Genetic structure and pollination biology of the FFH-protected *Gladiolus palustris* GAUDIN (Iridaceae) in Salzburg and adjacent areas

Masterarbeit

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The one process now going on that will take millions of years to correct is the loss of genetic and species diversity by the destruction of natural habitats. This is the folly our descendants are least likely to forgive us (Edward O. Wilson, 1984).

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Abstract

Due to the loss of potential habitats because of anthropogenic fragmentation many plant populations became small and isolated. Small populations are often facing negative genetic consequences like reduced genetic variation, genetic drift, founder effects, inbreeding depression and accumulation of deleterious mutations. These factors may lead on to a weaker future adaption potential to environmental changes, reduced plant fitness and consequential higher risk of becoming extinct. In this particular field, conservation genetics becomes enormously applicable. It targets the comprehension of dynamics of genes in populations via genetic methods to develop management plans for persistence and protection of the population. A plant species of conservational concern is the marsh gladiolus (*Gladiolus palustris*), which is critically endangered in Austria and therefore also in the federal state of Salzburg. The last occurrences in Salzburg are restricted to the base of the Untersberg. Many populations are rather small and geographically isolated.

The major aim of this thesis was to survey the genetic diversity and genetic structure within and among populations of *Gladiolus palustris* in Salzburg and adjacent areas. The questions if the geographical isolation of populations due to fragmentation of habitats is already visible in the genome and if small populations are facing genetic depletion should be answered.

For approaching these questions, DNA-material of seventeen gladiola populations of the northern base of the Untersberg were sampled, material of populations outside of the federal state of Salzburg were sent to Salzburg by colleagues. The AFLP-method and computer-based programmes were used to create DNA-fingerprints of the species. If there was genetic differentiation among the populations, a divergent development because of geographical isolation was conceivable.

No clear genetic groups could be identified. At three groups, the populations of Vorarlberg and Upper Austria were forming a separate cluster. Within Salzburg, most of the populations were genetically admixed; a few of them could explicitly be assigned to one of the three groups. Considering the gene diversity of populations in Salzburg it could be recognized that not the biggest populations were the most diverse ones, as one would expect, but rather small populations were having high levels of diversity. However, there was no significant correlation between the size of the populations and their gene diversities. Correlations between geographical and genetic distance were partially significant. Within Salzburg, no significant IBD (isolation by distance) could be determined. In summary I conclude that *Gladiolus palustris* is characterized by low genetic diversity, possibly due to the huge loss of potential habitats during the last two centuries.

Zusammenfassung

Durch den Verlust potentieller Lebensräume aufgrund menschlicher Fragmentierung der Landschaft wurden viele Pflanzenpopulationen stark dezimiert und oft auch räumlich voneinander isoliert. Solch kleine, isolierte Populationen sind durch negative genetische Konsequenzen gefährdet, wie beispielsweise durch verringerte genetische Variation, genetische Drift, Gründereffekt, Inzuchtdepression oder Ansammlung nachteiliger Mutationen. All diese Faktoren können zu einer verringerten künftigen Anpassungsfähigkeit an Umweltveränderungen führen und die generelle Fitness der Pflanzen verringern und somit auch das Aussterberisiko erhöhen. In diesem Bereich sind naturschutzgenetische Methoden („conservation genetics“) relevant, die zum Verständnis genetischer Prozesse in Populationen beitragen um Managementpläne zum Fortbestehen und zur Sicherung der Populationen zu erstellen. Eine Pflanzenart von großem naturschutzfachlichen Interesse ist die Sumpf-Gladiole (*Gladiolus palustris*), die in Österreich und somit auch im Bundesland Salzburg vom Aussterben bedroht ist. Die letzten Vorkommen der Sumpf-Gladiole beschränken sich in Salzburg auf das Vorland des Untersberges, viele Populationen sind eher klein und geografisch voneinander isoliert.

Das Hauptziel dieser Arbeit ist es, die genetische Struktur innerhalb sowie zwischen den Populationen der Sumpf-Gladiole in Salzburg und umliegender Gebieten zu untersuchen. Es soll festgestellt werden, ob die geografische Isolierung der Populationen durch Fragmentierung des Lebensraumes bereits Auswirkungen auf das Genom zeigen und ob kleine Populationen bereits von genetischer Verarmung betroffen sind.

Von siebzehn Sumpf-Gladiolen-Population im nördlichen Untersberg-Vorfeld wurden DNA-Proben gesammelt, von Populationen außerhalb des Bundeslandes Salzburg wurden Proben nach Salzburg geschickt. Mit der AFLP-Methode sowie mit computergestützten Analysen wurde ein genetisches Profil der Art erstellt. Sollte eine genetische Differenzierung zwischen den Populationen gefunden werden, wäre eine Auseinanderentwicklung aufgrund geografischer Isolierung zu vermuten.

Es konnten keine klaren genetischen Gruppen identifiziert werden. Bei einer Gruppenanzahl von drei Gruppen stellten sich jedoch Populationen aus Vorarlberg und Oberösterreich bereits als eigenständige Gruppen dar. Innerhalb von Salzburg sind die meisten Populationen genetisch durchmischt, einige wenige sind eindeutig einer der drei Gruppen zuzuordnen. Bei Betrachtung der genetischen Diversität von Populationen innerhalb von Salzburg ist feststellbar, dass nicht wie anzunehmen die größten Populationen die genetisch diversesten sind, sondern eher kleine Populationen hohe Diversitätswerte aufweisen. Es besteht jedoch kein signifikanter Zusammenhang zwischen Größe der Populationen und gentischer Diversität. Zusammenhänge zwischen geografischer und genetischer Distanz waren nur zum Teil signifikant, innerhalb von Salzburg konnte keine signifikante IBD (Isolation by Distance) festgestellt werden. Zusammenfassend lässt sich sagen,

dass *Gladiolus palustris* durch eine niedrige genetische Diversität gekennzeichnet ist, die möglicherweise durch den enormen Lebensraumverlust in den letzten Jahrhunderten bedingt ist.

1 Introduction

1.1 General introduction

Nature conservation is of great importance in the present age, as biodiversity continues to decline globally (PEARSON, 2016). There are plenty of arguments and ideologies for conserving nature comprehensively. Nature should be valuated as a basis of existence, as ongoing processes like e.g. decomposition, climate regulation, water purification and pollination ensure human existence permanently. Additionally, nature delivers nourishment and other resources and enables an enhancement of vitality and quality of living and is therefore of great importance for everyone, also for prospective generations. Besides, nature has a right to exist and we all have the ethical obligation to preserve it in its whole beauty and diversity as it has been produced by evolution over more than 3.5 billion years, aside from human interests (ALLENDORF & LUIKART, 2007; PEARSON, 2016). Humans are intervening into the environment at a very high speed and nature is not able to adapt to these fast anthropogenous changes, so we are facing a mostly human-induced mass extinction of plants and animals right now. 3.000 to 30.000 species go extinct annually (WOODRUFF, 2001). Regarding plant species, the IUCN (2001) estimates that 9-34% of major plant species are threatened with extinction over the next decades (ALLENDORF & LUIKART, 2007). Consequently, there is a need of nature conservation as well as species conservation; it is necessary to protect and conserve diversity on all relevant levels, namely on ecosystem diversity, species diversity and also genetic diversity level.

There are many global and local private associations and governmental agreements to protect nature. The Biodiversity Convention, Ramsar-Convention and Bern-Convention are supranational and governmental conventions to protect plants, animals and their habitats. At EU-level, nature conservation is regulated by two nature directives designed by the Directorate General on Environment of the European Commission, namely the “Birds Directive” and the “Habitats-Directive” (FFH-Directive). First named is responsible for the conservation of wild birds and the second directive protects endangered natural habitats and wild fauna and flora. The Directives include appendices, where habitats and rare species of community interest are listed. Every Member State of the European Union is committed to designate appropriate sites (“Natura 2000 sites”), to make reports about the conservation status and to avoid a degradation of conditions, in other words, to maintain a “Favourable Conservation Status” (OSTERMANN, 1998). The aim is to preserve nature as a habitat for species that enables the maintenance of large populations.

LEIMU et al. (2006) stated that fitness and genetic variation of populations were significantly positive correlated with population sizes. According to these authors, number of alleles, the proportion of polymorphic loci and the expected heterozygosity increased with population size. For some rare species, mean correlations between population size and fitness tended to be even stronger than for common species. However, many rare species are composed of small and/or isolated populations, what is coming along with several problems for the affected species, as discussed in the following paragraph.

Many populations have become small and isolated due to great loss of possible habitats because of recent anthropogenically induced fragmentation. Habitat fragmentation results in several smaller and spatially isolated patches of former continuous habitats (YOUNG et al., 1996). ELLSTRAND & ELAM (1993) identified consequences of fragmentation that may put rare plant species and small populations at a genetic risk. Small populations are predicted to be at a higher risk of facing negative genetic consequences like reduced genetic variation and population viability, due to genetic processes like genetic drift, founder effects, inbreeding depression and accumulation of deleterious alleles or mutations. Genetic drift, which causes a random change in allele frequencies within a population's gene pool may lead to larger and unpredictable fluctuations of allele frequencies in small populations, meanwhile in large populations, fluctuation effects are generally small. The chance of inbreeding, i.e. the mating of related individuals is increased as a result of small population size (ELLSTRAND & ELAM, 1993; OUBORG et al., 2006). Changes in allele frequencies, accumulation of harmful alleles or mutations, increased inbreeding and reduced genetic diversity may lead on to a weaker future adaption potential to environmental changes and reduced plant fitness (FRANKHAM, 1995; LEIMU et al., 2006; ALLENDORF & LUIKART, 2007). Therefore, small and isolated populations are at a greater risk of becoming extinct (SCHMIDT & JENSEN, 2000). This is a result of low levels of gene flow among isolated populations. Here, conservation genetics becomes applicable.

Conservation genetics targets the conservation of genetic diversity by understanding dynamics of genes in populations via applying genetic methods. First, genetic relationships within populations need to be understood, before management plans can be developed. As molecular techniques like e.g. allozymes, DNA sequencing, microsatellites, RAPD (random amplfified polymorphic DNA) and AFLP (amplified fragment length polymorphism) became available, they were applied to depict genetic relationships among individuals, populations and species (HAIG, 1998). AFLPs (VOS et al., 1995), a dominant DNA marker system based on polymerase chain reactions, are used for fast screening of genetic diversity and detecting polymorphism at DNA sequence level. This method has many benefits as many markers can be scored for many individuals at the same time, accompanied

with a high replicability and no requirement of any prior information about the genome (MUELLER & WOLFENBERGER, 1999; OUBORG et al., 2006; PAUN & SCHÖNSWETTER, 2012). PAUN & SCHÖNSWETTER (2012) state that AFLPs can be used to infer phylogenies and biogeographic patterns at population-level. LUCCHINI (2003) also tested the AFLP method as a tool for biodiversity conservation and management and came to the conclusion that it can be a very good molecular technique for conservation concerns.

The marsh gladiolus, *Gladiolus palustris* (GAUDIN), is an Iridaceae species with conservational concern, as it is critically endangered in Austria and in the federal state of Salzburg and is listed in the appendices II and IV of the Habitat-Directive of the EU (WITTMANN et al., 1996; SSYMANK et al., 2006). The main reason for its threat is the loss of suitable habitats as it primarily occurs in regularly late mown wet straw meadows which faced a dramatic decline in the last decades. In Salzburg, the last extant occurrences of the marsh gladiolus are at the northern base of the Untersberg (WITTMANN et al., 1987), in general populations show a negative development tendency in population sizes (Nowotny, personal communication). There are many rather small and isolated populations, which are threatened with extinction. Discussions with members of an association for ecological conservation "HALM" ("Heimisches Arten- und Lebensraummanagement") posed the demand of a genetic analysis of *Gladiolus palustris* populations of Salzburg. The aim was to detect the genetic diversity and relationships among populations of Salzburg and adjacent areas. Another aim was to examine, if there is already a genetic depletion due to geographical isolation and small population sizes. Ideally, results of this study may help to develop management plans for the marsh gladiolus in a cultural landscape, including *ex situ* cultivation and planting out of individuals to maintain and increase population sizes.

1.2 Study subject *Gladiolus palustris* GAUDIN

Gladiolus palustris, also called Marsh gladiolus, is a perennial species belonging to the family of Iridaceae. The current name "*Gladiolus palustris* GAUDIN" originates from the taxonomic author Jean François Aimé Philippe Gaudin, a Swiss priest and natural scientist. For the plant name, there are several synonyms, such as *Gladiolus boucheanus* Schltdl., *Gladiolus felicis* Z.Mirek, *Gladiolus felicis* var. *zmudae* Z.Mirek, *Gladiolus imbricatus* subsp. *parviflorus* K.Richt., *Gladiolus palustris* var. *vaudensis* Gaudin, *Gladiolus parviflorus* Berdau, *Gladiolus pratensis* A.Dietr. and *Gladiolus triphyllus* Bertol. (https://www.gbif.org/species/2750279).

1.2.1 Distribution of *Gladiolus palustris*

The distribution of *Gladiolus palustris* is restricted to the Northern Hemisphere (see Figure 1, MEUSEL et al., 1965), it is Illyrian, alpine-apenninic. Besides *Gladiolus imbricatus* and *G. illyricus*, *G. palustris* is the only species of the genus *Gladiolus* occurring in Central Europe (SCHMITT et al., 2010). It occurs in eastern France, Switzerland, Germany, Czech Republic, Slovakia, Poland, northern Italy, Austria, Hungary, in Balkan regions like Albania and Western Bulgaria and fragmentarily in Belarus, Ukraine and Russia (https://www.gbif.org/species/2750279; NOWOTNY, 2000).

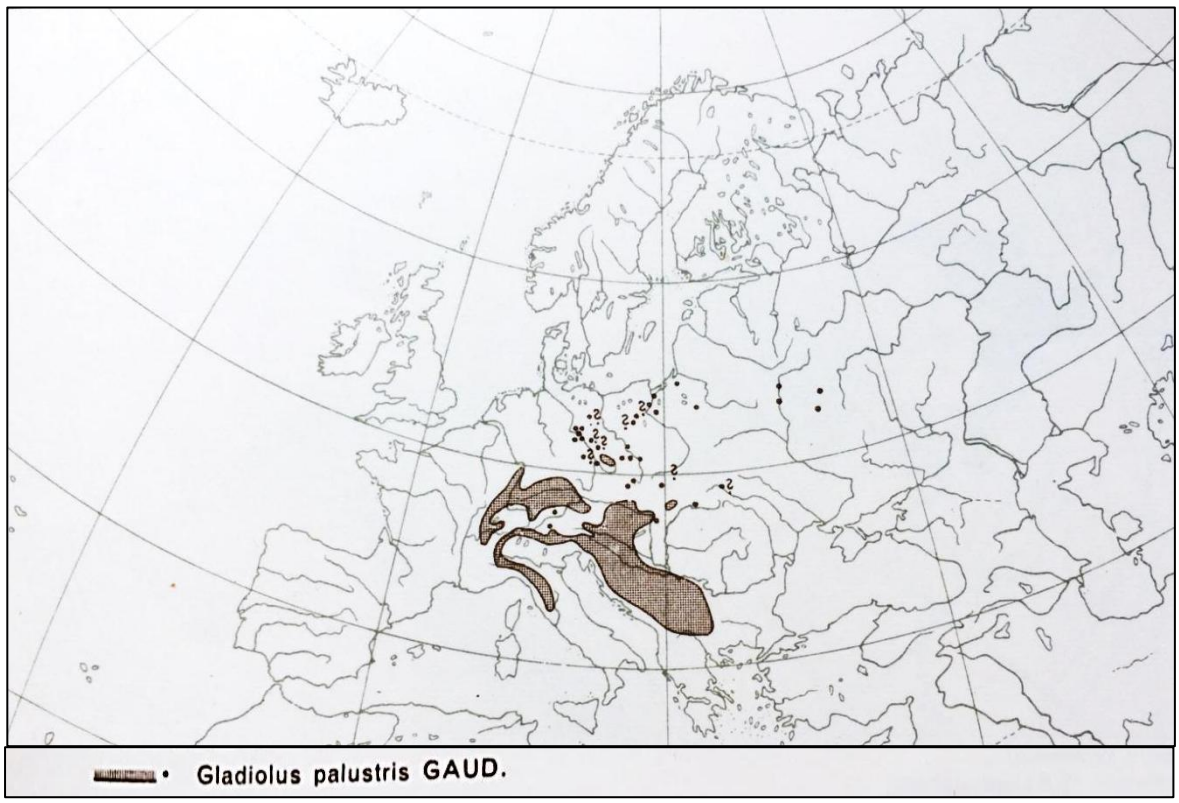


Fig. 1: Distribution map of *Gladiolus palustris* (taken from MEUSEL et al., 1965).

Regarding the distribution in Austria (see Figure 2), *Gladiolus palustris* occurs in Burgenland, Lower Austria, Upper Austria, Salzburg, North Tyrol and Vorarlberg (FISCHER et al., 2008) and uncertainly in Carinthia ("Floristische Kartierung Österreichs" derived from H. Niklfeld and L. Schratt-Ehrendorfer, University of Vienna). Figure 2 shows the distribution of *Gladiolus palustris* in Austria and adjacent areas of Germany and Switzerland.

The map has been compiled of information of H. Niklfeld (unpublished information, Vienna) and two websites, infoflora.ch of Switzerland and deutschlandflora.de of Germany. For a floristic mapping of Europe, a layer with mapping quadrants was used. If the species is present in the quadrant, there is a circle in the center of the quadrant. The distribution is scattered, there are cumulative occurrences in Vorarlberg, Bavaria and Swabia and in Salzburg, in between there are distribution gaps.

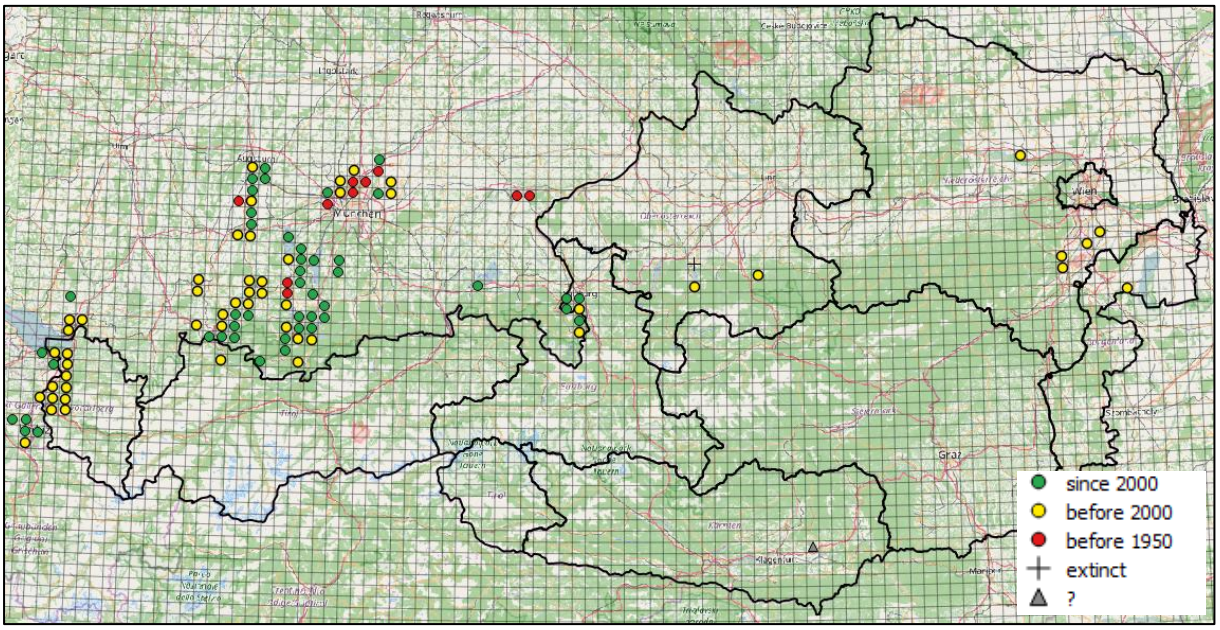


Fig. 2: Distribution of *Gladiolus palustris* in Austria an adjacent areas of Germany and Switzerland.

Figure 3 shows the known distribution of *Gladiolus palustris* in the state of Salzburg at the time of the publication of the distribution atlas of vascular plants of Salzburg by WITTMANN et al. (1987). Since the year 1945, the species is known in only three mapping quadrants, namely in the quadrants 8243/2, 8243/4 and 8244/1. These are the regions at the northern base of the Untersberg. The two blank spots represent vanished occurrences in the quadrant 7943/4 (Holzhausen, St. Georgen) and 8442/1 (Gumpinger Moos, surroundings of Lofer), which could not be confirmed since 1945. The species is considered extinct in these quadrants (NOWOTNY, 2000).

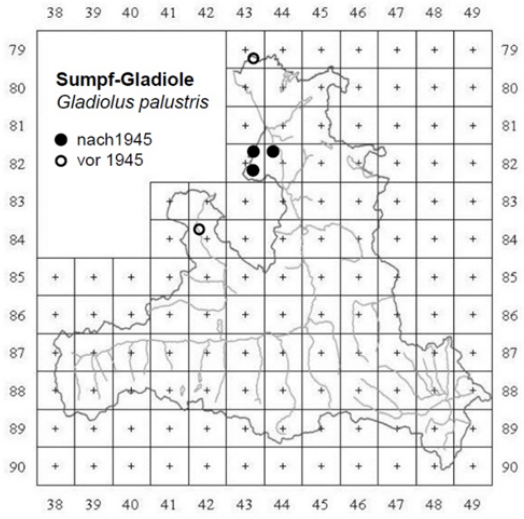


Fig. 3: Distribution map of *Gladiolus palustris* in Salzburg, obtained from the distribution atlas of vascular plants of Salzburg by Wittmann, Siebenbrunner, Pilsl & Heiselmayer 1987. The black spots are representing occurrences "starting in 1945" and the blank spots represent localities found "before 1945".

1.2.2 Biological features of *Gladiolus palustris*

Gladiolus palustris is a perennial and deciduous (summer-green) plant. It reaches a height of 30-60 cm (FISCHER et al., 2008). Its stem is foliate with lineal- lanceolate leaves of about 0.5-1cm breadth. The Marsh gladiolus has a geophytic growth form, which means that its hibernating organ is lying below the ground. This organ is an underground bulb, which is formed new annually for hibernation and is located in depths of 10-20 cm (HEGI, 1939; RIEGEL, 2010). The bulb cover has meshed filaments, with rotund or polyangular meshes (FISCHER et al., 2008; LAUBER & WAGNER, 2007).

G. palustris has a conspicuous, entomophilous one-sided flower spike (see Figure 4) with two to eight carmine red or purple flowers, which are up to three (four) centimeters long and have three stamina. Each flower has its own bracteole. The lower tepals are inside white with edging dark red longitudinal stripes (= nectar guide, see Figure 4.a); this is conducive to attract and lure pollinators and to give them orientation (SCHMALL, 2015). The lower flowers start to flower first (flowering bottom up), whereby the topmost flowers do not fully develop and often not produce seeds (RIEGEL, 2010). The flowering period of *Gladiolus palustr*is ranges between (May) June to July.

The main pollinators are bumble-bees (KNUTH, 1899). According to KNUTH (1899) and HERRMANN (2000) the plant inhibits self-pollination by protandry. During anthesis, the stamina are first below the still closed stigma. After draining the pollen, they start to shrink, while the stigma lowers itself through the stamens. The ovary is inferior and parted threepartly. Every part contains 10-15 ovules. If fertilization was successful, the ovules develop to winged brown propagules between the middle of August until the beginning of September. They are then dispersed by wind in the second half of September (HERRMANN, 2000; FISCHER et al., 2008). Vegetative reproduction via bulbs is also possible, but more the exception (SCHMITT et al., 2010). The time between germination and the first flowering of the marsh gladiola is at least three to four years.

Fig. 4: *Gladiolus palustris*. a) Inflorescence b) Flower in detail c) Stamina d) Side view of habitus e) Immature green capsule f) Mature chapped capsule with winged brown propagules [photo f) by G. Nowotny].

1.2.3 Habitat of *Gladiolus palustris*

The species occurs at different habitats, it is a character species of extensively managed purple moor grass bedding meadows (Molinion caeruleae, see Figure 5), however, it also occurs in regularly managed calcareous fens (Caricion davallianae), semi-dry calcareous grasslands (Bromion erecti) and also in currently unmanaged semi-dry pine forests (Erico-Pinion) on calcareous soils (HERRMANN, 2000; OBERDORFER, 2001; SCHMITT et al., 2010). It prefers moist and marshy meadows and fens but also can be found on more arid soils (LAUBER & WAGNER, 2007; FISCHER et al., 2008). The gladiola grows from the colline to montane altitudinal belt. According to ELLENBERG et al. (1992), *Gladiolus palustris* has following ecological characteristics: High demand of light (L=8), between morderate warm and warm temperatures (T=6), suboceanic (K=4), medium-moist to moist grounds (F=6), mostly showing lime (R=8), more occurring at very nitrogen-poor sites (N=2), not halotolerant (S=0), geophytic growth form (G).

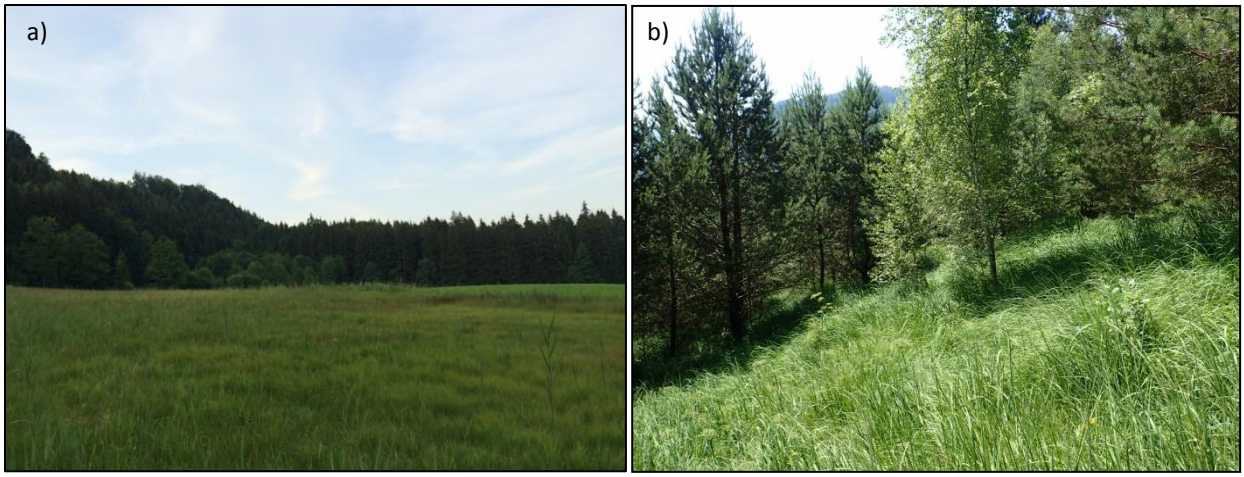


Fig. 5: Habitats of *Gladiolus palustris*. a) Typical bedding meadow. b) Semi-dry pine forest.

1.2.4 Conservation status and endangerment

In the course of the EU's eastward expansion in 2004, *Gladiolus palustris* was added to the Appendixes II and IV of the EU Habitats Directive. Therefore, Austria has a reporting obligation for the condition of this species. In the law of conservation of nature of Salzburg of 1999, paragraph 29 determines the specific protection of wildly growing plants. According to this law, *G. palustris* is listed within protection category B, which contains fully protected plant species in the State of Salzburg. "Fully protected" means that it is forbidden to damage or destroy the plants or to remove them from their habitat. Furthermore it is prohibited to deal, sell or buy the plants. Also, it is forbidden to damage the habitat in a way that prevents the plant from persisting (RIS, Salzburger Naturschutzgesetz 1999 - NSchG, StF: LGBl Nr 73/1999).

In the Red List of threatened fern- and flowering plants of the State of Salzburg (WITTMANN et al., 1996), *Gladiolus palustris* is listed as threatened with extinction (corresponds to the IUCN category "critically endangered") in Salzburg, which is category "1". Also, for whole Austria the plant is listed in this hazard level. Being critically endangered means that the survival of the species in the wild is unlikely if the threats are influencing it any longer or there are no supporting measures initiated. Species are ranked in this category if there are only single occurrences or very small and isolated populations left that are dependent on vulnerable habitats. Moreover, species that have faced a strong or very fast decline in population size are assigned in this category (WITTMANN et al., 1996). The main anthropogenic reasons for the threat of the species are the destruction of large areas of species-rich wetlands and fen meadows in Europe, leaving behind isolated patches of wetlands, often containing only small populations of former common species (SCHMIDT & JENSEN, 2000), as it is the case with *Gladiolus palustris*.

The habitat destruction happened in several ways, such as agricultural intensification (eutrophication, drainage and the forward bringing of the mowing date). Abandonment of agricultural land, scrub encroachment and reforestation are also responsible for the decline of the species (KORNECK et al., 1996; SCHMITT et al., 2010). These factors can lead to changes in vegetation and in competition status for *G. palustris* and other more competitive species can become dominant and suppress the marsh gladiolus (NOWOTNY, 2012; http://www.iucnredlist.org/details/162188/0). According to Günther Nowotny (personal communication), who is annually monitoring all populations in Salzburg, there is a negative tendency in the overall development of the species.

1.3 Aims of the study

1.3.1 Genetic structure within and among populations

Gladiolus palustris is threatened with extinction in Salzburg, mainly due to destruction of potential habitats. The base of the Untersberg is the last recent occurrence of this species in Salzburg (WITTMANN et al., 1987). Especially very small populations are threatened with extinction and many populations are isolated due to the great loss of possible habitats. Small and isolated populations might be threatened by inbreeding and inbreeding depression and an increasing genetic differentiation among the populations as a result of low levels of gene flow among isolated populations (SCHMIDT & JENSEN, 2000). Discussions with members of an association for ecological conservation "HALM" ("Heimisches Arten- und Lebensraummanagement", http://www.halm-salzburg.at/) posed the demand of a genetic analysis of *Gladiolus palustris* populations of Salzburg.
So the **first aim of this study was to survey the genetic structure within and among *Gladiolus palustris* populations in Salzburg and adjacent areas to answer the question if the geographical isolation had an impact on the genetic structure**. Is there already a genetic depletion due to isolation and small population sizes? This question is obvious, as genetic variation and population viability decrease with population isolation and small and isolated populations are at a greater risk of becoming extinct (SCHMIDT & JENSEN, 2000). It was decided to perform a dominant DNA marker system AFLP study (Amplified fragment length polymorphism, VOS et al., 1995) on all populations of Salzburg and adjacent areas to clarify these questions. The AFLP method was chosen because it does not require any prior information about the genome and is highly reproducible and sensitive for detecting polymorphism at DNA sequence level (PAUN & SCHÖNSWETTER, 2012). These authors state that AFLPs can be used to assess the genetic diversity within species and to infer phylogenies and biogeographic patterns at population-level, which is applicable for the interrogations concerning *Gladiolus palustris*.

1.3.2 Unique color variation in a single population

In Salzburg, *Gladiolus palustris* shows two different color morphs (bright pink and purple) at "Dürre Wiese" in Wals-Siezenheim, Gois (Nowotny, 2012). As the genetic AFLP study was conducted anyway, the **second aim of this study was to test if the difference within the color is reflected in patterns of genomic variation**. This would only be the case if gene flow is mainly present within the color morphs and is low among them. Flower color is very diverse throughout flowering plants but color variation within species is rather uncommon. Mostly, only a small color range is covered or only phenotypic plasticity is represented and the colors are not distinguished by pollinators. There are not many species that show a genetically based polymorphism (GIGORD et al., 2001). Studies on

rewardless orchids with color polymorphism showed that they are usually pollinated by naive insects, mostly bumblebees. Behavioral experiments revealed that the insects sample different color morphs in alternation because after visiting a rewardless flower they tend to switch to the different color morph (GIGORD et al., 2001). So, developing different color morphs may also be an attempt to circumvent pollinator limitation. In this case, the gladiola populations with different color morphs would not show a difference in genomic patterns. Rather, it is possible that they resemble each other even more, compared to different populations.

1.3.3 Pollination biology of *Gladiolus palustris*

Reproduction of *Gladiolus palustris* is typically insect-pollinated, bumble-bees are the main pollinators (KNUTH, 1899). The species forestalls self-pollination by protandry (KNUTH, 1899; HERRMANN, 2000), though self-pollination is theoretically possible (KIRCHNER et al., 1934). The **third aim is a small pollination biology study to test the self-compatibility of the plants and the effects of self pollination on the seed set**.

2 Material and Methods

2.1 Genetic structure within and among populations in Salzburg and adjacent areas

2.1.1 Sampling

For answering the question about genetic structure, 18 populations of *Gladiolus palustris* have been sampled in Salzburg at the northern foot of the Untersberg by myself with help of Nowotny Günther (Salzburg) and Bernhard Christian Schmall (Salzburg) in summer 2015 (see Figure 6). Ideally, leaf material has been collected of 15 individuals per population. Therefore, five centimeters of green leaves were cut and immediately stored in paper bags (MILFORD tea bags), which were then put in plastic bags with silica gel and dried. By doing this leaves were dried quickly to avoid degradation of DNA and preserve a high quality DNA. The silica gel was exchanged when it was saturated with water.

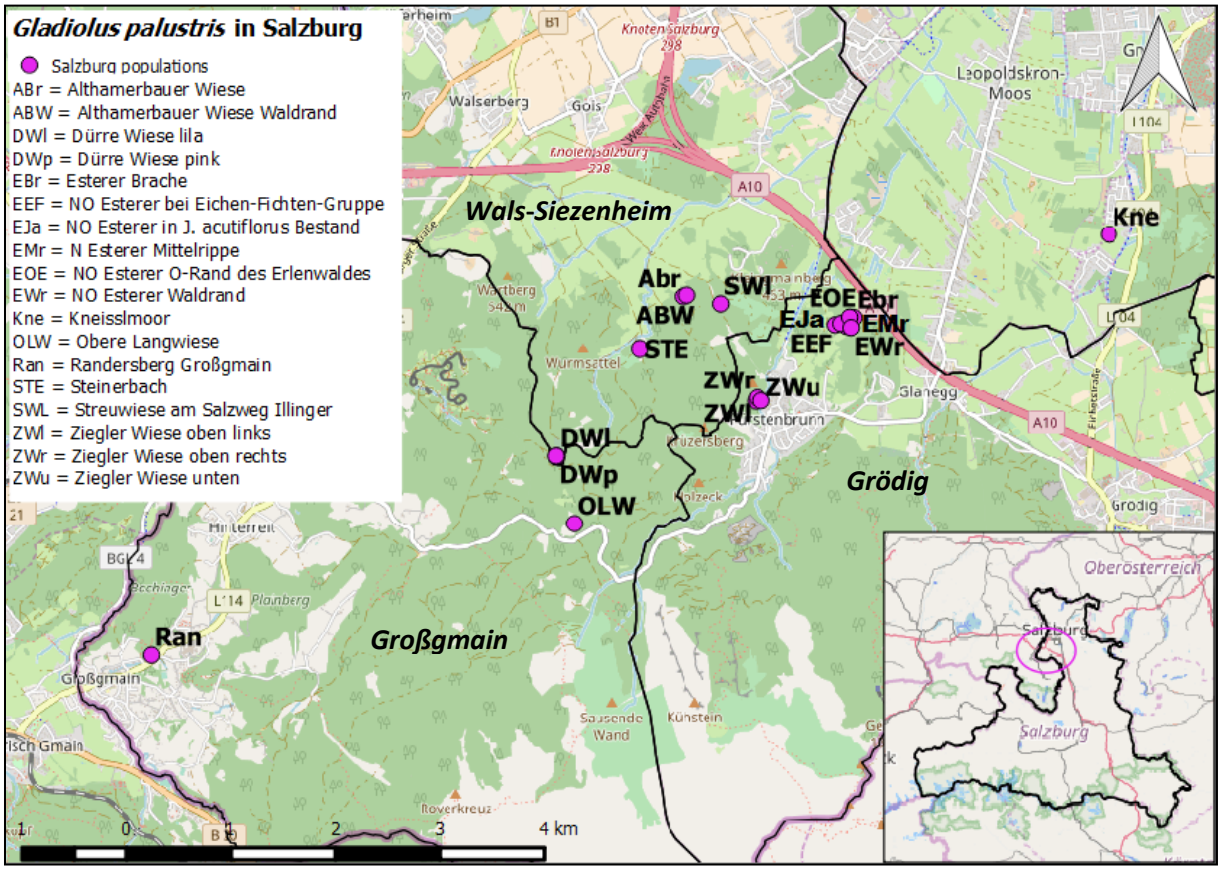


Fig. 6: Sampling locations of *Gladiolus palustris* populations in Salzburg shown using Quantum GIS. The used background map is OpenStreetMap (OSM). Details about the populations can be seen in Table 3 on the following pages. The black lines are representing the municipal boundaries.

Sometimes, it was not possible to sample 15 individuals per population, due to different reasons. Either the population size has been too small, or not enough individuals could be found because not-flowering plants are very difficult to spot. Another reason was that many plants were infected by a fungus and therefore could not be used for genetic fingerprinting.

For the Salzburg populations, Günther Nowotny kindly provided his unpublished results of annual counting from 2010 to 2016 of flowering individuals of *Gladiolus palustris*. He delivered minimum and maximum values for every population in this period of time and a mean value of the population sizes. For the two populations at "Dürre Wiese" (DWl and DWp) he made no differentiation between bright pink and purple (see Table 1).

Tab. 1: Population information of *Gladiolus palustris* in Salzburg. The average population size (mean value of the counted flowering plants from 2010 to 2016), the minimum and the maximum value were determined by Günther Nowotny. For more detailed information see Table 3.

Code	**Locality**	**Average population size**	**Min.**	**Max.**
Abr	Althamerbauer Wiese nähe Iris Hauptbestand rechts	29	6	64
ABW	Althamerbauer Wiese Waldrand (NW)	14	0	30
DW	Dürre Wiese lila colored	287	53	536
EBr	Dürre Wiese pink colored	9	4	15
EEF	Esterer Brache	5	2	10
EJa	NO Esterer bei Eichen-Fichten-Gruppe	39	0	107
EMr	NO Esterer in *Juncus acutiflorus* Bestand	47	10	122
EOE	N Esterer Mittelrippe/Geländestufe	4	1	8
EWr	NO Esterer O-Rand des Erlenwaldes	12	8	16
Kne	NO Esterer Waldrand	7	4	10
OLW	Kneisslmoor (Stadt)	16	6	35
Ran	Obere Langwiese	88	50	138
STE	Randersberg Großgmain	3	1	5
SWI	Steinerbach	237	70	335
ZWl	Streuwiese am Salzweg Illinger	26	12	39
ZWr	Ziegler Wiese oben links	59	42	93
ZWu	Ziegler Wiese oben rechts	33	18	53

Additionally to the populations collected in Salzburg, three populations in Upper Bavaria (Piding, Hammerstielwand) have been sampled by me and G. Nowotny. Other non-Salzburg populations have been sampled by colleagues, who are listed in the following Table 2. For the non-Salzburg populations, ten individuals per population were sampled.

Tab. 2: Voluntary collectors for non-Salzburg samples.

Region (Nr. of populations)	**Collector (Abbreviations)**	**Address**
Vorarlberg (2)	Beiser Andreas (AB)	Unterer Auweg 6, 6820-Frastanz
Upper Austria (1)	Arming Claudia (CA)	Firma/Bietergemeinschaft: TB CaREX
Upper Austria (1)	Maria & Norbert Pühringer (MP&NP)	Herrnberg 8, 4644 Scharnstein
Schwaben (1)	Stephan Günther (GS)	Lebensraum Lechtal e V., c/o Amt für Naturschutz, Dr.-Ziegenspeck-Weg 10, 86161 Augsburg
Upper Bavaria (2)	Kemmer Irmgard (IK)	Landratsamt Weilheim-Schongau, Untere Naturschutzbehörde, Pütrichstraße 8, 82362 Weilheim i.OB
Lower Bavaria (2)	Martin Scheuerer (MS)	Peter-Rosegger-Str. 10, 93152 Nittendorf

Furthermore, Andreas Tribsch (Salzburg) collected 12 samples of *Gladiolus imbricatus* of Styria, which were treated as an out-group population. In total, 310 individuals were available for analyses. Figure 7 shows all sampling locations outside of Salzburg. For detailed sampling information see Table 3.

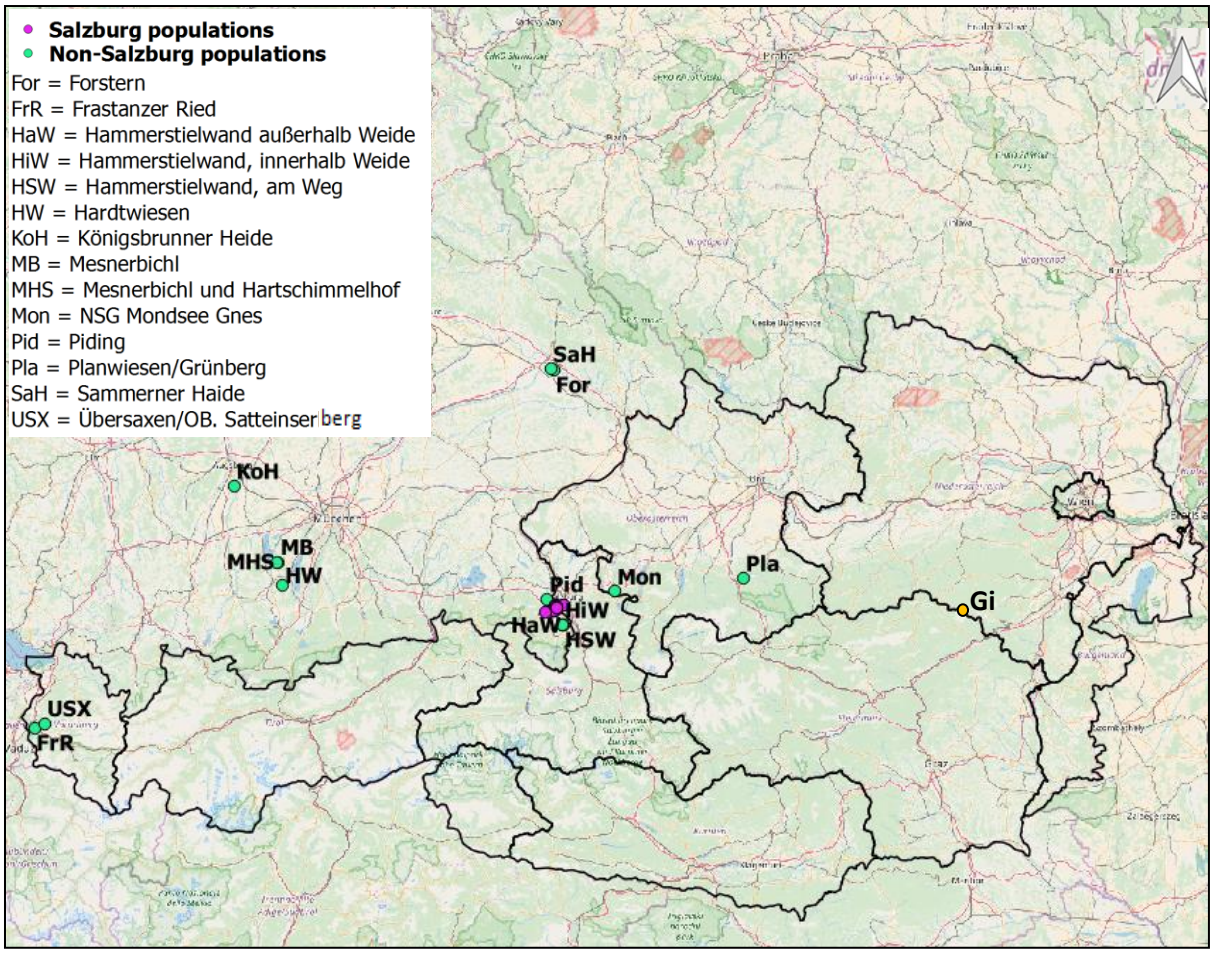


Fig.7: Sampling locations of all *Gladiolus palustris* populations outside of Salzburg mapped with Quantum GIS. The used background map is OpenStreetMap (OSM). Gi= *Gladiolus imbricatus*. Detailed information about the population can be seen in Table 3.

Tab. 3: Sampling locations and collection data of the *Gladiolus palustris* samples analyzed in this study. Pop = Population code used in several maps and figures and throughout the text. N: Number of genotyped individuals. Collectors: AT: Andreas Tribsch; BL: Bettina Leitner; BS: Bernhard Schmall; GN: Günther Nowotny; the others are already listed in Table 2. The last population Gi is *Gladiolus imbricatus*. Additional information after population abbreviation: * (used in color morph study), + (used in pollination biology study).

Pop	Country	State	Locality	N	Date	Collector	Longitude	Latitude
Abr	AUT	Salzburg	Althamerbauer Wiese nähe Iris Hauptbestand rechts	10	16.06.2015	BS & BL	12,98201	47,75799
ABW	AUT	Salzburg	Althamerbauer Wiese Waldrand (NW)	5	16.06.2015	BS & BL	12,98242	47,75809
DWl *	AUT	Salzburg	Dürre Wiese lila colored	16	21.06.2015	GN & BL	12,96586	47,74432
DWp *	AUT	Salzburg	Dürre Wiese pink colored	16	21.06.2015	GN & BL	12,96575	47,74434
EBr	AUT	Salzburg	Esterer Brache	4	26.06.2015	GN & BL	13,00374	47,75618
EEF	AUT	Salzburg	NO Esterer bei Eichen-Fichten-Gruppe	5	21.06.2015	GN & BL	13,00134	47,75555
EJa	AUT	Salzburg	NO Esterer in *Juncus acutiflorus* Bestand	10	21.06.2015	GN & BL	13,00280	47,75543
EMr	AUT	Salzburg	N Esterer Mittelrippe/Geländestufe	10	13.06.2015	GN & BL	13,00210	47,75567
EOE	AUT	Salzburg	NO Esterer O-Rand des Erlenwaldes	10	21.06.2015	GN & BL	13,00321	47,75621
EWr	AUT	Salzburg	NO Esterer Waldrand	10	21.06.2015	GN & BL	13,00339	47,75532
Kne	AUT	Salzburg	Kneisslmoor (Stadt)	5	21.06.2015	GN & BL	13,03621	47,76331
OLW	AUT	Salzburg	Obere Langwiese	10	21.06.2015	GN & BL	12,96807	47,73853
Ran +	AUT	Salzburg	Randersberg Großgmain	15	13.06.2015	GN & BL	12,91409	47,72731
STE	AUT	Salzburg	Steinerbach	1	21.06.2015	GN	12,97635	47,75355
SWl	AUT	Salzburg	Streuwiese am Salzweg Illinger	10	16.06.2015	BS & BL	12,98675	47,75735
ZWl +	AUT	Salzburg	Ziegler Wiese oben links	10	16.06.2015	GN & BL	12,99122	47,74900
ZWr +	AUT	Salzburg	Ziegler Wiese oben rechts	10	13.06.2015	GN & BL	12,99138	47,74928
ZWu	AUT	Salzburg	Ziegler Wiese unten	10	16.06.2015	GN & BL	12,99181	47,74903
For	D	Lower Bavaria	Forstern	8	12.07.2015	MS	12,96964	48,76768
FrR	AUT	Vorarlberg	Frastanzer Ried	10	07.07.2015	AB	9,61733	47,22329
HaW	D	Upper Bavaria	Hammerstielwand außerhalb Weide	10	05.07.2015	GN & BL	13,02896	47,67348
HiW	D	Upper Bavaria	Hammerstielwand, innerhalb Weide	9	05.07.2015	GN & BL	13,02820	47,67369
HSW	D	Upper Bavaria	Hammerstielwand, am Weg	2	05.07.2015	GN & BL	13,02763	47,67247
HW	D	Upper Bavaria	Hardtwiesen	10	09.07.2015	IK	11,21718	47,84698
KöH	D	Swabia	Königsbrunner Heide	10	16.07.2015	GS	10,90762	48,27307
MB	D	Upper Bavaria	Mesnerbichl	10	09.07.2015	IK	11,19350	47,94672
MHS	D	Upper Bavaria	Zwischen Mesnerbichl und Hartschimmelhof	4	09.07.2015	IK	11,18531	47,94704
Mon	AUT	Upper Austria	Naturschutzgebiet Mondsee Gnes	10	15.07.2015	CA	13,36639	47,81875
Pid	D	Upper Bavaria	Piding	15	07.07.2015	GN & BL	12,92826	47,78538
Pla	AUT	Upper Austria	Planwiesen/Grünberg	10	30.06.2015	MP & NP	14,19528	47,87583
SaH	D	Lower Bavaria	Sammerner Haide	10	12.07.2015	MS	12,95267	48,77297
ÜSX	AUT	Vorarlberg	Übersaxen/OB. Satteinserbg (??)	10	17.07.2015	AB	9,68721	47,24524
Gi_Stm	AUT	Styria	Steiermark, Nordöstliche Kalkalpen: Altenberg an der Rax, Kerngraben, ca. 400m W Gsoller, 960m	11	23.08.2015	AT	15,65833	47,68361

2.1.2 Genetic analyses

2.1.2.1 DNA-Extraction

The total genomic DNA of the 310 samples was extracted from silica gel-dried leaf tissue in December 2015 and January 2016 following the CTAB-protocol by MURRAY & THOMSON (1980), DOYLE & DOYLE (1987) and DOYLE & DOYLE (1990), modified by Matthias Affenzeller (Salzburg). First, a 2xCTAB-buffer according to the following prescription was prepared: 100 mM Tris-Cl (pH8.0), 1.4 M NaCl, 20 mM EDTA, 2 % CTAB, 1 % PVP-40. Then, the leaf material was grounded, wherefore about one cm^2 of the leaf was put in a 2ml Eppendorf tube together with two sterile steel beads. The Eppendorf tubes were put in special brackets and then put in a mixer mill (Retsch MM 301, Haan, Germany) at 30.000 Hz for 2 minutes. In this step, the leaf material was grinded into very fine particles. Afterwards, 400 µl of the already prepared 2xCTAB buffer was added plus 2 µl "RNase A" (10mg/ml stock, Thermo Scientific, Waltham, USA), an enzyme to degrade the RNA. The content of the Eppendorf tubes was vortexed fiercely, followed by an incubation step of ten minutes at 65°C on a thermoblock, ideally but not necessarily shaking the tube. During this step the plant cell walls were broken. After the incubation step the tubes were centrifuged (Labnet Spectrafuge 24D Microcentrifuge, Labnet International, Woodbridge, USA) for 5 minutes at full speed (around 13.000g) to force the debris to the lower part of the tube due to centrifugal force. The supernatant, which was containing the DNA, was transferred to a new Eppendorf tube. In the next step, 400 µl of a chloroform-isoamylalcohol (24:1) mixture was added. All tubes were vortexed again and then put in the centrifuge for another 5 minutes at full speed. In this step three different phases emerged due to a phase separation. The uppermost phase contained the watery DNA phase, the middle phase was composed of proteins and the lowest phase contained chloroform plus all fat-soluble parts of the plant. Ideally, the upper phase was clear and removed to a new Eppendorf tube without touching the protein phase. Afterwards approximately two-thirds of the available volume ice cold isopropanol was added to precipitate the DNA. Therefore, the tubes needed to be centrifuged at full speed for 20 minutes. During this step the DNA formed a small, not always visible pellet, which was sticking at the outside wall of the Eppendorf tube due to inertia. The supernatant was removed carefully and the remaining pellets were washed in 400 µl of 70% ethanol, where they became visible by turning whitish. All the pellets were dried at 50°C for at least 5 minutes or until the ethanol evaporated. Then, the pellets were resuspended in a volume of 30 µl of ddH_2O.

After the extraction agarose-gel-electrophoresis was conducted for all samples to check DNA concentration and quality. Therefore a 1 % agarose gel (Biozym Scientific GmbH, Hess, Germany; Biozym Biotech Trading GmbH, Vienna, Austria) was prepared, using the agarose powder, 1x TAE-

buffer and the Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH, Düren, Germany). A volume of three µl of the extracted DNA and three µl of a 6 x DNA Loading Dye (Thermo Scientific, Waltham, USA) were mixed and loaded on the gel. In the first slot three µl of a Gene ruler (DNA ladder 1 kB, Thermo Scientific, Waltham, USA) was applied as a marker. The running conditions of the gels were 120V, 400mA for 20 minutes.

Furthermore, the DNA concentrations of the samples were estimated by using a micro-volume UV-Vis Spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific, Waltham, USA), which allows measuring small amounts of DNA (in ng/µl). Therefore, 1.5 µl of each sample was tested. For further AFLP analysis, the concentration needed to be about 100ng/µl or below, so samples with higher concentrations were diluted accordingly.

2.1.2.2 Generating AFLPs

To screen the genetic diversity of the samples, amplified fragment length polymorphisms (AFLPs) was used, following a 4 step protocol by Vos et al. (1995) with modifications of Matthias Affenzeller. The samples were arranged in 96-well PCR plates (see an example in Appendix Table 1), whereby 48 samples always represented one run. In total, twelve runs were performed. Three wells of the 48 were used to check the quality of the AFLPs. For the "between-plates-replicate", a sample with a positive AFLP pattern was repeated in every run (marked yellow in Appendix Table 1). For the "within-plate-replicate", one sample was used twice in a run (marked orange in Appendix Table 1). Ideally, the replicates should show similar AFLP patterns which would indicate a high reproducibility within the runs. Additionally, every plate contained one negative control, where double distilled water was used instead of DNA to show possible contamination with foreign DNA.

The first step of the AFLP procedure was the restriction and ligation (RL, Appendix Table 2 shows the reaction mix), which were performed simultaneously. Therefore, the restriction enzymes MseI (50 u/µl; NEB) and EcoRI (80u/µl; Promega) were used to cut the genomic DNA. At the same time DNA adaptors were ligated to the restriction sites with the help of T4 DNA Ligase (3u/µl; Promega). According to the protocol of Vos et al. (1995) the adaptors EcoRI-1 5'-CTCGTAGACTGCGTACC-3', EcoRI-2 3'-CATCTGACGCATGGTTAA-5', MseI-1 5'-GACGATGAGTCCTGAG-3' and MseI-2 3'-TACTCAGGACTCAT-5' were used. 6 µl of the reaction mix were added to 5 µl of the genomic DNA. Restriction and ligation was conducted for 3 hours at 37°C in a GeneAMP® PRC System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Afterwards, eight samples (last column of the plate including positive controls and negative control) were checked on a 1% agarose gel at 120V for 20 minutes (see an example in Appendix Figure 1 a), whereby a smear of DNA fragments was expected. Then, the samples were diluted 1:20 with ddH_2O.

For the next step, the preselective polymerase chain reaction (PCR, see Appendix Table 3 for the reaction mix), two primers with one additional nucleotide 5'-EcoRI+A-3' primer (10microM) and 5'-MseI+C-3' primer (10microM) were used to reduce the number of different fragments. The sequences for the preselective PCR (VOS et al., 1995) are EcoRI + A 5'-GACTGCGTACCAATTC+**A**-3' and MseI + C 5'-GATGAGTCCTGAGTAA+**C**-3'.

8 µl of the reaction mix were applied in a 96-well PCR plate and 2 µl of the diluted restriction/ligation product were added. The used thermocycling program on the GeneAMP® PRC System 9700 was as follows: 2min – 72°C, 30x [30sec – 94°C, 30sec – 56°C, 1min – 72°C], 10min – 72°C. Afterwards the machine cooled down the samples to 4°C. After this step, the same eight samples were checked on a 1% agarose gel at 120V for 20 minutes (see an example in Appendix Figure 1 b) and diluted with ddH_2O 1:20.

The preselective PCR still yielded a huge number of fragments, which needed to be reduced with the selective PCR. In this step, three different primer combinations with each three selective bases were used. The primers with an EcoRI restriction site were labeled with a fluorescent color, the MseI primers were not labeled. To find out the best primer combinations, several combinations were tested (Appendix Table 4 shows the tested primer combinations). The primers which provided the best marker profiles and enough peaks were chosen for the selective PCR. For *Gladiolus palustris*, the primer combinations **6-FAM** (EcoRI+ACT_5'-GACTGCGTACCAATTC+**ACT**-3', MseI+CA_5'-GATGAGTCCTGAGTAA+**CA**-3'), **VIC** (EcoRI+AGG_5'-GACTGCGTACCAATTC+**AGG**-3', MseI+CT_5'-GATGAGTCCTGAGTAA+**CT**-3') and **NED** (EcoRI+AAC_5'-GACTGCGTACCAATTC+**AAC**-3', MseI+CT_5'-GATGAGTCCTGAGTAA+**CT**-3') showed the best distinct peaks and also the best number of peaks within the Fragment Profiler (Amersham Biosciences Corp, Piscataway, USA). MseI primers only had two additional nucleotides, because tests with three nucleotides showed that the number of frequents was reduced too much. 8 µl of the reaction mix (Appendix Table 5 shows the reaction mix) were applied in a 96-well PCR plate and 2 µl of the diluted preselective product were added. The used thermocycling program on the GeneAMP® PRC System 9700 was as follows: 2min – 94°C, 10x [20sec – 94°C, 30sec – 66°C, 2min – 72°C], 20x [20sec – 94°C, 30sec – 56°C, 2min – 72°C], 30min – 60°C. Afterwards the machine cooled down the samples to 4°C. Eight samples were again checked on an agarose gel (see an example in Appendix Figure 1 c).

The PCR products were purified with Sephadex™ G-50 Superfine (GE Healthcare Bio-Sciences, Uppsala, Sweden) to remove the primers and to reduce the concentration of ions. The Sephadex resin was applied into a 96-well MultiScreenHV filter plate (MAHVN45, Millipore, Molsheim, France) with help of a MultiScreen Column Loader (Millipore). Then 300 µl ddH_20 were added to each well and the Sephadex was left to swell at room temperature for three hours (or packed airtight overnight in the fridge). Subsequently the plate was centrifuged at 910 g for 5 minutes to remove unabsorbed

water. Afterwards, again 150 µl ddH_20 were added and the MultiScreenHV plate was centrifuged at 910 g for 5 minutes once more. After these steps the filtration plate was put on a sterile 96-well PCR plate. 6 µl of the NED-labeled, 3 µl of the VIC-labeled and 3 µl of the FAM-labeled PCR products of each sample were combined on the swollen Sephadex column using a multi-channel pipette (HTL Abimed Discovery). The filtration plate and the PCR plate were fixed consecutively with cellotape and centrifuged at 910 g for 5 minutes. This step yielded the purified selective PCR products, i.e. the purified labelled DNA. For electrophoresis 2 µl of each purified DNA sample were mixed with 12.9 µl ddH_2O and 0.1 µl ET400-R-ROX-MegaBace (Applied Biosystems) in a 96-well PCR plate (Cycleplate, Thermo Fisher Scientific). The DNA was denaturized in the GeneAMP® PRC System 9700 at 95°C for 3 minutes, then immediately stored on ice to keep the DNA single-stranded. Then the plates were centrifuged shortly to remove eventual air bladders. The samples were run on an automated 48-capillary MegaBace™ 1000 DNA Analysis System (GE Healthcare), following the manufacturers manual. Here, a capillary electrophoresis was performed, where DNA samples were placed at the negatively charged cathode and moved to the positively charged anode when voltage was applied. With the help of the capillary-gel-electrophoresis, the species-specific lengths of DNA fragments were yielded. A laser scanned the samples and the raw data was transformed to electropherograms. After each run, the samples were controlled with the program Fragment Profiler version 1.2 (GE Healthcare) to check for capillary failures and if all three selective amplifications performed well. Failed samples were repeated within the last run, together with the replicates.

2.1.2.3 AFLP data scoring

The program DAx version 8.0 (Van Mierlo Software Consultancy, Netherlands) was used for scoring the data based on the electropherograms, meaning the band intensity information from the electropherograms was extracted manually or automatically resulting in a binary matrix. Regions between 160-180bp and 320-340bp were excluded from scoring because the ET-ROX-standard did not work appropriately in these regions. First, the three differently labeled fragments (6-FAM, VIC and NED) and the ROX standard were separated by their fluorescence, using a color separation matrix (see Appendix Table 6). The program constructed a baseline to avoid noise signals from the electropherograms, which were subtracted by the baseline. With the ROX standard, the sizes of the fragments were calibrated because it contains the setting of automatic size calibration (ATC method). Three binning sheets were created and the peak search was conducted automatically by the program. The bins (also markers or loci) were set and after the automatic peak search each peak was checked manually for its integrity using the 'user defined bins' option. The AFLP electropherograms were scored as present (1) or absent (0), whereby (1) is the presence of a DNA fragment and (0) is its

absence at a specific position on the genome, resulting in a binary matrix. The binning sheets window allowed displaying peak height and peak area as well as the curves for each bin. The three 0/1 binary matrices of 6-FAM, VIC and NED were imported into Excel 2010 (Microsoft Office, Redmont, USA) and merged. The replicates were checked for mismatches. Different matrices were created, with and without the out-group of *Gladiolus imbricatus*. With the program PAST version 3.01 (PAleontological Statistics; HAMMER et al., 2001) some Neighbor Joining trees were constructed to identify samples that did not match the pattern.

2.1.2.4 Quantifying the error rate and pruning the binary matrix

In total, 309 samples (one sample was excluded because the DNA extraction did not work), 44 replicates and 6 "between-plates-replicates" have been analyzed. BONIN et al. (2007) states that the number of replicated individuals should be about 5-10% of the total number of samples to obtain an expressive error rate. In this study, the percentage of replicates is 16.18% (calculated for 50 replicates in 309 individuals). For calculating the error rate, the 50 successfully replicated samples were used. For each marker, the number of mismatches between replicate and reference sample, i.e. the fragment presence-absence patterns was recorded. After calculating the percentage of errors per marker, markers with more than 10% were removed. The total number of mismatches divided by the total number of phenotypic comparisons was stated as error rate (BONIN et al., 2004). It was calculated for each color separately and in total.

2.1.3 Genetic data analysis

2.1.3.1 Data matrices

Different data matrices for different analyses were produced, as it can be seen in the following Table 4. One matrix included the out-group population *G. imbricatus* and was used for building a tree, see chapter 3.1.3.1. Another matrix contained only populations of *G. palustris*, with modifications of populations (e.g. without population "STE", combination of populations "DWl" and "DWp" to "DW"). For a third matrix, markers which were present in every individual were excluded, i.e. only polymorphic markers were used.

Tab. 4: Different matrices for different analyses.

Nr.	Content	Populations	Programs used
Matrix 1	*G. palustris* & *G. imbricatus* – all markers	All populations	PAST version 3.01 (NJ)
Matrix 2	*G. palustris* - all markers	All populations of *G. palustris*	PAST version 3.01 (PCoA, NJ)
		Without population "STE"	SPLITSTREE4 version 4.13.1 (Neighbor-Net) GenAlEx (IBD)
		Without population "STE", combination of "DWl" and "DWp" to "DW"	R 3.1.2 (Genetic diversity and rarity)
		Combination of "DWl" and "DWp" to "DW"	Arlequin version 3.5.2.2 (AMOVA)
Matrix 3	*G. palustris* - only variable markers	All populations of *G. palustris*	STRUCTURE version 2.3.4 (Model-based clustering)

2.1.3.2 Genetic relationships (Trees) distance-based clustering methods

The program SPLITSTREE4 version 4.13.1 (HUSON & BRYANT, 2006) was used to construct a Neighbor-Net (NN) based on the genetic distance matrix of Dice coefficients (DICE, 1945). Therefore, Matrix 2 of Table 4 was used, where population "STE" was excluded because there was only one individual sampled in this population. Furthermore, an individual-based NeighborNet and an individual-based Neighbor-Joining-tree were constructed for *G. palustris* populations of Salzburg. Before using SPLITSTREE, an input file was created in R (R Development Core Team, 2014) with the function "Nexus" of the AFLPdat R Script of EHRICH (2010). With the program PAST version 3.01 (PAleontological Statistics; HAMMER et al., 2001), a Neighbor-Joining (NJ) clustering was conducted including the out-group species *G. imbricatus* (see Table 4, Matrix 1), based on Euclidean distances. The longer the branches of the tree, the more the clusters are separated from each other. Bootstrap values using 1000 replicates were computed to get statistical support of the clusters. Bootstrap values below 70% were not interpreted. Also, another Neighbor-Joining (NJ) clustering was conducted (using Dice coefficients) excluding the out-group to illustrate identical genotypes.

2.1.3.3 Principal Coordinates Analysis (PCoA)

The Principal Coordinate Analysis (PCoA) based on the genetic distances of the individuals was performed for the whole data-set (Salzburg populations and populations of adjacent areas) and also for the Salzburg populations only using the program PAST version 3.01 (PAleontological Statistics; HAMMER et al., 2001) with the Dice coefficient (DICE, 1945). All 125 markers (28 polymorphic, 97 monomorphic markers) were used (see Table 4, Matrix 2). The program calculates the Eigenvectors (coordinates) and Eigenvalues and similar individuals are clustered together.

2.1.3.4 Model-based clustering

The genetic structure of the dataset (excluding the outgroup *Gladiolus imbricatus*) was investigated with the program STRUCTURE (version 2.3.4; PRITCHARD et al., 2000). The program uses a Bayesian clustering approach with a Markov chain Monte Carlo (MCMC) algorithm and assumes linkage equilibrium and Hardy-Weinberg-equilibrium. The probabilities (P) of different numbers of genetically homogenous groups or clusters (K) are calculated and individuals are (probabilistically, Q) assigned to these groups. For the intraspecific approach of *Gladiolus palustris*, the admixture model was used, so that individuals could be probabilistically assigned to more than one population, if their genotypes were admixed. The other model, the no-admixture model assumes that all the genetic material of an individual originates from one of the K populations (PRITCHARD et al., 2000; FALUSH et al., 2007). Both the correlated and uncorrelated models for allele frequencies were applied. An input file for Structure was created in Excel, where every individual was duplicated in the matrix. For all further analysis the populations “ÜSX” and “KöH” were renamed in “USX” and “KoH” because the programs are not able to read special characters. In the settings the recessive allele model was chosen for the dominant marker systems in AFLPs. For an exploratory trial, all 295 individuals with ploidy level 2 and 28 polymorphic loci were used (monomorphic markers were excluded to reduce computation time; see Table 4, Matrix 3). The trial analysis was computed for K=1 to a maximum K=20, with a number of iterations of 10. The number of MCMC was set to 10 000 after a burn-in of 5000 iterations. For obtaining accurate estimations, the STRUCTURE analysis was performed again, both for admixture-correlated and admixture-uncorrelated and the number of MCMC was set to 50 000 after a burn-in of 20 000 iterations. Both models were checked for the resulting clusters at different K’s to see if the individuals were clustered together in the same way, independent of the model.

For determining the most probable number of genetic groups (K), two kinds of analyses were used. The first possibility to estimate the most likely number of groups was using the R-script STRUCTURE-SUM (EHRICH, 2009, unpublished). The function “Structure.simil” was used to calculate similarity

coefficients among each pair of STRUCTURE-runs according to NORDBORG et al. (2005), delivering tables and a figure. With the function "Structure.deltaK" the most likely number of K was estimated and four plots were calculated. Second, a web-based program called STRUCTURE HARVESTER (EARL & VONHOLDT, 2012) was used to analyze the results of STRUCTURE. To upload the result files of STRUCTURE to the website it was necessary to convert them into a ZIP-file. STRUCTURE HARVESTER produces several graphs and Evanno plots (EVANNO et al., 2005), which allow to identify the "most probable" number of genetic groups (K). The STRUCTURE HARVESTER also generates input files for the program CLUMPP "CLuster Matching and Permutation Program" (JAKOBSSON & ROSENBERG, 2007). Within CLUMPP, the independent runs for each K were matched up as closely as possible. Results of these analyses were graphically depicted with Excel 2010.

2.1.3.5 Genetic diversity and rarity

For calculating the genetic diversity of the complete data set the program R 3.1.2 (R Development Core Team, 2014) was used. One population (STE) was excluded from the analysis because it is composed of only one individual. The populations DWp and DWl were grouped together to the population "DW", because population size reckoning only exists for the whole population at the site "Dürre Wiese" (see Table 4, Matrix 2). With the R Script of EHRICH (2010), Nei's gene (NEI, 1987) was calculated for each population for each marker and then the average was taken. Therefore, the function "Diversity" was used. The program generates an output file containing a table with information about the proportion of variable markers and gene diversity of the populations. The function "Diversity.boot" also calculates Nei's gene diversity for each population and estimates the 95% confidence intervals (lower CI, upper CI) for the genetic diversities from bootstrapping over markers (1000 pseudo-replicates). Also, the rarity index (SCHÖNSWETTER & TRIBSCH, 2005; DW = Down weighed marker) was calculated with the R Script AFLPdat, using the function "Rarity". It is a measure of the amount of rare markers in each population. The higher the rarity, the more rare markers are present in a population. The program generates two output files (rarity index for individuals and for populations). To visualize the diversity and rarity of the data, GIS maps were created with Quantum GIS version 2.12.0. A correlation analysis was performed between the genetic diversity and rarity of Salzburg populations and the average population sizes (of Günther Nowotny) and the sample sizes of the Salzburg populations (see Table 1) using the function "corrplot" in R. The result was a heat map.

2.1.3.6 Isolation by distance (IBD)

For the testing the isolation by distance (IBD), a geographical distance matrix and a genetic distance matrix were required. The geographical distance matrix was created with the R Script of EHRICH (2010) with the function "Geodist". Populations "FrR" (Vorarlberg) and "Pla" (Upper Austria) show the biggest geographical distance of more than 300 kilometers. The program GenAlEx (PEAKALL & SMOUSE, 2012) was used to calculate Φ_{PT} for all pairs of populations, an analogue of F_{ST}. Population "STE" (containing only one population) was excluded of all analyses. Isolation by distance then was calculated by correlating the geographical distance against the genetic distance within a Mantel test (MANTEL, 1967). For the geographic distance, kilometers and the logarithm (log10) of the geographical distances in meters were used. For the genetic distance matrix, Φ_{PT}, $\Phi_{PT}/(1- \Phi_{PT})$ and the population average pairwise differences (derived from AMOVA, see Table 11 and 13) were used. 9999 random permutations were performed to evaluate the level of significance. There were made more Mantel-tests to clarify if there was a correlation between the pairwise F_{ST}s (or Φ_{PT}) and population average pairwise difference for the whole dataset and for Salzburg populations. Isolation by distance calculations were conducted for the whole dataset, for Salzburg populations including nearby populations and for Salzburg populations only (for group constellation see Table 5).

Tab. 5: Groups of populations used for the Isolation by distance (IBD).

Group	Populations	Number of populations
All populations	Abr, ABW, DW, EBr, EEF, EJa, EMr, EOE, EWr, For, FrR, HaW, HiW, HSW, HW, Kne, KoH, MB, MHS, Mon, OLW, Pid, Pla, Ran, SaH, USX , SWl, ZWl, ZWr, ZWu	30
Salzburg and nearby populations	Abr, ABW, DW, EBr, EEF, EJa, EMr, EOE, EWr, HaW, HiW, HSW, Kne, Mon, OLW, Pid, Ran, SWl, ZWl, ZWr, ZWu	21
Only Salzburg	Abr, ABW, DW, EBr, EEF, EJa, EMr, EOE, EWr, Kne, OLW, Ran, SWl, ZWl, ZWr, ZWu	16

2.1.3.7 Analyses of molecular variance (AMOVA)

An analysis of molecular variance (AMOVA) was performed via the program Arlequin version 3.5.2.2 (EXCOFFIER & LISCHER, 2010) to calculate the molecular variance among and within populations (three different F-values) to identify the amount of population genetic structure. The pairwise F_{ST}-value (fixation index) shows the variance among subpopulations relative to the total variance. The F_{SC}-value calculates the variance among subpopulations within populations. The F_{CT}-value reveals the variation among groups relative to the total variance. The input files for the program Arlequin were created

with the R-Script AFLPdat (EHRICH, 2010) using the function “Arlequin”. The number of permutations was set to 1000 to test the significance of variance. First, a non-hierarchical (2-level) AMOVA was performed for the complete dateset (the color-morph populations DWl and DWp were combined to population DW, as they are sharing the same habitat) and for Salzburg populations. Furthermore, different hierarchical AMOVAs (3-level) were performed for different scenarios of population groupings. Populations were grouped according to their geographical distribution (Salzburg – Non-Salzburg, Salzburg – Non-Salzburg separated in different regions, East-West) and also according to the groups identified with STRUCTURE for K=3 and K=7 (with and without strongly admixed populations).

2.2 Investigation of one population with color variation

2.2.1 Plant material

The population “DW” with two different color varieties is located at “Dürre Wiese” in Wals-Siezenheim, Gois (see Figure 6 and 8 and Table 3 for more details). At 21.06.2015, 16 of the bright pink (DWp) flowering individuals were sampled and also 16 of the purple (DWl) ones (few centimeters of the leaves). The plant material was stored as described above.

Fig. 8: Purple (left) and bright pink (right) individuals of *Gladiolus palustris* at “Dürre Wiese” in Wals-Siezenheim.

2.2.2 Data analysis

DNA extraction and AFLP analysis were conducted as described in chapter 2.1.2. After AFLP data scoring, a separate matrix was created which contained only the markers of the 32 individuals of "Dürre Wiese".

A Principal Coordinate Analysis (PCoA) based on the genetic distances of the individuals was performed for the 32 individuals using the program PAST version 3.01 (PAleontological Statistics; HAMMER et al., 2001) with the Dice coefficient (DICE, 1945). 121 markers were used. The program calculates the Eigenvectors (coordinates) and Eigenvalues and similar individuals are clustered together.

Furthermore, an AMOVA was conducted via the program Arlequin version 3.5.2.2 (EXCOFFIER & LISCHER, 2010) to calculate the molecular variance among and within the two populations. The input file for the program Arlequin was created with the R-Script AFLPdat (EHRICH, 2010) using the function "Arlequin". A non-hierarchical AMOVA was performed for the two populations (for 121 markers). 1000 permutations were calculated.

2.3 Pollination biology study

2.3.1 Experimental setting

With a pollination experiment, it was tested, if *Gladiolus palustris* could be self-pollinated. Therefore, breathable bags made of translucent curtain material were required to cover the flowers. Furthermore, wooden sticks of approximately 70cm height were used. On one end of the stick, two plastic rings cut from plastic bottles (diameter about 10cm) were stapled and the breathable bags were pulled over these plastic rings and tied up with a tearproof thread (see Figure 9).

Fig.9: Breathable bags of the pollination experiment setting. Top right forceps with stamen.

In total, 49 plants (at Ziegler-Wiese and Randersberg-Wiese) were covered as described. Thereof, 15 individuals, the "self-pollination-group" ("spontaneous selfing"), were covered before flowering. Another 15 individuals, the "self-compatibility group" ("forced selfing") were covered before flowering and artificially self-pollinated by hand with a paintbrush and a forceps. The remaining 19 individuals of the "control group" ("open pollination") were covered after flowering and possible cross-pollination by insects. However, for later analyses, each single flower was assigned to one of

the three groups (“spontaneous selfing”, “forced selfing”, “open pollination”), because due to irregular flowering of the flowers of every plant it was not possible to pollinate every single flower of the “self-compatibility-group” per hand. So, many flowers of this group are actually belonging to the “self-pollination-group”, as they were just covered and not treated by hand any further. After seed maturation, the bags were removed and the dry seed capsules were collected in late summer 2015 and stored in bags for later seed counting.

2.3.2 Evaluation of the data

The capsules were allocated to one of the three groups “spontaneous selfing”, “forced selfing” and “open pollination” and seeds per capsule were counted. It was distinguished between developed (fertile) seeds which were bigger and heavier and not fully developed (sterile) seeds. The number of possible opportunities for reproduction (flowers) was compared to the actually developed capsules. The data were separately analyzed concerning the different pollination treatments “self-pollination” (spontaneous selfing), “hand-pollination” (forced selfing) and the “cross-pollination” (open pollination). The mean numbers of fertile seeds per capsules were calculated for each treatment for all available flowers.

To test for differences between the treatments “spontaneous selfing”, “forced selfing” and “open pollination”, a linear mixed-effects model (LME4 package for R; BATES et al., 2015) was fitted with counting-results of capsules per flower and fertile seeds per capsule. Here, nested ANOVAs were conducted, as there were different treatments occurring for one individual. So, some individuals occurred more than once in the dataset, so the individuals were included as a random factor. To account for different sample sizes of individuals, a square root of them as weight in the linear mixed-effects models was used. This gives greater weight to larger sample sizes which are more reliable. These analyses were conducted for all data and for their means. The distribution of the data was neglected for this analysis and a normal distribution was assumed.

However, the data actually show a Poisson distribution instead of a normal distribution which was analyzed with a Shapiro-Wilk-test (SHAPIRO & WILK, 1965) in R. The linear mixed model was generalized according to this Poisson distribution. The plant individuals were again included as a random factor and the treatment as the fixed factor. This calculation was conducted only for the number of fertile seeds per capsule for all data. Furthermore, a pairwise comparison was conducted with the R package „multcomp“ to clarify if there was a significant difference between the treatments.

3 Results

3.1 Results of the investigation of the genetic structure within and among populations in Salzburg and adjacent areas

3.1.1 Results of the AFLP data

DNA extraction was conducted for 310 individuals, 309 were used for further analysis, because DNA extraction did not work for the outgroup sample "Gi_Stm_10", which was excluded. After generating the AFLPs and scoring the data, three samples (For_04, For_08, HiW_07) were excluded because their electropherograms were too weak and showed a great shift. The remaining 306 samples (295 individuals of *Gladiolus palustris*, 11 individuals of *G. imbricatus*) and the replicates (44 "within-plates-replicates", 6 "between-plates-replicates") were used for further genetic data analysis. First, the three primer combinations yielded 171 markers (55 FAM-markers, 56 VIC-markers and 60 NED-markers). Within each marker, the replicates were checked for mismatches and seven markers showed a high percentage of errors and where excluded for further analysis. So, the final number of markers was 164 (54 FAM-markers, 51 VIC-markers and 59 NED-markers). 39 markers were exclusively found in the outgroup species *Gladiolus imbricatus,* so for *G. palustris,* 125 markers (28 polymorphic, 97 monomorphic, percentage of polymorphic fragments PPF=22.4%) were left for further calculations. There were only 28 variable markers in the third matrix. The fragment lengths of the markers ranged from 105.2 – 409.6 bp in 6-FAM, 100.0 – 405.4 bp in VIC and 103.7 – 397.1 bp in NED. The average error rate for all three colors was 0.1829% (FAM: 0.148%, VIC: 0.039%, NED: 0.339%). Typical error rates should be between 2-5% and should not be higher than 10% (BONIN et al., 2007).

3.1.2 Identification of identical genotypes

Within the dataset, many identical genotypes within and also among populations were detected, as it can be seen in Table 6. 70 individuals had unique AFLP phenotypes and are not listed in the table. The remaining 225 individuals had redundant genotypes and shared their AFLP phenotype with at least one or more individuals. The NJ clustering as described in chapter 2.1.3.2 is shown in the Appendix Figures 2.a)-c).

Tab. 6: Identical genotypes of *G. palustris* individuals. Abbreviations see Table 3.

Individuals	Number of loci
Abr_02_2, KoH_08, SWI_01, SWI_02	118
Abr_03, Abr_04, Abr_05, Abr_06, ABW_01, ABW_02, ABW_04_2, ABW_05, For_05, SWI_03, ZWu_01	119
Abr_08, Abr_10, DWl_05, Ran_11	118

ABW_03, EEF_02_2, EWr_02, EWr_03, EWr_04, EWr_07, EWr_08, EWr_09_2, Ran_14, Ran_15, SaH_05_2, ZWl_06, ZWr_06, ZWr_08, ZWr_09, ZWu_03	118
DWl_04, Ran_13_2, ZWu_06_3, ZWu_07, ZWu_08	117
DWl_06_2, DWl_15	118
DWl_07, DWl_08_3, DWl_10, DWl_11, DWl_14, DWl_16, DWp_01, DWp_03, DWp_04, DWp_05, DWp_08, DWp_11, DWp_12, DWp_15, DWp_16	119
DWl_09, DWl_12, DWl_13, DWp_06, DWp_07, DWp_10, ZWr_04	118
DWp_02, DWp_09, DWp_13	117
Ebr_01, Ebr_03, For_03, For_06, HaW_07, Ran_07, SaH_01, SaH_02, SaH_03, SaH_04, SWl_06_2	120
EEF_01, ZWl_04, ZWr_05, ZWr_07_3	117
EEF_04, EOE_02	116
EJa_01_3, EJa_10, EOE_09, EOE_10, Mon_08	117
EJa_02_2, EJa_04, EJa_05, SWI_07	117
EJa_03_2, EJa_06, EOE_05, Ran_02, Ran_08, Ran_09	118
EJa_08, EOE_03_2, EOE_04, EOE_06_2	117
EMr_01, EMr_07, EMr_08	116
EMr_02_3, EMr_05	115
EMr_03, EMr_04, EMr_06	114
EMr_10, SWI_10	117
EOE_01, EOE_07	115
EWr_05, EWr_06	117
For_01, For_02, SaH_09	121
For_07_2, SaH_10, SWI_08	119
FrR_01, FrR_02, FrR_03, FrR_04, FrR_05, FrR_06, FrR_07_2, FrR_08, FrR_09_2, FrR_10, Pla_10, USX_01_2, USX_02, USX_03, USX_04, USX_05, USX_06, USX_07, USX_08_2, USX_10	117
HaW_01, HiW_03	117
HaW_02, HiW_08	119
HaW_05, HaW_09, Ran_04, Ran_06, Ran_10	119
HaW_06, HaW_10, Kne_05	119
HaW_08, HiW_05, SWI_05	119
HiW_01, HiW_06_3	117
HiW_09, MB_04	119
HSW_01, HSW_02	118
HW_03, MB_09	118
HW_05, MHS_03_2	117
HW_08, HW_09	118
Kne_01, SaH_06, SaH_07, SaH_08	120
KoH_01, KoH_06	118
KoH_02_2, KoH_07	116
MB_05, MHS_04	119
Mon_10, ZWl_03, ZWl_09, ZWl_10	119
OLW_01, OLW_02, OLW_03, OLW_04, OLW_05, OLW_06, OLW_07, OLW_10	119
Pid_01, Pid_09, Pid_10, Pid_11, Pid_13, Pid_15_2	116
Pid_02, Pid_03, Pid_04, Pid_05, Pid_06_2, Pid_07, Pid_08, Pid_12, Pid_14	115
Pla_01, Pla_03_2, Pla_06	115
Pla_02, Pla_04, Pla_05, Pla_07, Pla_08, Pla_09	114
SWI_04, ZWu_02, ZWu_10	118
SWI_09, ZWr_03	116
ZWl_05, ZWl_07	118
ZWr_01, ZWr_10	119

3.1.3 Results of the data analysis

3.1.3.1 Genetic relationships

The results of the Neighbor-Net analysis of all samples showed that only one population, namely population “Pla” (Planwiesen/Grünberg, Upper Austria) was slightly forming a separated cluster (see Figure 10). For all other populations there was no clear clustering visible. Regarding the results of the Neighbor-Joining-Clustering including the out-group species, they were clearly separated from the *G. palustris* populations. The bootstrap value for *Gladiolus imbricatus* was 100%, population “Pla” showed bootstrap value of 92% (here depicted in green, see Figure 11). All other populations showed values below 70%. Performing a Neighbor-Net and a Neighbor-Joining analysis for Salzburg populations only revealed unclear clustering; individuals were not grouped distinctly and had no bootstrap support (Figures can be seen in the Appendix Figure 3 & 4).

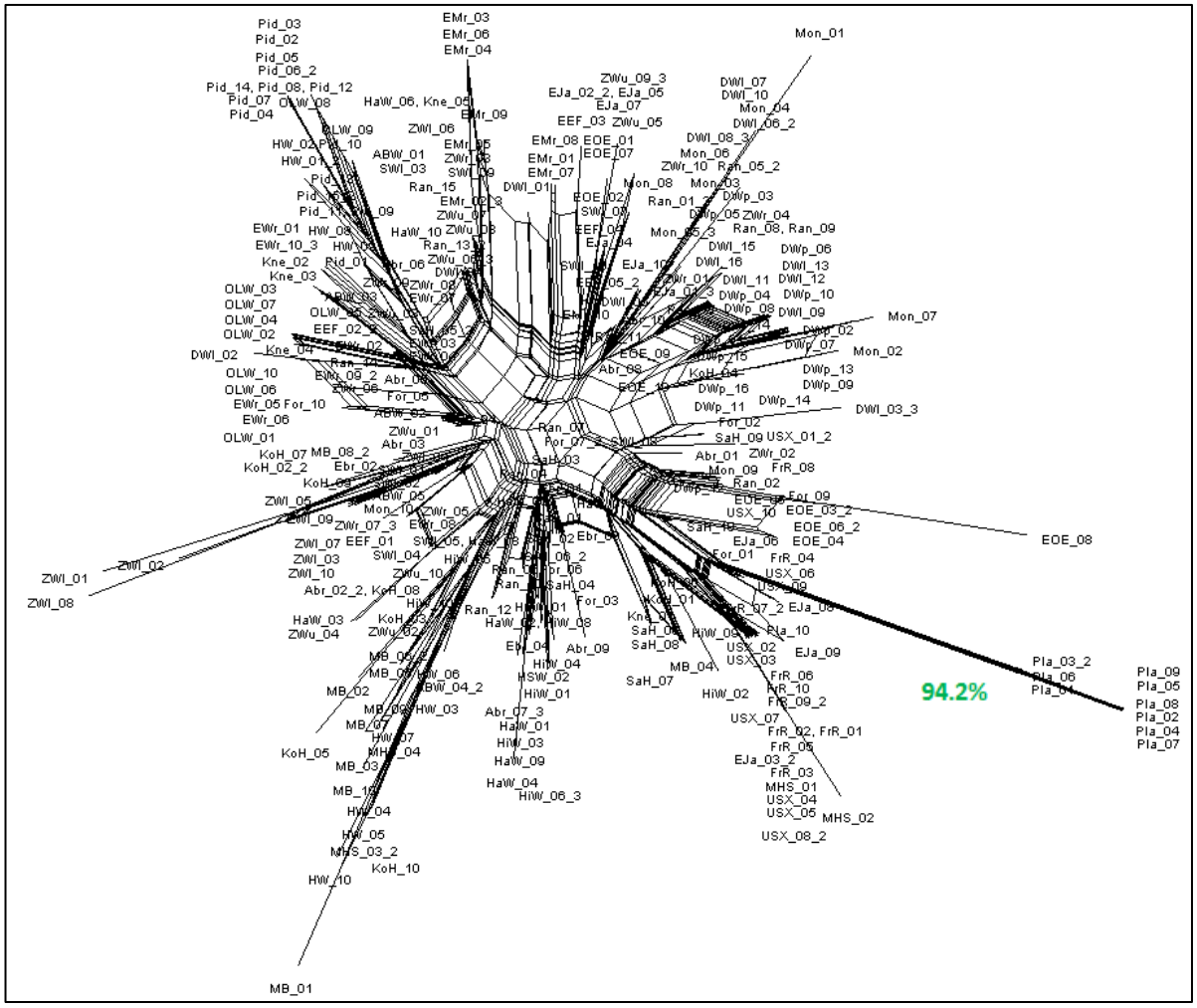


Fig. 10: Individual-based NeighborNet of all analyzed samples of *G. palustris*. The green value is the bootstrap value giving statistical support of the cluster “Pla”. Bootstraps below 70% are not interpreted and therefore not depicted in the figure.

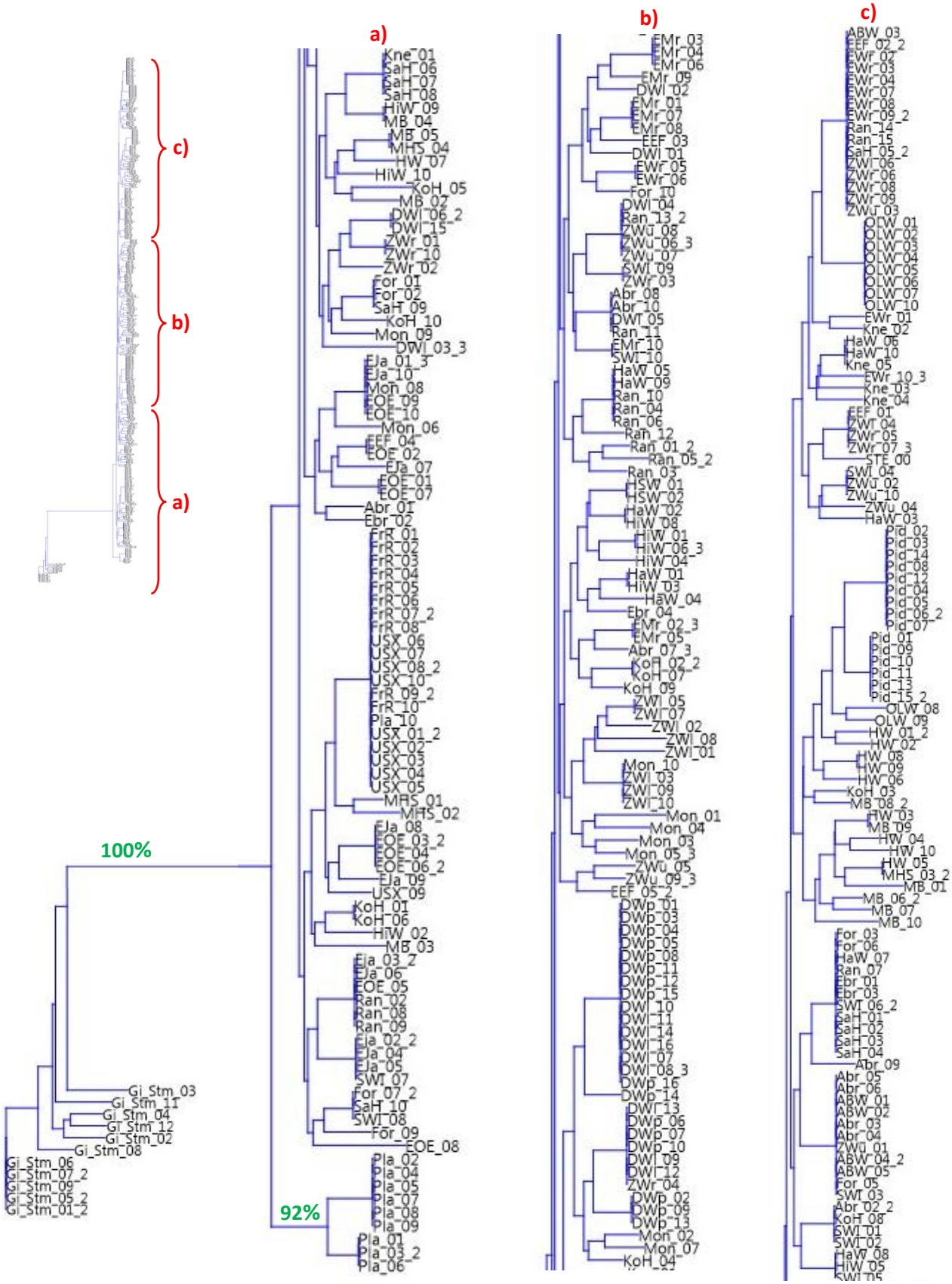


Fig. 11: Individual-based Neighbor-Joining-Clustering of all analyzed samples of G. *palustris* and *G. imbricatus.* Bootstrap values (here in green) below 70% are not interpreted and therefore not depicted in the figure. The out-group *G. imbricatus* is given as a single branch with 100% bootstrap support. Population "Pla" has a bootstrap support of 92%.

3.1.3.2 Results of the Principal Coordinates Analysis (PCoA)

The Principal Coordinate Analysis (PCoA) based on DICE coefficients was performed for 295 individuals, where the populations of Salzburg and the populations of Vorarlberg, Upper Austria and Germany were included in the analysis. The first two coordinates (axes) had an explanatory power of 33.38% (axis 1: 19.06%; axis 2: 14.32%). The third axis has an explanatory power of 10.67%. Because of many identical genotypes, there are fewer dots in the graphs, as identical individuals are overlying each other.

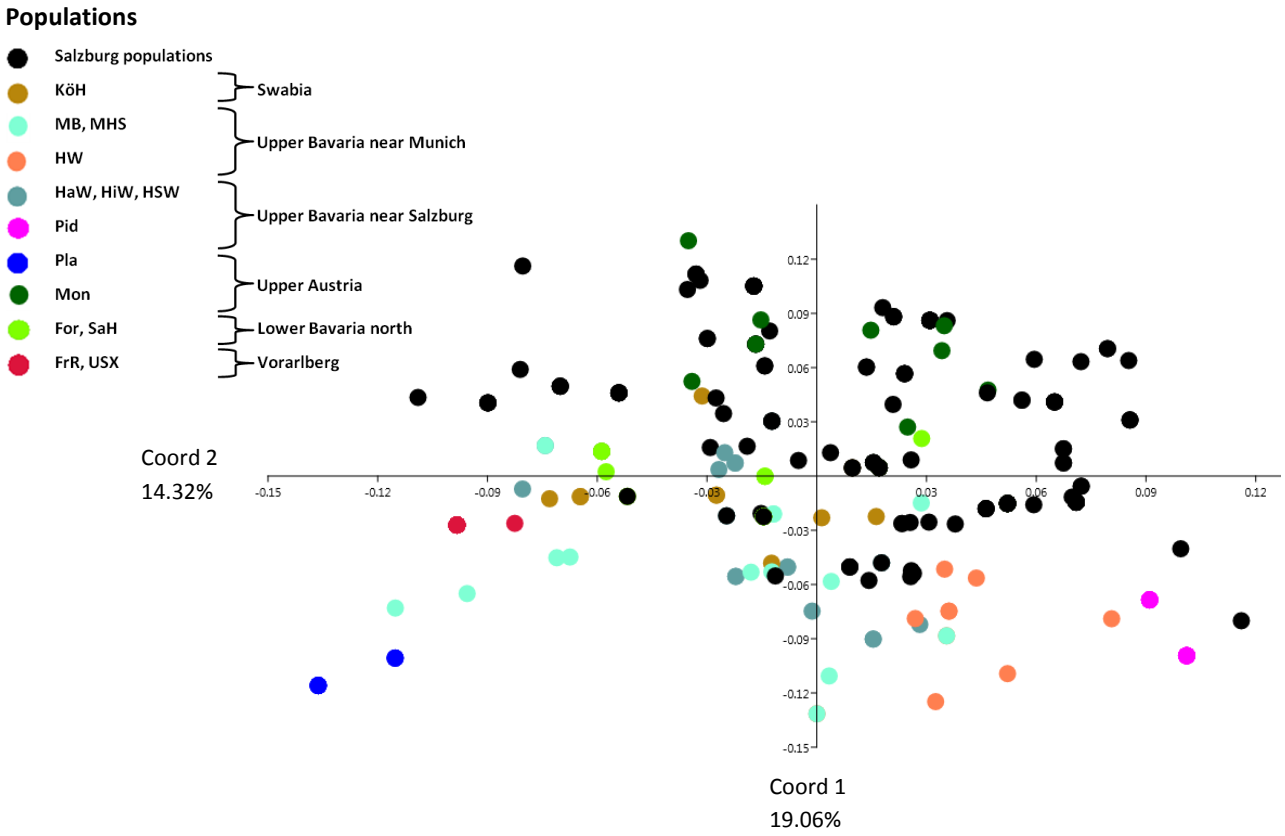


Fig. 12: Principal Coordinate Analysis of all individuals based on Dice coefficient. The first two axes comprise 33.379% of the total genetic variation. Salzburg populations are colored in black; the other populations are colored according to their geographical distribution.

Figure 12 shows that the individuals of population “Pla” (dark blue) were separated from the other populations, as was suggested in the Neighbor-Net and in the STRUCTURE results at K=7, where the population formed its own separate group. Also, the populations of Vorarlberg (red), “USX” and “FrR” were separated, as suggested by STRUCTURE. The individuals of Mondsee in Upper Austria (“Mon”, dark green) were scattered, but all above the first axis and therefore separated from the other population of Upper Austria (“Pla”). Generally, more than half of the Salzburg individuals were above axis 1.

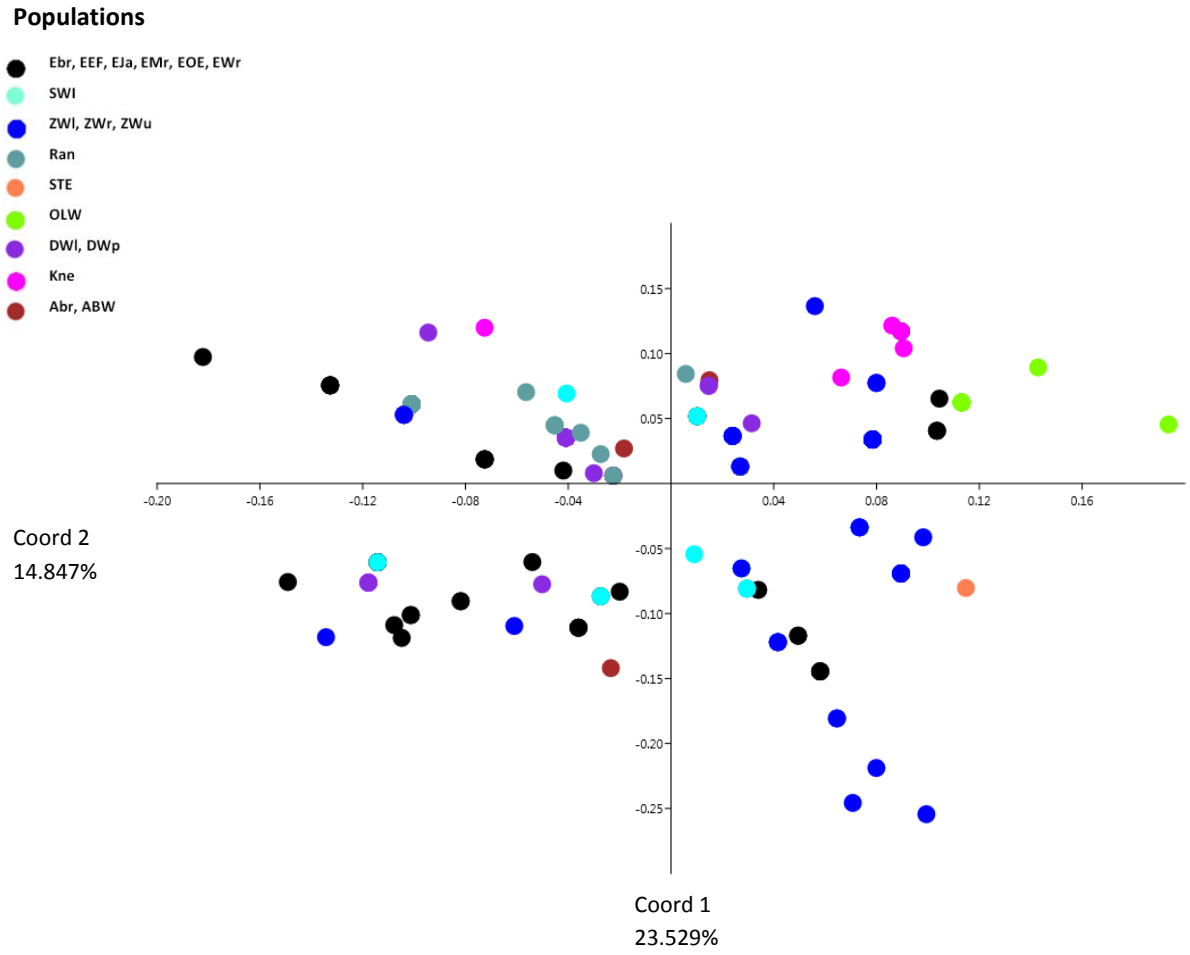


Fig. 13: Principal Coordinate Analysis of the Salzburg individuals only based on Dice coefficient. The first two axes comprise 38.376% of the total genetic variation. Populations are colored according to their geographical distribution.

The second Principal Coordinate Analysis (see Figure 13) was computed only on the 167 *Gladiolus* individuals of the populations of Salzburg. The first two coordinates (axes) had an explanatory power of 38.38% (axis 1: 23.53%; axis 2: 14.85%). The third axis had an explanatory power of 10.77%. The population “OLW” (Obere Langwiese, light green) was separated along the first and second axis and forming a separate cluster. Also, the population “Ran” (Randersberg, grey) was separated along the first axis. The individuals of “Kne” (Kneisslmoor, pink) were also a bit separated. The remaining populations were not forming any clear clusters and are scattered. As it was already mentioned a page before, the occurrence of identical genotypes led to a reduced number of dots, as individuals were overlying each other.

3.1.3.3 Results of the Bayesian clustering

For inferring the genetic structure of the dataset, 295 individuals of 32 *Gladiolus palustris* sampling sites were analyzed with the program STRUCTURE. The admixture model was assumed and both the correlated and uncorrelated allele frequencies were attempted. The comparison of the results of these STRUCTURE runs (K=1 to K=20, 10 replicates for each K, MCMC: 50000 after a burn-in of 20000 iterations) revealed that individuals were grouped in the same clusters, independent of correlated or uncorrelated allele frequencies. While evaluating probable solutions of K, the different K values were plotted against the likelihoods and the uncorrelated allele frequency model showed more clear results as can be seen in Figure 14 and was therefore chosen for further analysis (results of the correlated allele frequency model can be seen in the Appendix). The likelihoods (lnP) from K=1 to K=20 of the uncorrelated allele frequency model are shown in Table 7 (results of the correlated allele frequency model in Appendix Table 7). They were constantly increasing from K=1 to K=7 and then decreasing again with higher standard deviations. In the Evanno table output of the STRUCTURE HARVESTER K=16 was marked yellow, as it showed the highest DeltaK value, but this was due to outliers. Regardless of these outliers, the highest peak of mean DeltaK was at K=7 (here yellow), which would suggest that the most probable number of groups was 7. Also, the results K=2 and K=3 seemed to be meaningful.

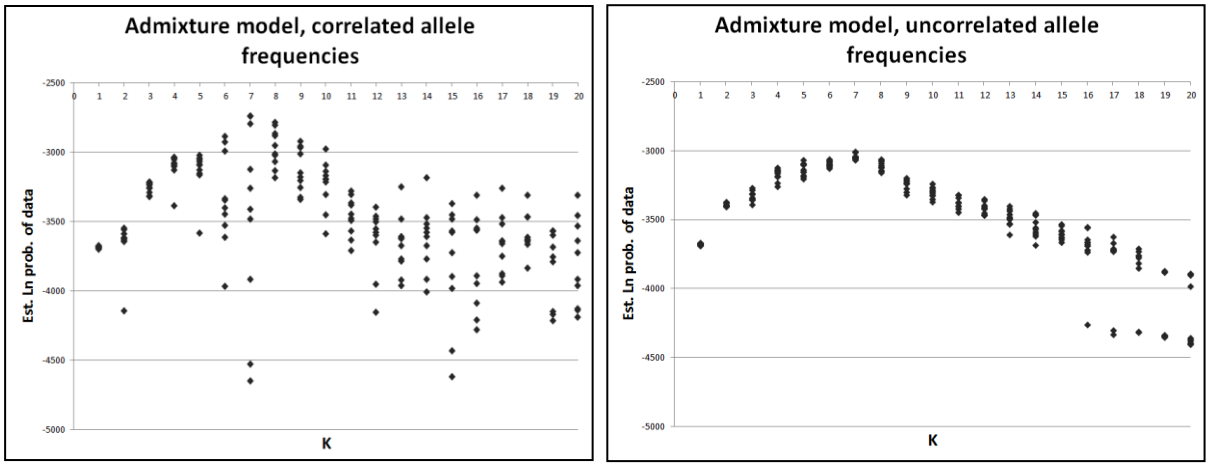


Fig. 14: Results of Bayesian cluster analysis. Logarithmic probability of the replicate runs is plotted against the number of K. Outliers were removed. Left: Correlated allele frequencies. Right: Uncorrelated allele frequencies (Microsoft Excel).

Tab. 7: Result of the Evanno method computed with the STRUCTURE HARVESTER of the STRUCTURE run with the admixture model and uncorrelated allele frequencies. Table for correlated allele frequencies in Appendix Table 7.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-3679.6600	7.0297	-	-	-
2	10	-3394.4400	11.6990	285.2200	224.7900	19.2144
3	10	-3334.0100	36.6955	60.4300	99.3400	2.7071
4	10	-3174.2400	43.7679	159.7700	124.1600	2.8368
5	10	-3138.6300	46.3785	35.6100	6.7700	0.1460
6	10	-3096.2500	21.7615	42.3800	8.2500	0.3791
7	10	-3045.6200	20.5162	50.6300	117.3000	5.7174
8	10	-3112.2900	34.8740	-66.6700	66.0100	1.8928
9	10	-3244.9700	42.0051	-132.6800	72.8700	1.7348
10	10	-3304.7800	39.5786	-59.8100	15.5400	0.3926
11	10	-3380.1300	43.0592	-75.3500	33.1200	0.7692
12	10	-3422.3600	43.2525	-42.2300	23.0300	0.5325
13	10	-3487.6200	61.1926	-65.2600	1.1000	0.0180
14	10	-3553.9800	76.5757	-66.3600	16.9600	0.2213
15	10	-3603.3800	43.7502	-49.4000	66.6000	1.5223
16	10	-3719.3800	200.9936	-116.0000	11621.0500	57.8180
17	10	-15456.4300	36737.2224	-11737.0500	11431.2800	0.3112
18	10	-15762.2000	37528.9210	-305.7700	11861.5800	0.3161
19	10	-4206.3900	225.8191	11555.8100	11548.5900	51.1410
20	10	-4199.1700	242.2618	7.2200	-	-

Evaluating the mean similarity coefficients and their standard deviation, the same numbers of groups were identified, as the highest values were at K=2 (0.98631, SD=0.00243), K=3 (0.92667, SD=0.04270) and K=7 (0.96251, SD=0.00284), which can also be seen in Figure 15, a), indicated with red arrows. The Evanno plots of the admixture model with correlated allele frequencies are shown in Appendix Figure 6.

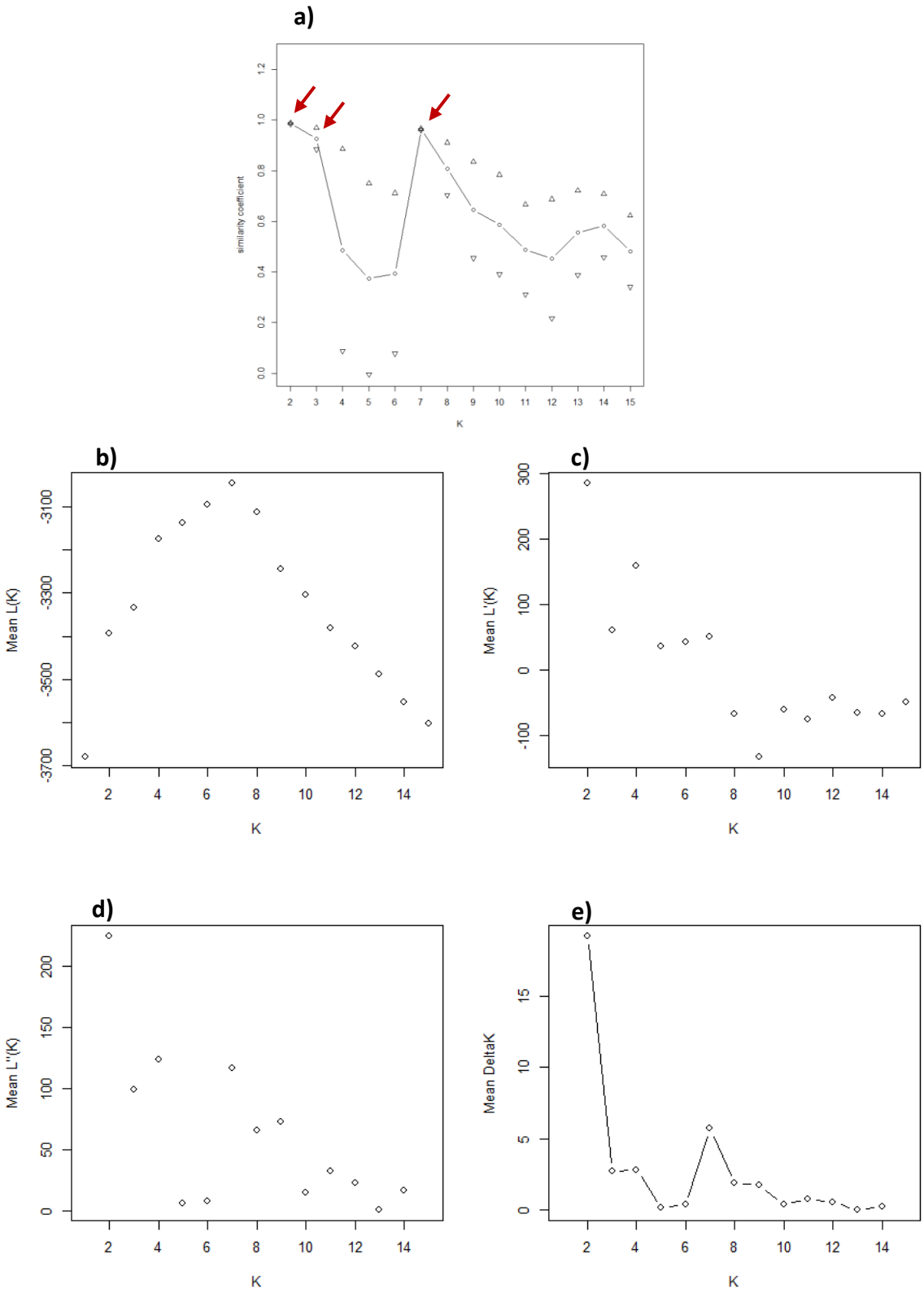


Fig. 15: Results of the STRUCTURE analysis of the admixture model with uncorrelated allele frequencies investigated with the R-script STRUCTURE-SUM (EHRICH 2009, unpublished). Data shown only from K=1 to K=15, data from K=1 to K=20 can be seen in Appendix Figure 5. a) The similarity coefficients are plotted against K. The circles indicate the mean values of the coefficients and the triangles the standard deviation. The similarity coefficients show peaks at meaningful values of K (marked with arrow). b) The mean L(K) is plotted against the number of groups (K). c) The mean L'(K) is plotted against the number of groups (K). d) The mean L''(K) is plotted against the number of groups (K). e) The mean DeltaK is plotted against the number of groups (K).

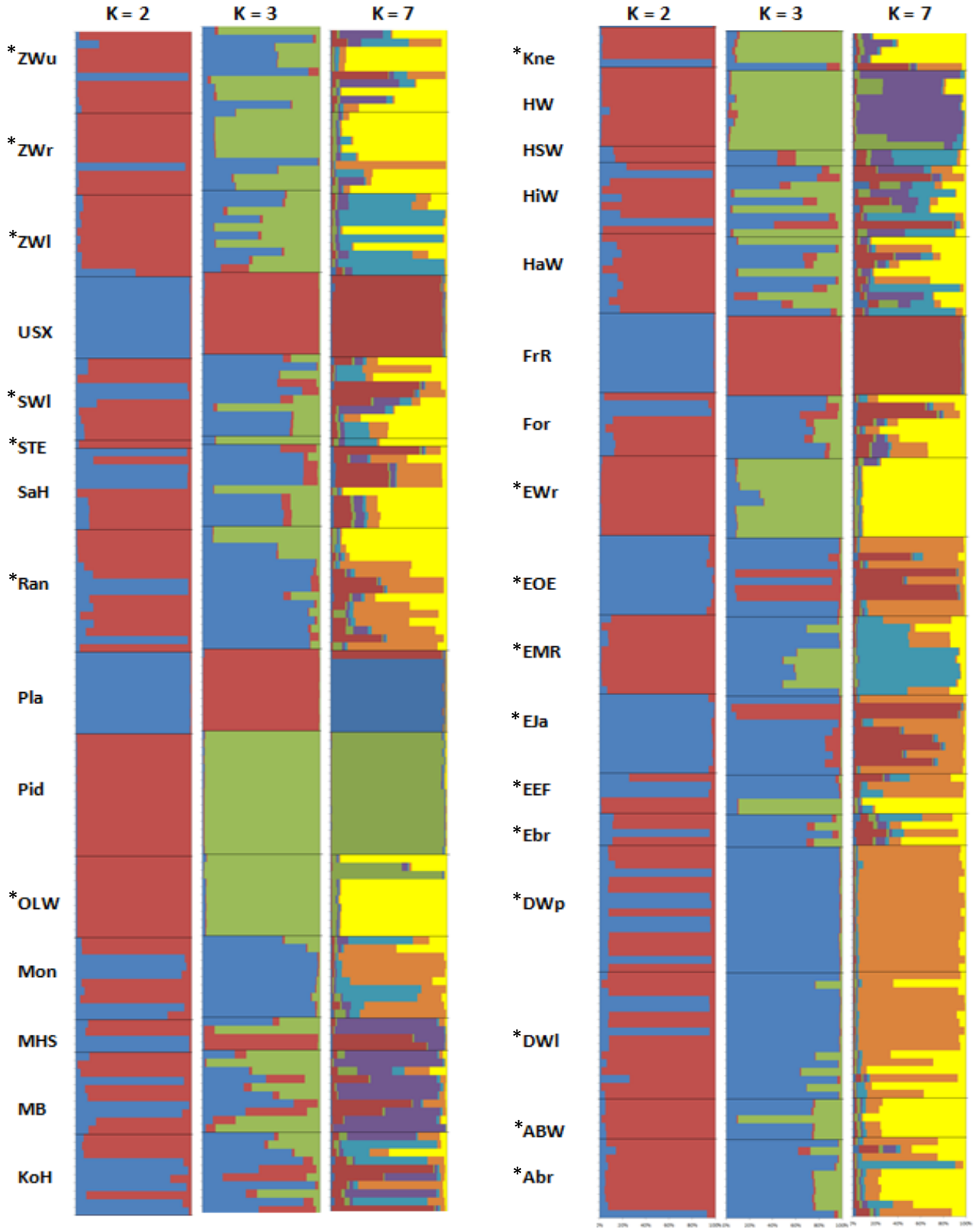


Fig. 16: Results of the genetic structure of the complete dataset (295 individuals, 32 populations) as indicated by STRUCTURE via a Bayesian clustering analysis using the admixture model with uncorrelated allele frequencies. The results are depicted for K=2, 3 and 7 for the run with the highest likelihood. For each K, the run with the highest likelihood is shown. The populations are separated in blocks according to their geografic distribution. Marked with a asterisk * refers to Salzburg populations. Results of the correlated allele frequencies in Appendix Figure 7.

The results of the STRUCTURE clustering of K=2, K=3 and K=7 can be seen in Figure 16. The matching up of the independent runs for each K as conducted with the program CLUMPP revealed nearly the same clustering as the run with the highest likelihood. So, for each K, the run with the highest likelihood was chosen to be depicted, also for the maps (background: OpenStreetMap OSWM) on the following pages. Regarding the clustering for K=2, six *Gladiolus* populations of Salzburg (ABW, EMr, Ewr, OLW, STE, ZWl) were entirely belonging to the same group (red), mostly to this cluster belonging were the Salzburg populations Abr, Ebr, SWl, ZWr and ZWu. The non-Salzburg populations HaW, HSW, Pid and HW were also entirely belonging to the same cluster, whereby the first three of them were geographically close to Salzburg. The Salzburg populations DWl, DWp, EEF, Kne and Ran were admixed. Non-Salzburg populations that were admixed are For, HiW, KöH, MB, MHS, Mon and SaH. Two populations of Salzburg, namely EJa and EOE, were entirely belonging to the second cluster (blue), as well as the non-Salzburg populations FrR, USX and Pla.

When considering the solution for K=3, the individuals were clustered as follows (Figure 17). First, only the non-Salzburg populations were considered. USX, Pla and FrR were again in a separate cluster, they were nearly totally belonging to the red cluster, this time without any populations of Salzburg. The population Mon was nearly totally belonging to the blue cluster, while Pid and HW were largely belonging to the green cluster. The non-Salzburg-populations MHS, MB, KoH, HSW, HiW and HaW were admixed, also SaH and For, whereby the latter were belonging to the blue cluster in a large part. Considering the populations of Salzburg, DWl and DWp, which were admixed in the K=2 model, were nearly entirely belonging to the blue cluster. Ran, Abr, ABW, SWI, EEF, EMr, EOE, EBr and EJa were partly (more than half) belonging to this cluster. Populations STE, OLW, Kne, EWr, ZWl, ZWr and ZWu were more than halfway through belonging to the green cluster. The red genetic cluster occurred to a larger extent (around 25%) in the populations EOE and EJa.

At K=7 (see Figure 18), on closer consideration of the non-Salzburg populations, the two populations of Vorarlberg USX and FrR were again belonging to the same cluster (red). The population of Mondsee “Mon” was mostly belonging to the orange cluster. The populations MHS, MB and HW were mostly belonging to the violet cluster. The population “Pla” was forming a separate group (dark blue) as well as “Pid” (green). The other non-Salzburg populations KöH, SaH, For, HiW, HaW and HSW (the latter is half belonging to the light blue group) were admixed. Considering populations of Salzburg, DWl and DWp were again nearly utterly in the same cluster (orange), as well as EOE and EJa (half orange, half red). Also partly (more than 25%) to the orange cluster belonging were Ran (admixed with yellow and red) and EEF (admixed with yellow). Most individuals of the population “EMr” and “ZWl” were belonging to a light blue group. Mostly to the yellow cluster belonging were the populations Abr, ABW, EWr, Kne, OLW, ZWr and ZWu. The remaining populations were admixed.

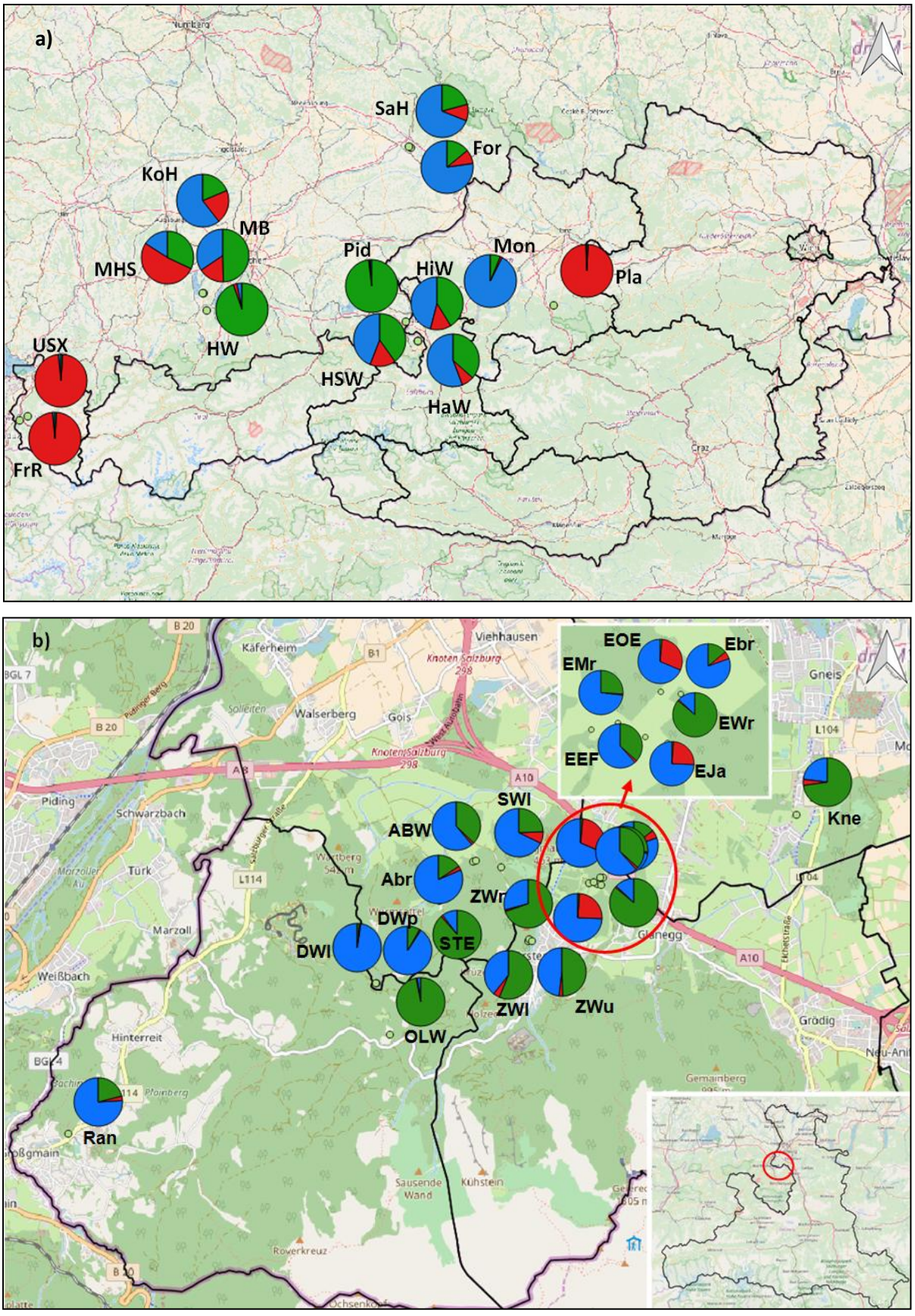


Fig. 17: Geographical distribution of the Bayesian clustering of K=3 of the *Gladiolus* populations. a) Non-Salzburg populations. b) Salzburg-populations.

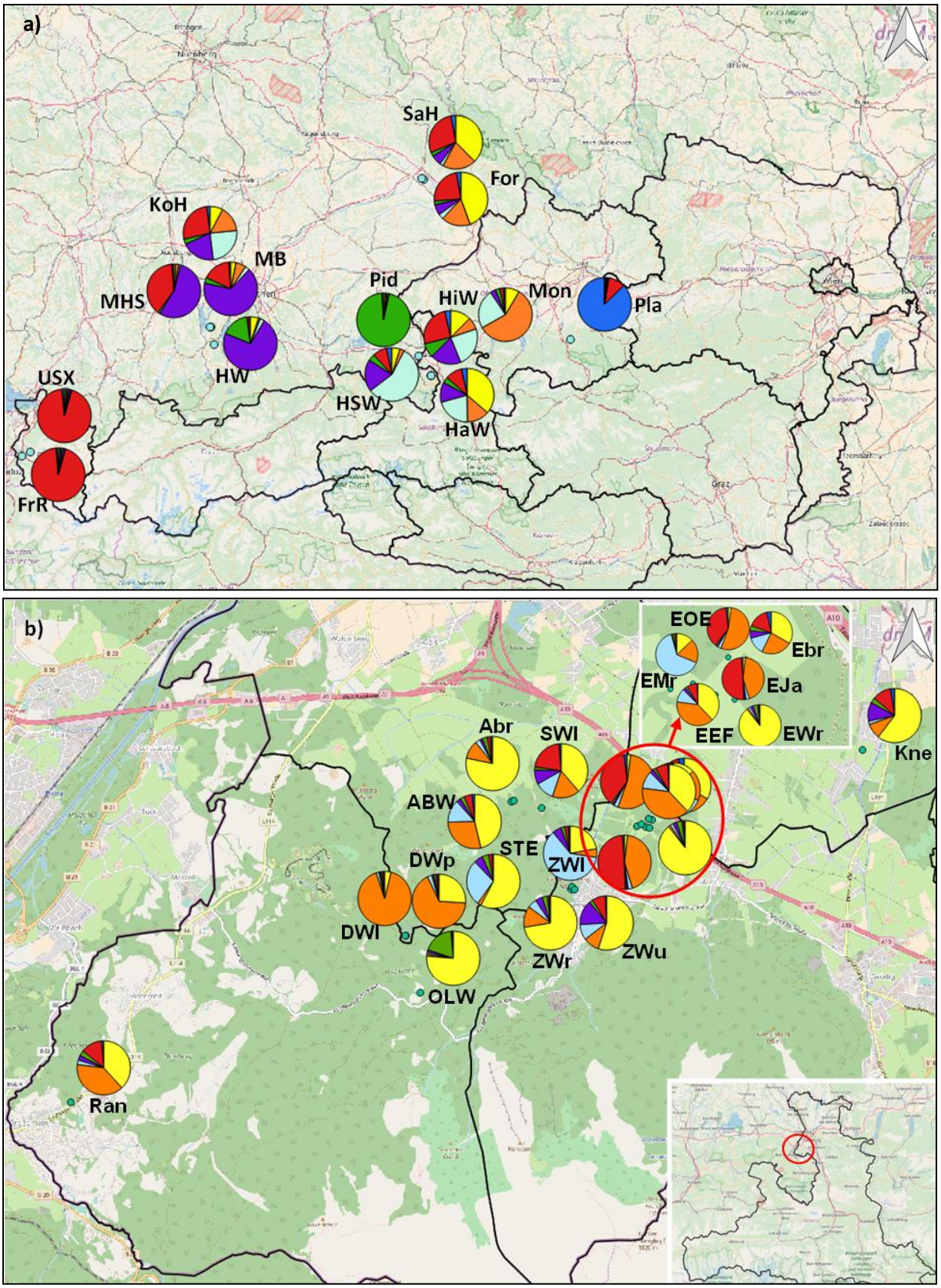


Fig. 18: Geographical distribution of the Bayesian clustering of K=7 of the *Gladiolus* populations. a) Non-Salzburg populations. b) Salzburg-populations.

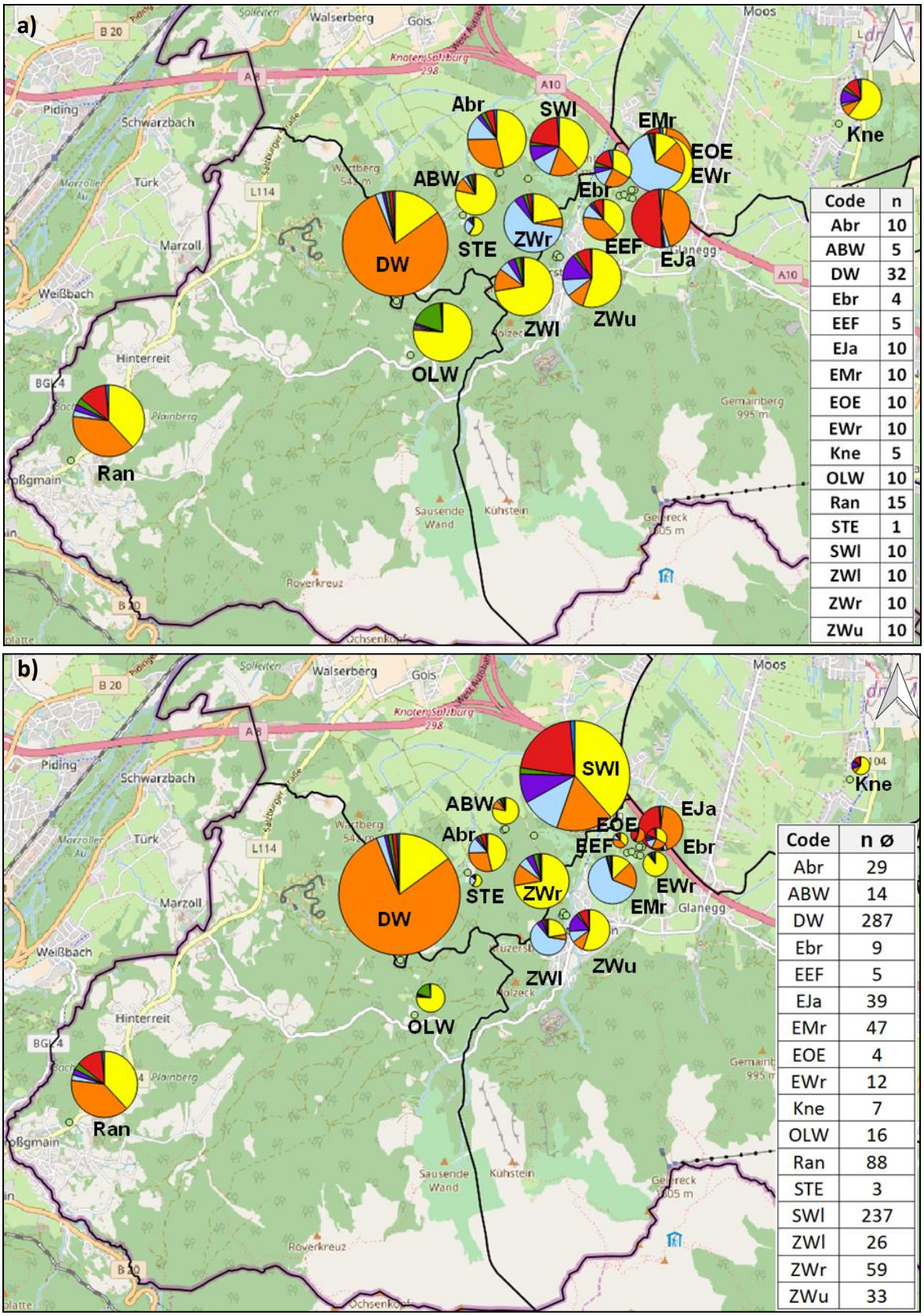


Fig. 19: Geographical distribution of the Bayesian clustering of K=7 of the *Gladiolus* populations in Salzburg. Circle size corresponds to population size. a) Circle size according to gentyped individuals. b) Circle size according to average population size (G. Nowotny, unpublished).

Figure 19 again shows the graphical distribution of the genetic clusters of K=7 (of the run with the highest likelihood), but this time according to the population sizes. For this graph, the populations DWl and DWp were assembled to populaiton “DW”, because there are no separate coutings for the color morphs available. In Figure 19, a) the circle size corresponds to the sample size. Most individuals (32) were sampled in the population “DW”, fewest (only one) in population “STE”. Figure 19, b) shows the same distribution but according to an average population size, determined by Günther Nowotny. The average sizes were calculated as mean values between a minimum and maximum count of Günther Nowowty in the recent years. Here, it can be seen that the populations “DW” and “SWl” are the biggest populations of Salzburg. “DW” however, despite its large population size, was rather uniform (mostly belonging to the orange cluster) while SWl was admixed. The Salzburg populations, which were occuring on the same meadows but were slightly separated geographicall were combined to “overview”-populations, as can be seen in Figure 20. The pattern stays more or less the same. The big population “ES” (combined out of EEF, EMr, EOE, Ebr, EWr, EJa) is most admixed.

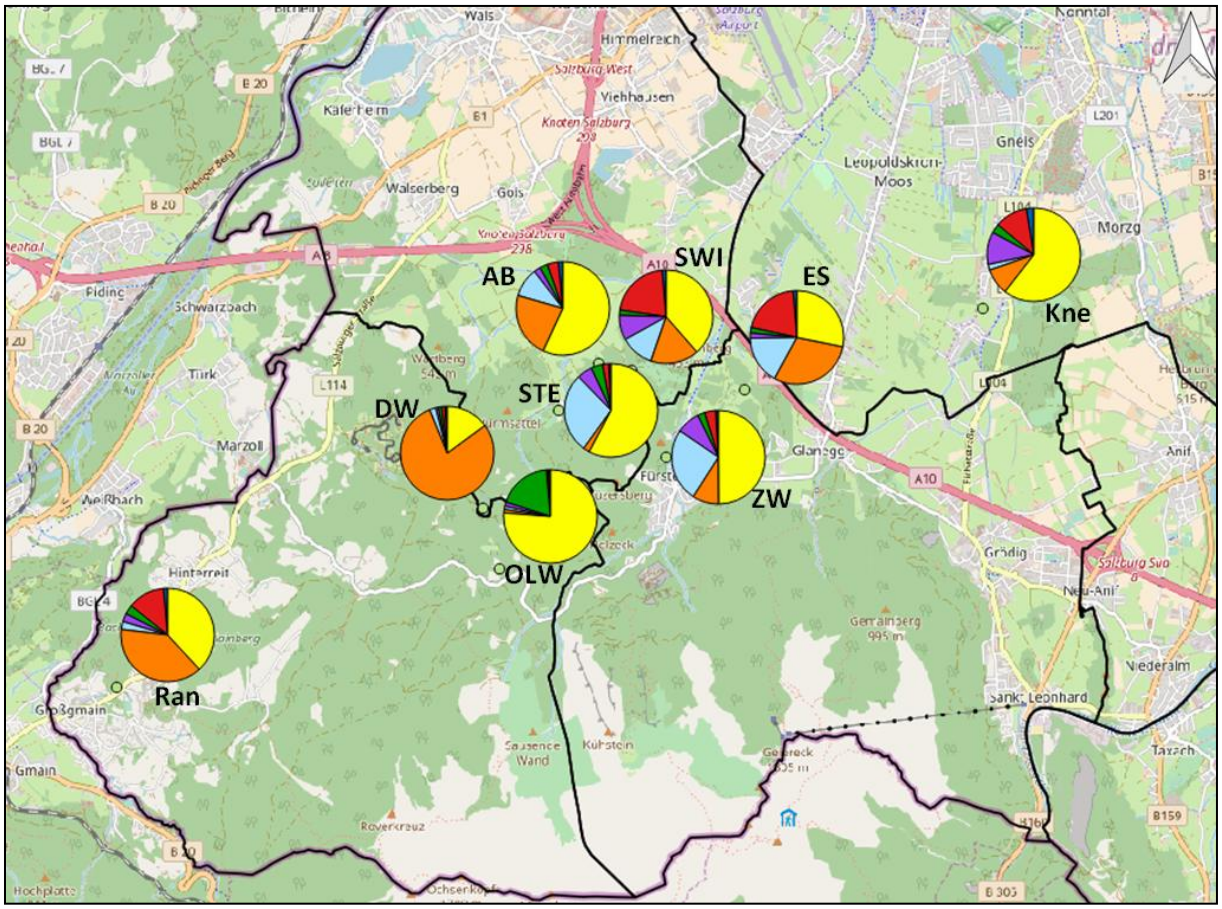


Fig. 20: Geographical distribution of the Bayesian clustering of K=7 of the combined *Gladiolus* populations. DW = DWl + DWp; ES = EEF + EMr +EOE + Ebr + EWr + EJa; ZW = ZWu + ZWl + ZWr; AB =AWB + Abr.

3.1.3.4 Results of the genetic diversity analysis

The Nei's genetic diversity and frequency down-weighted values (rarity) were calculated for all populations except population "STE", using the R functions of the AFLPdat-package. The results are summarized in Table 8.

Tab. 8: Genetic diversity and rarity calculated with AFLPdat. Abbreviations: N = Number of genotypes individuals, Nm=total number of markers in the population, PPF=percentage of polymorphic fragments, GD=gene diversity (Colour scale: the higher the gene diversity the darker/more intense the colour), CI low and CI upp = lower and upper boundary of the 95% confidence interval, R=Rarity (Colour scale: the higher the rarity the darker/more intense the colour). Populations are sorted alphabetically; * = Salzburg populations.

Pop	N	Nm	PPF	GD	CI low	CI upp	R
Abr*	10	120	0.048	0.01298	0.0032	0.0256	0.08189
ABW*	5	119	0.008	0.00320	0.0000	0.0096	0.08413
DW*	32	121	0.056	0.01073	0.0029	0.0208	0.12375
Ebr*	4	120	0.040	0.02000	0.0040	0.0400	0.08557
EEF*	5	120	0.048	0.02400	0.0064	0.0448	0.07412
EJa*	10	118	0.032	0.012620	0.0000	0.0261	0.07600
EMr*	10	117	0.024	0.012444	0.0000	0.0284	0.06650
EOE*	10	118	0.056	0.019556	0.0044	0.0363	0.07378
EWr*	10	119	0.032	0.007644	0.0016	0.0165	0.07939
For	8	121	0.032	0.013714	0.0034	0.0274	0.09286
FrR	10	117	0.000	0.00000	0.0000	0.0000	0.07771
HaW	10	120	0.048	0.01920	0.0048	0.0373	0.08457
HiW	9	121	0.056	0.02133	0.0076	0.0387	0.08577
HSW	2	118	0.000	0.00000	0.0000	0.0000	0.08249
HW	10	120	0.048	0.01778	0.0043	0.0338	0.09588
Kne*	5	122	0.048	0.01920	0.0064	0.0352	0.18951
KoH	10	121	0.064	0.02400	0.0076	0.0427	0.08357
MB	10	122	0.080	0.02951	0.0135	0.0492	0.10332
MHS	4	120	0.048	0.02533	0.0080	0.0480	0.08834
Mon	10	121	0.056	0.02507	0.0081	0.0468	0.11236
OLW*	10	119	0.016	0.00444	0.0000	0.0121	0.17885
Pid	15	116	0.008	0.00411	0.0000	0.0165	0.06964
Pla	10	118	0.040	0.01067	0.0016	0.0235	0.07059
Ran*	15	120	0.048	0.01676	0.0041	0.0317	0.08194
SaH	10	121	0.032	0.01173	0.0016	0.0261	0.09622
SWl*	10	120	0.040	0.01760	0.0037	0.0345	0.08118
USX	10	118	0.008	0.00160	0.0000	0.0048	0.07829
ZWl*	10	121	0.072	0.01973	0.0076	0.0354	0.15400
ZWr*	10	121	0.048	0.01564	0.0042	0.0305	0.08505
ZWu*	10	121	0.056	0.02311	0.0076	0.0421	0.08605
Mean	-	-	-	0.01479	-	-	0.09412

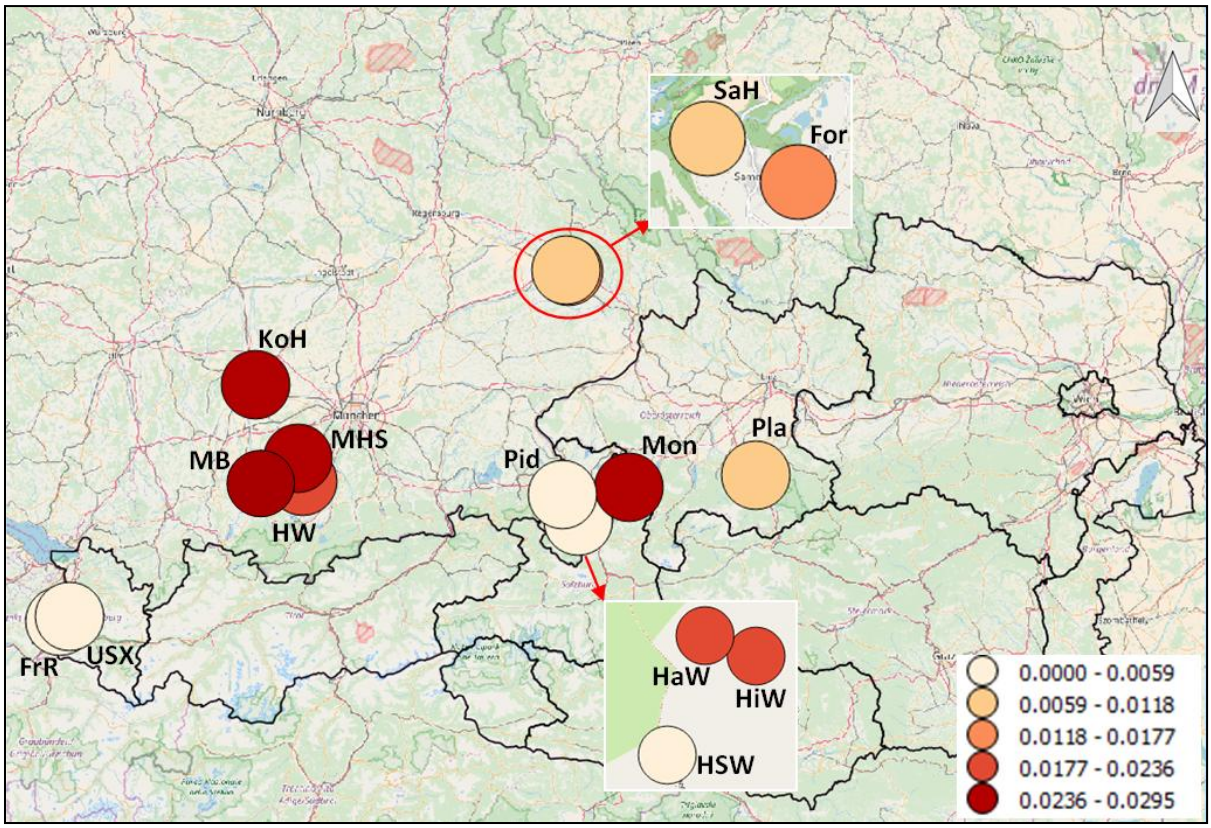


Fig. 21: Genetic diversity within populations expressed with Nei's gene diversity of the non-Salzburg populations of *Gladiolus palustris*.

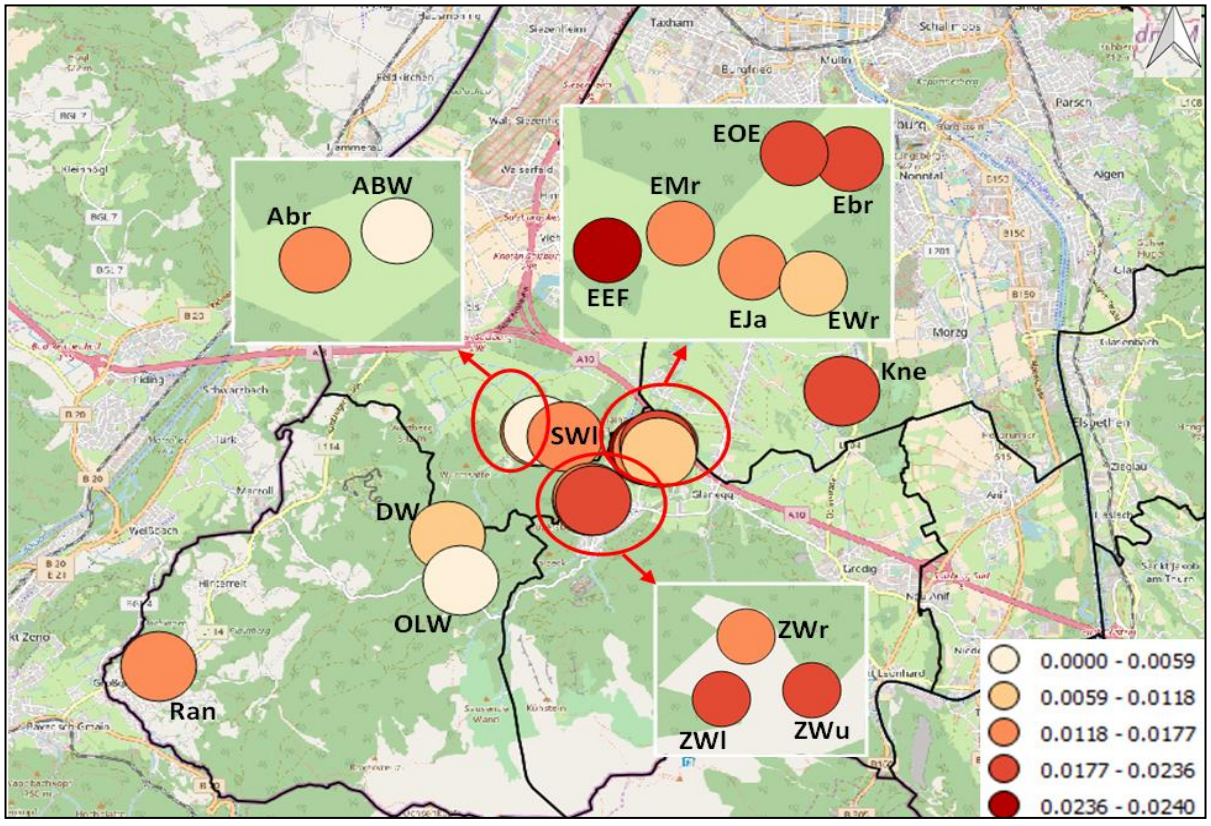


Fig. 22: Genetic diversity within populations expressed with Nei's gene diversity of the Salzburg populations of *Gladiolus palustris.*

The genetic diversity of the non-Salzburg populations of *Gladiolus palustris* ranged from 0 to 0.0295, as can be seen in Figure 21. The highest genetic diversity could be found in the populations KoH (Swabia), MB (Upper Bavaria), MHS (Upper Bavaria) and Mon (Upper Austria). Intermediate levels of diversity occurred in the populations For (Lower Bavaria), HW (Upper Bavaria), HiW (Upper Bavaria), HaW (Upper Bavaria), SaH (Lower Bavaria) and Pla (Upper Austria). The lowest genetic diversity occurred in the two populations of Vorarlberg, FrR and USX, in Pid (Upper Bavaria) and HSW (Upper Bavaria).

The genetic diversity within the *Gladiolus palustris* populations in Salzburg (see Figure 22) ranged from 0.0032 to 0.024 and was therefore lowest in population ABW and highest in EEF. The populations Kne, EOE, Ebr, ZWl, ZWu, ZWr, Ran, Abr, SWl, EMr and EJa showed an intermediate genetic diversity. Low genetic diversity occurred within populations ABW, OLW, DW (DWl and DWp together) and EWr. Considering the population sizes of the Salzburg populations (see Table 1), the populations SWl and DW were the biggest ones, but had intermediate and low genetic diversity, respectively. The population DW had an average population size of 287 individuals (53 at minimum, 536 at maximum) and SWl's average population size was 237 (70 at minimum, 335 at maximum). The smallest populations with average population sizes below 10 individuals were EOE, EEF, Kne and Ebr.

The genetic rarity was higher in the Salzburg populations. Within the non-Salzburg populations, the genetic rarity (DW) varied from 0.0696 to 0.1124 (see Figure 23). It was lowest in the populations Pid, Pla, FrR and USX and highest in the populations Mon, MB, SaH and HW. Within the Salzburg populations, the genetic rarity (DW) varied from 0.0665 to 0.1895 (see Figure 24). The lowest genetic rarity was found in five of the six populations at the "Esterer" tavern, namely in EMr, EOE, EEF, EJa and EWr. The genetic rarity was highest in the populations Kne, OLW, ZWl and DW.

The correlation analysis between genetic diversity and the average population sizes as well as the sample sizes showed a negative correlation without statistical significance (see Appendix Figure 8 and Appendix Table 8). Also with the rarity, there was no significant correlation.

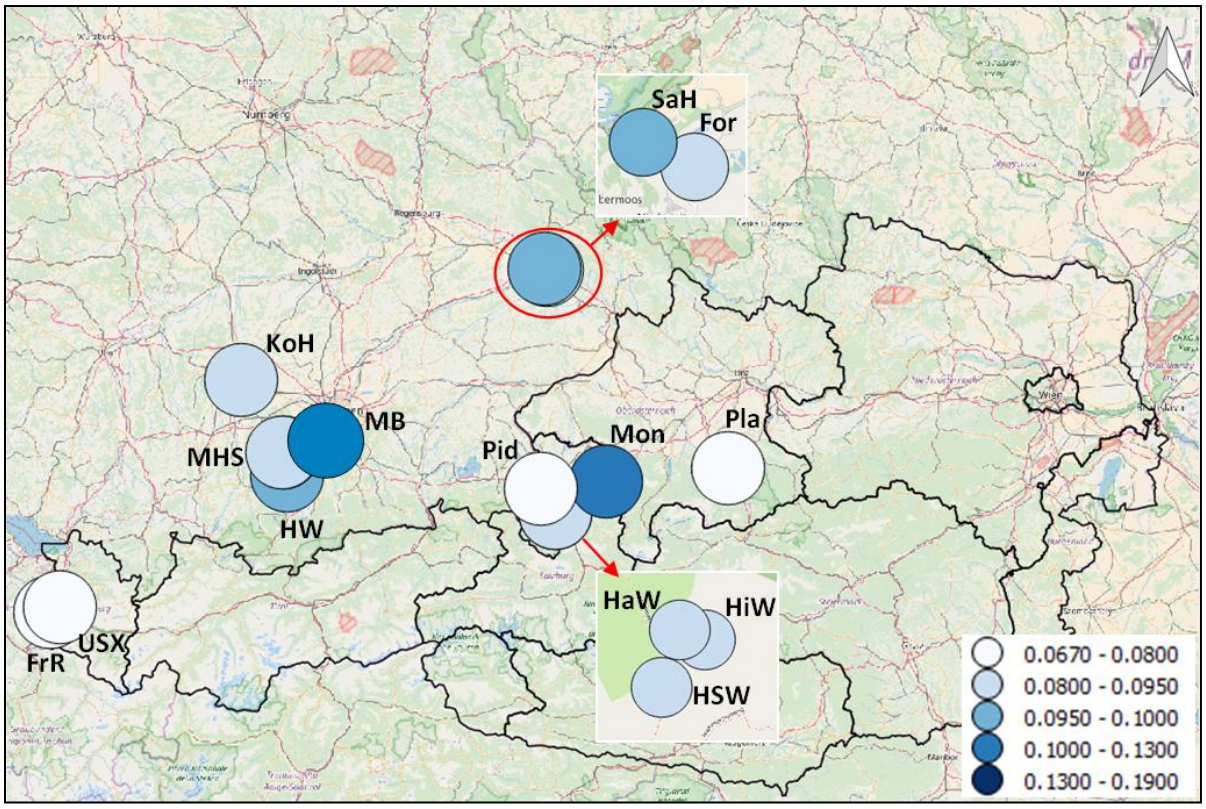


Fig. 23: Rarity index (DW) as a measure of the amount of rare markers in each population of the non-Salzburg populations.

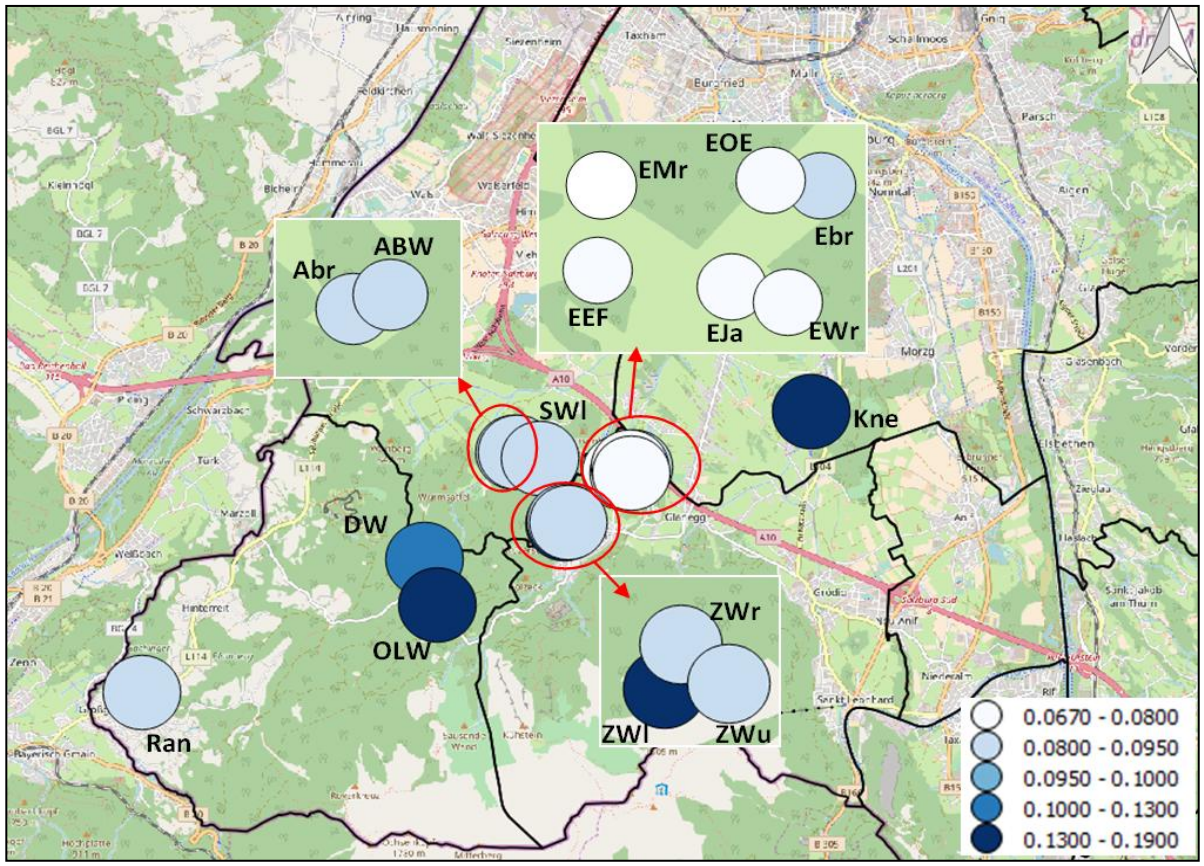


Fig. 24: Rarity index (DW) as a measure of the amount of rare markers in each population of the Salzburg populations.

3.1.3.5 Results of the AMOVA

The non-hierarchical AMOVA for the complete data set was highly significant (see Table 9). In Table 10 the genetic distances between the populations are shown. The genetic distance of the populations of Vorarlberg "FrR" and "USX" were big to the other populations, which was also confirmed by the p-values. Also, the populations of "Piding" (Pid), "Planwiesen" (Pla) and "Obere Langwiese" (OLW) had a high genetic distance to most of the other populations. The Salzburg "STE" (population with only one individual) had no significant pairwise F_{ST} values. Table 11 shows the population average pairwise differences.

Tab. 9: Non-hierarchical AMOVAs based on AFLPs of *Gladiolus palustris*. Abbreviations: D.f. = degrees of freedom; s = significant (P-value < 0.05)

Group	**Source of variation**	**D.f.**	**Sum of squares**	**Percentage of Variance**	**P-value**	**F_{ST} value**
All Samples	Among populations	30	378.038	58.12	0.00000+-0.00000 s	0.58121
	Within populations	264	236.315	41.88		
Only Salzburg	Among populations	16	146.028	49.33	0.00000+-0.00000 s	0.49331
	Within populations	150	132.798	50.67		

Tab. 10: Matrix of population pairwise F_{ST}s of all sampled *Gladiolus palustris* populations. Shaded in grey means no significant F_{ST} value (Significance Level=0.0500). The significant F_{ST} values are represented in a color scale whereby low values are shaded in yellow and higher values in orange and highest values in red.

	Abr	ABW	DW	Ebr	EEF	EJa	EMr	EOE	EWr	For	FrR	HaW	HiW	HSW	HW	Kne	KoH	MB	MHS	Mon	OLW	Pid	Pla	Ran	SaH	STE	SWl	USX	ZWl	ZWr	ZWu
Abr	0,000																														
ABW	0,018	0,000																													
DW	0,470	0,584	0,000																												
Ebr	0,080	0,301	0,547	0,000																											
EEF	0,185	0,356	0,467	0,122	0,000																										
EJa	0,509	0,685	0,570	0,353	0,286	0,000																									
EMr	0,559	0,694	0,634	0,567	0,242	0,636	0,000																								
EOE	0,416	0,555	0,501	0,286	0,231	-0,016	0,571	0,000																							
EWr	0,458	0,430	0,674	0,527	0,453	0,739	0,671	0,656	0,000																						
For	0,243	0,323	0,570	-0,028	0,354	0,524	0,671	0,446	0,525	0,000																					
FrR	0,802	0,970	0,826	0,779	0,727	0,708	0,866	0,641	0,910	0,780	0,000																				
HaW	0,292	0,396	0,589	-0,062	0,230	0,432	0,522	0,418	0,492	0,203	0,684	0,000																			
HiW	0,392	0,479	0,638	0,056	0,243	0,473	0,525	0,466	0,552	0,291	0,616	0,059	0,000																		
HSW	0,570	0,901	0,765	0,245	0,394	0,676	0,657	0,596	0,808	0,543	1,000	0,179	-0,114	0,000																	
HW	0,626	0,658	0,747	0,502	0,493	0,694	0,657	0,644	0,614	0,585	0,786	0,447	0,384	0,524	0,000																
Kne	0,445	0,478	0,659	0,171	0,357	0,583	0,671	0,511	0,381	0,225	0,818	0,167	0,254	0,530	0,439	0,000															
KoH	0,273	0,364	0,568	0,083	0,133	0,391	0,505	0,370	0,540	0,264	0,483	0,250	0,098	0,112	0,430	0,369	0,000														
MB	0,458	0,479	0,650	0,263	0,307	0,499	0,580	0,464	0,563	0,372	0,539	0,317	0,245	0,288	0,146	0,333	0,180	0,000													
MHS	0,613	0,697	0,755	0,404	0,414	0,587	0,698	0,503	0,734	0,541	0,643	0,457	0,370	0,478	0,296	0,487	0,262	-0,020	0,000												
Mon	0,313	0,409	0,365	0,293	0,234	0,411	0,490	0,339	0,574	0,425	0,715	0,428	0,427	0,484	0,564	0,499	0,312	0,382	0,509	0,000											
OLW	0,658	0,761	0,756	0,716	0,652	0,816	0,780	0,742	0,580	0,716	0,956	0,651	0,678	0,904	0,701	0,654	0,649	0,645	0,805	0,665	0,000										
Pid	0,774	0,857	0,820	0,803	0,782	0,867	0,819	0,808	0,775	0,815	0,959	0,741	0,736	0,898	0,675	0,788	0,718	0,692	0,829	0,737	0,839	0,000									
Pla	0,779	0,845	0,838	0,702	0,751	0,757	0,841	0,702	0,851	0,745	0,841	0,699	0,679	0,787	0,791	0,750	0,660	0,687	0,730	0,724	0,889	0,882	0,000								
Ran	0,216	0,333	0,421	0,094	0,227	0,334	0,536	0,261	0,465	0,267	0,730	0,181	0,387	0,551	0,563	0,316	0,398	0,446	0,561	0,371	0,623	0,728	0,743	0,000							
SaH	0,367	0,460	0,584	0,055	0,434	0,530	0,724	0,471	0,585	-0,024	0,771	0,239	0,295	0,596	0,594	0,199	0,293	0,380	0,561	0,465	0,741	0,827	0,750	0,314	0,000						
STE	0,537	0,857	0,734	0,412	0,063	0,713	0,568	0,579	0,618	0,619	1,000	0,429	0,314	1,000	0,471	0,429	0,268	0,291	0,449	0,430	0,832	0,888	0,855	0,545	0,681	0,000					
SWl	0,131	0,275	0,529	-0,011	-0,024	0,370	0,457	0,351	0,456	0,228	0,645	0,109	0,149	0,316	0,483	0,309	0,085	0,282	0,430	0,337	0,637	0,763	0,739	0,225	0,295	0,290	0,000				
USX	0,774	0,936	0,815	0,724	0,694	0,671	0,851	0,606	0,889	0,741	0,000	0,656	0,591	0,943	0,770	0,781	0,463	0,523	0,599	0,696	0,939	0,948	0,814	0,706	0,731	0,961	0,618	0,000			
ZWl	0,388	0,446	0,674	0,373	0,312	0,630	0,517	0,582	0,572	0,518	0,748	0,442	0,378	0,374	0,522	0,543	0,292	0,374	0,527	0,282	0,678	0,740	0,763	0,542	0,578	0,295	0,326	0,732	0,000		
ZWr	0,317	0,281	0,494	0,348	0,214	0,604	0,528	0,534	0,134	0,406	0,804	0,371	0,395	0,613	0,491	0,278	0,368	0,431	0,589	0,406	0,498	0,706	0,793	0,360	0,437	0,248	0,274	0,783	0,447	0,000	
ZWu	0,217	0,259	0,499	0,104	0,007	0,396	0,376	0,365	0,261	0,293	0,672	0,113	0,213	0,369	0,383	0,172	0,264	0,303	0,437	0,313	0,508	0,685	0,726	0,155	0,336	0,097	0,051	0,649	0,327	0,109	0,000

Tab. 11: Matrix of population average pairwise differences of all sampled *Gladiolus palustris* populations.

	Abr	ABW	DW	Ebr	EEF	EJa	EMr	EOE	EWr	For	FrR	HaW	HiW	HSW	HW	Kne	KoH	MB	MHS	Mon	OLW	Pid	Pla	Ran	SaH	STE	SWl	USX	ZWl	ZWr	ZWu
Abr	0,000																														
ABW	1,100	0,000																													
DW	2,719	2,681	0,000																												
Ebr	2,150	1,950	3,531	0,000																											
EEF	2,700	2,640	3,369	3,150	0,000																										
EJa	3,260	3,700	3,300	2,950	3,020	0,000																									
EMr	3,600	3,760	3,844	4,300	2,840	4,300	0,000																								
EOE	3,480	3,800	3,450	3,450	3,480	1,980	4,660	0,000																							
EWr	2,380	1,300	3,750	3,100	3,180	4,860	3,820	4,940	0,000																						
For	2,200	1,700	3,375	2,000	3,500	3,450	4,950	3,800	2,750	0,000																					
FrR	4,100	4,200	5,656	3,250	3,800	2,700	5,800	3,400	5,300	3,500	0,000																				
HaW	2,840	2,680	4,094	2,300	3,440	3,500	4,140	4,160	3,300	2,600	3,800	0,000																			
HiW	3,500	3,400	4,788	2,750	3,711	3,989	4,400	4,778	3,967	3,111	3,333	2,689	0,000																		
HSW	3,100	3,200	5,219	2,250	3,600	4,100	3,800	5,000	4,300	3,000	3,000	2,200	1,667	0,000																	
HW	5,140	4,600	6,300	4,650	4,960	6,200	5,500	6,560	4,120	4,800	5,200	4,180	3,956	3,800	0,000																
Kne	3,460	2,680	4,606	2,950	4,200	4,500	5,640	4,960	2,460	2,600	4,400	2,880	3,422	3,800	4,080	0,000															
KoH	3,180	3,100	4,369	3,050	3,460	3,760	4,600	4,320	4,300	3,250	2,900	3,600	3,144	2,500	4,580	4,380	0,000														
MB	4,900	4,680	5,906	4,400	4,920	5,260	6,240	5,720	5,320	4,400	4,000	4,460	4,222	4,000	3,460	4,760	4,080	0,000													
MHS	5,450	5,350	6,688	4,750	5,250	5,050	6,750	5,400	6,050	4,875	2,750	4,900	4,528	4,250	3,650	5,350	4,150	3,400	0,000												
Mon	3,460	3,500	3,175	4,100	4,020	4,000	4,600	4,220	4,800	4,300	5,500	4,840	5,078	4,900	6,140	5,700	4,460	5,520	6,400	0,000											
OLW	3,180	2,100	4,581	4,000	4,100	5,800	4,800	5,820	1,800	3,800	6,300	4,240	4,856	5,100	4,640	3,500	5,060	5,980	6,650	5,500	0,000										
Pid	4,300	3,400	5,881	4,850	5,400	7,100	5,200	6,800	3,100	5,100	7,600	5,000	5,156	4,600	3,800	4,800	5,500	6,000	6,350	6,100	3,300	0,000									
Pla	6,680	6,600	8,281	5,650	7,640	6,000	9,100	6,340	7,700	5,900	4,200	6,200	6,133	5,400	8,500	6,800	6,380	8,020	6,950	8,080	8,520	7,120	0,000								
Ran	2,393	2,227	2,848	2,483	3,160	2,793	4,013	3,053	2,980	2,633	4,533	2,733	3,822	3,933	4,920	3,227	4,153	5,053	5,450	4,073	3,820	4,800	6,933	0,000							
SaH	2,440	1,960	3,325	2,000	3,640	3,240	5,480	3,700	2,920	1,550	3,200	2,540	2,911	3,000	4,540	2,320	3,160	4,160	4,600	4,300	3,900	5,200	5,600	2,640	0,000						
STE	3,500	2,800	5,031	4,250	3,200	5,500	3,600	5,800	2,500	4,500	5,000	4,200	3,889	4,000	4,200	4,200	4,100	5,200	5,750	5,500	3,300	4,600	9,200	4,600	4,600	0,000					
SWl	2,200	2,020	3,463	2,300	2,500	3,000	3,460	3,580	2,900	2,550	3,100	2,580	2,856	2,500	4,280	3,300	2,840	4,100	4,450	4,020	3,800	5,100	6,760	2,767	2,600	3,100	0,000				
USX	4,040	4,100	5,581	3,200	3,860	2,700	5,900	3,360	5,200	3,400	0,100	3,780	3,389	3,100	5,260	4,300	2,980	4,080	2,850	5,480	6,200	7,500	4,120	4,433	3,100	5,100	3,140	0,000			
ZWl	3,340	3,020	5,156	3,950	3,900	5,460	4,160	5,880	4,000	4,400	4,900	4,360	4,122	3,100	4,900	5,340	3,860	4,920	5,700	3,900	4,700	5,100	8,020	4,927	4,660	3,500	3,460	4,980	0,000		
ZWr	2,620	1,840	3,069	3,300	3,040	4,460	3,720	4,720	1,680	3,100	5,000	3,460	3,800	4,200	4,100	2,960	3,920	4,960	5,700	4,280	2,500	3,800	7,940	3,173	3,040	2,600	2,860	4,960	4,000	0,000	
ZWu	2,880	2,520	3,744	3,050	2,960	3,700	3,560	4,200	2,600	3,300	4,400	2,980	3,533	3,600	4,140	3,240	4,000	4,720	5,300	4,380	3,500	4,800	7,700	2,920	3,280	3,200	2,680	4,400	3,980	2,720	0,000

Tab. 12: Matrix of population pairwise F_{ST}s of *Gladiolus palustris* populations of Salzburg. Shaded in grey means no significant F_{ST} value (Significance Level=0.0500). The significant F_{ST} values are represented in a color scale whereby low values are shaded in yellow and higher values in orange and highest values in red.

	Abr	ABW	DW	Ebr	EEF	EJa	EMr	EOE	EWr	Kne	OLW	Ran	STE	SWl	ZWl	ZWr	ZWu
Abr	0,000																
ABW	0,018	0,000															
DW	0,470	0,584	0,000														
Ebr	0,080	0,301	0,547	0,000													
EEF	0,185	0,356	0,467	0,122	0,000												
EJa	0,509	0,685	0,570	0,353	0,286	0,000											
EMr	0,559	0,694	0,634	0,567	0,242	0,636	0,000										
EOE	0,416	0,555	0,501	0,286	0,231	-0,016	0,571	0,000									
EWr	0,458	0,430	0,674	0,527	0,453	0,739	0,671	0,656	0,000								
Kne	0,445	0,478	0,659	0,171	0,357	0,583	0,671	0,511	0,381	0,000							
OLW	0,658	0,761	0,756	0,716	0,652	0,816	0,780	0,742	0,580	0,654	0,000						
Ran	0,216	0,333	0,421	0,094	0,227	0,334	0,536	0,261	0,465	0,316	0,623	0,000					
STE	0,537	0,857	0,734	0,412	0,063	0,713	0,568	0,579	0,618	0,429	0,832	0,545	0,000				
SWl	0,131	0,275	0,529	-0,011	-0,024	0,370	0,457	0,351	0,456	0,309	0,637	0,225	0,290	0,000			
ZWl	0,388	0,446	0,674	0,373	0,312	0,630	0,517	0,582	0,572	0,543	0,678	0,542	0,295	0,326	0,000		
ZWr	0,317	0,281	0,494	0,348	0,214	0,604	0,528	0,534	0,134	0,278	0,498	0,360	0,248	0,274	0,447	0,000	
ZWu	0,217	0,259	0,499	0,104	0,007	0,396	0,376	0,365	0,261	0,172	0,508	0,155	0,097	0,051	0,327	0,109	0,000

Considering only the populations of Salzburg (see Table 12), population “STE”, which is composed of only one individual, again had no significant pairwise F_{ST} values. Population “OLW” showed rather big genetic distances to the other populations of Salzburg, what was confirmed by the p-value. The most different from each other are the populations “OLW” and “EJa”. According to this result, the closest related populations are “SWl” and “Abr”, as they have the lowest pairwise F_{ST} value. They are followed by “ZWr” and “EWr”. Table 13 shows the population average pairwise differences.

Tab. 13: Matrix of population average pairwise differences of *Gladiolus palustris* populations of Salzburg.

	Abr	ABW	DW	Ebr	EEF	EJa	EMr	EOE	EWr	Kne	OLW	Ran	SWl	ZWl	ZWr	ZWu
Abr	0,000															
ABW	1,100	0,000														
DW	2,719	2,681	0,000													
Ebr	2,150	1,950	3,531	0,000												
EEF	2,700	2,640	3,369	3,150	0,000											
EJa	3,260	3,700	3,300	2,950	3,020	0,000										
EMr	3,600	3,760	3,844	4,300	2,840	4,300	0,000									
EOE	3,480	3,800	3,450	3,450	3,480	1,980	4,660	0,000								
EWr	2,380	1,300	3,750	3,100	3,180	4,860	3,820	4,940	0,000							
Kne	3,460	2,680	4,606	2,950	4,200	4,500	5,640	4,960	2,460	0,000						
OLW	3,180	2,100	4,581	4,000	4,100	5,800	4,800	5,820	1,800	3,500	0,000					
Ran	2,393	2,227	2,848	2,483	3,160	2,793	4,013	3,053	2,980	3,227	3,820	0,000				
SWl	2,200	2,020	3,463	2,300	2,500	3,000	3,460	3,580	2,900	3,300	3,800	2,767	0,000			
ZWl	3,340	3,020	5,156	3,950	3,900	5,460	4,160	5,880	4,000	5,340	4,700	4,927	3,460	0,000		
ZWr	2,620	1,840	3,069	3,300	3,040	4,460	3,720	4,720	1,680	2,960	2,500	3,173	2,860	4,000	0,000	
ZWu	2,880	2,520	3,744	3,050	2,960	3,700	3,560	4,200	2,600	3,240	3,500	2,920	2,680	3,980	2,720	0,000

Tab. 14: Groups composition used for the hierarchical AMOVAs.

Groups	**Populations**
Salzburg	Abr, ABW, DW (DWl+DWp), EBr, EEF, EJa, EMr, EOE, EWr, Kne, OLW, Ran, STE, SWl, ZWl, ZWr, ZWu
Non-Sbg	For, FrR, HaW, HiW, HSW, HW, KoH, MB, MHS, Mon, Pid, Pla, SaH, USX
Salzburg	Abr, ABW, DW (DWl+DWp), EBr, EEF, EJa, EMr, EOE, EWr, Kne, OLW, Ran, STE, SWl, ZWl, ZWr, ZWu
Non-Sbg_separated	
Vorarlberg	FrR, USX
Lower Bavaria	For, SaH
West Germany	HW, KoH, MB, MHS
East Germany	HaW, HiW, HSW, Pid
Upper Austria	Mon, Pla
East	Abr, ABW, DW (DWl+DWp), EBr, EEF, EJa, EMr, EOE, EWr, For, HaW, HiW, HSW, Kne, Mon, OLW, Pid, Pla, Ran, SaH, STE, SWl, ZWl, ZWr, ZWu
West	FrR, HW, KoH, MB, MHS, USX
Following Structure K=3	
Red	FrR, MHS, Pla, USX
Blue	Abr, ABW, DW, Ebr, EEF, EJa, EMr, EOE, For, HaW, HiW, HSW, HW, KoH, MB, Mon, Ran, SaH, SWl
Green	EWr, Kne, OLW, Pid, STE, ZWl, ZWr, ZWu
Following Structure K=7 (with admixed)	
Red	FrR, USX, EJa
Dark blue	Pla
Green	Pid
Yellow	Abr, ABW, EWr, Kne, OLW, STE, SWl, ZWr, ZWu
Orange	DW, EOE, Mon
Light blue	EMr, HSW, ZWl
Purple	HW, MHS, MB
Strong admixed	Ebr, EEF, For, HaW, HiW, KoH, Ran, SaH
Following Structure K=7 (without admixed)	
Red	FrR, USX, EJa
Dark blue	Pla
Green	Pid
Yellow	Abr, ABW, EWr, Kne, OLW, STE, SWl, ZWr, ZWu
Orange	DW, EOE, Mon
Light blue	EMr, HSW, ZWl
Purple	HW, MHS, MB

Tab. 15: Hierarchical AMOVAs based on AFLPs of *Gladiolus palustris*. Abbreviations: D.f. = degrees of freedom; s = significant (P-value < 0.05).

Groups	Source of variation	D.f.	Sum of squares	Percentage of Variance	P-values	Fixation indices	
Salzburg	Among groups	1	53.776	12.12	0.00098+-0.00098 s	F_{CT}	0.12120
Non-Sbg	Among populations	29	324.262	48.47	0.00000+-0.00000 s	F_{SC}	0.55160
	Within populations	264	236.315	39.41	0.00000+-0.00000 s	F_{ST}	0.60595
Salzburg	Among groups	5	126.378	16.62	0.00098+-0.00098 s	F_{CT}	0.16617
Non-Sbg_	Among populations	25	251.660	43.83	0.00000+-0.00000 s	F_{SC}	0.52560
separated	Within populations	264	236.315	39.56	0.00000+-0.00000 s	F_{ST}	0.60444
	Among groups	1	54.718	19.60	0.00000+-0.00000 s	F_{CT}	0.19600
East - West	Among populations	29	323.320	44.17	0.00000+-0.00000 s	F_{SC}	0.54935
	Within populations	264	236.315	36.23	0.00000+-0.00000 s	F_{ST}	0.63767
Structure K=3	Among groups	2	113.969	25.31	0.00000+-0.00000 s	F_{CT}	0.25313
	Among populations	28	264.069	37.72	0.00000+-0.00000 s	F_{SC}	0.50508
	Within populations	264	236.315	36.96	0.00000+-0.00000 s	F_{ST}	0.63036
Structure K=7 (with admixed)	Among groups	7	255.069	37.44	0.00000+-0.00000 s	F_{CT}	0.37439
	Among populations	23	122.969	22.83	0.00000+-0.00000 s	F_{SC}	0.36497
	Within populations	264	236.315	39.73	0.00000+-0.00000 s	F_{ST}	0.60272
Structure K=7 (without admixed)	Among groups	6	241.483	43.74	0.00000+-0.00000 s	F_{CT}	0.43744
	Among populations	16	92.811	23.29	0.00000+-0.00000 s	F_{SC}	0.41403
	Within populations	201	164.331	32.96	0.00000+-0.00000 s	F_{ST}	0.67035

For the hierarchical AMOVAS, populations were grouped according to different scenarios (see Table 14). For the STRUCTURE clusters, the biggest STRUCTURE group amount was chosen for allocating the populations to the groups used for the AMOVAs. The results of these AMOVAs are listed in Table 15. The hierarchical AMOVA between Salzburg and Non-Salzburg populations were highly significant and accounted for 12.12% of variation among the groups. Similar values were obtained for the comparison of Salzburg populations against Non-Salzburg populations separated according to their geographical distribution. The AMOVAs performed following the STRUCTURE clusters were all highly significant. For K=3, 25.31% of variation among groups was accounted, for K=7 (with strongly admixed populations) the value was 37.44% and for K=7 (without strongly admixed populations) it was even higher (F_{CT}=43.74%).

3.1.3.6 Isolation by distance

According to the results of the Mantel test (see Table 16), for the dataset including all populations the correlation coefficient (r) was moderately high and positively significant (p-value < 0.05) for all settings (see Figure 25). The moderately high correlation coefficient indicates a moderate isolation by distance (IBD)-pattern, meaning that nearby populations tend to be more similar to each other.

For the dataset with Salzburg and nearby populations, only the correlation between kilometers (and log10 (meters)) against the population average pairwise differences showed a significant moderately high correlation (r=0.299 and r=0.287, both with $p < 0.05$, see Figure 26). The other correlations had a rather low correlation coefficient but without statistical significance. Decreasing the dataset to only Salzburg populations of *Gladiolus palustris*, the correlation coefficients became rather small but again without statistical significance. The correlation between the pairwise F_{ST}s (or Φ_{PT}) and population average pairwise differences was significantly positive both for the whole dataset (r=0.6382, P=0.000) and for only Salzburg populations (r= 0.6510, P=0.000).

Tab. 16: r=correlation coefficient (Rxy in GenAlEx output), P-value = statistical significance (shaded green if significant), b= regression coefficient, R^2= Coefficient of determination. The composition of the three categories "All populations", "Salzburg and nearby populations" and "Only Salzburg" can be seen in Table 5 on page 25.

All populations	**r (Rxy)**	**P-value**	**b**	**R^2**	**Regression line**
Km - Φ_{PT}	0.3709	0.0056	0.0009	R^2 = 0.1376	y = 0.0009x + 0.4275
Km - Φ_{PT}/(1- Φ_{PT})	0.3444	0.0007	0.0127	R^2 = 0.1186	y = 0.0127x + 0.8643
Km - population average pairwise differences	0.2717	0.0316	0.0042	R^2 = 0.0738	y = 0.0042x + 3.7935
Log10(m) - Φ_{PT}	0.3295	0.0102	0.0757	R^2 = 0.1086	y = 0.0757x + 0.1787
Log10(m) - Φ_{PT}/(1- Φ_{PT})	0.2334	0.0042	0.7828	R^2 = 0.0545	y = 0.7828x – 1.4511
Log10(m) - Population average pairwise differences	0.3676	0.0014	0.5104	R^2 = 0,1351	y = 0.5104x + 1.9317
Salzburg and nearby populations	**r (Rxy)**	**P-value**	**b**	**R^2**	**Regression line**
Km - Φ_{PT}	0.0371	0.3860	0.0010	R^2 = 0.0014	y = 0.001x + 0.4463
Km - Φ_{PT}/(1- Φ_{PT})	0.0190	0.3532	0.0035	R^2 = 0.0004	y = 0.0035x + 1.2835
Km - population average pairwise differences	0.2994	0.0473	0.0376	R^2 = 0.0896	y = 0.0376x + 3.421
Log10(m) - Φ_{PT}	0.2024	0.0926	0.0633	R^2 = 0.041	y = 0.0633x + 0.2326
Log10(m) - Φ_{PT}/(1- Φ_{PT})	0.1691	0.1164	0.3620	R^2 = 0.0286	y = 0.362x + 0.0471
Log10(m) - Population average pairwise differences	0.2875	0.0137	0.4249	R^2 = 0.0826	y = 0.4249x + 2.2031
Only Salzburg	**r (Rxy)**	**P-value**	**b**	**R^2**	**Regression line**
Km - Φ_{PT}	0,0469	0.4004	0.0045	R^2 = 0.0022	y = 0.0045x + 0.412
Km - Φ_{PT}/(1- Φ_{PT})	0.0254	0.4103	0.0100	R^2 = 0.0006	y = 0.01x + 0.9621
Km - population average pairwise differences	-0.0040	0.5120	-0.0018	R^2 = 2E-05	y = -0.0018x + 3.4159
Log10(m) - Φ_{PT}	0.1497	0.1790	0.0497	R^2 = 0.0224	y = 0.0497x + 0.2683
Log10(m) - Φ_{PT}/(1- Φ_{PT})	0.1070	0.2602	0.1457	R^2 = 0.0114	y = 0.1457x + 0.5335
Log10(m) - Population average pairwise differences	0.0230	0.4449	0.0368	R^2 = 0.0005	y = 0.0368x + 3.2977

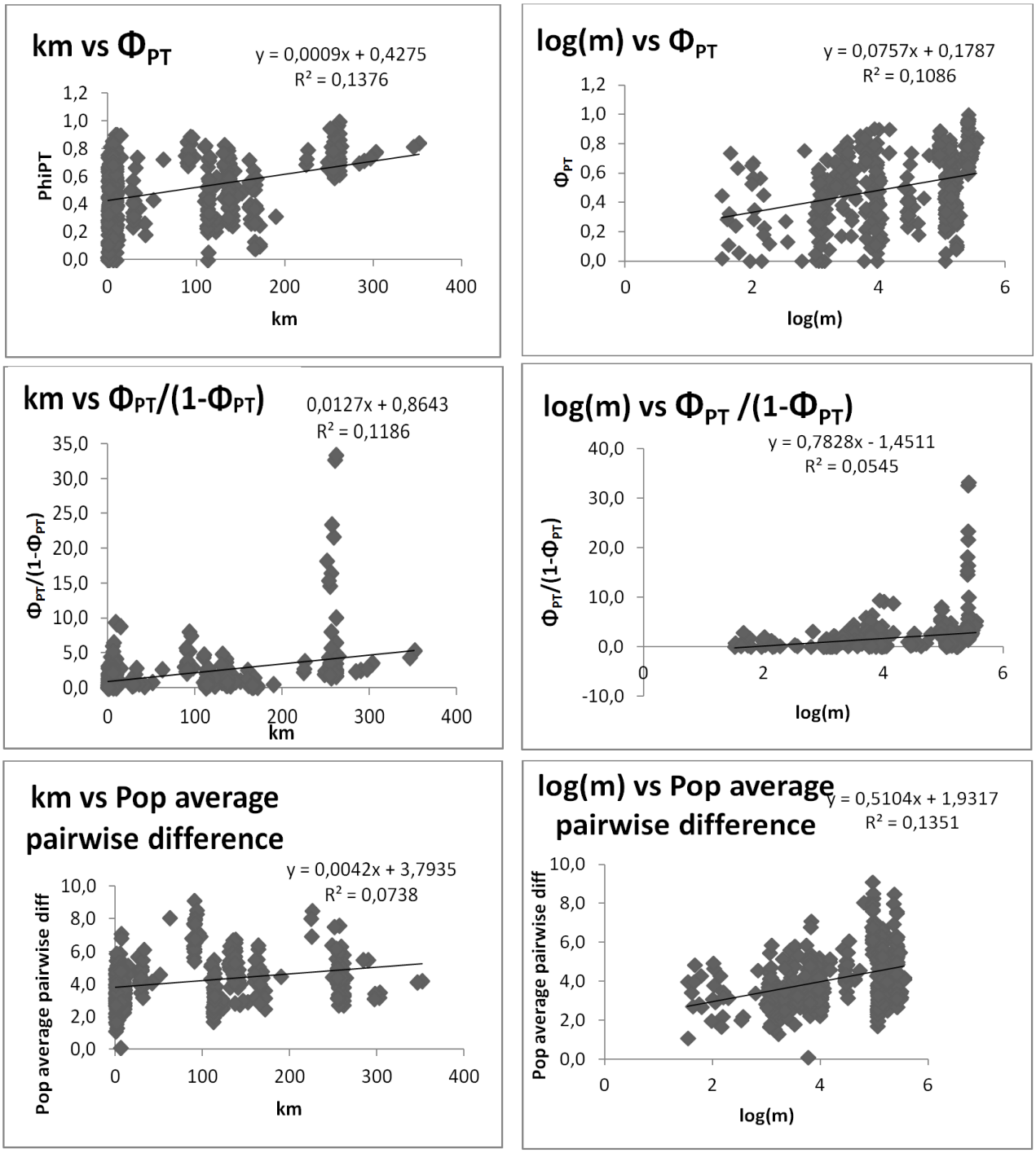


Fig. 25: Significant results (p-value < 0.05) of the Mantel-test for the whole dataset. 9999 random permutations were performed to evaluate the level of significance.

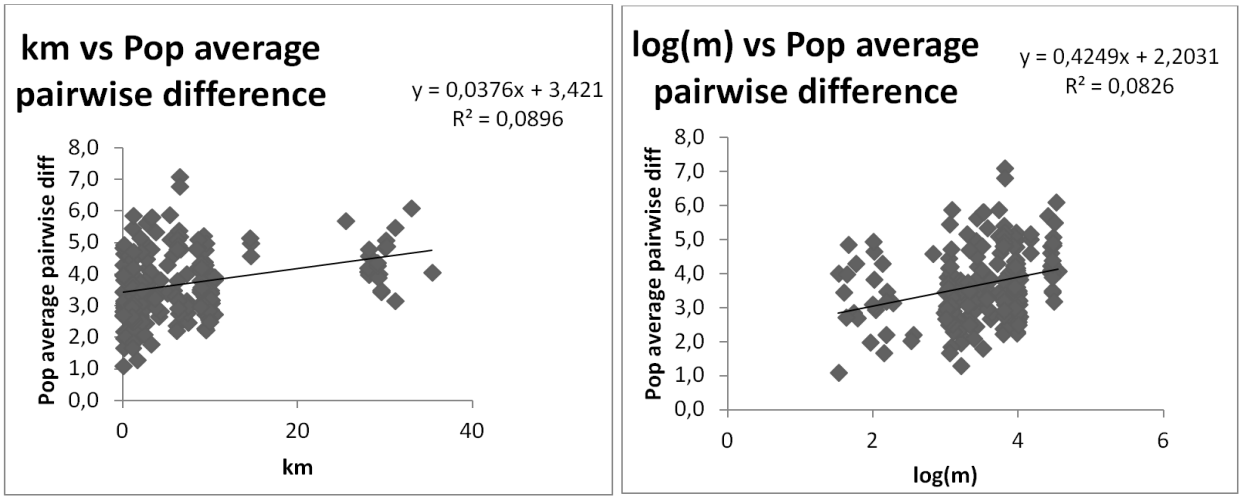


Fig. 26: Significant results (p-value < 0.05) of the Mantel-test for the dataset containing populations of Salzburg and nearby populations. 9999 random permutations were performed to evaluate the level of significance. Not significant results (p-value > 0.05) are not graphically shown.

3.2 Results of the investigation of one population with color variation

3.2.1 Results of the AFLP data

Generating the AFLPs and scoring for the two populations yielded 121 markers (40 FAM-markers, 37 VIC-markers, 44 NED-markers), whereas seven markers are polymorphic and 114 markers are monomorphic. Population “DWl” has six polymorphic markers and eight AFLP phenotypes, whereas population “DWp” has only two polymorphic markers and four AFLP phenotypes.

3.2.2 Results of the data analysis

The PCoA based on DICE coefficients was performed for 32 individuals. The first two coordinates (axes) had an explanatory power of 65.393% (axis 1: 49.062%; axis 2: 16.331%). The third axis had an explanatory power of 8.606%.

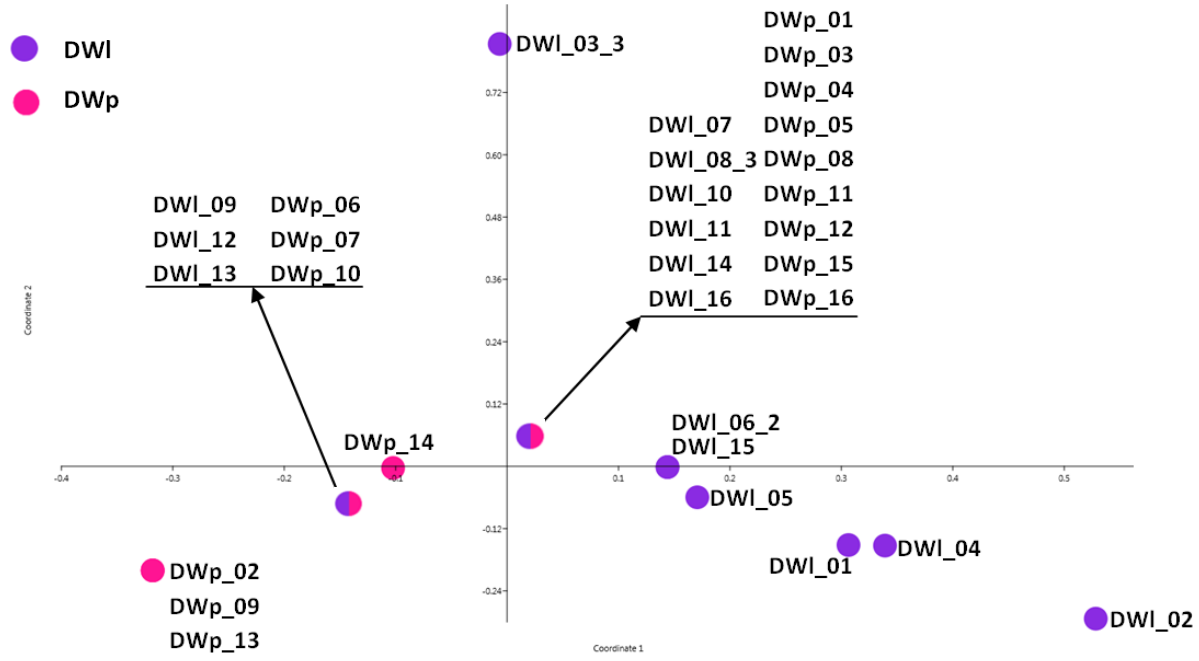


Fig. 27: Principal Coordinate analysis of bright pink (DWp) and purple (DWl) individuals based on Dice coefficient. The first two axes comprise 65.393% of the total genetic variation.

In Figure 27 the result of the PCoA of the sampled 16 bright pink and 16 purple flowering individuals of *Gladiolus palustris* of the “Dürre Wiese” in Wals-Siezenheim is shown. There were fewer dots than individuals because individuals were often genetically identical and dots were overlaying each other, even if they were not of the same color. Some purple flowering individuals are partially separated from the bright pink ones by the first (vertical) axis.

For the AMOVA, the input matrix for the program Arlequin contained the two populations “DWp” (16 bright pink individuals) and “DWl” (16 purple individuals). The result of the performed non-hierarchical AMOVA (see Table 17) was significant. The percentage of variation among populations was 11.79%, the F_{ST} value was 0.11795. This result was supported by the p-value which was 0.02444 (+/- 0.00603).

Tab. 17: Non-hierarchical AMOVA based on AFLPs of *Gladiolus palustris*. Abbreviations: D.f. = degrees of freedom; s = significant (P-value < 0.05).

Group	**Source of variation**	**D.f.**	**Sum of squares**	**Percentage of Variance**	**P-value**	**F_{ST} value**
“Dürre Wiese”	Among populations	1	1.969	11.79	0.02444 +- 0.00603	0.11795
	Within populations	30	18.812	88.21	s	

3.3 Results of the pollination biology study

Seeds per capsule were counted and categorized in fertile and sterile seeds (see Figure 28). Results of the counting of the pollination biology study are listed in Table 18. In total, data (number of inflorescences and capsules) were available for 30 individuals of *G. palustris*, or 167 single flowers (83 developed capsules), respectively, see Table 18. The remaining 19 plant individuals got lost during summer, because of game bite or dieback, for instance.

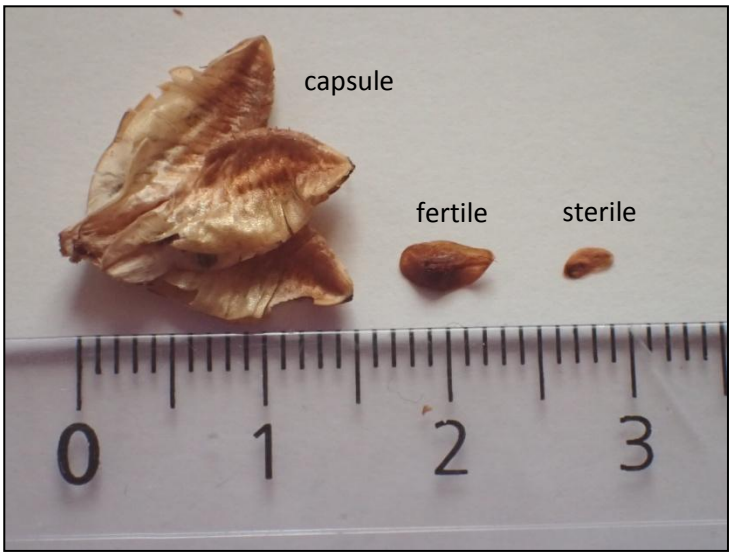


Fig. 28: Content of a capsule, sterile and fertile seeds of *Gladiolus palustris*.

The mean number of fertile seeds per capsule was calculated as the total number of developed fertile seeds per capsule divided by the number of capsules. The percentage of fruit set was calculated as the total number of capsules divided by the total number of flowers per individual, times 100. Afterwards, the mean of this data per treatment was calculated. For the flowers which were covered before flowering ("spontaneous selfing"), the percentage of fruit set was lowest with a value of 32.12%. They also had the lowest number of fertile seeds per capsule (7.66). The hand self-pollinated flowers ("forced selfing") showed a percentage of fruit set of 55.52% with 16.5 fertile seeds per capsule on average. The highest percentage of fruit set showed the flowers which were covered after flowering ("open-pollination") with a value 71.12% but they had a lower mean number of fertile seeds per capsule (12.8) than the hand-pollinated flowers.

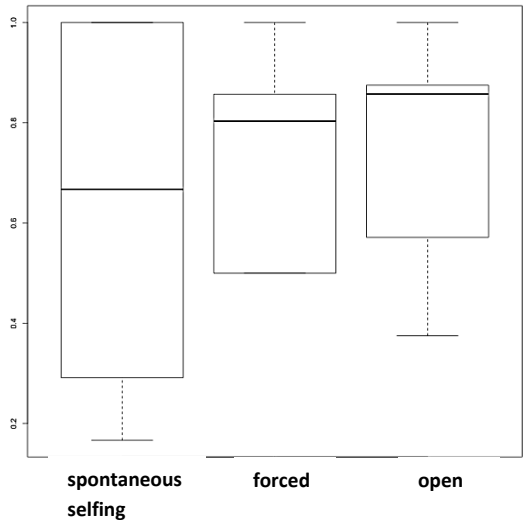
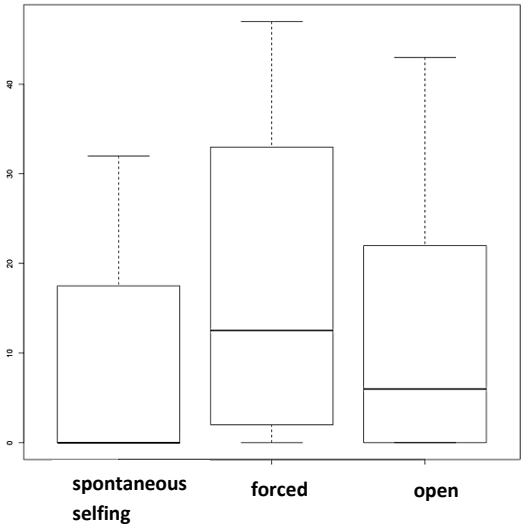
Tab. 18: Reckoning values of the pollination biology study.

Treatment	Nr. of flowers	Nr. of developed capsules	Mean number of fertile seeds per capsule	Percentage of fruit set
"spontaneous selfing"	90	32	7.656	32.120
"forced selfing "	30	18	16.500	55.519
"open pollination "	47	33	12.848	71.122

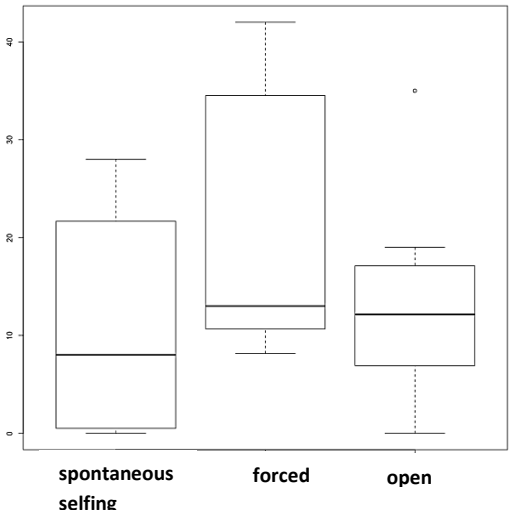
The results of the ANOVA with the linear mixed-effects model are depicted in the following two Tables (see Table 19 and 20). Considering all values, there is a highly significant (P-value = 3.582e-11) difference in the number of capsules per flower depending on the treatment. The dependence of the

number of fertile seeds per capsule on the treatment is not statistically significant (P-value = 0.09451). For the dataset considering the mean values, both the capsules per flower and the fertile seeds per capsule show a statistically supported dependence on the treatment. The capsules per flower differ significantly dependent on the treatment. For the fertile seeds per capsule, a highly significant difference exists, depending on the treatment.

Tab. 19: Results of the linear mixed-effects model for all samples.

All values	
Capsules per flower – treatment	Fertile seeds per capsule – treatment
	
X^2_1 = 48.105, P = 3.582e-11 ***	X^2_1 = 4.7181, P = 0.09451

Tab. 20: Results of the linear mixed-effects model for mean values.

Mean values	
Capsules per flower – treatment	Fertile seeds per capsule – treatment
	
X^2_1 = 7.5587, P = 0.02284 *	X^2_1 = 16.06, P = 0.0003256 ***

The linear mixed model generalized according to the Poisson distribution (of all values) revealed that there was a significant difference in the number of fertile seeds per capsule according to the treatment. The pairwise comparison showed that there was a highly significant difference in the number of fertile seeds between the treatments “forced selfing” and “spontaneous selfing” with a p-value of P = e-04. The other comparisons showed no significant differences.

4 Discussion

The main aim of my study was to analyze genetic diversity patterns and underlying processes of *Gladiolus palustris* in Salzburg and adjacent areas to be able to make a statement about the genetic condition of this threatened species. Another aim was to clarify if there was a genetic hint for the occurring color morphs in one population in Salzburg (Wals-Siezenheim, Gois). Additionally, I wanted to test if the species was actually self-compatible and if there were differences in self- and cross-pollination regarding the size and characteristics of the fruit set.

4.1 Genetic structure of *Gladiolus palustris* in Salzburg and adjacent areas

4.1.1 Genetic variation in *Gladiolus palustris*

The genetic variation of *Gladiolus palustris* can be regarded as very low. The scoring of the three AFLP primer combinations of 295 individuals (32 populations) of *G. palustris* resulted in 125 markers, of which only 28 were polymorphic (PPF=22.4%), what is rather low. The remaining 97 markers were monomorphic, i.e. present in every individual of the sample set. Remarkably, only 70 individuals had unique AFLP phenotypes (i.e. fragment combinations). The AFLP phenotypes of the remaining 225 individuals were redundant and appeared at least twice in the dataset. In Table 6 the 50 genotypes are listed, whereby they contain not less than two individuals up to twenty. Not only individuals of the same populations shared the same AFLP phenotypes, many individuals of different populations were genetically overlapping each other, as for example the individuals EMr_10 and SWl_10 were sharing the same AFLP phenotype. Differences between the genotype groups often emerged because there was just one absent or present locus. It has been shown that some rare species with low variation have a high proportion of monomorphic markers (MORDEN & LOEFFLER, 1999; NYBOM & BARTISH, 2000), what would be applicable to *G. palustris*.

There are some other studies, where a higher percentage of polymorphic makers was found in rare plants. For example, REICHEL et al. (2016) conducted an AFLP study on *Laserpitium prutenicum,* an European plant which faced an enormous fragmentation of its habitat and a decline in population number during the last 70 years. The authors yielded a percentage of polymorphic loci of 45% and interpreted it as relatively high. SCHMIDT & JENSEN (2000) examined the genetic structure and AFLP variation of populations of *Pedicularis palustris,* also called European purple lousewort, a plant which only occurs on the Northern hemisphere in Europe in natural, moderately managed fen meadows. The former common purple lousewort experienced a strong decline in its abundance because of changes in land-use during the last decades – a situation similar to *Gladiolus palustris*. The AFLP study with four primer combinations on 129 individuals revealed 262 loci of which 64% were

polymorphic. Only two individuals showed the same AFLP phenotype, so in total, the authors received 128 AFLP phenotypes, what is, compared to my study on *G. palustris*, quite a lot.

One question to pose here is, if AFLPs were an inappropriate method for surveying the genetic structure of the marsh gladiola. The Amplified Fragment Length Polymorphism method has been broadly used in systematic, population genetics, DNA fingerprinting and quantitative trait loci (QTL) because of its many advantages such as rapid generation of hundreds of DNA markers, time and cost efficiency, replicability and high resolution (MUELLER & WOLFENBERGER, 1999). Another advantage of this technique is that only small amounts of tissue are needed, which is accruing for rare plants like *G. palustris* (VOS et al., 1995; SCHMIDT & JENSEN, 2000). Nevertheless, there are also disadvantages. Mostly dominant markers are generated, making a distinction between individuals being heterozygous or homozygous for the dominant allele impossible (FOLL et al., 2010). Furthermore, they often have lower levels of polymorphism than other methods (GAGGIOTTI, 2010). This might be problematic because the accuracy and resolution of measurements on genetic distances increases with the number of loci used (TRAVIS et al., 1996; SCHMIDT & JENSEN, 2000). However, in my study, the marker profiles in the Fragment Profiler mostly showed distinct peaks, what made me conclude that the method was appropriate and that the observed low variation is real and not due to the method chosen. The authors SCHMIDT & JENSEN (2000) of the AFLP study on *Pedicularis palustris* had fewer individuals but a bigger number of loci, which might possibly be dependent on the one more primer combination they used. Possibly, the usage of more than three primer combinations would be preferable for a better resolution of the AFLP markers and a better illustration of the genetic structure and diversity of *Gladiolus palustris*.

Another hint that the low percentage of polymorphic loci is real and not a product of inappropriate methods was given by a different study on *Gladiolus palustris* and *Gladiolus imbricatus*. The authors analyzed the natural hybrid between the both species and also used AFLPs. They detected 61 fragments, which were only present in *G. palustris* and of this, 24.59 % were polymorphic (SZCZEPANIAK et al., 2016). A further supporting study on *G. palustris* in Poland, where the species is also threatened with extinction, revealed similar results. The authors got a PPF of 10% for the marsh gladiola; genetic variability for the marsh gladiola was four times lower than for *Gladiolus imbricatus* (CIEŚLAK et al., 2014).

4.1.2 Genetic structure of *Gladiolus palustris* in Salzburg and adjacent areas

The result of the STRUCTURE analysis identified three numbers of groupings, namely at K=2, K=3 and K=7, where mean DeltaK and similarity coefficients were highest. At K=2, many populations are admixed, but there are some which are entirely belonging to one of the two clusters. Already, we can recognize some geographical allocation. When K=3, the populations of Vorarlberg (“FrR” and “USX”)

are forming a distinct cluster together with the population of Upper Austria ("Pla"). At K=7, the two populations of Vorarlberg formed their own cluster, as well as the population of Upper Austria was forming its own cluster. This result proved that there is some geographical distribution pattern. To a small extent, this is also confirmed by the results of the following analyses. Comparing the STRUCTURE results to the results of the NeighborNet and the PCoA, only the population "Pla" (Upper Austria) was separated again from the other populations, whereas the two populations of Vorarlberg were more or less scattered amongst the other populations. Also the bootstrap values in the NeighborNet did not support any certain grouping of populations, except the separation of "Pla". All other individuals were more or less scattered, individuals of different populations were overlapping each other, even if their distances were great, for example SaH_10 and SWl_08 (113km). Interestingly, populations which were forming separate groups in STRUCTURE at K=7, are having fewer AFLP phenotypes than other populations. For example, population "Pla" has 10 individuals but only three AFLP phenotypes, whereas only two of them are visible in the PCoA (dark blue). Also, the two populations of Vorarlberg (20 individuals) are just present in two AFLP phenotypes. In general, there is some differentiation between the populations and there also seems to be some geographical separation. The STRUCTURE results at K=7 look quite colorful, but one has to be careful not to overestimate the diversity of the genetic structure, because the estimated allele frequencies depend on a low number of variable loci. Populations of Vorarlberg and Planwiesen in Upper Austria have a few alleles less than the other populations and therefore are separated in the analyses. For example, individuals of "Pla" only have 115 fragments on average, whereas more admixed populations like for instance "HaW" have 119 fragments. So, as already mentioned, differences between the genotype groups often emerged because of just one absent or present locus, what should always be kept in mind while viewing the results.

4.1.3 Gene diversity and genetic rarity

The estimated Nei's gene diversity (GD; NEI, 1987) at population level of *Gladiolus palustris* was quite low as the mean value of gene diversity across all populations was 0.01479. According to RIEGEL (2010), the population in Königsbrunn (KoH) is the biggest one in Central Europe. This population has one of the highest gene diversities (0.02400) in my study. As big population sizes usually contribute to higher genetic diversities (SZCZECIŃSKA et al., 2016), my result of a higher genetic diversity in this population would be matching into this theory. However, I do not have population sizes for the non-Salzburg populations, wherefore I could not perform a correlation analysis between population size and gene diversity. The lowest genetic diversity occurred in the two populations of Vorarlberg FrR (0.0000) and USX (0.0016), in Piding Pid (0.0041) and HSW (0.0000) in Upper Bavaria. This would fit

to the previous results of STRUCTURE, where FrR and USX were forming a separate cluster, as well as Pid, as they are having fewer alleles than other populations.

Regarding the populations of Salzburg, the gene diversity within the *Gladiolus palustris* populations ranged from 0.0032 to 0.024. They were lowest in population ABW (0.0032) and highest in EEF (0.0240). As the populations DW and SWl were the biggest ones in Salzburg, with an average population size of 287 and 237, respectively, one would expect the highest gene diversities within these populations. A performed correlation analysis between gene diversities and mean values of the population sizes yielded controversial results – there was suggested a negative correlation but without statistical significance. Therefore, there was no significant correlation between these two factors. The two biggest populations showed rather intermediate and low gene diversities. However, there are comparative values of other authors necessary to classify the genetic diversity as "high", "moderate" or "low". In a paper of JAROS et al. (2016), there is talk of a generally low within-population gene diversity of values between 0.026 to 0.124 and an averaged gene diversity of 0.072 across all populations of an epiphytic orchid in Madagascar and La Réunion. In this study, the percentage of polymorphic fragments (PPF) was 86.3%. Compared to this result, the gene diversity of *G. palustris* is very low, as well as the PPF.

There is a discrepancy between the biology of *Gladiolus palustris* and the "expected diversity values" according to NYBOM & BARTISH (2000). These authors studied the effects of life history traits and sampling strategies on genetic diversity obtained with RAPD markers by summarizing results of numerous other authors. As life history traits they used taxonomic status, life form, geographic distribution range, breeding system, seed dispersal mechanism and successional state (NYBOM & BARTISH, 2000). They found that the mean within-population gene diversity showed a significant association with taxonomic status, breeding system and successional state. In a comparable study by REISCH & BERNHARDT-RÖMERMANN (2014) the impact of study design and life history traits on genetic variation of plants determined with AFLPs were analyzed. Similar to NYBOM & BARTISH (2000), the authors used life history traits like taxonomic status, life span, frequency, mating system and pollination vector (REISCH & BERNHARDT-RÖMERMANN, 2014). They found a significant association of the mean gene diversity with the frequency of the plant and the mating system.

In the study of NYBOM & BARTISH (2000) the mean within-population gene diversity was highest for gymnosperms, the value was at 0.386. Monocotyledon plants, as *G. palustris* is, should have a mean within-population gene diversity of about 0.190. My results of gene diversities, ranging from 0.0000 to 0.02951, are far below these values. Also, the breeding system was significantly associated with the mean within-population gene diversity. According to NYBOM & BARTISH (2000), outcrossing plants have the highest gene diversity (0.260), followed by plants with a mixed mating system (0.219). Selfing plants are coming in last with a mean within-population gene diversity of 0.091. REISCH &

BERNHARDT-RÖMERMANN (2014) obtained resembling gene diversity results for outcrossing (0.19), mixed (0.18) and selfing (0.09) plants. Also HAMRICK & GODT (1996) found that outcrossing species seemed to be more genetically diverse than selfing species and had a lower genetic differentiation amongst the populations. According to these results, *G. palustris* would fall into the category of a selfing plant because of its low mean within-population gene diversity of 0.01479. However, it is out-crossing and seems to inhibit self-pollination by protandry (KNUTH, 1899; OHRI & KHOSHOO, 1981; HERRMANN, 2000). In my pollination biology experiment I got the evidence that the marsh gladiola has a "mixed mating" system, mostly avoids self-pollination but is not self-incompatible. Also, *G. palustris* is able to reproduce vegetatively via bulbs, but this is more the exception (SCHMITT et al., 2010).

As *Gladiolus palustris* is threatened by habitat fragmentation, what has stronger negative effects on within-population genetic diversity of outcrossing species than selfing species (AGUILAR et al., 2008) I was wondering if there was going on a change in the breeding system from a former out-crossing to a selfing breeding system. Ecological forces can determine, whether a species' mating system will be selected to change (CHARLESWORTH, 2006). Outcrossing, insect-pollinated plants are dependent on the density of plant individuals and also on the density of pollinators (CHARLESWORTH, 2006). If these factors are limited for the marsh gladiola, why should there be no change to a mainly inbreeding mating system? Clearly, this would be a long lasting process.

Furthermore, NYBOM & BARTISH (2000) found that the mean within-population gene diversity was associated with successional state. A late successional status goes along with higher mean within-population gene diversity (0.287). The gene diversity is decreasing from mid successional status (0.195) to early successional status (0.166). Here, too, my results are converse, as *G. palustris* is a late successional plant but has very low gene diversities.

Also, the frequency down weighed marker value (DW), or rarity index, was calculated (SCHÖNSWETTER & TRIBSCH, 2005; EHRICH, 2010). According to SCHÖNSWETTER & TRIBSCH (2005), high values of the frequency-down-weighted marker values are expected in populations which are long-term isolated. In such populations, an accumulation of rare markers due to mutations could have taken place. In contrast, it is expected that newly developed populations are having lower values. So, the rarity index might be helpful for analyzing population history (SCHÖNSWETTER & TRIBSCH, 2005).

For non-Salzburg *G. palustris* populations, it varied from 0.0696 to 0.1124. It was lowest in the populations Pid (0.06964), Pla (0.07059), FrR (0.07771) and USX (0.07829). As expected, these are the populations, which were already forming separate clusters in the STRUCTURE results and having fewer alleles than other populations.

In Salzburg populations, the genetic rarity was generally higher than outside of Salzburg; it varied from 0.0665 to 0.1895. The lowest genetic rarity was found in the populations at the "Esterer" tavern, namely in EEF (0.07412), EJa (0.07600), EMr (0.06650), EOE (0.07378) and EWr (0.07939). It

was highest in the populations Kne (0.18951), OLW (0.17885), ZWl (0.15400) and DW (0.12375). There is no certain pattern in the Salzburg populations as it already was the same with the gene diversities. Furthermore, population size does not seem to play an important role, as the populations with the highest rarity indices have quite different sizes. While Kne (7), OLW (16) and ZWL (26) have rather small average population sizes, population DW is the biggest in Salzburg with more than 280 individuals on average but has a lower rarity index than the three aforementioned. However, all the values, even the highest ones for *G. palustris* are quite low – this would suggest that the populations were not isolated over a long period of time but rather newly developed. This would fit to the fact that the habitat of the marsh gladiola has decreased enormously in the last decades (see discussion below).

4.1.4 Differentiation (F_{ST}) of *Gladiolus palustris* and isolation by distance (IBD)

Regarding all populations involved, the non-hierarchical AMOVA indicates that more than half of the total genetic variance can be found among the populations (58.12%), in contrast to within populations (41.88%). Considering only the Salzburg populations, about half of the total genetic variance is explained among the populations (49.33%) and the other half within the populations (50.67%). Both analyses of molecular variance are highly significant and supported by the p-values ($P<0.001$). The F_{ST} values for all populations (0.58121) and for Salzburg populations (0.49331) are quite high. The F_{ST} value can range from zero to one, whereas a value of one would depict completely differentiated populations where all the genetic differentiation is explained within the populations. A value of zero indicates that two populations are randomly mating, panmictic, respectively (BALLOUX & LUGON-MOULIN, 2002; WEISING et al., 2005). Also, the matrix of the population pairwise F_{ST} values (Table 10) shows partially very high values.

A study on *G. imbricatus* revealed F_{ST} values much lower than the values I obtained for *G. palustris*, of about 0.27 (CIEŚLAK et al., 2014). These results are fitting better to the differentiation values for different life history traits of NYBOM & BARTISH (2000). They found significant associations of AMOVA-derived F_{ST} values with life form of plants, geographic range, breeding system and successional state (NYBOM & BARTISH, 2000). REISCH & BERNHARDT-RÖMERMANN 2014 found a significant association between F_{ST} values and frequency and mating system of plant species. Regarding the life forms of plants, long-lived woody species should have lower differentiation amongst populations than short-lived herbaceous and annuals plants (HAMRICK & GODT, 1989; MAHY et al, 1997). According to NYBOM & BARTISH (2000) the F_{ST} values for long-lived perennials are expected to be around 0.25, for short-lived perennials around 0.39 and for annuals around 0.70. Comparing the population pairwise F_{ST} values of *G. palustris* to this categorization, my study species would rather be ranged within annuals plants than with perennials. Also, the association with the geographic range does not fit to the situation of

the marsh gladiola. According to the authors, the highest F_{ST} values are found in regional (0.43) and widespread (0.42) species, followed by species with a narrow geographical range (0.34) and endemic species (0.20). *Gladiolus palustris* is anything but widespread; still, its F_{ST} values would indicate this. REISCH & BERNHARDT-RÖMERMANN 2014 state that for a rare species the F_{ST} value should be around 0.34, whereas common species usually have F_{ST} values around 0.20, indicating that rare species have a higher differentiation. Regarding the breeding system, for a species with a mixed mating system favouring out-crossing but also being capable of self-pollinating, the AMOVA-derived F_{ST} value for the marsh gladiola should be around 0.27-0.28. Again, as with the life forms, the F_{ST} values of *G. palustris* argue for a selfing plant, as selfing species tend to have F_{ST} values around 0.7 (NYBOM & BARTISH, 2000). REISCH & BERNHARDT-RÖMERMANN (2014) yielded a similar tendency - outcrossing plants have the lowest F_{ST} values (0.20), followed by plants with a mixed mating system (0.28) and selfing plants (0.53). Last, early successional states (0.45) and mid successional states (0.40) have higher F_{ST} values than late successional states (0.21) (NYBOM & BARTISH, 2000). This is again contrary to the marsh gladiola, as it actually falls in the category of late successional plants.

SCHMIDT & JENSEN (2000) yielded a high genetic differentiation between most pairs of populations of *Pedicularis palustris.* Their values ranged from 0.27 to 0.89 and there was no significant correlation between the pairwise genetic distances and pairwise geographic distances. They obtained an amount of variance among the populations of 44%, the median distance between pairs of populations was 40.75km (SCHMIDT & JENSEN, 2000). They conclude that because of the high amount of genetic differentiation among the populations much of the overall genetic variation of the species would be lost if management of the residual populations only focused on large populations and if small populations decline or become extinct (SCHMIDT & JENSEN, 2000). However, the F_{ST} values I obtained for *Gladiolus palustris* cannot be applied one-to-one.

According to the formula $F_{ST}=(H_T-H_S)/H_T$, the high F_{ST} values are a result of the low gene diversities in the subpopulations (WEISING et al., 2005). This coincides, as most populations with the lowest gene diversities are having the highest population pairwise F_{ST} values, like populations FrR, OLW, Pid, Pla and USX. The high genetic differentiation among populations and weak Isolation by Distance (see later) might be a result of high genetic drift in subpopulations, assuming a low genetic exchange, or gene flow, respectively, between the populations. In some rare species, there has been found genetic differentiation among populations as a result of low levels of gene flow among the isolated populations (RAIJMANN et al., 1994; SCHMIDT & JENSEN, 2000). RAIJMANN et al. (1994) suggest that the limited gene flow of the rare plant species *Gentiana pneumonanthe* is probably related to the behavior of its main pollinator, the bumblebee *Bombus pascuorum*. The maximum distance (about 250m) this bumblebees travel each day in search for pollen is far below the distance (>800m) between most gentian populations (KWAK et al., 1991; RAIJMANN et al., 1994). The mean distance

between the *Gladiolus palustris* populations in Salzburg is 2.238 kilometers (mean derived from the geographical distance matrix). The marsh gladiola is mainly pollinated by the bumblebee *Bombus hortorum* (KNUTH, 1899; HERRMANN, 2000). HAGEN et al. (2011) examined the space use behavior of bumblebees by radio-tracking and found that *Bombus hortorum* flies distances up to 2.5 kilometers in a rather short time of one to four days. So, theoretically, gladiola populations of Salzburg are within the bounds of possibility to reach for the pollinators. However, some populations are farther away, as for example "Ran" in Großgmain and "Kne" in district Leopoldskroner Moos have greater distances and are therefore expected to have fewer exchanges with the other populations.

The Isolation by Distance (IBD) for all populations was significant (p-value < 0.05) in all settings. The correlation coefficient ranged from 0.2717 to 0.3709. This indicates a moderately high isolation, meaning that nearby populations tend to be more similar to each other and genetic differentiation increases with rising geographical distances. Here, the populations of Vorarlberg are included, which are more than 250 kilometers apart of the other populations. As already mentioned, they were forming a separate cluster in STRUCTURE, have very low gene diversities and fewest rare alleles; probably they have just a few alleles less than the other populations and therefore contribute to the isolation by distance. On a regional scale with Salzburg populations and nearby populations including HaW, HiW, HSW, Mon and Pid, only the correlations of kilometers with the population average pairwise differences were significant. The trend of the regression line was flatter and the correlation coefficient was 0.299, and 0.287, respectively. Considering only the populations of Salzburg, there is no significant isolation by distance, whereby the trend of the regression line would have been further sagging, in one setting even negatively. The non-existence of IBD-effects fits to the previous results - Salzburg populations do not show any specific diversity pattern amongst the populations and are not grouped into defined clusters. Similar genotypes are occurring in different populations. This can indicate gene flow and exchange between the populations, at least in earlier times, when the habitat of *Gladiolus palustris* was still intact and interconnected. Habitat destruction led to isolation and strong genetic drift in subpopulations and can reduce plant fitness due to pollinator limitation and increased inbreeding (FISCHER & MATTHIES, 1997).

4.2 Discussion of the color variation

The occurrence of two color morphs at "Dürre Wiese" in Wals-Siezenheim in a roughly 50:50 proportion was described by NOWOTNY (2012). The meadow is located in the middle of a commercial forest; the nearest next population is population "OLW", which is about 980m away. All other populations are more than 1.8 kilometers away. NOWOTNY (2012) describes the meadow as centre of biodiversity with typical plant species of fens with a management of a bedding meadow. In the year 2008, the population was at its highest level with 1834 flowering individuals (NOWOTNY, 2012). Then, a new landowner mowed the meadow during summer, which led on to a dramatic decline in the population size. In the year 2011 only 53 individuals were flowering but the landowner was enlightened about the FFH-status of the species and ever since the population is able to recover (NOWOTNY, 2012). The average population size of the species from 2010 to 2016 is 287 flowering individuals (Nowotny, personal communication). The color morphs quickly aroused peoples' interest, as, to my knowledge, this phenomenon only occurs in Salzburg and the cause of it is unclear. Also, in literature, the color is mostly described as bright pink (carmine red, purple red), SCHMALL (2015) also mentioned the occurrence of lilac (purple) plants but refers to the same population as NOWOTNY (2012).

The flower color is very diverse throughout flowering plants but color variation within species is rather uncommon (WANG et al., 2013). WEISS (1995) reported about a floral color change, where plants were changing the colors of the flowers during the flowering process, for example *Lobularia maritima*. This may increase the pollination success by increasing the interactions of plant and pollinator (WEISS, 1995). However, this concept is not negotiable to the color morphs of *Gladiolus palustris*, because the color of the individuals is fixed and maintained throughout the flowering period.

On further reflection, we perused different approaches for an explanation of the appearance of two color morphs. Firstly, it could be possible that the difference within the color is reflected in patterns of genetic variation. If this would be the case, gene flow between color morphs was mainly present within the color morphs and low among them. The second approach was that pollinators might switch between bright pink and purple individuals. Consequently, gene flow among the two color morphs would be strongly present, leading to a genetic resemblance. This is often a strategy of deceptive plants, which do not offer nectar. There are only few species which show a genetically based polymorphism (GIGORD et al., 2001). GIGORD et al. (2001) showed for a rewardless orchid *Dactylorhiza sambucina* with color polymorphism that they are mostly pollinated by naive bumblebees. According to behavioral experiments the insects sample different color morphs in alternation, because after visiting a rewardless flower they tend to switch to the different color morph (GIGORD et al., 2001). Flower color polymorphism is also occurring in *Iris lutescens*, which

appears in purple and yellow color morphs and does not produce any nectar reward for pollinators (WANG et al., 2013). WANG et al. (2013) say that different selection pressures like precipitation, soil, temperature, herbivores and pathogens can also indirectly influence color traits. Furthermore, many floral diversifications (concerning morphology, color, patterning and scent) can occur when plants undergo an adaptive shift between pollinator classes, also called the pollinator-shift model by Verne Grant and Ledyard Stebbins (ELLIS & JOHNSON, 2009). However, according to KIRCHNER et al. (1934), *Gladiolus palustris* is not only attractive to insects because of the conspicuous flower color and the nectar guide but also because of its' generous sweet nectar production. So, pollinators are not fooled by the plant species and should have no reason to switch to another plant with a different color.

My results of the AFLPs and scoring of the 32 sampled individuals (16 bright pink [DWp] and 16 purple [DWl] individuals) yielded 121 markers. The special color morph "DWl" had a higher proportion of polymorphic markers and also more haplotypes than the regular color morph. In the Principal Coordinate Analysis, the first two coordinates (axes) had an explanatory power of 65.393%. Although some purple individuals were partially separated from the bright pink ones by the first (vertical) axis, there were also many genetically identical individuals, even if they were of different colors. The result of the performed non-hierarchical AMOVA was significant. The percentage of variation among populations was 11.79%; the F_{ST} value was 0.11795, so some variation is explained among the morphs. On the other hand, regarding the results of STRUCTURE at K=7 (see Figure 13 and 15), the two populations both had big amounts of the separated orange cluster, suggesting that they are rather more similar to each other than to the other populations. This would fit the theory that they make pollinator switch between them, probably to increase pollination success in the case of pollinator limitation. To some extent, the color morphs are explained by genetic differentiation among the populations, but certainly, other factors are involved as well.

The difference in the two colors is not so conspicuous, at least for the human's eye, so the assertions about pollinator behavior regarding the color are solely speculations. It would be interesting to measure the reflectance spectra of the colors and to examine, if the difference in the colors is more distinct and perceived differently by trichromatic species, such as bumblebees. Additionally, a further genetic analysis including more individuals of both color morphs would be interesting to get a better resolution of the results. Also, examination of soil parameters and a study of present pollinators might be interesting.

4.3 Discussion of the pollination biology

According to literature (KNUTH, 1899; HERRMANN, 2000), *Gladiolus palustris* can forestall self-pollination by protandry. HERRMANN (2000) describes that during anthesis the anthers of the three stamens are located below the still closed stigma. After draining of the pollen, the stamens start shrinking and the stigma starts opening and lowering itself through the level of the stamens. During this last stage, the tepals are already starting to decompose (HERRMANN, 2000). Alternatively, further literature also describes the protandry but states that self-pollination seems to be a frequent phenomenon in the genus *Gladiolus*, also in *Gladiolus palustris* (KIRCHNER et al., 1934). KIRCHNER et al. (1934) described an experiment, where *G. palustris* was grown in a greenhouse, excluding pollinators and showed an "equally abundant" fructification. Also NOWOTNY (2000) and SCHMALL (2015) indicate that self-pollination is possible.

Within my pollination experiment I wanted to confirm the statements about self-pollination and to analyze if there were differences in self- and cross-pollination regarding the size of the fruit set. My results support the literature, as *G. palustris* was self-compatible and also spontaneously selfing in my experimental settings. However, not all gladiola species are self-compatible, OHRI & KHOSHOO (1981) report about many self-incompatible species in the genus *Gladiolus*. Plants of all three groups "spontaneous selfing", "forced selfing" and "open pollination" formed capsules with seeds. The percentage of fruit set and the mean number of fertile seeds per capsule were lowest for the flowers of the "spontaneous selfing" group. This result is not surprising, as flower color and morphology indicate pollination via insects and self-pollination may be rather the stopgap and therefore just sufficient to provide little offspring. The percentage of fruit set was highest in the "open pollination" flowers, indicating that insect pollinated flowers used the most opportunities to build a capsule. The following result is astonishing as it indicates that flowers of "open pollination" have a lower mean number of fertile seeds per capsule than flowers of the "forced selfing" group. This contrasts with the previous result of a higher percentage of fruit set for the insect pollinated ("open pollination") flowers. This would imply that in the presence of pollinators, the fruit set is better and more capsules are developed, but fewer seeds are in the capsules. Perhaps this is a hint that there is a pollinator limitation in Salzburg, at least during the period of the experiment in 2015, indicating that more pollinators would be required to efficiently pollinate the flowers.

The pollination mechanism and the breeding system were also examined in garden gladiola by OHRI & KHOSHOO (1981). They authors point out that the flower morphology indicates out breeding. In an experiment with hand-pollinated, natural cross-pollinated and bagged flowers they found that natural cross-pollinated showed a lower capsule formation and attributed it to an absent of insects. Hand-self- and cross-pollinated flowers had a higher pod development (OHRI & KHOSHOO, 1981). YALEK (2016) examined the floral biology and hybridization in cultivated gladiola and also found a higher

seed set in forced cross-pollinated plants compared to open pollinated plants. In contrast to my results, the author did not obtain seeds under controlled selfing conditions.

A further interpretation of my data was difficult, as I often applied more than one treatment on a single plant, meaning that some flowers of an individual were treated with the forced selfing, the others belonged to the spontaneous selfing group. This was due to irregular flowering of the flowers of every plant and I was not able to get hold of every flower at its flowering time. In the fields I did not consider this to be of importance, but statistical analyses with the data are controversial. So are the results of the nested ANOVA with the linear mixed-effects model. Here, a statistical evaluation was performed; individuals were included as a random factor, as some individuals occurred more than once in the dataset. Greater weight was given to larger sample sizes. Considering all values, there was a highly significant difference in the number of capsules per flower depending on the treatment, but no significant difference concerning the fertile seeds per capsule. Calculating with the mean values, both capsules per flower and fertile seeds per capsule differed according to the treatment. However, the validity of this result is to doubt as a Gaussian distribution would be the foundation for the ANOVA, but is not the case. Discussions with academics did not lead to a distinct opinion how to statistically interpret my results. Also, the experimental set-up is lacking a fourth treatment group, namely the “forced cross-pollination”. Furthermore, it would be of great interest to conduct a seed germination test with seeds of the different treatments. A further study on this topic would be interesting to help developing a management plan for the best reproductive success of *Gladiolus palustris*, also in respect of progeny and cultivation for reintroduction.

4.4 Habitat fragmentation, potential consequences and conservation

4.4.1 Habitat fragmentation and decline of *Gladiolus palustris* in Salzburg

Many natural areas have been transformed into anthropogenic landscapes during the last two centuries due to the development of human civilization (AGUILAR et al., 2008). Habitat fragmentation comes along with loss of habitats, a reduction of the size of habitats and an increased isolation of remaining habitats and also populations, respectively (ANDREN, 1994; HONNAY & JACQUEMYN, 2007; AGUILAR et al., 2008).

It is possible that this huge loss of potential habitats contributed or even led to the low genetic diversity of the marsh gladiola due to genetic drift in isolated populations. For Salzburg, the only recent occurrence of *Gladiolus palustris* is the foothills of the Untersberg (WITTMANN et al., 1987). A historical map of the 18th century shows that the region between the city in the north (in the left of the map; see Figure 29) and the "Untersberg" in the south was composed of large fens and swamps, cultivation and agricultural usage were already recognizable, but extremely slight. In all probability, these areas were once mown wet meadows, which depicted a great habitat for today rare species like *G. palustris*. The pink dots represent the current occurrences of *G. palustris* in this region, in Kneisslmoor, Ziegler Wiese and in the meadows of the Esterer tavern. The other recent occurrences are not in this map excerpt. For that reason, another map has been prepared, using the "Franciszäische Kataster", a land survey register initiated by Emperor Franz I in 1830, now provided by SAGIS (Salzburger Geographisches Informationssystem). In this Figure 30, all recent populations of *Gladiolus palustris* in Salzburg are depicted on the historical map.

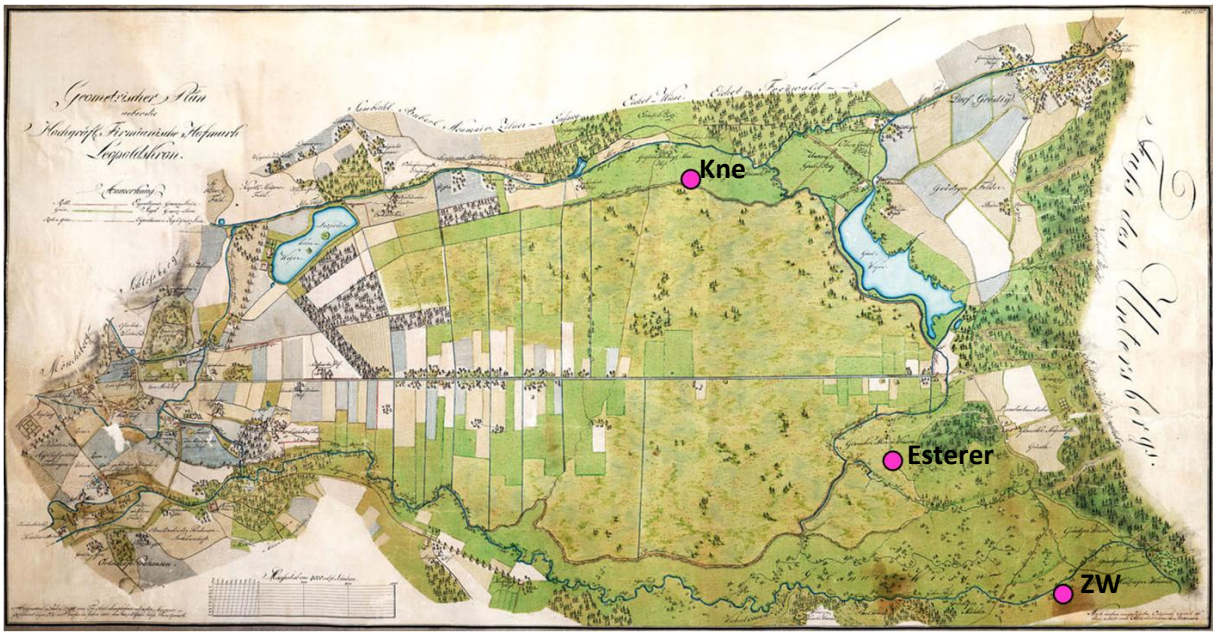


Fig. 29: Historical map excerpt of the Northern base of the Untersberg (LANGLECHNER & WEIS 1798-1803). This map is oriented to the east.

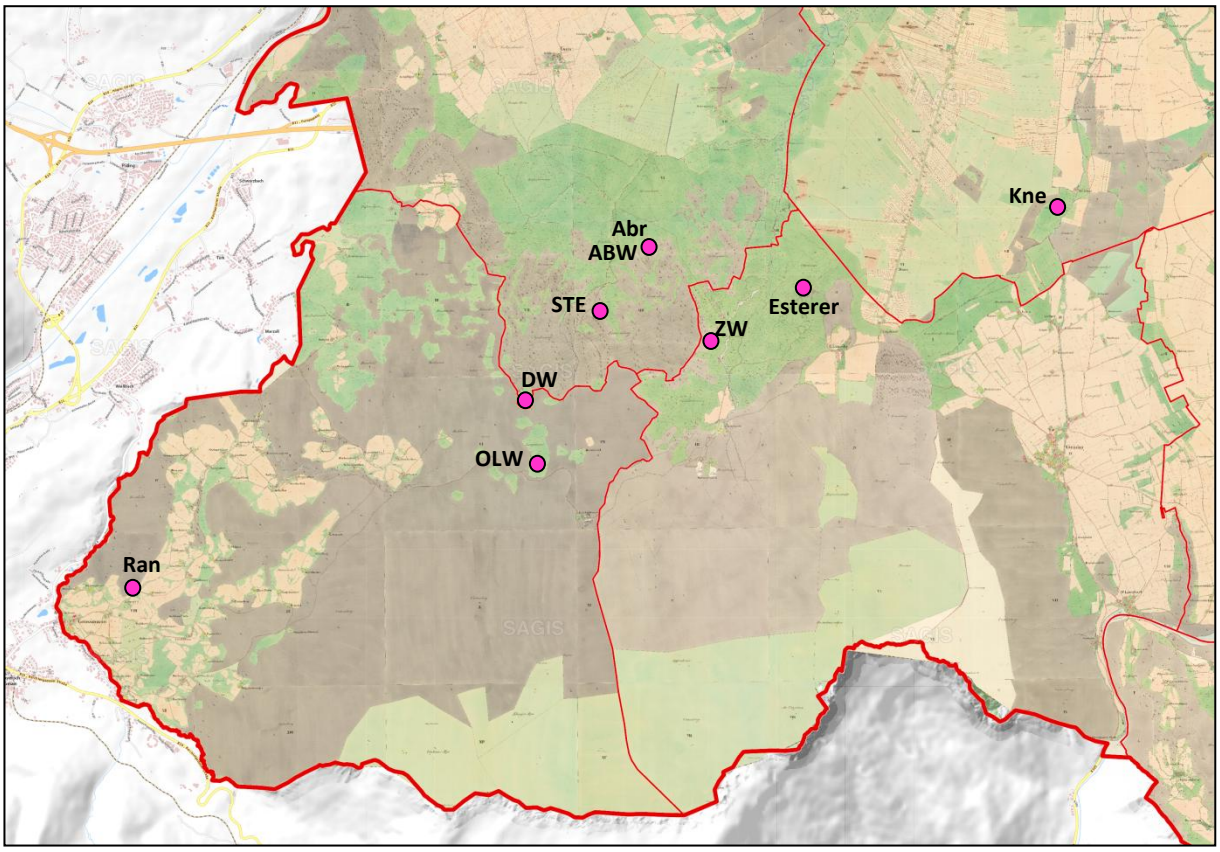


Fig. 30: Historical map excerpt of the Northern base of the Untersberg (Quelle SAGIS, Salzburger Geographisches Informationssystem). This map is oriented to the north.

According to historical literature of HINTERHUBER & HINTERHUBER (1851), *G. palustris* was occurring "on the bog meadows near Glanegg and Viehausen. SAUTER (1866) described the marsh gladiola on turf-containing meadows beyond the "Lazarettwäldchen" to Glanegg. As the "Lazarettwäldchen" is located in the city of Salzburg, Sauters' text means that the marsh gladiola was occurring in the meadows south of Salzburg till Glanegg. He also stated that the species was already very rare because the bulbs were collected as "Johanns Häupel" against wounds (SAUTER, 1866). NOWOTNY & TRÖSTER (2002) summarized additional historical literature from 1797 until 1958 of BRAUNE (1797) and HINTERHUBER & PICHELMAYR (1899), who gave hints for occurrences of the marsh gladiola. LEEDER & REITER (1958) reported about occurrences in troops in the area of the stream Glan.

Comparing current aerial photos (see Figure 31 and 32) of this region it is immediately clear that the habitat "late mown wet straw meadows" has decreased enormously in the last decades. Large sections of this area are built-up municipal areas, airport ground, motorway and intensively managed cultural fields and meadows (LEITNER et al., 2015).

Fig. 31: Current ortho-foto excerpt of the Northern base of the Untersberg (Quelle SAGIS, Salzburger Geographisches Informationssystem, 2013). This map is oriented to the east.

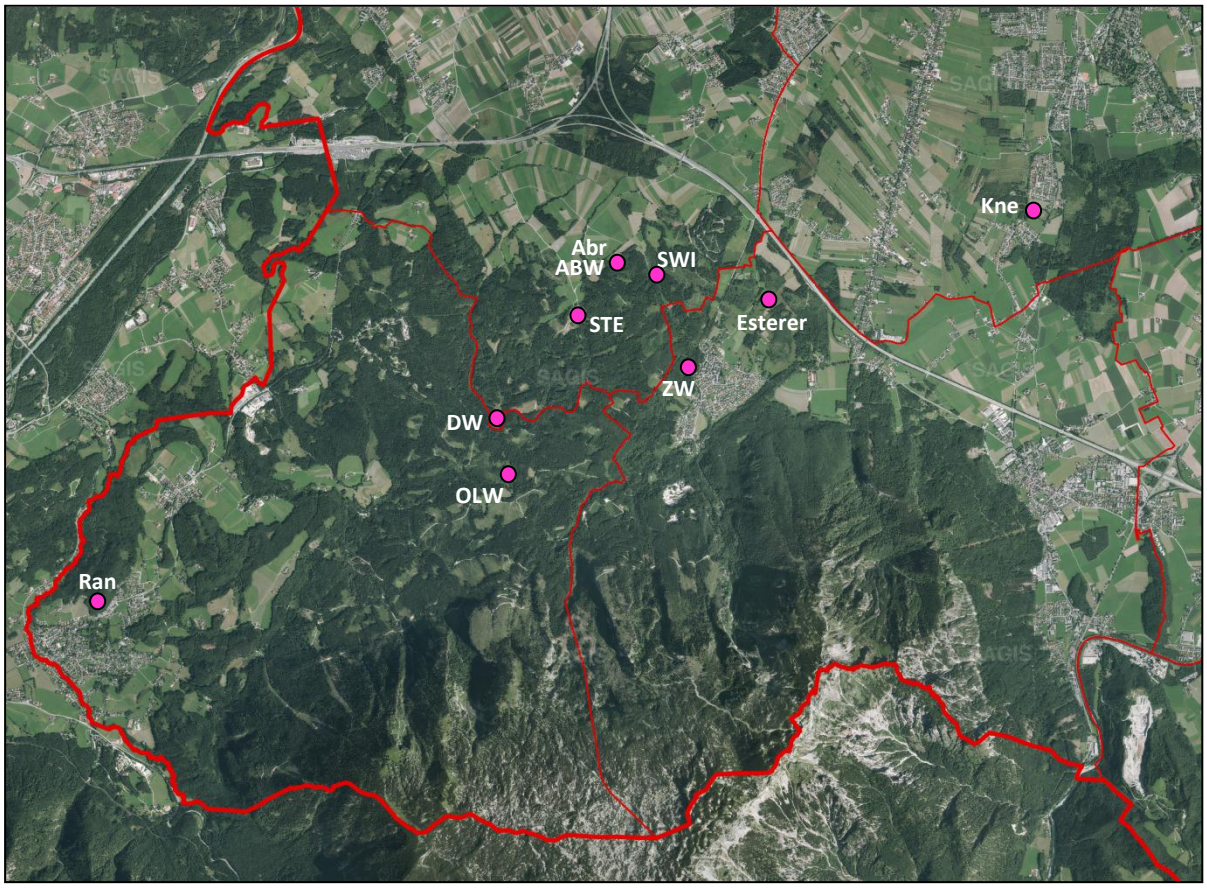


Fig. 32: Current ortho-foto excerpt of the Northern base of the Untersberg (Quelle SAGIS, Salzburger Geographisches Informationssystem, 2013). This map is oriented to the north.

NOWOTNY & TRÖSTER (2002) reported a decline in the total number of flowering specimen of the marsh gladiola from at least 1200 to 462 (year 2000) and 391 (year 2001) since the eighties of the twentieth century. Also WITTMANN (1989) reported a negative tendency and a decline in the abundance of the marsh gladiola. Simultaneously, there was a decline of the sites of *Gladiolus palustris* from 21 (year 1982) to eight in 2001 and a reduction in the growth area from 45 ha (year 1953; WEINMEISTER, 1984) to 3.5-4 ha, what equals a reduction of area by four fifths (NOWOTNY & TRÖSTER, 2002). The recent populations are mostly remnants of the former extended "Fürstenbrunner", "Walser" and "Goiser" bedding meadows (municipals Grödig and Wals-Siezenheim). In the municipal area of Großgmain, two populations were discovered 1999 by Oliver Stöhr, but one got destroyed by road construction (STÖHR, 2001; NOWOTNY & TRÖSTER, 2002).
According to NOWOTNY & TRÖSTER (2002) the loss of biotopes due to intensification of agricultural measures, afforestation or fallow lying of land after cultivation is responsible for the decrease in the stock development of the marsh gladiola. NOWOTNY & TRÖSTER (2002) guessed that the total population might already have reached a critical size, which makes the extinction of the species in the future likely. They also doubted the genetic exchange between the populations. So what are the general consequences we have to expect because of the habitat destruction of *Gladiolus palustris* and the associated decline and downsizing of populations?

4.4.2 Potential consequences of habitat loss for *Gladiolus palustris*

The expected consequences of a loss of populations and a reduction in population size are eroded genetic variability due to genetic bottlenecks, random genetic drift, interpopulation genetic divergence, increased inbreeding and reduced interpopulation gene flow (YOUNG et al., 1996; OUBORG et al., 2006; AGUILAR et al., 2008). Furthermore, a lower proportion of polymorphic loci and a reduced number of alleles per locus would be expected (AGUILAR et al., 2008). All these factors, influencing the populations over a longer period of time, can reduce the ability of populations to adapt to environmental changes and therefore drive populations towards extinction. AGUILAR et al. (2008) state several life-history traits which make species more susceptible to habitat fragmentation. According to them, short-lived species are more vulnerable to habitat fragmentation. Furthermore, generative reproduction can also be prejudicial to species suffering from habitat fragmentation, as clonally reproducing plants are able to persist at circumstances, which do not allow a completion of the usual life cycle, even during time periods with negative population growth rate (HONNAY & BOSSUYT, 2005). Furthermore, diploids are more sensitive to lose genetic diversity due to genetic drift after habitat fragmentation than autotetraploids are (AGUILAR et al., 2008). Habitat fragmentation and therefore reduced population sizes have strong negative effects on the within-population diversity of species with a self-incompatible or mainly outcrossing self-compatible mating system,

because most of their genetic variability is situated within the populations (AGUILAR et al., 2008). Therefore, they lose more alleles and polymorphic loci due to genetic bottlenecks than non-outcrossing self-compatible and selfing plants (AGUILAR et al., 2008). In addition, plants which are pollinated by animals and whose seeds are dispersed by animals are potentially more hit by habitat fragmentation than plants with abiotic pollination and seed-dispersal. This is because habitat fragmentation not only affects the plants but also the pollinators (KWAK et al., 1998). There is also a difference in rare and common species; naturally rare species are less susceptible to lose genetic diversity because of habitat fragmentation than common species (HONNAY & JACQUEMYN, 2007; AGUILAR et al., 2008). REISCH & BERNHARDT-RÖMERMANN (2014) found that common species have the highest mean gene diversities (0.20), followed by moderately common species (0.14) and lastly rare species (0.12). This is due to the fact that common species are usually less isolated than rare species and therefore have a greater amount of gene flow (REISCH & BERNHARDT-RÖMERMANN, 2014). The marsh gladiola is a perennial plant (SZCZEPANIAK et al., 2016) and should therefore be less vulnerable to habitat fragmentation, as it takes more time to elapse several generations (at least three to four years between germination and first flowering) than in short-lived species (YOUNG et al., 1996; AGUILAR et al., 2008). Furthermore, the species has a generative reproduction but can also reproduce vegetative via bulbs (SCHMITT et al., 2010). This ability may help to buffer the negative genetic effects of habitat fragmentation, because the time between generations can be delayed. *Gladiolus palustris* is tetraploid with 2n=4x=60 (SZCZEPANIAK et al., 2016) and should therefore be less vulnerable to effects of genetic drift than diploids, as polyploidy plants often exhibit greater vitality. The marsh gladiola has a mixed mating system, it has conspicuous flowers and is therefore pollinated by insects, but self-fertilization is also possible. AGUILAR et al. (2008) state that outcrossing plants usually have higher within-population genetic variation than selfing plants; the latter do have more genetic variation among populations (HAMRICK & GODT, 1989; AGUILAR et al., 2008). Habitat fragmentation followed by a decrease in the effective population size would have stronger negative effects on the within-populations genetic diversity of an outcrossing species (AGUILAR et al., 2008). The genetic bottleneck is more severe, the fewer individuals are surviving after habitat fragmentation (NEI et al., 1975; AGUILAR et al., 2008). AGUILAR et al. (2008) suggest that fragmented plant populations are suffering because of changes in their mating patterns towards increased selfing, which can lead to an increase in the inbreeding coefficient (YOUNG et al., 1996; AGUILAR et al., 2008). Furthermore, they found an indication that adult individuals in fragmented populations are more frequently mating autogamous and/or with related individuals (AGUILAR et al., 2008). Populations subjected to fragmentation over several generations often also have a reduced heterozygosity due to random genetic drift and also inbreeding (AGUILAR et al., 2008).

The marsh gladiola is insect-pollinated, mainly by bumblebees and the propagules are dispersed by wind (KNUTH, 1899; FISCHER et al., 2008) or gravity, respectively. In a fragmented landscape, the level of genetic connectivity among fragments depends on attributes of pollinators, their abundance, composition and behavior. Therefore, plants which are dependent on animals are expected to be more vulnerable to habitat fragmentation and show decreased genetic connectivity rather than abiotically pollinated and dispersed plant species (NATHAN & MULLER-LANDAU, 2000; AGUILAR et al., 2008).

Finally, species which are naturally rare are less susceptible to lose genetic diversity because of habitat fragmentation. Here, the question rises if the marsh gladiola was always a naturally rare species or was it once common and became rare? The latter coincides with the conservation status of the species as it is threatened with extinction. Also, as discussed above, historical literature revealed that *G. palustris* was more frequent and became rarer during the last centuries, although it probably never was a fairly common plant because of its restriction to very particular habitats. Species which are generally having a narrow geographical range, particular habitats or small populations tend to be less diverse than widespread common species (HAMRICK & GODT, 1989; ELLSTRAND & ELAM, 1993; AGUILAR et al., 2008). Consequently, common species are more hit by habitat fragmentation and the loss of genetic diversity due to genetic bottlenecks.

We classify the marsh gladiola to a once more common and now really rare plant by cause of anthropogenic activity in recent ecological times. Therefore, according to literature, it is expected to undergo stronger effects because of the habitat fragmentation on its genetic diversity than a naturally rare species (AGUILAR et al., 2008). Furthermore, AGUILAR et al. (2008) indicates that even more common species are at a potential risk in consequence of habitat fragmentation and they suggest that the emphasis of nature conservation should not only focus on rare species but also on common plants.

Habitat fragmentation is a complex procedure with numerous factors simultaneously involved contributing to a reduced genetic diversity (OUBORG et al., 2006; AGUILAR et al., 2008). Of course, population size is an important factor. Even more substantial is the degree of population isolation and the quality and characteristics of the surrounding areas, which enable gene flow among fragmented populations (AGUILAR et al., 2008). The presence and amount of gene flow among residual populations plays an important role by determining the genetic consequences of habitat fragmentation, because even moderate or relatively low levels of gene flow can ease the loss of genetic diversity due to preventing genetic drift (SORK et al., 1999; AGUILAR et al., 2008). Another important factor is the time and the number of generations under the fragmented conditions. AGUILAR et al. (2008) summarized that species which occurred in more than 100-year-old fragmented systems showed significantly stronger negative effects on genetic diversity than in younger

fragmented areas. In the following chapter, implications for the conservation of this conspicuous species are summarized.

4.4.3 Implications for conservation of *Gladiolus palustris*

Ecological, demographic and genetic factors are interacting by influencing a species' risk of becoming extinct (AGUILAR et al., 2008). It is necessary to protect and conserve diversity on all relevant levels, namely on ecosystem diversity, species diversity and also genetic diversity level. Several disciplines as population genetics, landscape ecology and plant-animal interaction ecology need to be merged to sustainably maintain species and their evolutionary potential in a continually changing anthropogenic landscape (AGUILAR et al., 2008). *Gladiolus palustris* is very vulnerable to anthropogenic interventions, the main threat is the change and the loss of its habitat due to changes in agricultural land use. NOWOTNY (2012) summarized the contributing factors: Agricultural intensification (eutrophication, forward bringing of the mowing date and enhancement of the mowing frequency), drainage, abandonment of agricultural land, scrub encroachment and reforestation. The habitats of the recent occurrences of the marsh gladiola in Salzburg are protected by the habitat protection § 24 of the Salzburger Naturschutzgesetz of 1999 and most of them are situated in the landscape-and plant reserve Untersberg (Wittmann, 1989; Nowotny, 2000). Also, the species itself is fully protected in Salzburg and also FFH-protected. Nevertheless, the landowner is not obligated to manage his land - so the gladiola cannot be saved from the abandonment of agricultural land; an appropriate management is not guaranteed because of the conservation status.

NOWOTNY (2012) states that for a sustainable conservation of the species it is important to protect the remnant populations and their habitats and to support the dispersal to former and potential sites. For the preservation of the species it would be important to gather all recent and former occurrences of the marsh gladiola, as well as potential suitable habitats. Furthermore, the current populations should be monitored, counted annually and their development observed as well as their endangerment assessed. The preservation of the main habitat of the species, the extensively managed purple moor grass bedding meadows is of great importance for the continued existence of the species, as it is dependent on the extensive management. The mowing of the species should not take place before September, because seed maturation and also the displacement of nutrients to the bulb lasts up to six weeks after flowering (RIEGEL, 2010).

To implement the preservation of these habitats, several tools and management strategies are available (NOWOTNY & TRÖSTER, 2002). Firstly, in Salzburg there is the possibility of a nature conservation contract, which grants the landowner money, if his land is extensively managed with a late-autumn mowing and buffer zones to adjacent intensive grasslands. Furthermore, many populations are preserved only because of voluntary work, as for example through the association

for ecological conservation “HALM” (NOWOTNY, 2012). Also, the ex-situ-cultivation of the species and resettlement at former or potential sites contributes to the maintenance of the species. Currently, “HALM” is cultivating *Gladiolus palustris* for later resettlement in the Botanical Garden of the University of Salzburg (Loiperdinger, 2014; SCHMALL et al., 2015). It may be antvantageous to introduce individuals of further distant populations, for example of Königsbrunner Heide in Swabia to increase the genetic diversity of the populations.

SCHMIDT & JENSEN (2000) stated that for *Pedicularis palustris* not only large populations should be protected, but also the small populations because of the high genetic differentiation among the populations. *Gladiolus palustris* has an extremely low overall diversity; many individuals of different populations are sharing the same AFLP genotype, suggesting that the extinction of a single small population would not considerably alter the overall genetic variation. Nevertheless, all sites of the marsh gladiola should be protected as they depict valuable habitats and can be considered as an indicator of habitats of a good quality. Thus, the preservation and proper management of the habitats accrues not only the marsh gladiola but also other threatened and rare species like for example *Iris sibirica, Gentiana pneumonanthe, Dianthus superbus* ssp*. superbus*. (NOWOTNY & TRÖSTER, 2002).

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7 Appendix

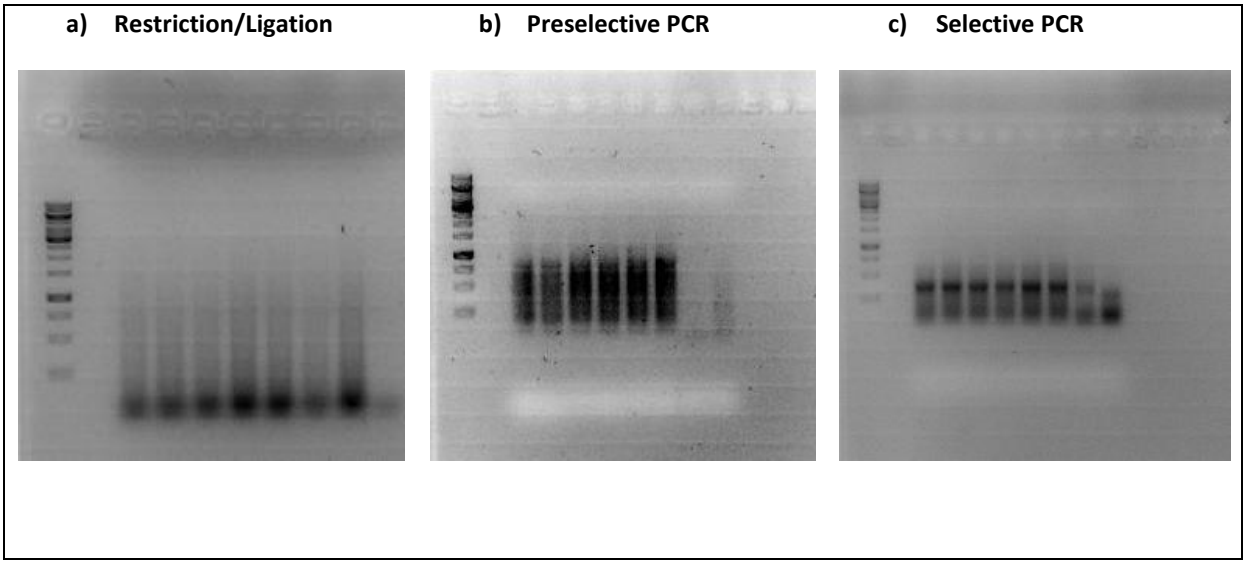
Appendix Table 1: Example of half of a 96-well PCR plate. The yellow sample represents the "between-plates-replicate"; the orange samples are the "within-plate-replicate". The meaning of the abbreviations can be seen in Table 3.

A	EWr_05	EMr_03	EJa_01	EJa_09	EOE_02	EOE_10
B	EWr_06	EMr_04	EJa_02	EJa_10	EOE_03	STE_00
C	EWr_07	EMr_05	EJa_03	EEF_01	EOE_04	DWl_01
D	EWr_08	EMr_06	EJa_04	EEF_02	EOE_05	DWl_02
E	EWr_09	EMr_07	EJa_05	EEF_03	EOE_06	DWl_03
F	EWr_10	EMr_08	EJa_06	EEF_04	EOE_07	EMr_08_Rep
G	EMr_01	EMr_09	EJa_07	EEF_05	EOE_08	ZWu_03_Rep5
H	EMr_02	EMr_10	EJa_08	EOE_01	EOE_09	Neg_04
	1	**2**	**3**	**4**	**5**	**6**

Appendix Table 2: Reaction mix for restriction and ligation for one DNA sample

Reagent	**Supplier**	**Volume [µl]**
Double distilled water	-	0.83
10x T4 Ligase buffer	Promega, Madison, WI USA	1.10
0.5M NaCl	-	1.10
BSA (1mg/ml)	Fermentas	0.55
MseI adaptor (50µM)	MWG Biotech AG	1.00
EcoRI adaptor (5µM)	MWG Biotech AG	1.00
MseI enzyme (50u/µl)	New England Biolabs ®Inc.	0.02
EcoRI enzyme (80u/ µl)	Promega, Madison, WI USA	0.10
T4 DNA Ligase (3u/µl)	Promega, Madison, WI USA	0.30
Total		**6.00**

Appendix Figure 1: Gel pictures of restriction and ligation (a), preselective (b) and selective (c) PCR. The smear in a) emerges because of DNA fragments cut into different lengths. The eight slot contains the negative control.



Appendix Table 3: Reaction mix for the preselective PCR for one DNA sample

Reagent	**Supplier**	**Volume [µl]**
Double distilled water	-	4.95
5x Green GoTaq Buffer	Promega, Madison, WI USA	2.00
10mM dNTP	Promega, Madison, WI USA	0.22
EcoRI + A primer (10microM)	??	0.29
MseL + C primer (10microM)	??	0.29
GoTaq G2 dilution (1:10)	??	0.25
Total		**8.00**

Appendix Table 4: Tested primer combinations for selective PCR

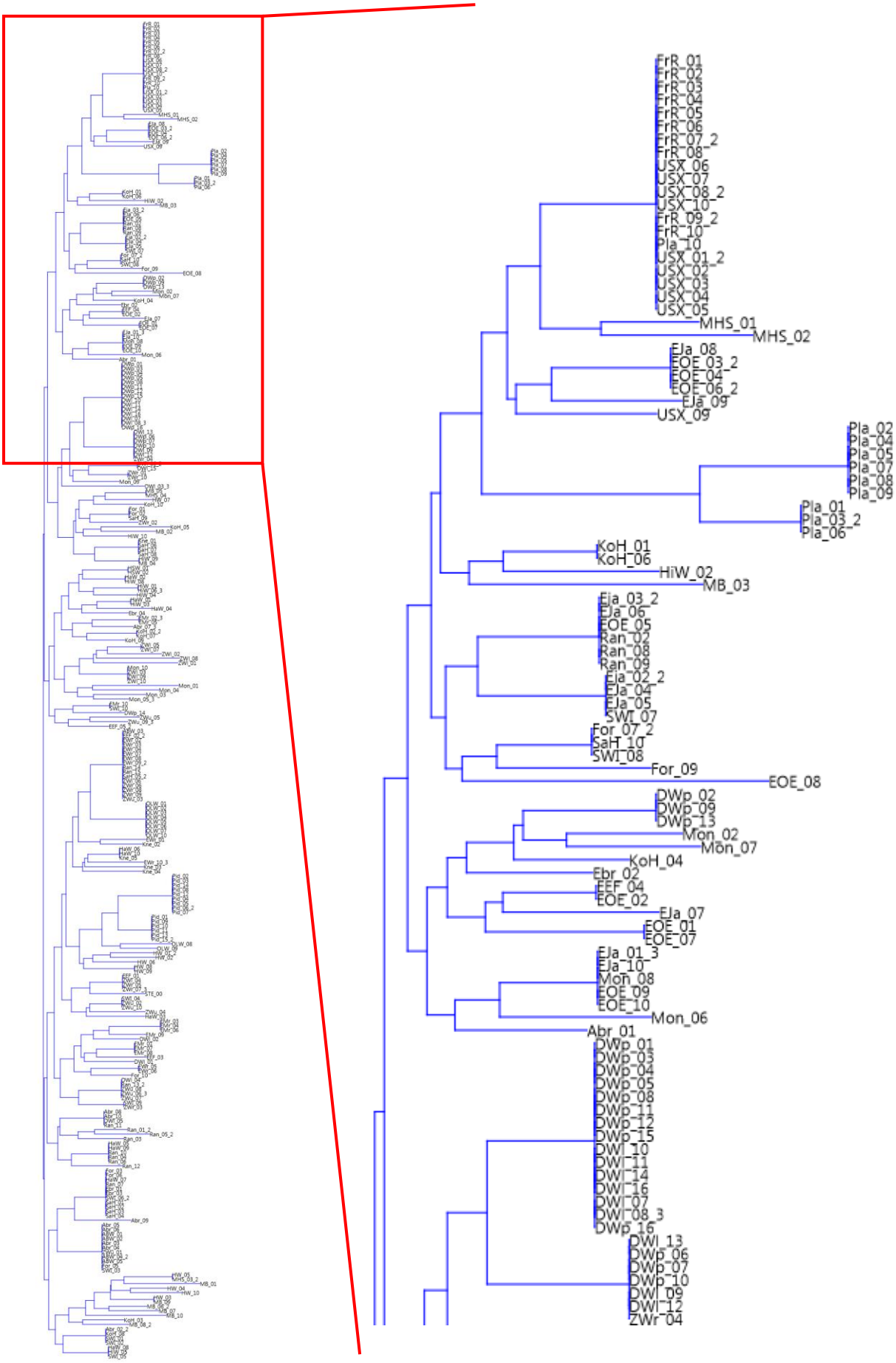
Primer number	**Primer name**	**DNA sequence**
FAM1	EcoRI+ACA	5'-GACTGCGTACCAATTCACA-3'
	MseI+CAC	5'-GATGAGTCCTGAGTAACAC-3'
FAM2	EcoRI+ACT	5'-GACTGCGTACCAATTCACT-3'
	MseI+CAT	5'-GATGAGTCCTGAGTAACAT-3'
FAM3	EcoRI+AGA	5'-GACTGCGTACCAATTCAGA-3'
	MseI+CTC	5'-GATGAGTCCTGAGTAACTC-3'
FAM4	EcoRI+ACA	5'-GACTGCGTACCAATTCACA-3'
	MseI+CC	5'-GATGAGTCCTGAGTAACC-3'
FAM5	EcoRI+ACT	5'-GACTGCGTACCAATTCACT-3'
	MseI+CA	5'-GATGAGTCCTGAGTAACA-3'
FAM6	EcoRI+AGA	5'-GACTGCGTACCAATTCAGA-3'
	MseI+CG	5'-GATGAGTCCTGAGTAACG-3'
VIC1	EcoRI+AGG	5'-GACTGCGTACCAATTCAGG-3'
	MseI+CTC	5'-GATGAGTCCTGAGTAACTC-3'
VIC2	EcoRI+AAG	5'-GACTGCGTACCAATTCAAG-3'
	MseI+CTT	5'-GATGAGTCCTGAGTAACTT-3'
VIC3	EcoRI+AGG	5'-GACTGCGTACCAATTCAGG-3'
	MseI+CAA	5'-GATGAGTCCTGAGTAACAA-3'
VIC4	EcoRI+AGG	5'-GACTGCGTACCAATTCAGG-3'
	MseI+CT	5'-GATGAGTCCTGAGTAACT-3'
VIC5	EcoRI+AAG	5'-GACTGCGTACCAATTCAAG-3'
	MseI+CT	5'-GATGAGTCCTGAGTAACT-3'
VIC6	EcoRI+ACC	5'-GACTGCGTACCAATTCACC-3'
	MseI+CG	5'-GATGAGTCCTGAGTAACG-3'
NED1	EcoRI+AAC	5'-GACTGCGTACCAATTCAAC-3'
	MseI+CTT	5'-GATGAGTCCTGAGTAACTT-3'
NED2	EcoRI+AGC	5'-GACTGCGTACCAATTCAGC-3'
	MseI+CTG	5'-GATGAGTCCTGAGTAACTG-3'
NED3	EcoRI+ACC	5'-GACTGCGTACCAATTCACC-3'
	MseI+CAG	5'-GATGAGTCCTGAGTAACAG-3'
NED4	EcoRI+AAC	5'-GACTGCGTACCAATTCAAC-3'
	MseI+CT	5'-GATGAGTCCTGAGTAACT-3'
NED5	EcoRI+AGC	5'-GACTGCGTACCAATTCAGC-3'
	MseI+CC	5'-GATGAGTCCTGAGTAACC-3'
NED6	EcoRI+ACC	5'-GACTGCGTACCAATTCACC-3'
	MseI+CG	5'-GATGAGTCCTGAGTAACG-3'

Appendix Table 5: Reaction mix for the selective PCR for one DNA sample for one color

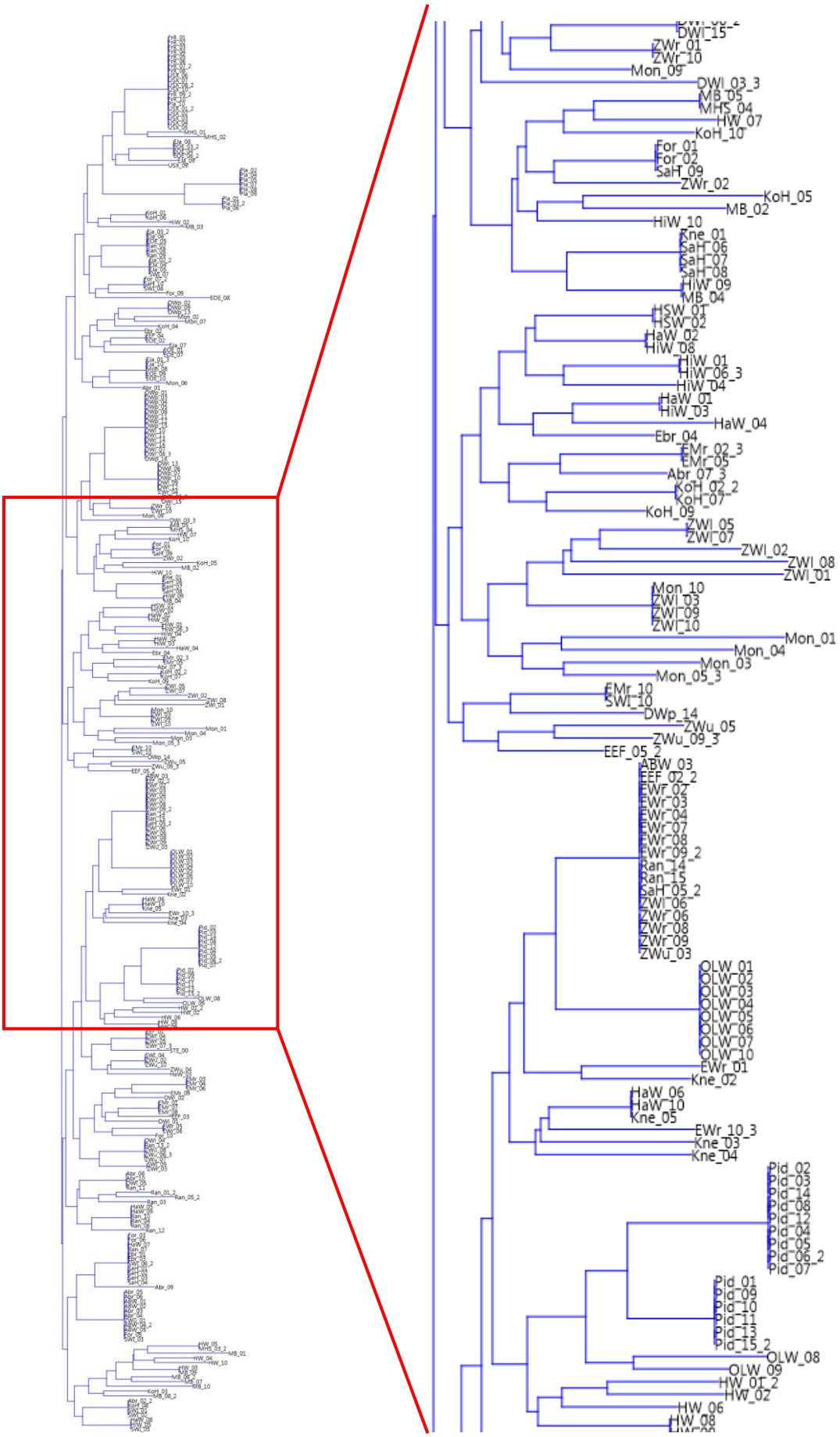
Reagent	**Supplier**	**Volume [µl]**
Double distilled water	-	5.04
5x Green GoTaq Buffer	Promega, Madison, WI USA	2.00
10mM dNTP	Promega, Madison, WI USA	0.22
EcoRI primer (labeled! 10microM)	MWG-Biotech AG	0.27
MseL primer (10microM)	MWG-Biotech AG	0.27
GoTaq G2 dilution (1:5)	??	0.20
Total		**8.00**

Appendix Table 6: Color separation matrix used for the program DAx

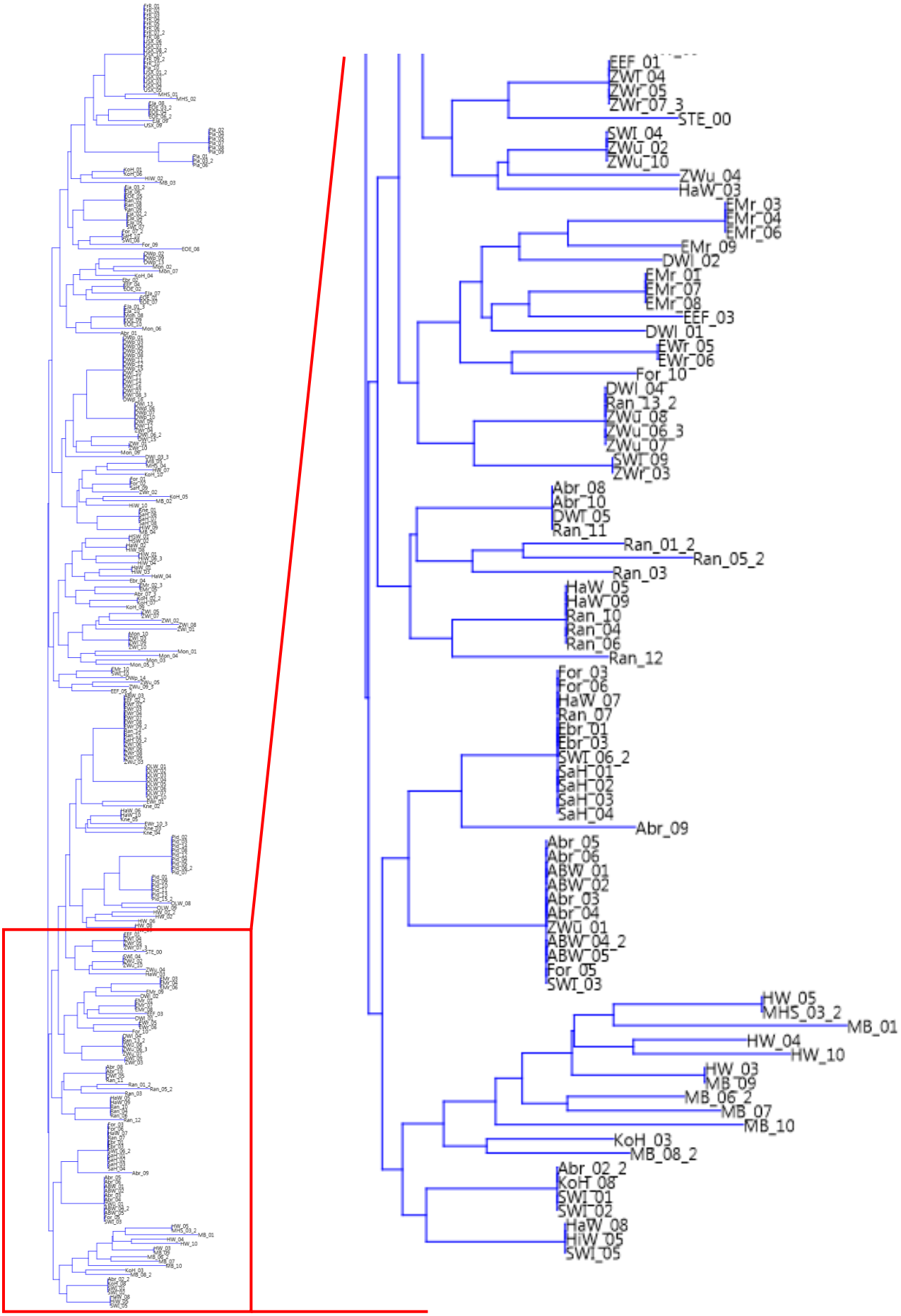
1.0000	0.0687	0.1256	0.0629
0.1034	1.0000	0.0009	0.0169
0.6104	0.0283	1.0000	0.5081
0.0069	0.0519	0.4296	1.0000



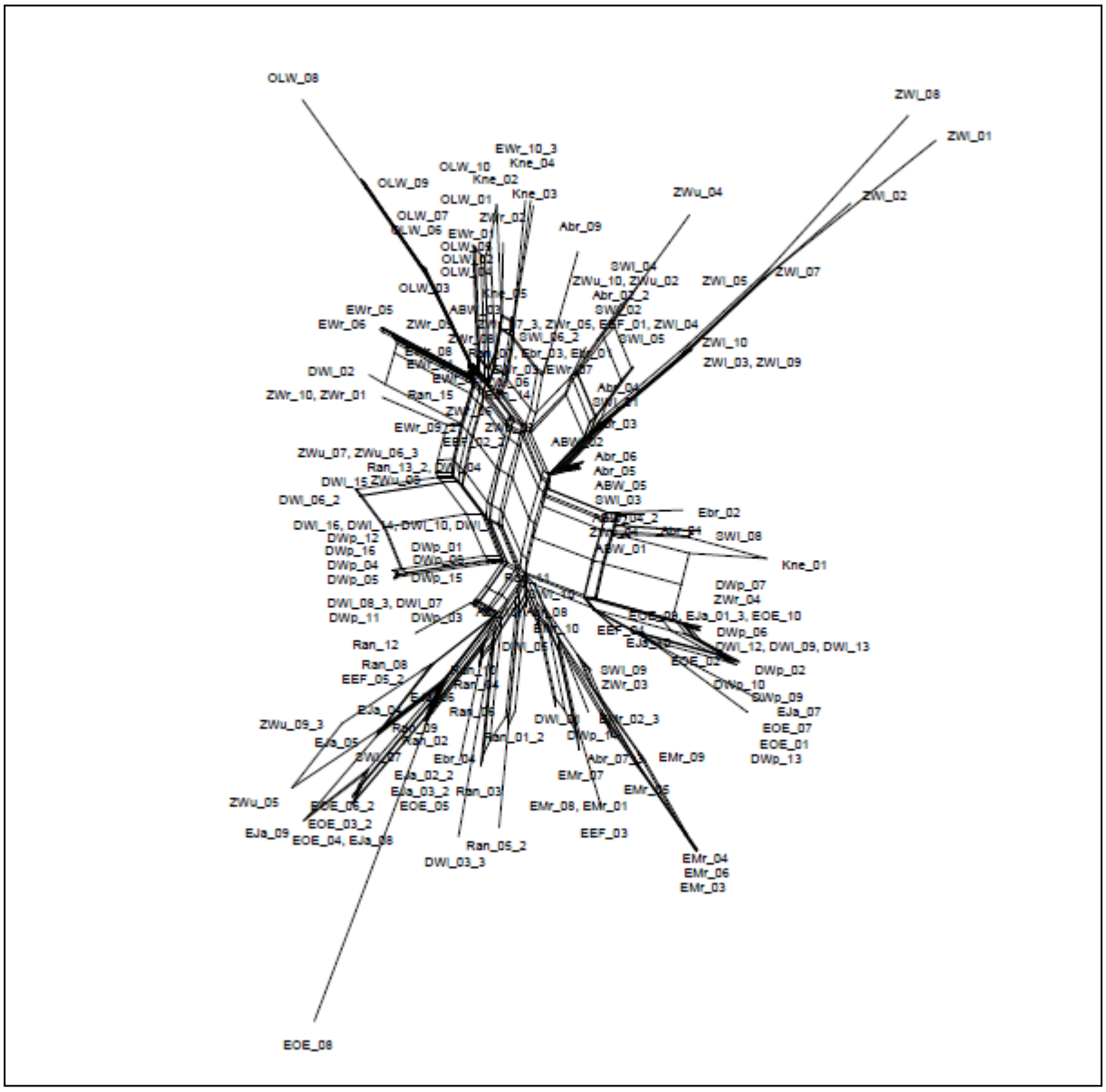
Appendix Figure 2.a): Neighbor-Joining (NJ) clustering of *G. palustris* to reveal identical genotypes



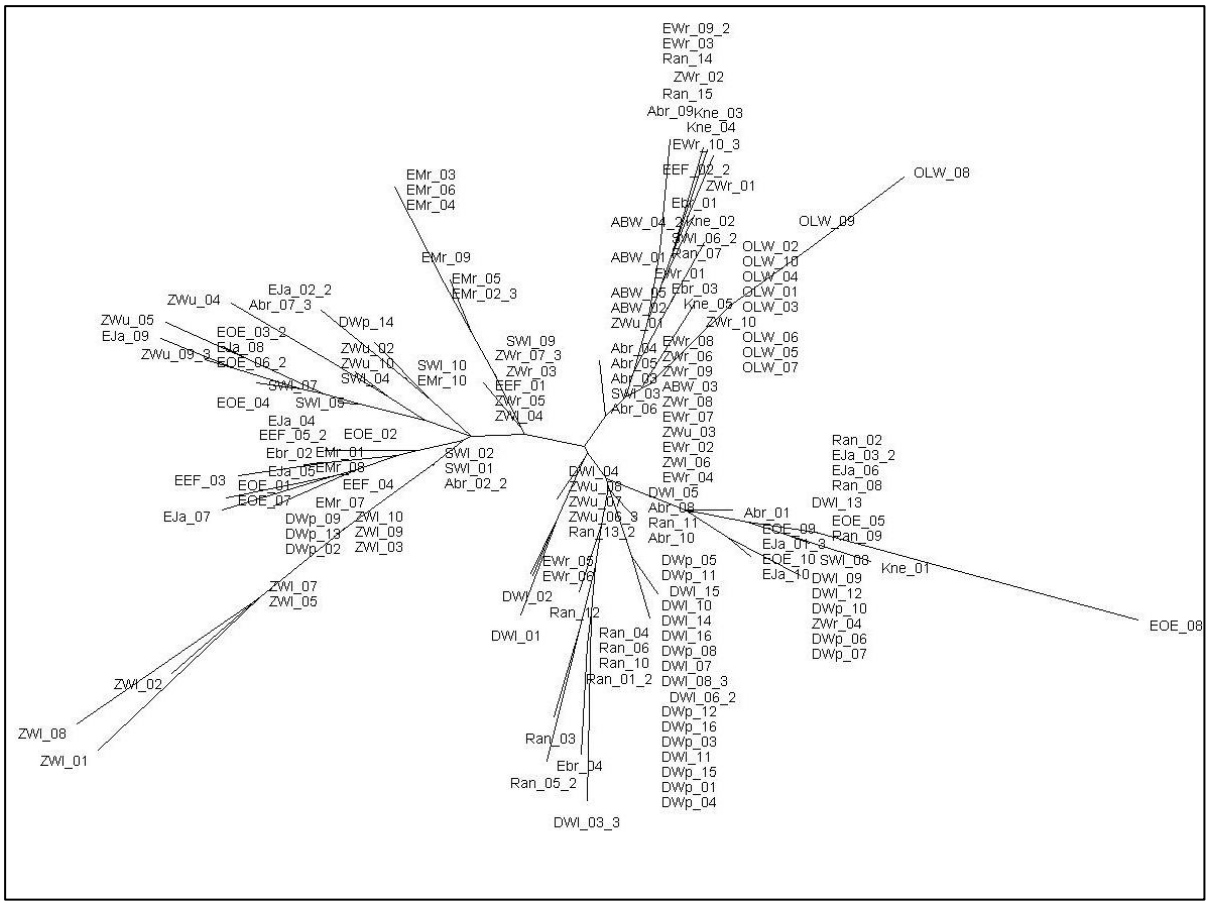
Appendix Figure 2.b): Neighbor-Joining (NJ) clustering of *G. palustris* to reveal identical genotypes



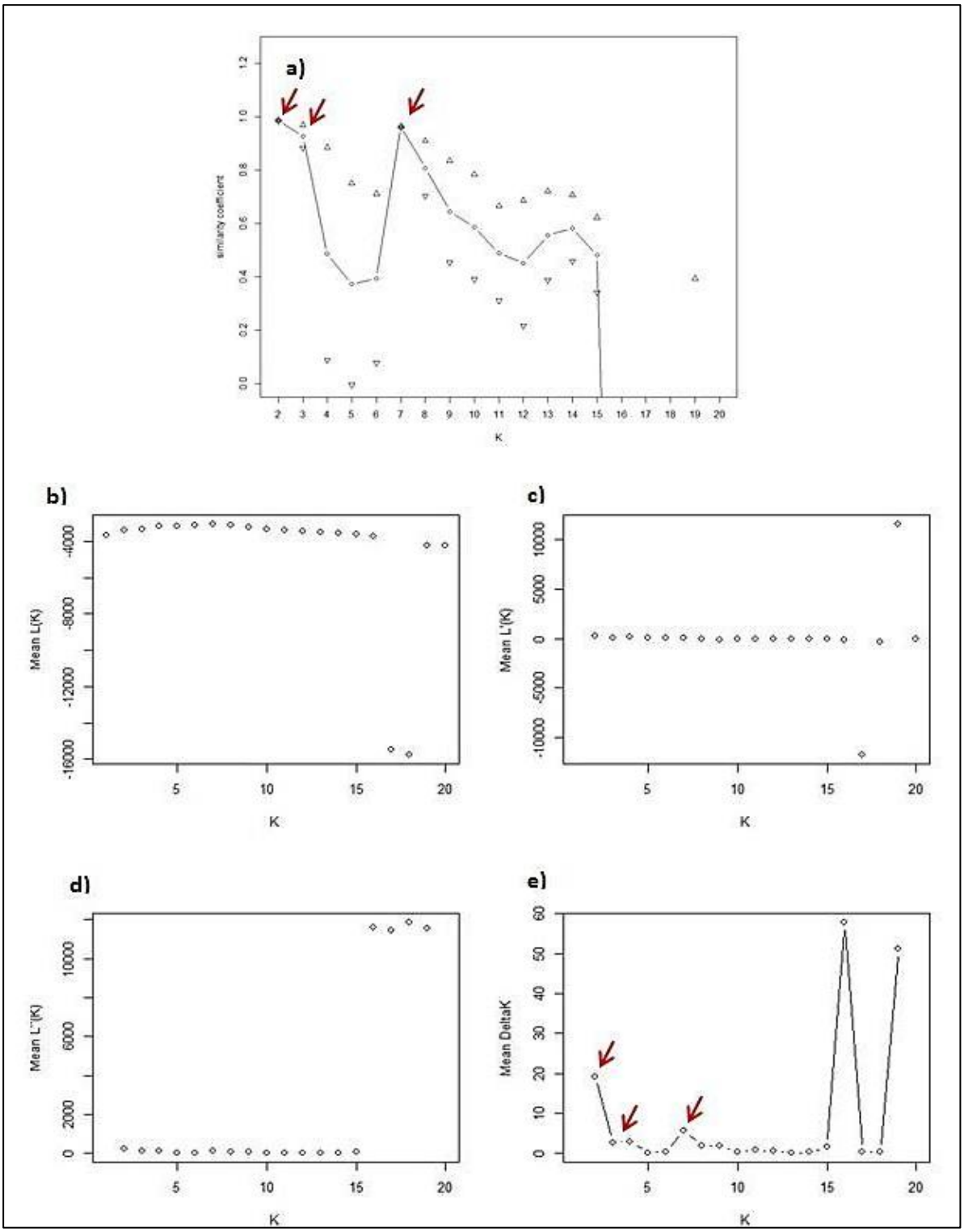
Appendix Figure 2.c): Neighbor-Joining (NJ) clustering of *G. palustris* to reveal identical genotypes



Appendix Figure 3: 10: Individual-based NeighborNet of *G. palustris* populations of Salzburg. Bootstraps below 70% are not interpreted and therefore not depicted in the figure.



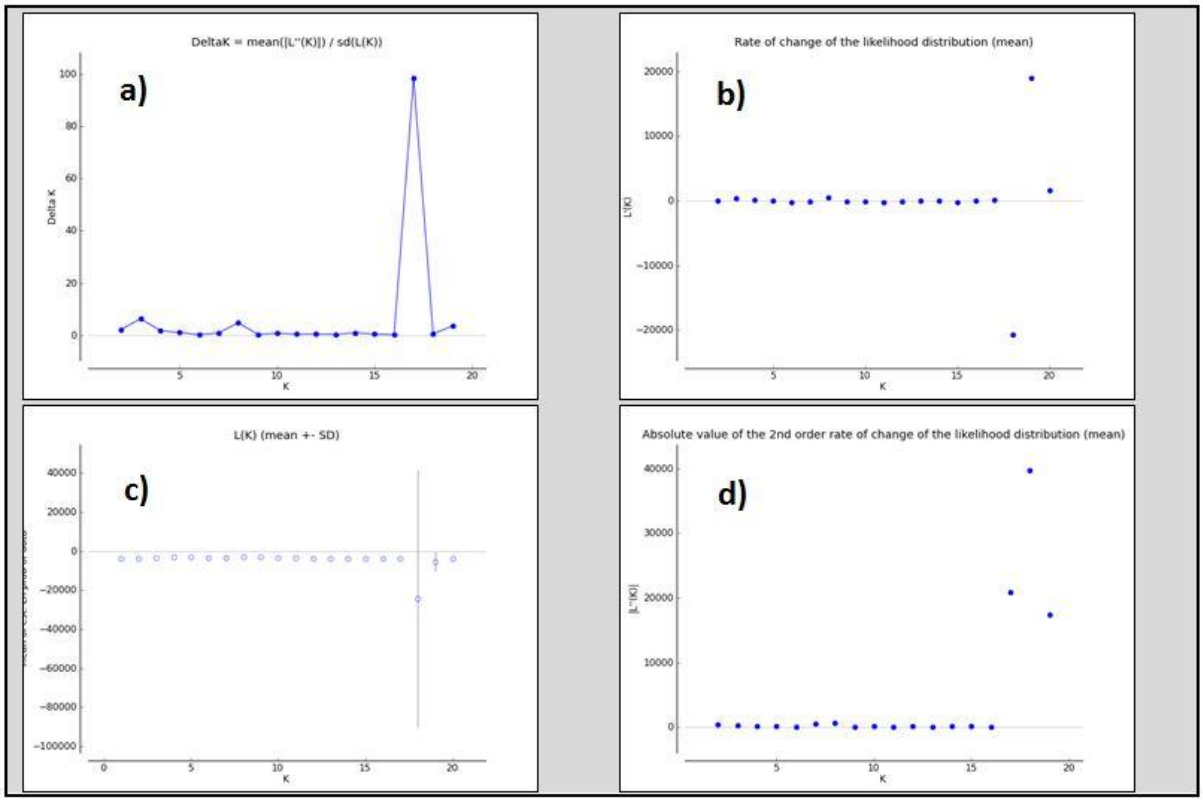
Appendix Figure 4: Individual-based Neighbor Joining of *G. palustris* populations of Salzburg. Bootstrap values below 70% are not interpreted and therefore not depicted in the figure.



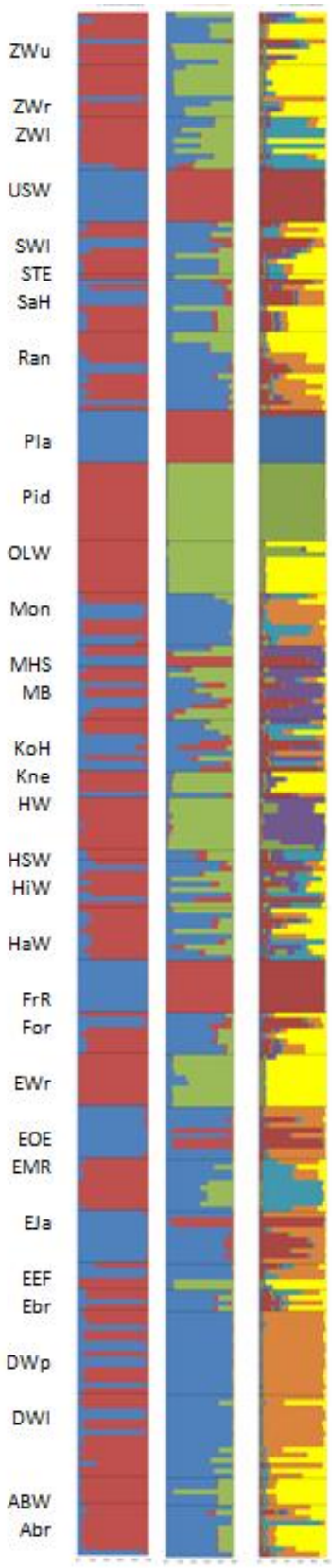
Appendix Figure 5: Results of the STRUCTURE analysis of the admixture model with uncorrelated allele frequencies investigated with the R-script STRUCTURE-SUM (EHRICH 2009, unpublished). a) The similarity coefficients are plotted against K. The circles indicate the mean values of the coefficients and the triangles the standard deviation. The similarity coefficients show peaks at meaningful values of K (marked with arrow). b) The mean L(K) is plotted against the number of groups (K). c) The mean L'(K) is plotted against the number of groups (K). d) The mean L''(K) is plotted against the number of groups (K). e) The mean DeltaK is plotted against the number of groups (K).

Appendix Table 7: Result of the Evanno method computed with the STRUCTURE HARVESTER of the STRUCTURE run with the admixture model and correlated allele frequencies.

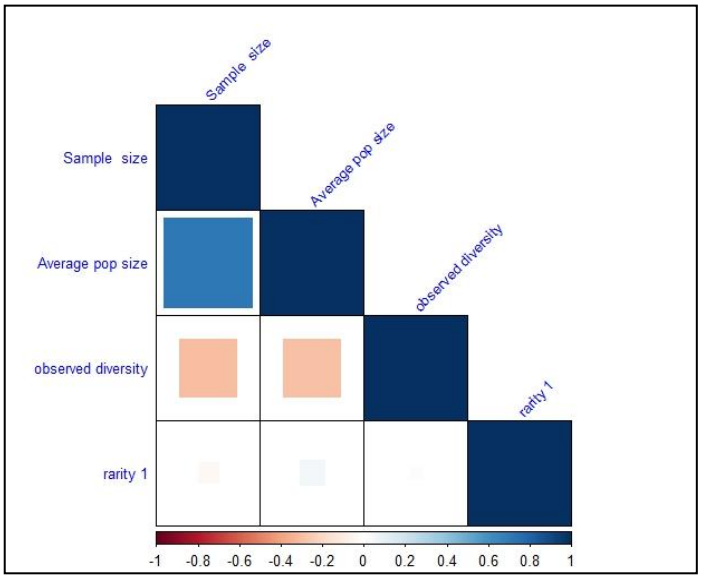
K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-3684.4900	8.8202	-	-	-
2	10	-3661.9200	173.7021	22.570000	381.720000	2.197556
3	10	-3257.6300	39.7042	404.290000	252.340000	6.355501
4	10	-3105.6800	103.5549	151.950000	186.710000	1.803005
5	10	-3140.4400	163.4351	-34.760000	170.070000	1.040596
6	10	-3345.2700	336.0685	-204.830000	84.430000	0.251229
7	10	-3465.6700	700.2158	-120.400000	614.730000	0.877915
8	10	-2971.3400	137.1766	494.330000	655.610000	4.779312
9	10	-3132.6200	156.2091	-161.280000	60.970000	0.390310
10	10	-3232.9300	177.0739	-100.310000	133.670000	0.754883
11	10	-3466.9100	139.9501	-233.980000	67.040000	0.479028
12	10	-3633.8500	238.1674	-166.940000	129.670000	0.544449
13	10	-3671.1200	208.6483	-37.270000	79.880000	0.382845
14	10	-3628.5100	234.1080	42.610000	226.150000	0.966007
15	10	-3812.0500	425.1818	-183.540000	205.390000	0.483064
16	10	-3790.2000	337.7277	21.850000	102.730000	0.304180
17	10	-3665.6200	211.9110	124.580000	20841.180000	98.348723
18	10	-24382.2200	65761.6067	-20716.60000	39710.480000	0.603855
19	10	-5388.3400	4921.6305	18993.880000	17406.910000	3.536818
20	10	-3801.3700	312.1971	1586.970000	-	-



Appendix Figure 6: Evanno plots computed with the STRUCTURE HARVESTER of the STRUCTURE run with the admixture model and correlated allele frequency model. Plot a) mean values of DeltaK; plot b) rate of change of the likelihood distribution; plot c) mean likelihood L(K) and variance per K value (mean ± SD) and plot d) absolute value of the 2nd order rate of change of the likelihood distribution.



Appendix Figure 7: Results of the genetic structure of the complete dataset (295 individuals, 32 populations) as indicated by STRUCTURE via a Bayesian clustering analysis using the admixture model with correlated allele frequencies. The results are depicted for K=2, 3 and 7 for the admixture model with correlated allele frequencies. For each K, the run with the highest likelihood is shown. The populations are separated in blocks according to their geografic distribution.



Appendix Figure 8: Results of the correlation analysis between sample size and average popzulation size to genetic diversity and rarity of the Salzburg populations of *Gladiolus palustris.*

Appendix Table 8: Results of the correlation analysis.
** = highly significant

	Sample size	Average pop. size	observed diversity	rarity
Sample size	1.00			
Average pop. size	0.72 **	1.00		
observed diversity	-0.30	-0.30	1.00	
rarity	-0.04	0.06	0.01	1.00

Eidesstattliche Erklärung

Ich erkläre hiermit eidesstattlich [durch meine eigenhändige Unterschrift], dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Alle Stellen, die wörtlich oder inhaltlich den angegebenen Quellen entnommen wurden, sind als solche kenntlich gemacht.
Die vorliegende Arbeit wurde bisher in gleicher oder ähnlicher Form noch nicht als Bachelor-/ Master-/ Diplomarbeit/ Dissertation eingereicht.

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Datum, Unterschrift