus* populations of the Eastern Central Alps. Katrin Wilfing (2013) observed a significant difference in the ecology of the two subspecies, which can easily be explained by the pore sizes and resulting differences in humidity of the limestone and marble soils, as well as by the acidic conditions of the Koralpe. These structural differences might be causing the white colour of the petals. An ecological impact on the morphology of *E. glaberratus* subsp. *candidus* could explain the lack in divergence inside the genetic structure (**Figure 2 & 3**).

The co-ancestry reveals a clustering by locality (Appendix: **Figure S.6**). The sampled populations were all restricted to small areas. At most of the spots, only a few individuals could be found per population. Alpine environments and patchy distribution of habitats lead to spatial isolation with decreased gene flow and smaller population sizes (Stöcklin *et al.*, 2009). Regarding the observed common ancestry pattern, bottleneck populations may have caused a fixation of some genetic variations. Additionally, the isolation by distance can be seen as a main driver for the low genetic differentiation in the Eastern Central Alps (**Figure 5**). Excluding the Eisentalhöhe and Speiereck populations from the data, led to an increase of the isolation by distance signal, which reveals that the genetic variation in these two outlier populations is caused by another reason instead of a geographical isolation.

This study focuses only on a small spatial scale regarding that the sampling localities of the *glabratus* subspecies were only between 30 km and 150 km distant from the *candidus* subspecies. By including populations from more remote locations the similarity of the *candidus* subspecies to the Eastern Central Alps *glabratus* populations may become more significant.

All in all, combining the morphometric and ecological analyses of Katrin Wilfing (2013) and the results of the population genetics of the two subspecies of this study leads to the rejection of defining *E. glabratus* subsp. *candidus* as an own subspecies due to the strong similarity between these two taxa. Huber (1993) categorised the Koralpe populations as a subspecies based on the geographical separation and the homogenous morphology. These two criteria can be used to categorise the Koralpe population as a variety of *Erigeron glabratus*.

The local endemic *E. glabratus* subsp. *candidus* is listed as an endangered species in Austria (Komposch *et al.*, 2016) due to its local restriction. As a conclusion, considering to reject it as a subspecies will lead to the loss of the conservational status of the taxon. The Koralpe is a habitat for many endemics and therefore it is necessary to protect the whole area of the mountain range. The separation of species due to isolation as well as differentiation of populations takes tens of thousands to millions of years (Dynesius and Jansson, 2000). For speciation processes, the populations need to subsist during the time of evolving genetic divergence and reproductive barriers. There are evidences for isolation by distance and for the fixation of certain morphological traits in the Koralpe individuals, even though we cannot

identify them as genetically separated subspecies, yet. However, the individuals of the Koralpe might be evolving in a sub-speciation process, right now and this is why it is substantial to protect their habitat.

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Appendix

Table S.1: Sampling information of all 70 individuals, including Sampling numbers, altitude [m], Coordinates [Decimal Degree], name of the location, collector and the abbreviation of the population

Sampling Number	Species	Altitude	Coordinates (Northing, Easting)	Location	Collector	Population
CH01	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1683	47.58238, 15.23443	Hochschwab (Bürgeralm)	C.Hedderich	Bue
CH02	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1683	47.58246, 15.23437	Hochschwab (Bürgeralm)	C.Hedderich	Bue
CH03	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1684	47.58263, 15.23455	Hochschwab (Bürgeralm)	C.Hedderich	Bue
CH04	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1684	47.58262, 15.23472	Hochschwab (Bürgeralm)	C.Hedderich	Bue
CH05	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1683	47.58236, 15.23462	Hochschwab (Bürgeralm)	C.Hedderich	Bue
CH06	<i>Erigeron glabratus</i> subsp. <i>candidus</i>	1763	46.81473, 14.9901	Koralpe (Bärentalkar)	C.Hedderich	Kor (Can)
CH07	<i>Erigeron glabratus</i> subsp. <i>candidus</i>	1763	46.81473, 14.99004	Koralpe (Bärentalkar)	C.Hedderich	Kor (Can)
CH08	<i>Erigeron glabratus</i> subsp. <i>candidus</i>	1763	46.81471, 14.99007	Koralpe (Bärentalkar)	C.Hedderich	Kor (Can)
CH09	<i>Erigeron glabratus</i> subsp. <i>candidus</i>	1764	46.81472, 14.99005	Koralpe (Bärentalkar)	C.Hedderich	Kor (Can)
CH10	<i>Erigeron glabratus</i> subsp. <i>candidus</i>	1764	46.81471, 14.99005	Koralpe (Bärentalkar)	C.Hedderich	Kor (Can)
CH11	<i>Erigeron glabratus</i> subsp. <i>candidus</i>	1764	46.81474, 14.99002	Koralpe (Bärentalkar)	C.Hedderich	Kor (Can)
CH12	<i>Erigeron glabratus</i> subsp. <i>candidus</i>	1764	46.8147, 14.99001	Koralpe (Bärentalkar)	C.Hedderich	Kor (Can)
CH13	<i>Erigeron glabratus</i> subsp. <i>candidus</i>	1738	46.81413, 14.991	Koralpe (Bärentalkar)	C.Hedderich	Kor (Can)
CH14	<i>Erigeron glabratus</i> subsp. <i>candidus</i>	1739	46.81413, 14.99099	Koralpe (Bärentalkar)	C.Hedderich	Kor (Can)
CH15	<i>Erigeron glabratus</i> subsp. <i>candidus</i>	1739	46.81412, 14.991	Koralpe (Bärentalkar)	C.Hedderich	Kor (Can)
CH16	<i>Erigeron glabratus</i> subsp. <i>candidus</i>	1737	46.81412, 14.99099	Koralpe (Bärentalkar)	C.Hedderich	Kor (Can)
CH17	<i>Erigeron glabratus</i> subsp. <i>candidus</i>	1737	46.80661, 14.98549	Koralpe (Bärentalkar)	C.Hedderich	Kor (Can)
CH18	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1275	47.34021, 15.42685	Grazer Bergland, Tyrnauer Alm	C. Berg	Gra
CH19	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1984	47.12922, 13.63962	Speiereck	C. Hedderich	Spei
CH20	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1985	47.12921, 13.63965	Speiereck	C. Hedderich	Spei
CH21	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1985	47.12921, 13.63968	Speiereck	C. Hedderich	Spei
CH22	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1983	47.12913, 13.63964	Speiereck	C. Hedderich	Spei
CH23	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1983	47.12911, 13.63967	Speiereck	C. Hedderich	Spei
CH24	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1983	47.12913, 13.63979	Speiereck	C. Hedderich	Spei
CH25	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1983	47.12913, 13.63983	Speiereck	C. Hedderich	Spei
CH26	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2109	46.93791, 13.76136	Eisenthalhöhe	C. Hedderich	Eis
CH27	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2109	46.93786, 13.76138	Eisenthalhöhe	C. Hedderich	Eis
CH28	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2108	46.93782, 13.7614	Eisenthalhöhe	C. Hedderich	Eis

CH29	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2108	46.93784, 13.76148	Eisentalhöhe		C. Hedderich	Eis
CH30	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2108	46.93795, 13.76143	Eisentalhöhe		C. Hedderich	Eis
CH31	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2104	46.93759, 13.76102	Eisentalhöhe		C. Hedderich	Eis
CH32	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2104	46.93753, 13.76103	Eisentalhöhe		C. Hedderich	Eis
CH33	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1767	46.59537, 13.70513	Dobratsch		C. Hedderich	Dob
CH34	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1768	46.59534, 13.70506	Dobratsch		C. Hedderich	Dob
CH35	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1770	46.59531, 13.70496	Dobratsch		C. Hedderich	Dob
CH36	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1771	46.59528, 13.70497	Dobratsch		C. Hedderich	Dob
CH37	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1771	46.59523, 13.70492	Dobratsch		C. Hedderich	Dob
CH38	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2011	46.88955, 13.79901	Schiestelnock		C. Hedderich	Schi
CH39	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2012	46.88954, 13.79903	Schiestelnock		C. Hedderich	Schi
CH40	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2015	46.88954, 13.79901	Schiestelnock		C. Hedderich	Schi
CH41	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2015	46.88956, 13.79913	Schiestelnock		C. Hedderich	Schi
CH42	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1742	46.48048, 13.52326	Monte (Italien)	Lussari	C. Hedderich	ML
CH43	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1742	46.48049, 13.52326	Monte (Italien)	Lussari	C. Hedderich	ML
CH44	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1743	46.48048, 13.52325	Monte (Italien)	Lussari	C. Hedderich	ML
CH45	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1743	46.4805, 13.52327	Monte (Italien)	Lussari	C. Hedderich	ML
CH46	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1435	47.19797, 15.45932	Schöckl		C. Hedderich	Schoe
CH47	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1435	47.19797, 15.45937	Schöckl		C. Hedderich	Schoe
CH48	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1436	47.19794, 15.45934	Schöckl		C. Hedderich	Schoe
CH49	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1440	47.19783, 15.46019	Schöckl		C. Hedderich	Schoe
CH50	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1440	47.1978, 15.46025	Schöckl		C. Hedderich	Schoe
CH51	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1440	47.19777, 15.46025	Schöckl		C. Hedderich	Schoe
CH52	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1440	47.19775, 15.46033	Schöckl		C. Hedderich	Schoe
CH53	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1690	47.0808, 14.9245	Gaberl		C. Hedderich	Gab
CH54	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1690	47.08078, 14.92446	Gaberl		C. Hedderich	Gab
CH55	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1703	47.08035, 14.92428	Gaberl		C. Hedderich	Gab
CH56	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1703	47.08031, 14.92425	Gaberl		C. Hedderich	Gab
CH57	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1704	47.08017, 14.92435	Gaberl		C. Hedderich	Gab
CH58	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1704	47.07647, 14.92986	Gaberl		C. Hedderich	Gab
CH59	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2030	46.50454, 14.76655	Petzen		C. Hedderich	Pet
CH60	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2030	46.50454, 14.76659	Petzen		C. Hedderich	Pet
CH61	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2033	46.50452, 14.76665	Petzen		C. Hedderich	Pet
CH62	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2033	46.5046, 14.76666	Petzen		C. Hedderich	Pet
CH63	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2034	46.50475, 14.76661	Petzen		C. Hedderich	Pet
CH64	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2034	46.50475, 14.76665	Petzen		C. Hedderich	Pet

CH65	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	2034	46.50478, 14.76675	Petzen	C. Hedderich	Pet
CH66	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1876	47.70248, 15.59528	Schneealpe	C. Berg	Schne
CH67	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1876	47.70248, 15.59529	Schneealpe	C. Berg	Schne
CH68	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1876	47.70248, 15.59530	Schneealpe	C. Berg	Schne
CH69	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1876	47.70248, 15.59531	Schneealpe	C. Berg	Schne
CH70	<i>Erigeron glabratus</i> subsp. <i>glabratus</i>	1876	47.70248, 15.59532	Schneealpe	C. Berg	Schne

Table S.2: Compounds of the Sorbitol buffer.

Sorbitol buffer (pH 8)

Tris-HCL	100 mM	12.114 g/L
Ethylenediaminetetraacetic acid (EDTA)	5 mM	1.461 g/L
Sorbitol	0.32 M	63.77 g/L

Table S.3: Summary of all six sub-libraries including the sampled individual (ID and number), the DNA quantity [ng/μl], the barcode of the adapters, as well as the numbers of the P1 and P2-adapters.

	No.	Sample	ng/ul	volume/150ng	for 40 ul	P1-	P2	P1-2	P1-	P2		
		number			H2O	1			1			
sub-library 1	1	1	EglaSchne1	66	2,52	59,52	-19,5	14	2	ACTGAT	507	2
	2	2	EglaSchne1	66	2,52	59,52	-19,5	15	2	TGACCA	507	2
	3	3	EglaBü4	4	3,52	42,61	-2,6	16	2	ACTGAT	506	2
	4	4	EglaBü4	4	3,52	42,61	-2,6	17	2	TGACCA	506	2
	5	5	EglaBü5	5	5,98	25,08	14,9	18	2	GAGTGG	506	2
	6	6	EglaSchi2	39	7,68	19,53	20,5	19	2	CTCATC	506	2
	7	7	EglaSchne4	69	7,9	18,99	21,0	20	2	GAGTGG	507	2
	8	8	EglaBü1	1	8,8	17,05	23,0	21	2	CTCATC	507	2
	9	9	EglaSchne2	67	9,16	16,38	23,6	22	2	ACTGAT	508	2
	10	10	EglaSchne3	68	10,8	13,89	26,1	23	2	TGACCA	508	2
	11	11	EcanKor2	7	13,6	11,03	29,0	24	2	GAGTGG	508	2
	12	12	EcanKor6	11	13,9	10,79	29,2	25	2	CTCATC	508	2
sub-library 2	1	13	EglaSchi3	40	14,3	10,49	29,5	14	3	ACTGAT	507	3
	2	14	EglaSchoe2	47	19	7,89	32,1	15	3	TGACCA	507	3
	3	15	EglaSchne5	70	19,1	7,85	32,1	16	3	ACTGAT	506	3
	4	16	EcanKor5	10	21,6	6,94	33,1	17	3	TGACCA	506	3
	5	17	EglaSpei6	24	22,2	6,76	33,2	18	3	GAGTGG	506	3
	6	18	EglaSchoe3			6,7		19	3	CTCATC	506	3

sub-library 3	7	19	EglaSchoe5	50	22,4	6,70	33,3	20	3	GAGTGG	507	3
	8	20	EcanKor4	9	22,6	6,64	33,4	21	3	CTCATC	507	3
	9	21	EglaSpei5	23	22,8	6,58	33,4	22	3	ACTGAT	508	3
	10	22	EglaML4	45	23	6,52	33,5	23	3	TGACCA	508	3
	11	23	EglaPet7	65	23,2	6,47	33,5	24	3	GAGTGG	508	3
	12	24	EglaML3	44	25	6,00	34,0	25	3	CTCATC	508	3
	1	25	EglaML2	43	26,6	5,64	34,4	14	4	ACTGAT	507	4
	2	26	EcanKor11	16	27,2	5,51	34,5	15	4	TGACCA	507	4
	3	27	EcanKor3	8	28,2	5,32	34,7	16	4	ACTGAT	506	4
	4	28	EcanKor10	15	28,2	5,32	34,7	17	4	TGACCA	506	4
	5	29	EglaGab4	56	28,6	5,24	34,8	18	4	GAGTGG	506	4
	6	30	EglaDob5	37	29,4	5,10	34,9	19	4	CTCATC	506	4
sub-library 4	7	31	EcanKor12	17	31	4,84	35,2	20	4	GAGTGG	507	4
	8	32	EcanKor8	13	32	4,69	35,3	21	4	CTCATC	507	4
	9	33	EglaDob2	34	34,2	4,39	35,6	22	4	ACTGAT	508	4
	10	34	EglaDob1	33	34,4	4,36	35,6	23	4	TGACCA	508	4
	11	35	EglaEis7	32	34,8	4,31	35,7	24	4	GAGTGG	508	4
	12	36	EglaPet4	62	36,4	4,12	35,9	25	4	CTCATC	508	4
	1	37	EglaSchi4	41	38,8	3,87	36,1	14	6	ACTGAT	507	6
	2	38	EglaSchi1	38	40	3,75	36,3	15	6	TGACCA	507	6
	3	39	EglaPet6	64	41,4	3,62	36,4	16	6	ACTGAT	506	6
	4	40	EcanKor1	6	42	3,57	36,4	17	6	TGACCA	506	6
	5	41	EcanKor9	14	42,2	3,55	36,4	18	6	GAGTGG	506	6
	6	42	EglaSpei4	22	42,6	3,52	36,5	19	6	CTCATC	506	6
sub-library 5	7	43	EglaSchoe7	52	43,6	3,44	36,6	20	6	GAGTGG	507	6
	8	44	EglaGab3	55	44,4	3,38	36,6	21	6	CTCATC	507	6
	9	45	EglaBü3	3	47,2	3,18	36,8	22	6	ACTGAT	508	6
	10	46	EcanKor7	12	47,8	3,14	36,9	23	6	TGACCA	508	6
	11	47	EglaML1	42	48,4	3,10	36,9	24	6	GAGTGG	508	6
	12	48	EglaEis5	30	49,2	3,05	37,0	25	6	CTCATC	508	6
	1	49	EglaEis3	28	49,8	3,01	37,0	14	9	ACTGAT	507	9
	2	50	EglaBü2	2	55,2	2,72	37,3	15	9	TGACCA	507	9
	3	51	EglaGab2	54	60,6	2,48	37,5	16	9	ACTGAT	506	9
	4	52	EglaSpei7	25	62,4	2,40	37,6	17	9	TGACCA	506	9
	5	53	EglaPet5	63	62,6	2,40	37,6	18	9	GAGTGG	506	9
	6	54	EglaEis6	31	62,8	2,39	37,6	19	9	CTCATC	506	9
Sub-library 6	7	55	EglaGab5	57	63	2,38	37,6	20	9	GAGTGG	507	9
	8	56	EglaGra1	18	63,2	2,37	37,6	21	9	CTCATC	507	9
	9	57	EglaDob3	35	64	2,34	37,7	22	9	ACTGAT	508	9
	10	58	EglaSpei3	21	66,2	2,27	37,7	23	9	TGACCA	508	9
	11	59	EglaPet2	60	66,6	2,25	37,7	24	9	GAGTGG	508	9
	12	60	EglaGab1	53	69,6	2,16	37,8	25	9	CTCATC	508	9
	1	61	EglaSchoe1	46	70,8	2,12	37,9	14	10	ACTGAT	507	10
	2	62	EglaSpei1	19	73,6	2,04	38,0	15	10	TGACCA	507	10
	3	63	EglaSchoe4	49	76,8	1,95	38,0	16	10	ACTGAT	506	10
	4	64	EglaEis1	26	78	1,92	38,1	17	10	TGACCA	506	10
	5	65	EglaEis4	29	78,2	1,92	38,1	18	10	GAGTGG	506	10
	6	66	EglaGab6	58	85	1,76	38,2	19	10	CTCATC	506	10

7	67	EglaEis2	27	91,4	1,64	38,4	20	10	GAGTGG	507	10
8	68	EglaPet3	61	95,2	1,58	38,4	21	10	CTCATC	507	10
9	69	EglaPet1	59	97,8	1,53	38,5	22	10	ACTGAT	508	10
10	70	EglaSpei2	20	99,4	1,51	38,5	23	10	TGACCA	508	10
11	71	EglaDob4	36	102	1,47	38,5	24	10	GAGTGG	508	10
12	72	EglaSchoe6	51	110	1,36	38,6	25	10	CTCATC	508	10
No.		Sample number	ng/ul	volume/150ng	for 40 ul	P1- H2O	P2	P1-2	P1- 1	P2	

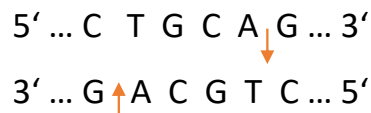


Figure S.1: Recognition and cutting sites of the restriction enzyme *PstI* (New England Biolabs).

Table S.4: Quantified DNA of the six sub-libraries using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific). The measured DNA (2nd sub-library) content was used to calculate the sample volume of the other sub-library for the following steps. The DNA was diluted in ddH₂O.

Sub-library	quantified DNA [μ l/ng]	sample [μ l]	ddH ₂ O [μ l]
1	1.75	all	/
2	2.08	all	/
3	3.14	13.6	6.9
4	3.28	13.0	7.5
5	5.18	8.23	12.27
6	3.36	12.7	7.81

Table S.5: Measured DNA concentration on Qubit 3.0 Fluorometer (Thermo Fisher Scientific) at different stages of the RADseq library preparation.

	DNA quantity [μ l/ng]
before PCR	4.32
after PCR	before size selection 69.00
after PCR	after size selection 14.80

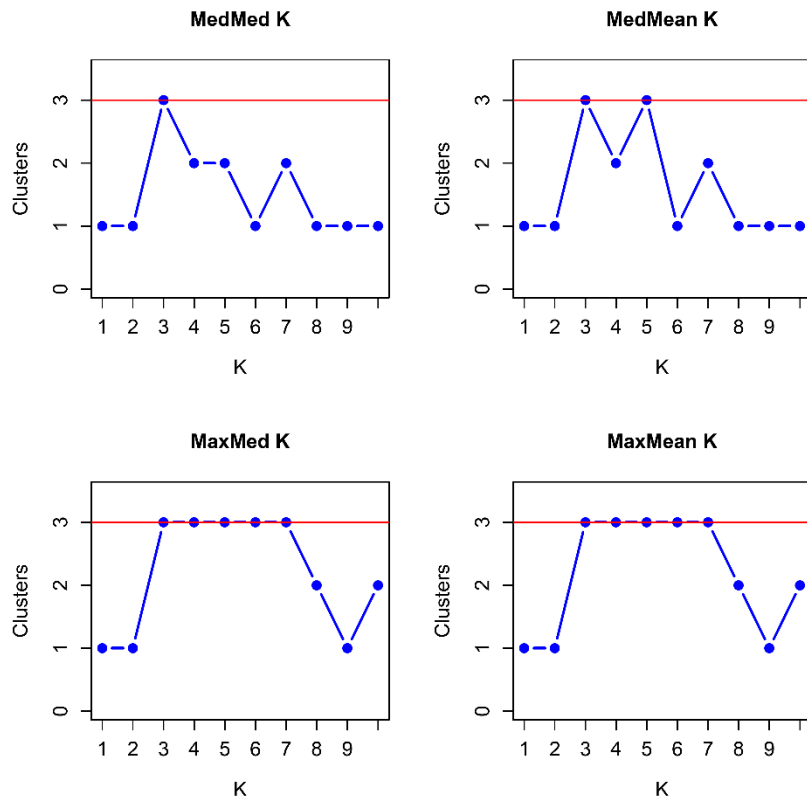
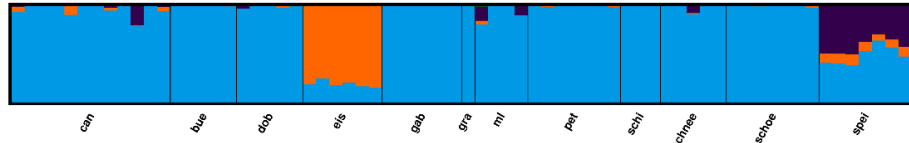
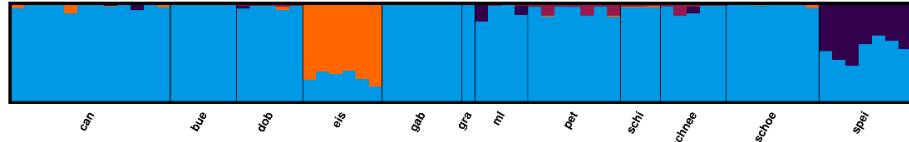


Figure S.2: Results of the FastSTRUCTURE analysis representing the estimators MedMed K, MaxMed K, MaxMean K and MedMeanK, which are based on the number of clusters that are included in at least on sampling locality/population illustrated in StructureSelector.

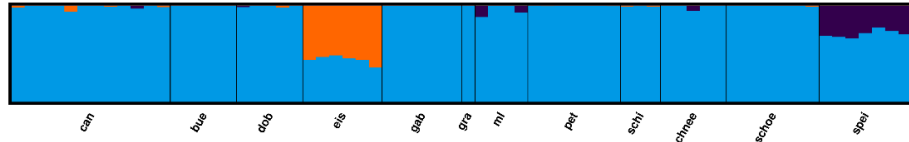
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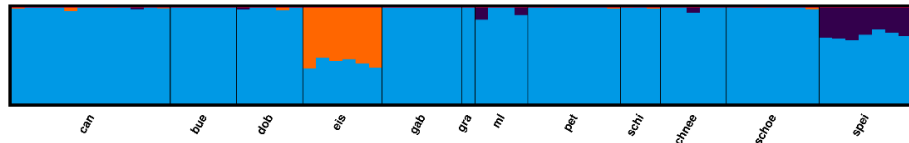
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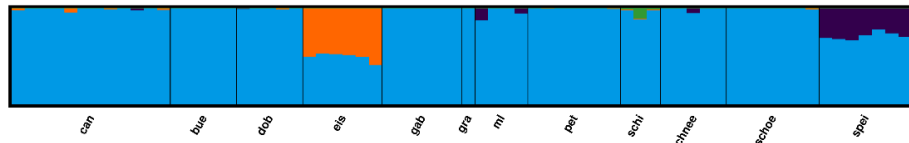
K=6



K=7



K=8



K=9

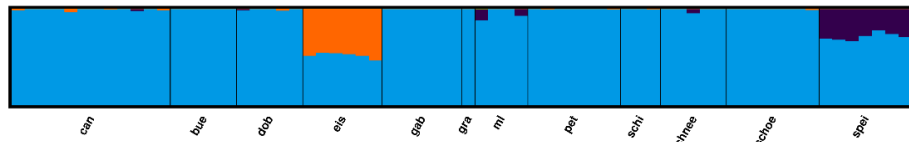


Figure S.3: Results of the FastSTRUCTURE analysis representing different estimated K clusters.

Table S.6: Arlequin Output, including the type of substitutions: Number of Transitions, Number of Transversions, as well as the total number of substitutions within each population, number of private alleles and the molecular diversity indices Theta S (Θ_S) and Pi (π).

Population	can	bue	dob	eis	gab	ml	pet	sch	schne	schoe	spei
Number of Transitions	2142	2215	2277	2733	2177	2423	2439	1925	2298	2301	2972
Number of Transversions	1531	1545	1588	1869	1538	1672	1708	1280	1571	1668	2043
Number of Substitutions	3673	3760	3865	4602	3715	4095	4147	3205	3869	3969	5015
Number of Private Substitution Sites	32	85	136	313	118	63	195	113	77	64	565
Pi	668,413	1442,363	1481,032	2095,678	1441,45	1776,178	1314,432	1634,682	1451,685	1177,866	2191,697
Theta_S	983,587	1329,106	1366,222	1523,90295	1230,182	1579,339	1304,033	1403,650	1367,636	1248,061	1576,978
s.d. Theta_S	316,142	534,255	549,164	582,482	470,285	677,070	478,520	657,529	549,732	457,997	578,601

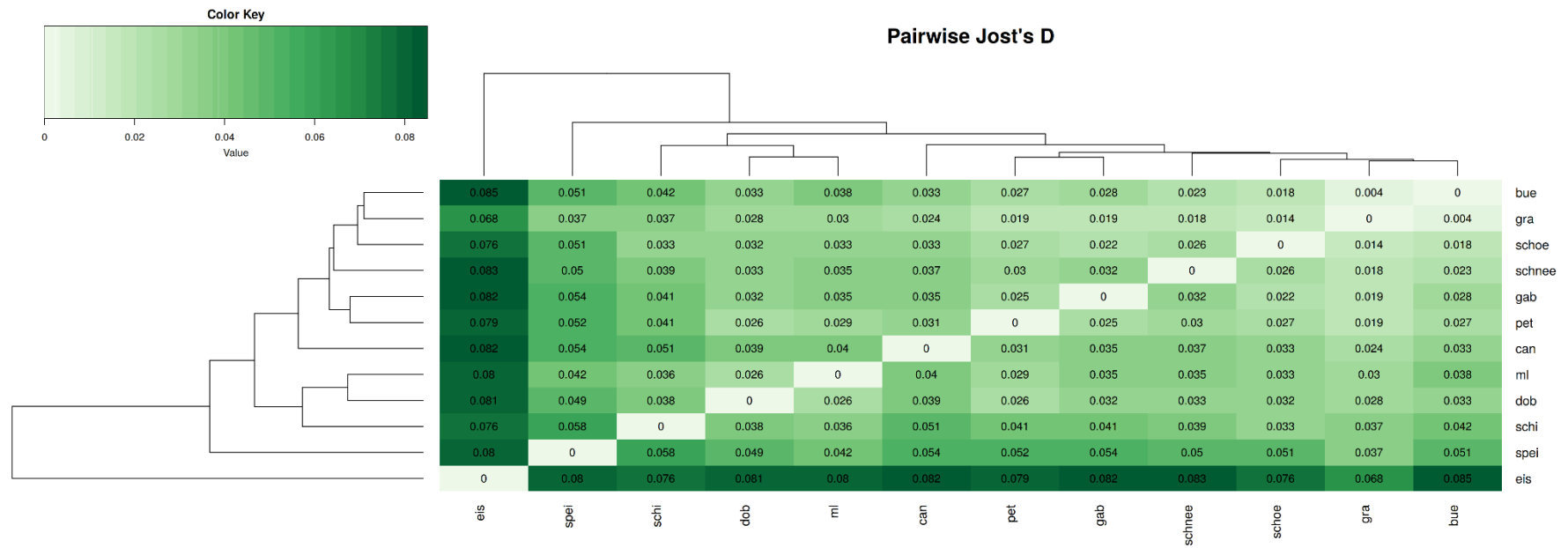


Figure S.4: Results of the Pairwise Jost's D_T statistics represented as a heatmap.

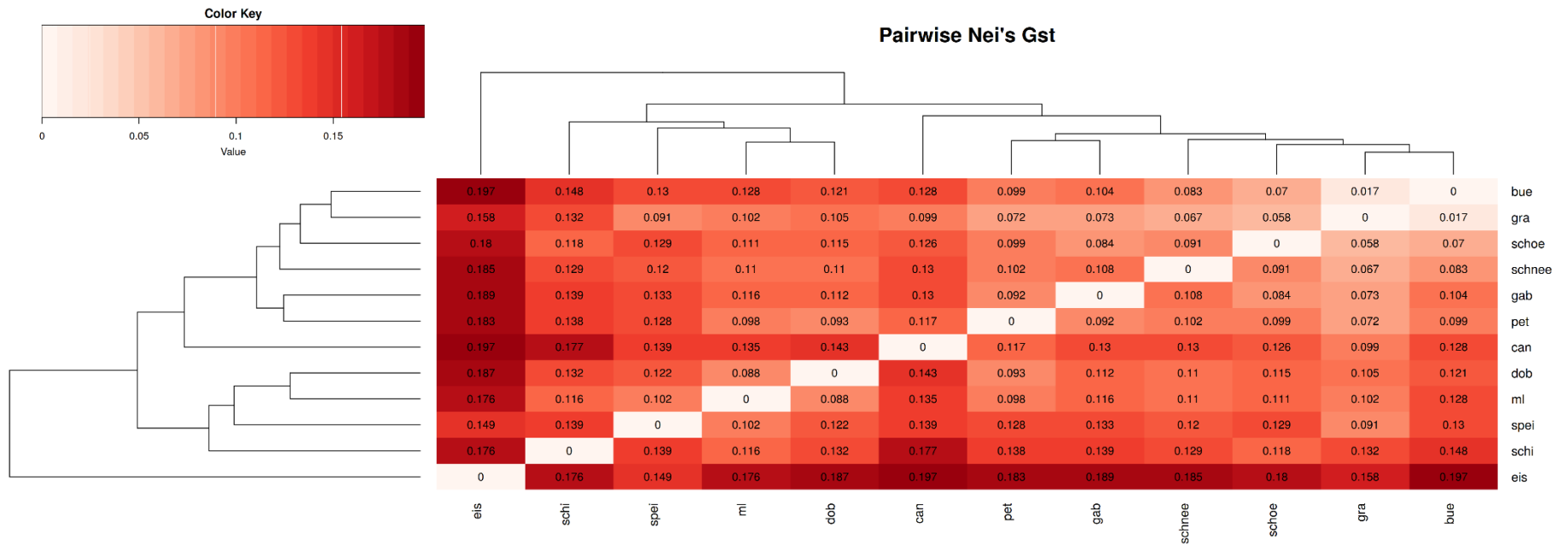


Figure S.5: Results of the Pairwise G_{ST} statistics represented as a heatmap.

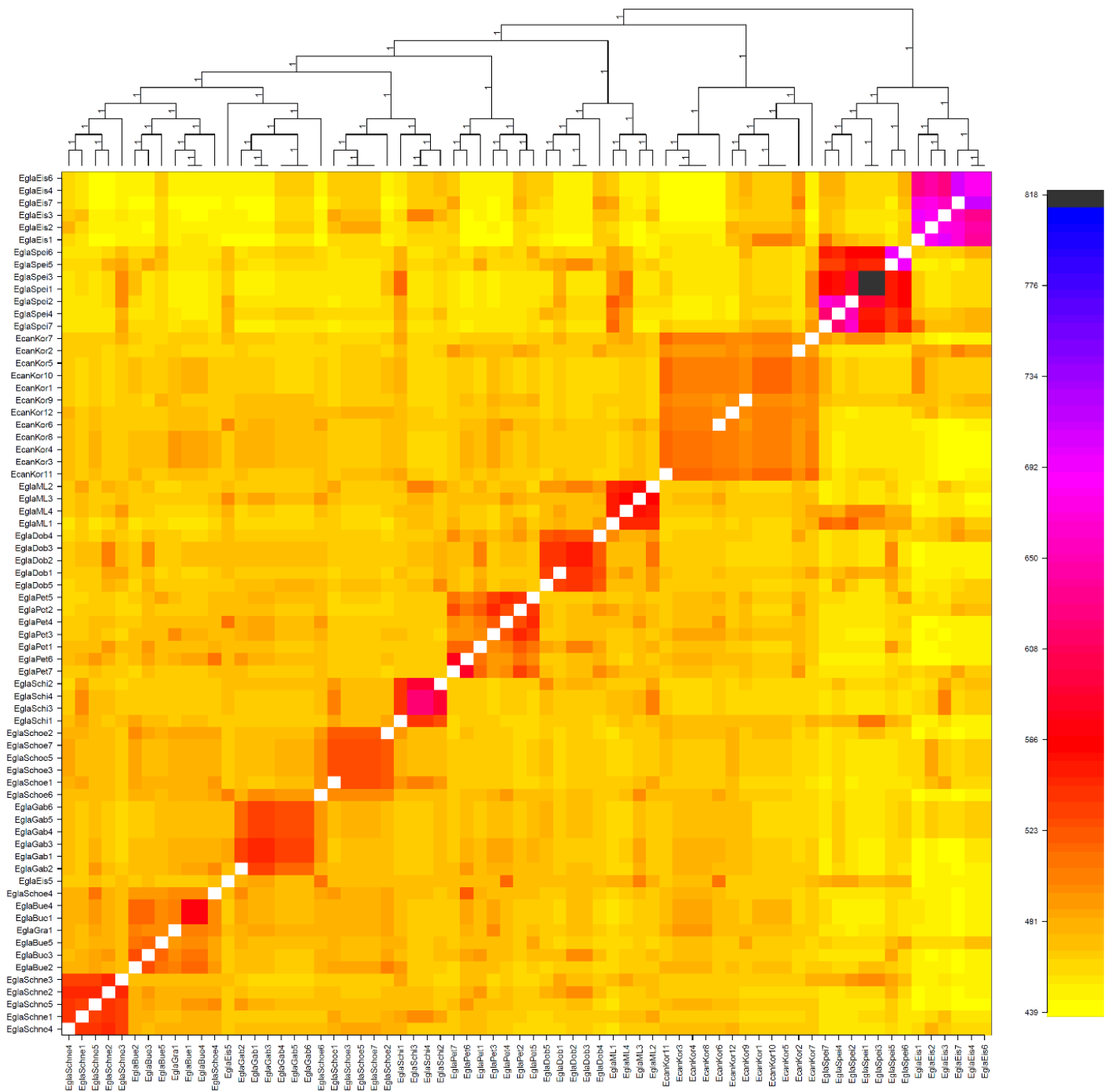


Figure S.6: Results of the Co-Ancestry analysis using FineRADStructure. The analysis includes all 70 individuals. Co-ancestry matrix is represented as a heatmap and the cladogram is illustrating the relationships between the population, which is focusing on recent coalescence.