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Master Thesis

Prospects and Limitations of a Genetic Diversity Monitoring in Nature Conservation

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The Marbled White (*Melanargia galathea*). © Sabine Brodbeck, WSL

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General Introduction

Genetic diversity is the variation of heritable information that is stored as DNA within living cells and is one major level of biodiversity. During recent decades, it has been shown that genetic diversity has a direct impact on all levels of an ecosystem, including the fitness of individuals, the resistance of populations to biotic and abiotic changes and even on large scale element cycles. Several studies detected a positive correlation between heterozygosity and the fitness of individuals, known as the heterozygosity-fitness correlation (HFC, Chapman et al. 2009, David 1998). In diploid organisms, the heritable information is stored in two sets of DNA. Although these two sets are homologous to each other, they do not necessarily contain identical DNA sequences. Heterozygosity describes the proportion of analyzed locations (loci) in the genome that exhibit two different variants (alleles). Therefore, the HFC states that a high proportion of heterozygous loci increases the fitness of an individual. For instance, Olano-Marin et al. (2011) studied a population of blue tit (*Cyanistes caeruleus*) and detected a positive effect of heterozygosity on clutch size, the number of eggs sired by males and the number of recruits produced by males and females.

Beside this effect on the fitness of single individuals, effects at the population level has been detected. Reusch et al. (2005) and Hughes & Stachowicz (2004) measured an increased resistance towards heat stress and faster recovery after grazing in seagrass (*Zostera marina*) when multiple genotypes were present within a population compared to only single genotypes. Honey bee (*Apis* sp.) colonies with multiple patrilines were able to maintain more stable brood nest temperatures compared to colonies with a single patriline (Jones et al. 2004). In agriculture, common rice (*Oryza sativa*) has been shown to be less susceptible to blast disease (*Magnaporthe grisea*) when multiple genotypes has been planted within the same field (Zhu et al. 2000). From the opposite point of view, mixed genotype infections of a trematode parasite have been shown to have higher infection success in fish compared to single genotype infections (Karvonen et al. 2012). Genetic diversity even shapes social systems. The invasive Argentine ant (*Linepithema humile*) has formed supercolonies as result of genetic uniformity, which has not been observed before in the native range and is one explanation for the successful colonization of California, USA (Tsutsui et al. 2003). Genetic diversity is also of relevance at the community level. The loss of species diversity of long-lived herbs was reduced when multiple genotypes of the respective species were present within the community (Booth & Grime 2003). The abundance and species diversity in adjacent trophic levels have been influenced by intraspecific genetic diversity when epifaunal species were more abundant in genetically rich populations of seagrass (Reusch et al. 2005) and higher species diversities of rice pathogens were observed in rice fields of multiple genotypes (Zhu et al. 2000). In a study by Crutsinger et al. (2008), the occurrence of multiple genotypes in the tall goldenrod (*Solidago altissima*) was connected with a higher resistance towards invasions by other plant species. Genetic diversity provides not only multiple possibilities to react on change but can act as driver of element cycles. Litter decay in a mixture of five *Populus* genotypes (Salicaceae) was faster than would have been expected from the single genotype decay rates and is therefore driven by genetic diversity (Schweitzer et al. 2005). Furthermore, genetic diversity is one precondition for speciation (Reed & Frankham 2003, Fisher 1930) and correlates with organismal complexity (Lynch & Conery 2003).

Although the processes behind these detected relationships are diverse and also negative consequences for genetically diverse populations have been detected (de Roode et al. 2005), efforts in nature conservation as well as of breeding programs in agriculture generally aim to preserve and support high levels of genetic diversity. As a consequence of ratifying the "Convention on Biological Diversity" in 1993, the Federal Office for the Environment in Switzerland has launched the Biodiversity Monitoring (BDM) to measure the recent state of biological diversity as well as changes over time. The BDM comprises several monitoring programs, which aim to quantify, among others, the diversity of species within habitats (indicator Z9) and landscapes (indicator Z7). Within this scope, monitoring of genetic diversity is conducted for livestock breeds and crop plant varieties (indicator Z1, indicator Z2). In contrast, the monitoring of genetic diversity for feral species has not been conducted by now. One important reason for this gap within the BDM is of financial character. Genetic analyses are expensive and continuing monitoring of several species across Switzerland was unaffordable at the beginning of the program. However, due to recent technological advances, the costs have decreased rapidly within the last decade and the monitoring of genetic diversity might become affordable in the near future.

With the aim to monitor genetic diversity, it is important to distinguish between two different types of diversity (Holderegger et al. 2006). Adaptive genetic diversity describes diversity driven by selection. Differences in the nucleotide sequence not only change the expression of a protein, but can have a direct effect on fitness. To monitor adaptive genetic diversity, it would be necessary to sequence a large part of the genome of many individuals from several populations within a given species, find the coding regions of genes relevant for selection and accumulate knowledge about the conditions under which these genes or alleles are favorable (Hansen et al. 2012). Although progress has been made concerning the identification of adaptive genes in the last decade, and costs for DNA sequencing have decreased by several orders of magnitudes, the knowledge is mainly restricted to a few model organisms and in general is still scarce. A large-scale monitoring of adaptive genetic diversity is therefore not realistic within the near future. The second type of genetic diversity is called neutral genetic diversity and stands for genetic diversity that is not linked to selection (Kimura 1984) and has no effect on fitness. This kind of genetic diversity is much easier to measure and quantify, but the link to fitness, adaptation and the evolutionary potential of a species is rather indirect. Several empirical studies detected a positive correlation between heterozygosity and fitness of populations (HFC), also for heterozygosity at neutral loci (Olano-Marin et al. 2011). In the latter case, HFC might refer to the fitness cost of homozygosity caused by deleterious recessive alleles that is resulting in the nonrandom association of diploid genotypes in zygotes (general effect hypothesis, Chapman et al. 2009). Neutral genetic markers should enable to detect reduced heterozygosity with negative consequences for fitness, to distinguish between single and multiple genotype populations and detect major differences in genetic diversity on the population level. Nevertheless, adaptive genetic diversity is only measured indirectly and high levels of neutral diversity do not necessarily correspond to high levels of adaptive diversity. Beside the richness within single populations, the differentiation between populations can be estimated with neutral markers. Several studies have shown similar or even larger values of differentiation in adaptive traits (Q_{ST}) compared to differentiation at neutral loci (F_{ST}) although correlations were weak (Leinonen et al. 2008). Therefore, a differentiation at neutral loci should roughly correspond to differentiation at the adaptive level (McKay & Latta 2002). A further goal of a genetic diversity monitoring program

could be the detection of evolutionarily significant units (ESUs) as described by Ryder (1986). ESUs were described as "*a group of conspecific populations that has substantial reproductive isolation, which has led to adaptive differences so that the populations represent a significant evolutionary component of the species*" (Palsbøll et al. 2007). These ESUs are a prior protection target for nature conservation and therefore are of high relevance for genetic monitoring efforts (Crandall et al. 2000). Despite these empirical data and theories, the relationship between neutral genetic diversity and fitness, adaptation and evolutionary potential remains indirect (Holderegger et al. 2006) and therefore indistinct.

The present study was set up to test the feasibility of a genetic diversity monitoring program within the BDM and detect the potentials and limitations of such an effort. As first step, microsatellite markers for a butterfly species (*Melanargia galathea*) were developed to measure neutral genetic diversity. This work was conducted during the Compulsory Work Experience over six months in the winter term 13/14. Second, a population genetic analysis on samples of *M. galathea* from Switzerland was conducted to detect genetic diversity within individuals, within populations, between populations, and on the landscape level and to detect the causes of these patterns. Finally, a simulation model was used as a tool to assess the probability of some causes and test the applicability of such models for monitoring efforts concerning genetic diversity.

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Microsatellite Marker Development for *Melanargia galathea* (Lepidoptera: Nymphalidae)

Introduction

In general, there are two types of markers to measure neutral genetic diversity. Dominant genetic markers, like random amplified polymorphic DNAs (RAPDs) or amplified fragment length polymorphisms (AFLP) create presence/absence data and do not allow the detection of heterozygote genotypes. As a result, the important measure of observed heterozygosity (H_O) cannot be calculated from dominant markers, and conclusions on inbreeding are difficult and less sensitive. Therefore, co-dominant markers are more suited as they allow for distinction between homozygote and heterozygote genotypes. Besides allozymes or single nucleotide polymorphism (SNP), microsatellites, also referred to as simple sequence repeats (SSRs), have been frequently used in the last years. Microsatellites are tandem repeats of short nucleotide motifs of 1-6 base pairs (bp) and are ubiquitous in eukaryotic genomes (Kelkar et al. 2010). In the following, a single DNA sequence with a microsatellite motif of ATC and six repetitions is pictured in two different notations:

ATGCCCGGATCAATCTGATCATCATCATCATCATCCGAAGGCGTTACAT (1)

ATGCCCGGATCAATCTG $(ATC)_6$ CGAAGGCGTTACAT

Alleles of microsatellites differ in the number of repeats of the respective motif, which can be measured using a PCR approach with fluorescently labeled primers and (capillary) electrophoresis. The popularity and power of this genetic marker is based on their high variability (i.e., high mutation rates) and compliance to Mendelian inheritance. Nevertheless, we have to be aware of the drawbacks of microsatellites. The knowledge on the evolution and the mutation mechanism of microsatellites is still incomplete. Mutation rates vary not only between organisms and loci, but also between alleles. Microsatellites may not be necessarily neutral and differences in sequence length may not be caused by differences in repeat length per se (Zhang & Hewitt 2003). As DNA is not only present in the cell nucleus but in other cell organelles like mitochondria or chloroplasts too, the question arises which DNA should be studied. Mitochondrial and chloroplast DNA ($_{mt}$DNA, $_{cp}$DNA) is of small size and inherited solely by one of the two gametes, therefore lacking recombination. In contrast, nuclear DNA ($_{n}$DNA) stores most of the DNA and is, in sexually reproducing organisms, inherited bi-parentally with one chromosome set from the father and the other from the mother. Therefore, neutral genetic markers applied to $_{n}$DNA should be more meaningful to estimate within species genetic diversity (Zhang & Hewitt 2003).

For lepidopteran species, the development of microsatellite markers has shown to be difficult. Using DNA libraries, Sarhan (2006) and Ji et al. (2003) both found only five successfully developed markers for *Melitaea cinxia* and *Helicoverpa armigera.* Anthony et al. (2001) developed four reliable microsatellite markers for *Lycaeides melissa samuelis* with a DNA library. Using 454 sequencing data of *Euphydryas aurinia*, Smee et al. (2013) were able to develop seven markers (from 74 tested primer pairs). Sinama et al. (2011) described three markers (out of tested 96 pairs) that were transferable among populations of *E. aurinia*. However, there are several studies that reached relatively high numbers of developed markers (Van't Hof et al. 2005, Flanagan et al.

2002, Tang et al. 2014). Many of these markers showed considerable heterozygosity deficiency, signs of linkage disequilibrium or have been tested only for a single population. Therefore, the number of applicable and meaningful microsatellite markers is expected to be low for butterflies. The reasons for these difficulties are most likely not the low abundance and diversity of microsatellite loci in lepidopterans (Van't Hof et al. 2007, Reddy et al. 1999). One possible explanation is a high variability in the flanking regions beside microsatellite motifs that lead to heterogeneous primer binding sites and a high number of null alleles. Mutations can hinder primer binding and insertions or deletions (Indels) shift the fragment length of the PCR-product outside of the expected range. A further hypothesis on the observed difficulties encompass microsatellite DNA families (MDF's). Members of MDF's are loci that share similar or identical flanking regions of microsatellite motifs (2) and occur in large numbers within lepidopteran genomes (Van't Hof et al. 2007). MDF's are expected to be created by transposable elements (Tay et al. 2010, Coates et al. 2010) which can be verified using a library for known transposons or alignments for ab initio repeat identification (Joly-Lopez & Bureau 2014). Coates et al. (2010) detected a family of *Helitron*-like transposons with a semi-replicative mechanism in which one strand is transferred within the genome and complemented by the host repair system (Joly-Lopez & Bureau 2014). Two different loci of the same microsatellite DNA family are represented in the following:

$$\begin{array}{l} \text{ATGCCCGGATCAATCTG}\,(\text{ATC})_6\,\text{CGAAGGCGTTACATCGGTACGAC} \\ \text{ATGCCCGGATCAATCTG}\,(\text{ATC})_6\,\text{CTACATCGGGTACGAATGAGGCC} \end{array} \qquad (2)$$

While symmetrical MDF's share similar sequences on both sides of the microsatellite motif, asymmetrical MDF's (2) share similarities only on one side (Van't Hof et al. 2007). As result, primers can bind several times within the genome and uninterpretable banding patterns are created. Although MDF's could be useful for different kinds of questions (Anderson et al. 2007, Zhang 2004), it is recommended to avoid MDF's for microsatellite marker development (Zhang 2004, Meglécz et al. 2004).

Materials and Methods

Sampling & DNA Extraction

During the monitoring program of the BDM in 2013, the employees collected tissue samples from *Melanargia galathea* for DNA analysis by removing one hind leg from living individuals before the butterflies were released again. According to Koscinski et al. (2011), Crawford et al. (2013), and Marschalek et al. (2013), non-lethal tissue sampling is not detrimental, as wing wear or leg loss occur naturally because of, e.g., bird attacks. After sampling, the tissue samples were placed within glassine bags, stored in the freezer, sent to WSL in Birmensdorf at the end of the monitoring season, where they were finally stored at -22°C. For DNA extraction, the tissue samples were grinded within 1.5 ml Eppendorf-tubes (single extraction) or collection microtubes (plate extraction) from Qiagen (Venlo, Netherlands) using two stainless steel beads (31 mm in diameter) and a mixer mill (Retsch MM 300) for three minutes with a frequency of 30 Hz. DNA was extracted using the Qiagen DNeasy Tissue Kit for single extractions and the Qiagen DNeasy 96 Blood & Tissue Kit for plate extractions according to the manufacturer's protocol. We departed from the proposed protocol of Qiagen as we conducted the final elution step two times with

respectively 100 µl of Buffer AE. DNA concentrations were measured with an Eppendorf BIOPhotometer and DNA quality was estimated with an agarose gel electrophoresis approach using EC Vision.

Sequencing & Sequence Selection

The extracted DNA of three individuals was used for 454 Shotgun Sequencing (1/16 run) using a GS FLX Titanium system from Roche at Microsynth AG, Balgach. Out of the resulting FASTA file, sequences with perfect microsatellite motifs (minimum repeat lengths of 8, 6 and 6 for di-, tri- and tetranucleotides) and suitable primer binding sites were extracted with the software msatcommander 0.8.2 for MS Windows (Faircloth 2008). Primers were developed with PRIMER3 (Rozen & Skaletsky 2000) implemented in the software msatcommander, using following default parameters. Amplification of products within a size range of 100–500 bp, an optimal melting temperature of 60.0 °C (range 57.0-62.0 °C), an optimal GC content of 50% with possession of at least one bp GC clamp, low levels of self- or pair-complementarity and maximum end-stability (DG) of 8.0. Furthermore, a manual selection was conducted to exclude sequences with mononucleotide motifs longer than five repeats. Using the software CLC Main Workbench 6.5.1 (CLC Bio, http://www.clcbio.com, Aarhus, Denmark) an alignment of this selected sequences was performed to test for unwanted double draws of sequences and detect microsatellite DNA families (MDF's). Instead of excluding all sequences with similar flanking regions and thus members of MDF's, as proposed by Meglécz et al. (2004) and Zhang (2004), we also ordered primer pairs for such sequences. In case of asymmetric MDF, when similarities in the flanking regions were found only for one side, we labelled the primers in the non-repetitive flanking region (3) with the aim to detect only the PCR products that contain the more specific primer outside of repetitive flanking sequences.

ATGCCCGGATCAATCTG $(ATC)_6$ CGAAGGCGTTACATCGGTACGAC (3)

ATGCGCGTATCAATCTG $(ATC)_6$ CGAAGGCGTTACATCGGTACGAC

(labelled primer) -symmetric- (unlabelled primer)

ATGCCCGGATCAATCTG $(ATC)_6$ GGCTTCACATGTAGACGAGGCAC

(unlabelled primer) -asymmetric- (labelled primer)

Microsatellite DNA families were categorized as symmetric when similarities existed on both sides of the microsatellite motif, even when these similarities were of short length. In case of symmetric microsatellite DNA families, we tried to place the primers outside, at the edge of MDF or in regions with high abundance of single-nucleotide polymorphisms (SNPs) or Indels with the aim to create uniquely binding primers for a locus (3). As microsatellite DNA families share identical or similar flanking regions at different loci, a single marker could be applied to several loci and the unlabeled primer within the repetitive flanking region was tested for as many loci as possible. This approach could be beneficial as it reduces the number of ordered primers during primer development and could result in higher number of markers when a primer can bind successfully within a DNA family.

Primer Testing and Application

The procedure of microsatellite marker development was conducted on basis of the proposed methods of Schoebel et al. (2013). We applied the ordered primers on DNA from seven individuals from Soglio, Switzerland, to test their practicability at a single location and on DNA from further seven individuals from different locations (cantons of BL, GR, JU, TG, TI, VD, VS) to test for applicability across locations before the fluorescently labeled primers were ordered. We chose a 10 µl PCR-protocol (Appendix 1) using a PCR kit from Qiagen containing a HotStarTaq *Plus* DNA Polymerase, a M13 (-21) labelling (Schuelke 2000) with a FAM-dye and an adjusted PCR program on a Veriti 96 Well Thermal Cycler from Applied Biosystems (Foster City, USA). The PCR program starts with an initial activation of the hot-start polymerase heating up on 94°C for 15 minutes. Subsequently, 30 cycles of denaturation (94°C for 30 s), annealing (57°C for 90 s), and elongation (72°C for 60 s) were conducted, followed by 8 similar cycles with an annealing temperature of 53°C. The final elongation step was conducted over 30 minutes with a temperature of 72°C. PCR products were diluted 1:2 with purified water (10 µl PCR product + 10 µl H_2O). This dilution (1 µl) was mixed with 10 µl of a Hi-Di-Formamide-ROX400-mix (10 µl of size standard ROX400 within 1 ml of Hi-Di-Formamide). The measurements were conducted on an ABI 3130 Genetic Analyzer (Applied Biosystems) and electropherograms were analyzed using GeneMapper 5 (Applied Biosystems).

Labelled primers were ordered from Microsynth AG (Balgach) using fluorescent dyes (FAM, ATTO532, ATTO565, ATTO550) with the aim to create two multiplex sets. These primers were applied to all samples using standard PCR protocols following the manufacturer's protocol from Qiagen. Initial activation of the hot-start polymerase was conducted by heating up to temperatures of 95°C for 5 minutes. Subsequently, 30 cycles of denaturation (95°C for 30 s), annealing (60°C for 90 s), and elongation (72°C for 30 s) were conducted, followed by a final elongation step over 30 minutes with 60°C. Electropherograms were analyzed using GeneMapper 5 and the R package MsatAllele 1.05 (Alberto 2009) to create cumulative allele size distribution and test the feasibility of automated binning.

Null Alleles, Linkage Disequilibrium and Probability of Identity

The resulting genotype tables from GeneMapper were exported and transformed with CONVERT 1.31 (Glaubitz 2004). GENEPOP 4.2.2 (Rousset 2008) was used to test for null alleles (Pemberton et al. 1995) and linkage disequilibrium (LD) at each location for each marker. These analyses were conducted only for locations with a minimum sample size of ten individuals. Lower sample numbers provide no accurate picture of allele frequencies or heterozygosity within a location and might lead to wrong estimates in null allele frequency and occurrence of LD. GENEPOP uses the maximum likelihood approach of the EM algorithm (Dempster & Laird 1977, Hartl & Clark 1989, Kalinowski & Taper 2006) to estimate the null allele frequency (menu option 8.1). The software LINKDOS (Ganier-Géré & Dillmann 1992) is implemented in GENEPOP and conducts a pairwise test for linkage disequilibrium. Three analyzed individuals had the same genotype at each of the nine markers. To estimate if such a result is probable to occur by chance and test the specificity of the marker set, the probability of identity (PI) was calculated at each location with at least ten sampled individuals using GenAlEx 6.501 (Peakall & Smouse 2012, Peakall & Smouse 2006).

Results

Sampling, DNA Isolation & Sequencing

Legs of 424 individuals of *M. galathea* from 56 locations were sampled across Switzerland in the course of the summer 2013 between the 18th of June and the 22nd of August. At 18 locations ten or more individuals could be caught. As a single leg of *M. galathea* has an average weight of 0.5 mg, the mean concentration of extracted DNA was 7.4 ng/µl and thus sufficient for microsatellite analysis. As result, the quality assessment was difficult to interpret because of low-contrast banding patterns. The 454 sequencing resulted in a total of 37 707 reads with an average length of 376 bp (median of 411 bp) and 14 186 875 bp in total. Thirty-four, 210, and 294 sequences exhibited di-,tri-, and tetra-microsatellite motifs with sufficient length together with suitable primer binding sites. Out of these sequences, a further pre-selection was conducted manually to exclude sequences with multiple mononucleotide motifs beside the microsatellite region.

Alignment & Primer Design

We found microsatellite motifs with symmetric and asymmetric microsatellite DNA families as well as sequences without any sign of such DNA families in the alignments (Figure 1). Within the similar flanking regions, several indels (insertions or deletions) and mutations could be observed.

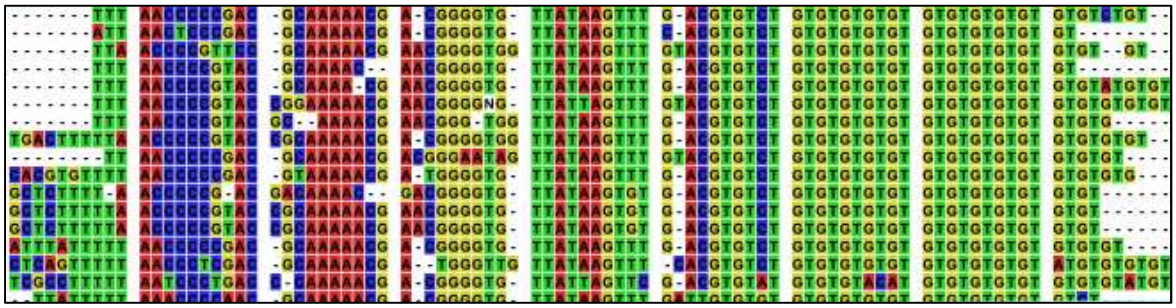

Figure 1: Detail of an alignment of sequences (each row represents a single sequence) with microsatellite motifs (repeats of the motif GT in the right sector). Beside microsatellite motifs, almost identical flanking regions exist (on the left side). Because the complementary flanking regions do not possess such similarities, it is most likely that these sequences are different loci and are not replicates of the same locus.

In total, we ordered 118 primers from Microsynth AG, Balgach with lengths of approx. 20 bp and M13 (-21) tails for fluorescent dye labelling (Schuelke 2000). Using these 118 single primers, we tested 61 primer combinations for 13 loci with symmetric microsatellite families, 16 loci with asymmetric DNA families and 32 without any sign for microsatellite DNA families.

Marker Properties

Nine out of 61 tested marker combinations were judged as promising, and fluorescently labelled primers were ordered and applied to all 424 samples (Table 1). Five of the finally selected nine markers were designed for loci with no sign for MDF's and four primers were designed for loci of microsatellite DNA families. The marker D9L2 is targeting a member of an asymmetric MDF and the markers 48TK, 522M, and 7QTP were designed for loci of the same symmetric MDF. In these cases, the labelled primers could be positioned outside or at the edge of the repetitive sequences. The markers 48TK and 7QTP were not only designed for the same MDF but share the same unlabeled primer within the repetitive flanking sequence. Loci with MDF's do not seem to differ in

polymorphism from loci without MDF's, although the low number of samples impedes a statistical analysis. During the design of the primer multiplexes, several combinations were not working. Most of the primer fragments had similar lengths and it was rarely possible to choose the same dye two times within the same multiplex (Table 1).

Table 1: Characteristics of the nine microsatellite markers developed for *Melanargia galathea*. Given are the mean length of the PCR products (*pl*), the number of different alleles *(na*), the proportion of differences between alleles of one bp length (*di*), and the mean observed heterozygosity (H_o) for each locus across all 421 samples. Additionally, the used dye for labelling (*Dye*), the sequences of the labeled and unlabeled primers, the multiplex membership (*Mu*), and the absence (*No*) or characteristics of present MDF's (*MDF*) are listed (*Sym* - Symmetrical MDF, *Asym* - Asymmetrical MDF). The two primers with a positive test on null alleles are not included within this table.

ID	Motif	pl	na	di	H_o	Mu	MDF	Unlabeled primer	Labeled Primer	Dye
C2S5	ATG	186	14	0.62	0.47	1	No	GTCGATAACTTTGTTA AGTCCTAATCC	TGCGTTGATGATACG TTGGC	FAM
952H	ATC	226	22	0.67	0.67	1	No	GGTTGGCGCTGCTTA GAAAG	TGCTCCCACCATTTCT ATCTG	ATTO 532
BBJK	ATC	200	21	0.45	0.66	1	No	CCCTTGCAGACAGGT CACG	CTGTAAAGCCCAGCC CAAAG	ATTO 565
522M	AC	348	23	0.64	0.72	2	*Sym*	CCCTGACATTCGCCAT CTTG	AAGAACCTCGACAGC TGCC	FAM
48TK	AC	204	38	0.70	0.71	2	*Sym*	GCATTCAAATCGGTCC ACCC *	CTCTTGCTTACATCCA ACACCA	ATTO 532
BS05	GGT	209	11	0.30	0.17	2	No	AGTCGCTACAGGCTA CGTG	CGTTAAAGTCCGTCA ACGC	ATTO 565
7QTP	AC	220	22	0.14	0.67	2	*Sym*	GCATTCAAATCGGTCC ACCC *	ACCGCCCAAGACCAT CATG	FAM
5LN0	GTGC	280	13	0.42	0.24	3	No	CGATAATAACGCTTAC ACTTAGGAAC	AATCCCGAGGGAATC GTGG	ATTO 550
D9L2	ATG	204	11	0.30	0.68	3	Asym	GTGAAGTCTGCCAAT CCGC	GAACAGTGGTACACC GTGAG	M13 (-21)

Ghost peaks overlaid peaks of other loci and prohibited accurate allele calling. One primer was not working with the fluorescent dye (marker D9L2) and one within the multiplex (marker 5LN0). Therefore, we ended up with three multiplexes, two regular multiplexes and one pseudo-multiplex. For the pseudo-multiplex, the PCR was conducted for the markers 5LN0 and D9L2

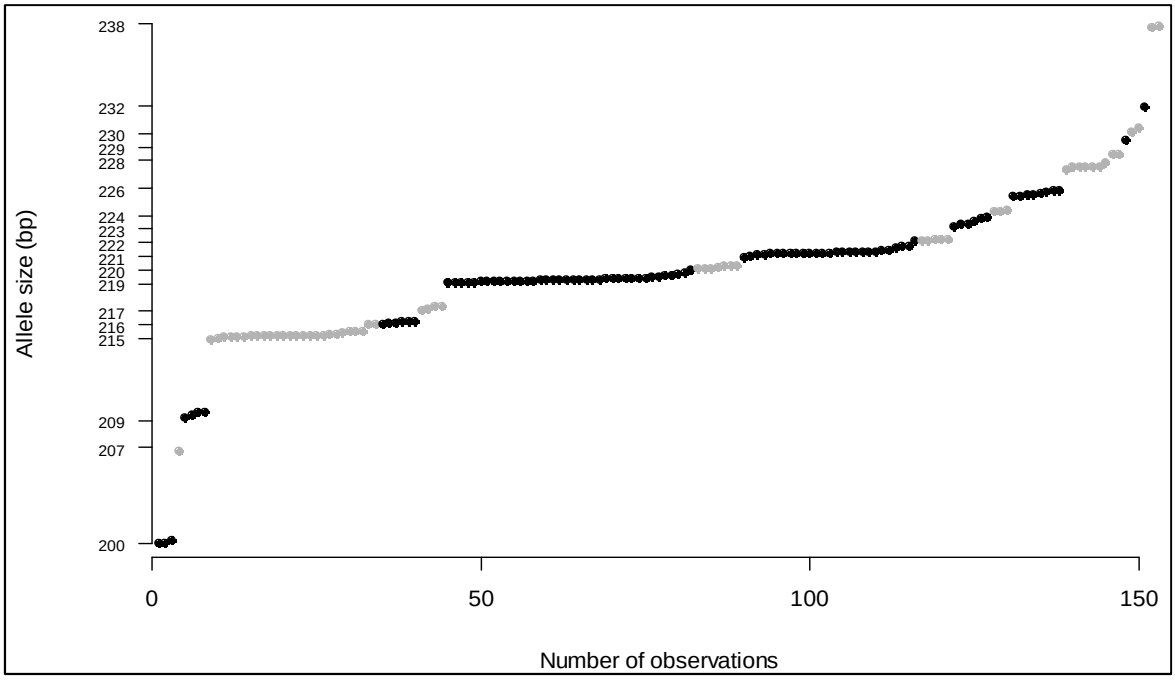

Figure 2: Cumulative allele size distribution of the marker 7QTP at a subset of samples created with the R package MSatAllele 1.05 (Alberto 2009). The two different colors help to differ between neighboring alleles and display the allele limits that were set by the automated binning procedure. Several alleles were separated only by a length of one bp and a distinction between alleles was difficult.

separately and the PCR products were then mixed for electrophoresis. Concerning the allele size distribution, many markers exhibited differences between allele lengths of one base pair which is in contrast to the motif lengths and the resulting expected distance between alleles. In many cases, the transition between alleles was more or less linear and the discrimination between alleles difficult. In our example, stutter patterns were no help to determine the borderline between alleles, as it was possible for oak trees (Gugerli et al. 2008). The use of the automated binning procedure of the R package *MSatAllele* 1.05 (Alberto 2009) provided no satisfying results (see Figure 2, regard allele 216 and 222). Consequently, the bin limits were set manually based on the overlay of the electropherograms in GENEMAPPER 5. There was no difference in the abundance of 1bp distances among loci of MDF and loci without MDF's (Table 1).

Null Alleles, Linkage Disequilibrium and Probability of Identity

For the two markers BS05 and 5LN0, high frequencies of null alleles were detected (Figure 3). As result, we excluded these two markers from the further analysis.

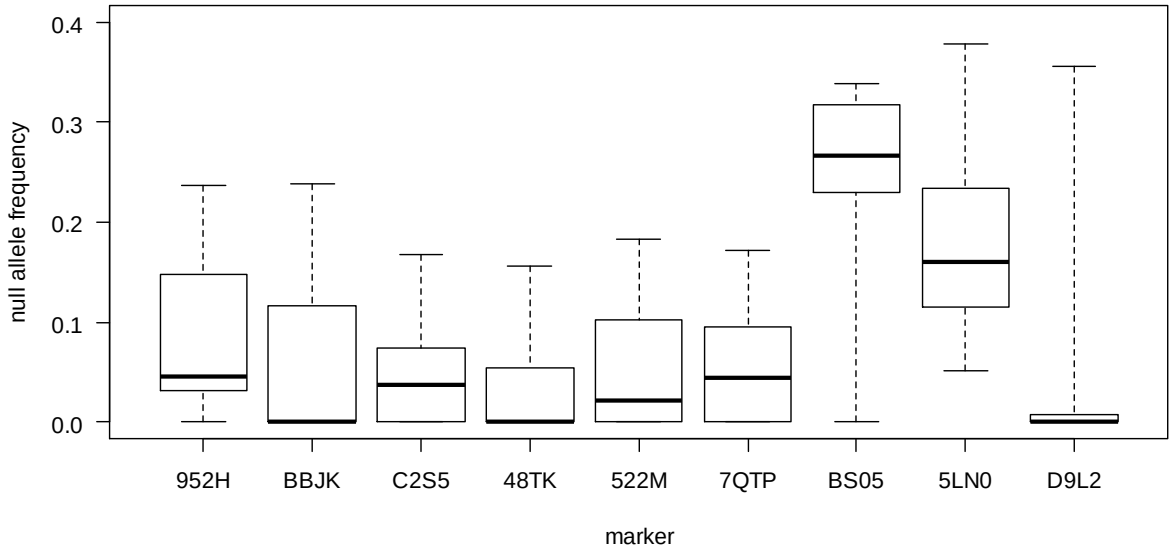

Figure 3: Estimated null allele frequency by the software GENEPOP using the maximum likelihood approach of the EM algorithm (Dempster & Laird 1977, Hartl & Clark 1989, Kalinowski & Taper 2006). Null allele frequency was estimated for each marker in locations with a minimum of ten samples (18 locations). The bottom and top of the box are the first and third quartiles, the band inside the box is the second quartile (the median) and the whiskers extend to the minimum and maximum of all data.

In contrast to null alleles, no sign for linkage disequilibrium between markers was detected (Table 2). The estimated probability of identity (PI) was PI=2.9E-08 and revealed that it is not probable to find the same genotype two times within our sample set (PIsibs=8.7E-04; expected number of identical genotypes=1.3E-05; expected number of identical genotypes for sibs=2.5E-02). This result is based on seven markers applied on 18 locations with 281 individuals. Although the total number of analyzed samples is larger than this subset, it is still unlikely that these individuals are not the result of errors that occurred during sampling, DNA extraction and analysis. As result, these three identical individuals were removed from the further analysis.

Table 2: Result of a pairwise test for linkage disequilibrium using the software LINKDOS (implemented in GENEPOP). For this analysis, all locations with a minimum of ten samples were included using the seven markers without sign of null alleles. The null hypothesis in this test is: "Genotypes at one locus are independent from genotypes at the other locus". The p-value for each locus pair was estimated using Fisher's combined probability test for the log likelihood ratio statistic (G-test) of each locus pair at each location.

Locus pair	chi2	df	p-value	Locus pair	Chi2	Df	p-value
952H & BBJK	21.37	36	0.975	BBJK & 7QTP	32.64	34	0.534
952H & C2S5	31.04	36	0.704	C2S5 & 7QTP	43.13	34	0.136
BBJK & C2S5	28.81	36	0.797	48TK & 7QTP	33.00	34	0.516
952H & 48TK	12.19	34	1.000	522M & 7QTP	14.94	34	0.998
BBJK & 48TK	30.57	36	0.724	952H & D9L2	20.99	36	0.978
C2S5 & 48TK	23.69	36	0.943	BBJK & D9L2	24.12	36	0.935
952H & 522M	24.09	36	0.935	C2S5 & D9L2	35.47	36	0.494
BBJK & 522M	30.86	36	0.711	48TK & D9L2	20.49	36	0.982
C2S5 & 522M	28.21	36	0.820	522M & D9L2	39.32	36	0.323
48TK & 522M	26.36	36	0.880	7QTP & D9L2	18.83	34	0.984
952H & 7QTP	24.91	34	0.872				

Discussion

The applied procedure for tissue sampling and DNA extraction seems to work. Already one butterfly leg was sufficient to extract enough DNA for microsatellite analysis. Nevertheless, the DNA extraction contains a considerable risk as no second sample exists as backup to repeat the extraction in case of a failed first attempt. This is especially valid for plate extractions. Although challenging, seven microsatellite markers were developed for the lepidopteran species *Melanargia galathea*. The markers exhibit comparatively high levels of polymorphism (Table 1) and should allow to distinguish between each individual as the probability of identity was very low. Therefore, double sampling of the same individual by accident should be detectable. In contrast of the statement of Zhang (2004) and Van't Hof et al. (2007), microsatellite markers could be successfully developed for microsatellite DNA families (four of the seven final markers). We were even able to profit from the similar flanking regions and used the same unlabeled primer for two different loci of the same microsatellite DNA family (MDF). In general, this finding highlights the importance of alignments during the process of marker development. Together with the test for linkage disequilibrium, alignments are beneficial to detect marker pairs that have been designed for the same locus by accident. It would be helpful to use bioinformatics tools to search for microsatellite DNA families within all reads (and not within the preselected sequences by Primer3) to detect MDF's correctly and estimate their abundance. A meta-analysis could be an instrument to test if there is a difference in the polymorphism and abundance of successfully developed markers within or outside of MDF's for studies using NGS-technologies. The number of one bp differences between alleles is not differing among loci with and without MDF and the causes of these one bp distances between alleles seem to be independent of MDF. It could be beneficial to choose next generation sequencing techniques that provide high read lengths to detect MDF's and regions beside them.

Although microsatellites are expected to be neutral (Kelkar et al. 2010), several exceptions have been detected (Martin et al. 2005, Li et al. 2004). Despite being laborious, an important next step could be a study to test for neutrality. A further important question concerns the applicability for

samples within an extended range (outside of Switzerland) and it might be of interest, whether these markers could be applied to the sister species *Melanargia lachesis*. For future marker development, more attention should be paid to the connection between transposable elements and microsatellite motifs in lepidopterans. What is the impact of transposable elements on microsatellite evolution and the diversity patterns? Recent studies have detected regular associations of transposable elements (TEs) with epigenetic silencing mechanisms (e.g., methylation of DNA and histones, small RNAs) that can also concern neighboring DNA regions (Joly-Lopez & Bureau 2014, Ito & Kakutani 2014). Such epigenetic silencing mechanisms might have an impact on the application of microsatellite markers and could be a reason for the problematic microsatellite development in lepidopterans. A step of demethylation before PCR may be a solution to overcome this problem.

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Genetic Diversity of the Butterfly *Melanargia galathea* in Switzerland

Introduction

Beside the adaptive and neutral components of genetic diversity, different hierarchical levels of genetic diversity can be separated similar to the levels of species diversity introduced by Whittaker (1972). Genetic diversity could be split up into diversity within individuals, diversity within populations (alpha diversity), between populations (beta diversity) and at landscape level (gamma diversity). This differentiation could be beneficial as the controlling factors of the different levels vary and consequences of increased or reduced genetic diversity differ between levels. A resemblance between species diversity and genetic diversity was already described by Vellend & Geber (2005). A descriptor of neutral genetic diversity within individuals is the observed heterozygosity H_o. The observed heterozygosity represents the probability that an individual sampled from the population will be heterozygous at a locus and is applicable for diploid organism (Templeton 1994). H_o can be averaged over several loci to obtain a multi-locus measure and is expected to be the relevant measure to estimate the link between genetic diversity and the fitness of a single individual (see HFC). The neutral genetic diversity within populations (alpha diversity) could be quantified with Nei's gene diversity H_s (sometimes misleadingly called expected heterozygosity H_e). H_s indicates the probability that two alleles of a locus drawn at random from the gene pool are different (Nei 1987, Templeton 1994). Despite several advantages, this measure does not correspond directly to the intuitive concept of diversity (Jost 2007, Jost 2008), especially when we want to compare populations in space and time. Regarding a population with 30 different equally common alleles, a drop to 15 different equally common alleles would lead to a change in H_s from 0.967 to 0.933 (a decline of 3.5 %) although half of the alleles got lost. Jost (2008) concluded that "*neither heterozygosity [nor Shannon entropy] precisely match the intuitive concept of diversity"* and *"ratio comparisons are invalid when diversity is equated with heterozygosity [or entropy]*". The number of alleles (N), often called allelic richness, reflect such changes correctly but has other downsides (Templeton 1994). As N is highly dependent on sample size, a rarefaction approach has to be used to correct for different sample sizes (Mousadik & Petit 1996) and we have to be aware that it is not possible to compare results when the number of alleles of rarefaction (min.n) differ between data sets. The genetic diversity between populations which is corresponding to beta diversity is called differentiation. Although F_{ST} is the measure for differentiation that has been applied most often in the past, several drawbacks have been detected. It has been shown that F_{ST} is dependent on the within population diversity H_s (Carreras-Carbonell et al. 2006) and is not able to range between zero and one in every set of population (Jost 2008). This is in conflict with the intended relationship between the different levels of diversity. In case of species diversity, either an additive definition ($\alpha + \beta = \gamma$) or a multiplicative definition ($\alpha + \beta = \gamma$) was used in the past (Templeton 1994). As result of both definitions, alpha should be independent of beta (Wilson & Shmida 1984) as "*it would be impossible to compare beta diversities of regions whose alpha diversities differed*" (Jost 2007). As

measure for genetic differentiation that is independent from genetic alpha diversity (H_s) the measure D_{est} was introduced by Jost (2008). When we want to quantify the genetic diversity at landscape level, the extent of a landscape of interest for a monitoring of genetic gamma diversity has to be defined first. It would make no sense to define the area of interest solely on the species geographic distribution but it should be rather tried to detect and monitor evolutionarily significant units (ESU). This concept was brought to attention by Ryder (1986) to identify distinct genetic lineages for conservation when resources are limited and taxonomy does not reflect underlying genetic diversity. Moritz (1994) distinguish between ESUs on the genetic level as they have to be reciprocally monophyletic for $_{mt}$DNA alleles and significantly divergent in allele frequencies at nuclear loci. As we decided to monitor nuclear DNA, we should aim to detect and characterize sets of populations that differ considerably in allele frequencies. As tools to distinguish ESU, pairwise measures of differentiation, allele or haplotype trees, wombling, and Bayesian clustering approaches (e.g., STRUCTURE) could be useful. The measures rarefied allelic richness (N_r) and observed heterozygosity (H_o) can be used to measure the diversity within ESUs and test for differences in diversity between ESUs. A further measure for the diversity within and differentiation between populations or clusters could be the number of private alleles. Private alleles were defined as alleles that are only present within a single population or cluster and correspond to endemic species in the context of species diversity. Some authors use the term private allelic richness and use it as a measure for distinctiveness (Kalinowski 2004). As the number of private alleles is highly dependent on sample size the application of a rarefaction method was proposed by Kalinowski (2004).

Several factors seem to influence neutral genetic diversity, including the mutation rate, genetic drift, demographic processes (population size, sex ratio, mating system) and gene flow (Young et al. 1996, Frankham 1996, Frankham 1995). As result, the impact of anthropogenic activities on genetic diversity could act in various ways, including landscape fragmentation (reduced gene flow), habitat destruction (long-term decreases in population size), disturbances (short-term changes in population sizes), the translocation of species (artificial gene) or artificial selection (increasing the abundance of specific genotypes at the expense of others) (Prober & Brown 1994, Frankham 1996, Scribner et al. 2001, Farwig et al. 2008, Montalvo & Ellstrand 2001). Although the quantification of biological diversity in space and time is the prime intention of monitoring efforts (Yoccoz et al. 2001), monitoring could further be used to identify the controlling factors of diversity. Although landscape fragmentation is a widespread problem in Central Europe and landscape genetic tools are available to detect reduced gene flow (Manel & Holderegger 2013), the study design of the BDM almost impede such approaches. As not all populations are sampled within Switzerland and population between two BDM sites can influence gene flow, changes in differentiation might not reflect changes in the conductivity of the landscape . In contrast, a link between habitat quality or size and genetic diversity could be detected for the case that gene flow with neighboring populations is low (Scribner et al. 2001, Pitra et al. 2011, Frankham 1996). When we assume that a habitat with high quality and size is connected with large population sizes, a high genetic diversity should be linked to suitable habitat. As precondition for such an approach, a positive relationship between effective population size and within population genetic diversity (N_r) should exist.

The aims of this study include the detection of the current state and spatial patterns of neutral genetic diversity at the different hierarchical levels. Furthermore, the causes for these patterns are tried to be estimated and the feasibility of a habitat suitability analysis using genetic diversity

data is assessed. Conclusions for nature conservation and future monitoring programs of genetic diversity are tried to be drawn.

Materials and Methods

The Biodiversity Monitoring Switzerland

As part of the Biodiversity Monitoring Switzerland (BDM), butterflies are monitored intensively across Switzerland since 2003 to estimate the species diversity within landscapes (Z7). For this purpose, a regular sampling grid of circa 520 quadratic areas with a size of one square-kilometer each was set up (Figure 4). In the Jura mountains and in Southern Switzerland, the sampling grid was compressed to overcome statistical problems. Every five years, each square of the grid is monitored by employees who walk along a transect of 2.5 km length forth and back seven times during the flight season between 10:30 am and 5 pm. Transects in alpine areas are monitored only four times per season. The transect walks are conducted between end of April and end of September depending on region and altitude (Koordinationsstelle Biodiversitäts-Monitoring Schweiz 2008).

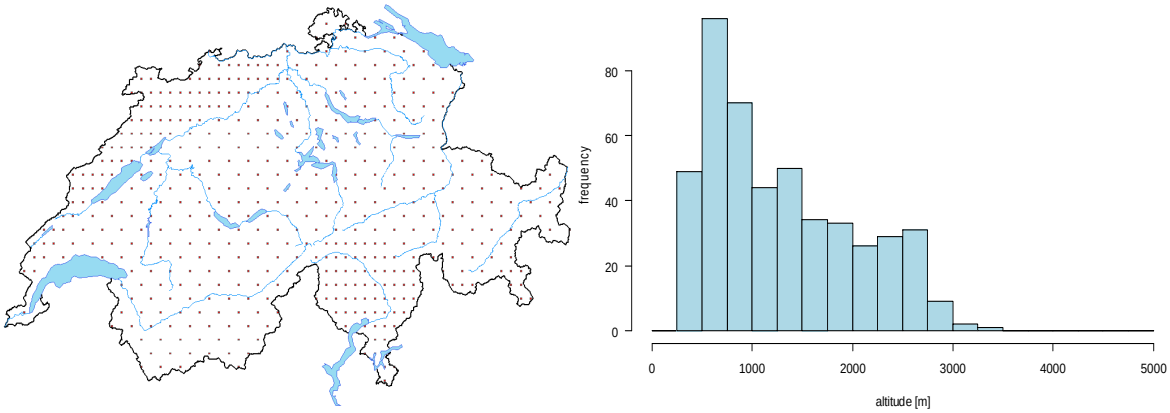

Figure 4 : Positions of the study areas of the BDM for the Z7 program to monitor diversity of species in landscapes. The study areas of one square-kilometer are pictured in red. Not all of the pictured squares can be monitored for butterflies as some of the squares lay in inaccessible areas in the mountains. The histogram on the right site reflects the abundance of BDM study sites monitored for butterflies according to the altitude (m asl). Within the Z7 BDM program, butterflies are monitored intensively up to altitudes of 2750 m asl.

During the transect walks temperature has to be higher than 13°C, wind intensity should not be larger than 3 Bf and the sun has to shine on 80% of the transect route (Koordinationsstelle Biodiversitäts-Monitoring Schweiz 2008). All butterfly imagines within a distance of five meters beside the path are recorded. To get tissue from *M. galathea* for DNA analysis, individuals of this species are caught and a single hind leg was removed before the butterfly was released again. According to Koscinski et al. (2011), Crawford et al. (2013) and Marschalek et al. (2013), non-lethal tissue sampling is not detrimental, as wing wear or leg loss occur naturally because of, e.g., bird attacks. In the first season, the employees were asked to collect tissue from 15 individuals per transect if available. The samples were stored in the freezer till the end of the monitoring season and sent to WSL in Birmensdorf altogether. At WSL, the tissue samples were stored at -22°C until DNA extraction was conducted.

The Study Species

Figure 5: A Marbled White photographed in Val Codera, Italy. © Sabine Brodbeck, WSL

The Marbled White, *Melanargia galathea* (Linnaeus, 1758), is a lepidopteran of the family Satyridae. The species is present over large parts of Europe and the Maghreb. The distribution borders in Europe range from Poland, Denmark, Belgium, Southern England and France down to the Pyrenees, Southern Italy, the Balkan region and the Southern Caspian region. In recent years, an expansion of the Marbled White at the northern distribution range could be observed (Schmitt et al. 2006, Ebert & Rennwald 1991b). On the Iberian Peninsula, the Marbled White is replaced by the sibling species *M. lachesis* (Hübner, 1790). The two species exist nearly allopatrically, but do occur syntopically in some areas (Habel et al. 2008). Using allozyme markers, refugia of *Melanargia galathea* during the last glacial has been detected in the Maghreb, Southern Italy and the Balkan region (Schmitt et al. 2006, Habel et al. 2005, Habel et al. 2008). The highest genetic diversity was detected in Morocco with several private alleles beside the complete representation of the alleles found in Europe. The differentiation among populations in Morocco was higher than the differentiation between populations across Europe (Habel et al. 2008). It is expected that the differentiation within Europe emerged during the last

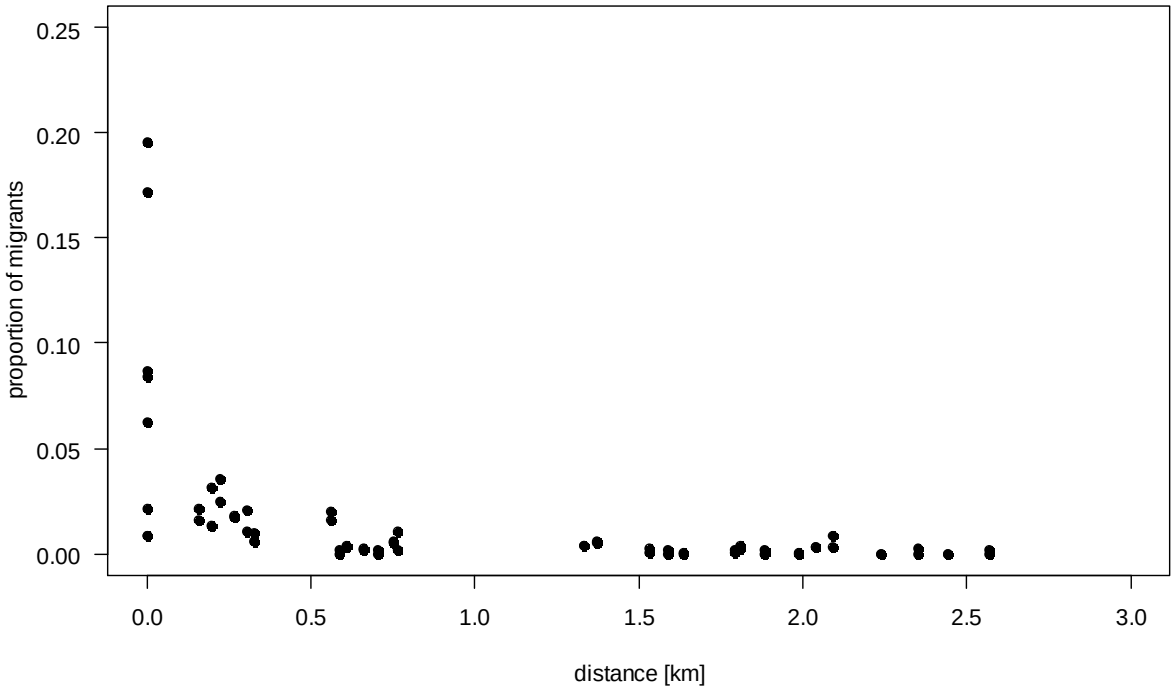

Figure 6: Dispersal events observed by Baguette et al. (2000) in Southern Belgium in dependence of the distance between the patch of first capture and that of recapture. The proportion of migrants represents the number of migrants between plots divided by the total number of recaptured individuals. The events of no dispersal, when marked butterflies have been recaptured within the same patch, have been included in the plot at a distance of zero.

glacial period, when this butterfly species could persist in Italy and the Southern Balkan region. During earlier glaciations, the Marbled White is assumed to have been restricted to North-Western Africa and has recolonized Europe via Tunisia, Sicilia and Italy (Habel et al. 2008).

Baguette et al. (2000) studied the dispersal ability of *M. galathea* using a mark–release–recapture approach in Southern Belgium. The study system consisted of eight patches with sizes between 0.3 and 2.4 ha with pairwise distances ranging from 158 to 2568 m. In total, 4041 individuals of *M. galathea* were marked and 1173 individuals recaptured. From the 1173 recaptured individuals, 432 individuals were observed to change the patch (36 %) with a maximum distance of dispersal of 2568 m following a negative exponential function (Figure 6).

In Switzerland *Melanargia galathea* is present in almost every region except for the Inn valley and alpine areas (based on the data of the BDM since 2003, see Figure 7 and Figure 8).

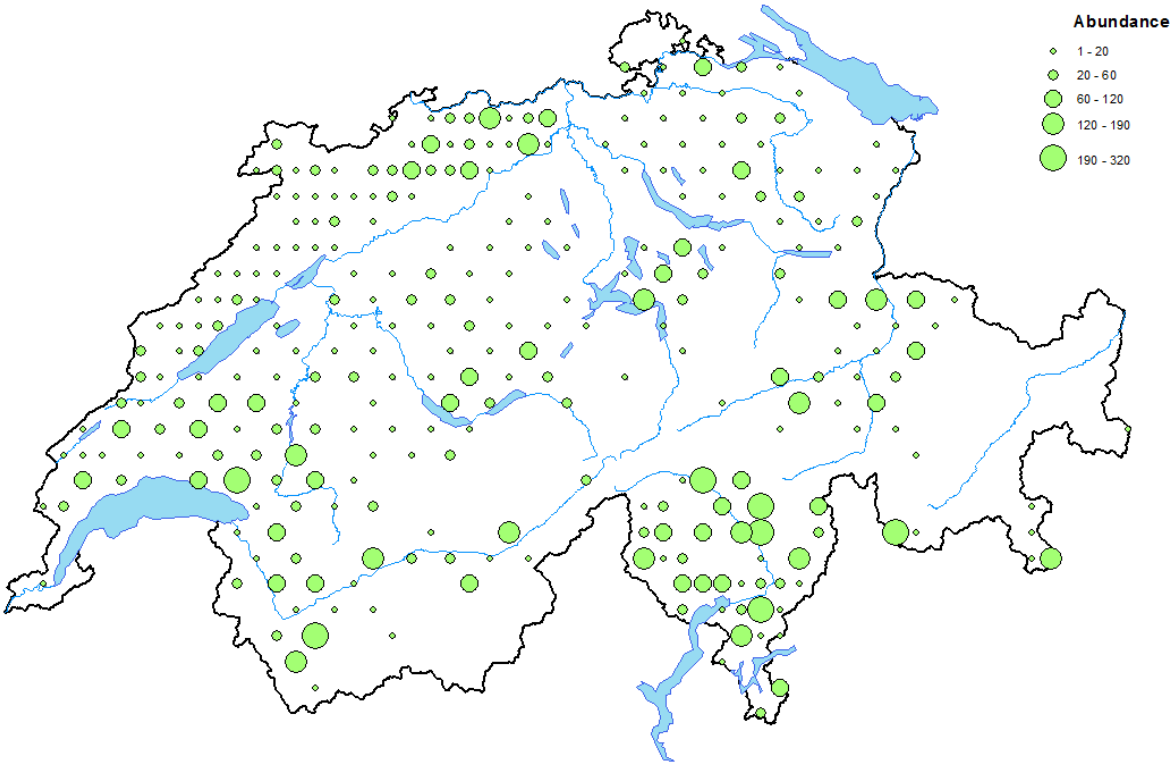

Figure 7: The abundance of *M. galathea* in Switzerland estimated by BDM transect counts between 2003 and 2012. The abundance per location and season was estimated by Hintermann & Weber (Reinach) following a special data evaluation method. Using this measure, the arithmetic mean between seasons was calculated. Except for the Inn valley and alpine areas, *M. galathea* has been detected in every regions of Switzerland

Concerning the vertical distribution, *M. galathea* has been recorded mainly between altitudes of 250 and 2250 m asl (Figure 8) and there seems to be an altitudinal limitation for this species as the BDM monitors butterflies intensively up to a height of 2750 m asl (Figure 4).

The life-cycle of the Marbled White starts in spring when the caterpillars emerge from hibernation and feed (in Central Europe) on grass species like *Bromus erectus*, *Festuca rubra*, *Brachypodium pinnatum* or *Dactylis glomerata* (Ebert & Rennwald 1991b). While young caterpillars feed during daytime mainly on the meristem of weakly sclerotized grass species, older larvae prefer to feed during the night on more sclerotized plants (Fartmann & Hermann 2006). Depending of latitude and altitude, the imagines fly between end of May and September. The preferred nectar plants for imagines of the Marbled White in Central Europe are *Centaurea jacea*, *Centaurea scabiosa*, *Cirsium tuberosum, Scabiosa columbaria* and *Knautia arvensis*.

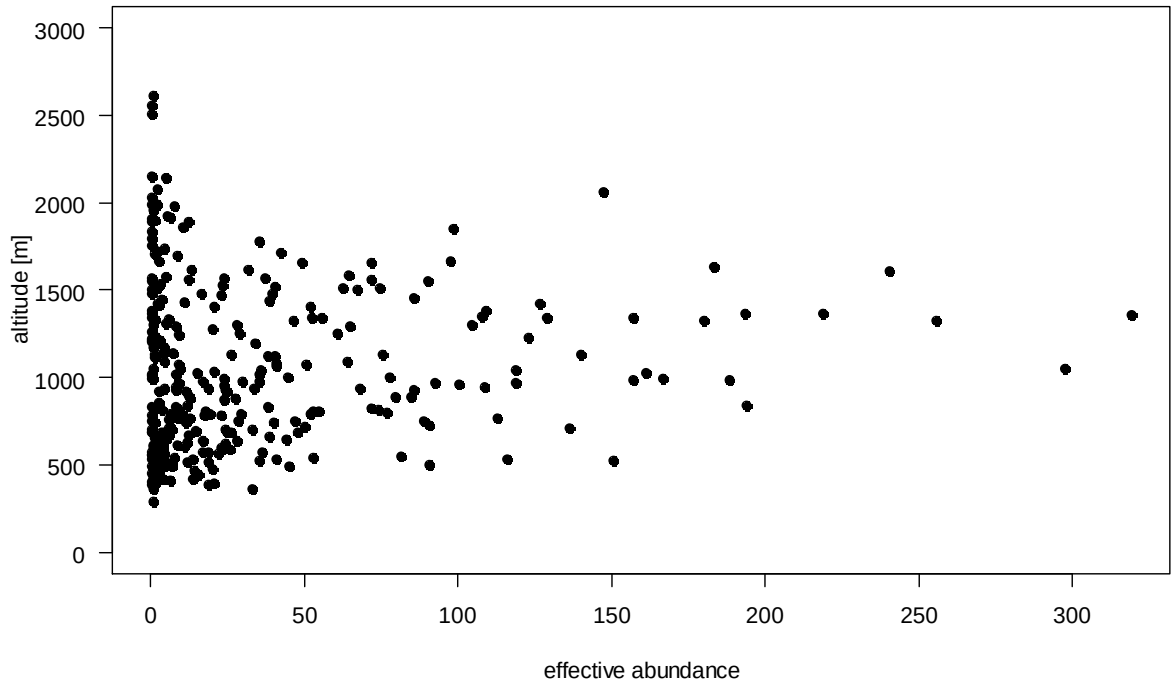

Figure 8: The abundance of *M. galathea* within BDM squares between 2003 and 2012 has been plotted according to the altitude (m asl) of the respective square. The abundance has been estimated by Hintermann & Weber (Reinach, CH) using a special data evaluation method. This butterfly species has been detected regularly up to altitudes of 2250 m asl.

Larger plants with purple color and large florescence are thus favored (Ebert & Rennwald 1991b). Although a high number of possible nectar plants have been detected (>60), the Marbled White was described as stenanth by Ebert & Rennwald (1991b), therefore with a narrow nectar plant spectrum. During their search for females, male imagines show the typical behavior of a patrolling species. Males search for females while flying lowly over the larval habitat of grassland in undirected manner. Other areas are covered linearly with increased velocity (Ebert & Rennwald 1991b). For the oviposition, females scatter single eggs over unmowed grass, where the emerging caterpillar hibernates almost immediately after emergence. Typical habitats of the Marbled White are grasslands with the preferred feeding plants for caterpillars and nectar plants for the imagines. Additionally, margins of shrubs and fringes, forest edges and trails, embankments and meadows with scattered fruit trees can be populated by the Marbled White. Dry meadows seem to be preferred but wet locations are also occupied. Although larvae are feeding during night and mowing is no danger for them, extensive usage of grassland reduces the supply with nectar plants and unmowed areas for oviposition (Ebert & Rennwald 1991b) as it has been detected as threat for most butterflies (van Swaay & Warren 2006).

Genetic Markers

For DNA extraction, the tissue samples were grinded within 1.5 ml Eppendorf-tubes for single extraction or collection microtubes from Qiagen (Venlo, Netherlands) for plate extraction, using two stainless steel beads (31 mm in diameter) and a mixer mill (Retsch MM 300) with a frequency of 30 Hz for three minutes. DNA was extracted using the Qiagen DNeasy Tissue Kit for single extractions and the Qiagen DNeasy 96 Blood & Tissue Kit for plate extractions following the manufacturer's protocol, except for conducting the final elution step with two times with respectively 100 µl of Buffer AE. Seven microsatellite markers were applied to estimate neutral genetic diversity (Table 1). Primers were labelled with fluorescent dyes (FAM, ATTO532, ATTO565, ATTO550) using three sets of multiplexes (two regular multiplexes, one pseudo-multiplex). A 10 µl

PCR-protocol was chosen with a PCR kit from Qiagen (containing a HotStarTaq *Plus* DNA Polymerase) and a Veriti 96 Well Thermal Cycler (Applied Biosystems). A ABI 3130 Genetic Analyzer (Applied Biosystems) was used for electrophorese and electropherograms were analyzed using GeneMapper 5 (Applied Biosystems). For further details, please refer to the previous section.

Genetic Measures

To estimate measures of genetic diversity, the package *hierfstat* version 0.04-10 (Goudet 2005) in R 3.1.0 (R Development Core Team 2014) in combination with the integrated development environment RStudio (RStudio 2014) was used. For comparisons among locations, only locations with at least ten samples were included. The function *basic.stats* was used to calculate the observed heterozygosity H_O, the gene diversity H_S, and F_{IS} for each marker and location, with the arithmetic mean and the media to get a multi-locus measure of genetic diversity at each location (Templeton 1994). The rarefied allelic richness (N_r) was calculated for each marker at each location using the rarefaction method of Hurlbert (1971) as it was applied by Mousadik & Petit (1996) using the R function *allelic.richness* and the arithmetic mean and median to obtain a multi-locus measure (Templeton 1994). The minimum number of alleles for rarefaction (min.n) was set to 18 as *M. galathea* is a diploid organism and the minimum number of genotyped individuals was nine (as result of some occurring null alleles). The values of N_r, H_s, H_o, and F_{IS} at each location were tested for normal distribution by a Shapiro-Wilk-test with the function *shapiro.test* (R-package stats) and checked visually using QQ-plots and density distribution plots. The homogeneity of variance was tested with a Bartlett-test using the function *bartlett.test* (R-package stats). In case of normal distribution of N_r and H_s values within locations and a homogeneity of variances, an analysis of variance (ANOVA) was conducted with the function *aov* (R-package stats) to test for differences in H_o, H_s and N_r between locations. When the conditions for an ANOVA were not met, a Kruskall-Wallis-test was conducted using the function *kruskal.test* (R-package stats). For F_{IS}, a test for significant deviation from zero was conducted using a two-sided t-test (df=6) with H_A of "true mean is not equal to zero" for each location. A one-sample Wilcoxon signed-rank test (two-sided) with the function *wilcox.test* (R-package stats) was used in cases of not normal distributed data of F_{IS}.

The measure D_{est} was calculated applying the function *basic.stats* for each pair of locations. The Euclidian distance was calculated using R and the coordinates of each BDM square (Swiss Coordinate System 1903). As the STRUCTURE results indicate the Alps as important barrier, the maximal altitude that has to be covered to get from one location to the other was included to test for isolation by altitude. Using ArcGIS 10.2 (ESRI, Redlands, USA), a two-sided buffer of two kilometers along the linear connection between each location was created (Tool: *Buffer* – Analysis). Elevation data were gained from the Shuttle Radar Topography Mission (SRTM) with a resolution of 90 m (maps 38_03 and 39_03) (Jarvis et al. 2008), merged together (Tool: *Mosaic to new Raster* – Data Management), clipped to smaller size (Tool: *Clip* – Data Management), aggregated for lower resolution (Tool: *Aggregate* – Spatial Analyst, aggregation technique: MAXIMUM) and transferred into polygons (Tool: *Raster to Polygon* – Conversion). This polygon file was intersected (Tool: *Intersect* – Analysis) with the buffer-polygons and a summary statistic was calculated to gain the maximum altitude within each buffer-polygon (Tool: *Summary statistics* – Analysis). As result of the high type-1 error rates of Mantel tests (Balkenhol et al. 2009) a multiple regression on distance matrices (MRM) was conducted to test for isolation by distance

(IBD) and isolation by altitude (IBA) following Legendre et al. (1994). From the R package ecodist (Goslee & Urban 2007) the function *MRM* with default parameters (nperm = 1000, mrank = FALSE) was used to test for a linear relationship between D/(1-D) and ln(distance) or ln(maximum altitude) following Rousset (1997). Additionally, a Mantel correlogram was conducted to estimate the range of IBD and IBA using the R function *mgram* (package ecodist) and utilizing the default parameters (nperm = 1000, mrank = FALSE, nboot = 500, pboot = 0.9, cboot = 0.95, alternative = two.sided, trace = FALSE). In this function, the number of classes was determined using Sturge's rule and the step size is calculated based on the number of classes and the range of the independent variable. The MRM and the correlogram were conducted for all samples together as well as within the largest detected cluster to exclude the impact of major barriers between clusters.

To get a measure for genetic diversity on the landscape level, the mean rarefied allelic richness (N_r) was calculated within each cluster as set of locations defined on the basis of STRUCTURE results using the function *allelic.richness* with min.n=68. Differentiation (D_{est}) between each cluster was calculated using the R function *basic.stats*. Because of the low number of locations with sufficient sample size in some clusters, it was not possible to calculate the within cluster differentiation. A moving window approach was used to account for the large number of locations with only few sampled individuals. For each BDM square (Z7), all analyzed individuals within a distance of 20 km were used to calculate N_r with the function *allelic.richness* (min.n=18) as long as at least ten individuals are included. These data were extrapolated for Switzerland with a spherical Kriging approach (Tool: *Kriging* – Spatial Analyst. Method: Ordinary; Semivariogramm Model: Spherical; Search Radius: Variable; Output Cell Size: 1000 (m); Number of Points: 12) using ArcGIS 10.2 (ESRI, Redlands, USA). To get a further measure for the diversity within and differentiation between populations and clusters the number of private alleles was estimated (using a self-written R script). Private alleles were defined as alleles occurring only within a single location or cluster.

STRUCTURE

The software STRUCTURE 2.3.1 (Pritchard et al. 2000) was used on the cluster Hera at WSL (CentOS Linux 5.5 in 64bit mode (x86-64)) to identify genetic clusters. As the samples were collected within an area of one square-kilometer and sample locations are at least 15 km apart from each other (max. distance is 306 km), the LOCRPRIOR model (Hubisz et al. 2009) was chosen to "*use the sampling locations as prior information to assist the clustering*" (locprior=1, locispop=1, lociprioriniti=1.0, maxlociprior=20.0). Nevertheless, it is realistic that exchange between locations happens and the ADMIXTURE model was used (noadmix=0) to follow the assumption that an individual has inherited some fraction of his/her genome from ancestors in different population. As exchange between locations seems to be realistic, we assume that allele frequencies are likely to be similar between different locations (freqscorr=1) (Falush et al. 2003). Ten repetitions were done for each number of clusters (K) between K=2 and K=10. The software STRUCTURE HARVESTER (Earl & vonHoldt 2012), CLUMPP 1.1.2 for Windows (Jakobsson & Rosenberg 2007) and DISTRUCT 1.1 (Rosenberg 2004) were used to collate, uniformly label and visualize the results gained from STRUCTURE. To estimate the true number of clusters (K) from STRUCTURE results, STRUCTURE HARVESTER (Earl & vonHoldt 2012) calculates several measures based on log-likelihood. When the true number of K is reached, the mean of estimated log-likelihood of the data L(K) is expected to reach a plateau and variance between runs increases

afterwards (Evanno et al. 2005). As this estimation is not objective and precise, the difference between successive likelihood values L'(K) = L(K)-L(K-1) could be useful to indicate the K with the highest increase in log-likelihood. *Delta K* is the difference |L'(K)-L'(k+1)| divided by the standard deviation of L(K) and reach a maximum value when true K is reached (Evanno et al. 2005).

Data on the First Records of *Melanargia galathea* in Switzerland

Data were obtained from the Swiss Biological Records Center (Centre Suisse de Cartographie de la Faune) to detect if certain areas of Switzerland have been colonized within the last century. The data are concatenated to squares of 5x5 km and the earliest record for each square was extracted.

Habitat Suitability Analysis

As precondition for a relationship between genetic diversity and habitat suitability, a correlation between genetic diversity and population size has to exist. Theory predicts a linear relationship between the effective number of alleles and the logarithm of population size (Frankham 1996, Soulé 1976). The rarefied allelic richness N_r was calculated as described in the section before for locaitons with at least ten sampled individuals. The population size for each location was estimated from BDM transect counts by Hintermann & Weber (Reinach, CH) using a special data evaluation procedure. The R package *lm* (package stats) was used for a linear regression analysis with the logarithm of population size as independent variable and genetic diversity as dependent variable. For the case that cluster differ significantly in their genetic diversity independently from population size (e.g., founder events, bottlenecks), a parallel analysis was conducted with measures of N_r standardized for each cluster using following formula:

$$N_r\_standardized = \frac{(N_r - mean N_r\_cluster)}{sdv(N_r\_cluster)} \quad (4)$$

As only few locations were sampled with more than ten individuals in some clusters (two locations in the Rhone valley cluster and one location in the Rhine valley cluster), a good estimate for the standard deviation of N_r and thus a normalization was not possible. To overcome this problem, we estimated N_r for all locations with at least six sampled individuals and standardized these diversity measures. Afterwards, we selected the data from locations with at least ten individuals and tested them for a relationship between sample size and genetic diversity.

As result of this expected relationship between population size and within population genetic diversity, it is reasonable to assume there is a relationship between the size and quality of habitat and genetic diversity. Parameters that could describe suitable habitat for *Melanargia galathea* include the presence and size of habitat types (e.g., extensively managed grassland), abiotic characteristics (e.g., temperature, precipitation) and spatial structures (e.g., forest structure, exposition). Parameters of habitat type and size were obtained from the "Arealstatistik nach Nomenklatur 2004 – Standard" (Humbel et al. 2010) for each BDM-square of one square-kilometer size. The data were downloaded from the paleo server at WSL and analyzed with the software ArcGis 10.02 (ESRI, Redlands, USA). We used the Arealstatistik-Data from 2004-2009 with 72 categories and a resolution of 100x100 m. The point data were transferred to raster data (Tool: *Point to Raster* - Conversion) and further to polygon data (Tool: *Raster to Polygone* - Conversion). Subsequently, Arealstatistik-data for each BDM-square were extracted (Tool: *Intersect* - Analysis) and placed into the attribute table. Some squares crosses the border of

Switzerland and were smaller in size as we have no Arealstatistik-Data outside of Switzerland. To correct for that bias, the data were normalized for the total size of the Arealstatistik-data per location (area of category / total area of Arealstatistik-data) using the software R. As *M. galathea* has a preferred diet of grass species of the family Poacea as larval food plants and forbs as nectar plants of the imagines, the habitat of *M. galathea* should be restricted to areas where both food types occur. As such data do not exist with a sufficient resolution in the Arealstatistik, the preferred habitat type of *M. galathea* can be described best as extensively managed grassland (Ebert & Rennwald 1991a). Out of the 72 categories from the Arealstatistik-Data, four categories were built from several categories that were expected to be relevant descriptors of habitat size and quality for *Melanargia galathea*:

Table 3: Habitat parameters were derived from Arealstatistik 2004-2009 (Humbel et al. 2010) with 72 categories. Several categories have been concatenated to four habitat types that are assumed to be beneficial or detrimental for *M. galathea*. Categories that could contain feeding and nectar plants of *M. galathea* and represent extensively managed areas are concatenated into the descriptor Extensively Managed Grassland and Shrubbery. In contrast, the habitat types Intensively Managed Grassland and Forest represent areas of intensive anthropogenic use and are lacking suitable feeding and nectar plants.

	ID in Arealstatistik 09	Description
Intensively managed grassland	16, 18, 21, 23, 30, 31, 32, 33, 34, 35, 36, 37, 39, 40, 41	Grassland beside roads, rails and airports, parkway, sport facilities, camping places, cemetery, garden plot, agricultural areas, wine yards.
Extensively managed grassland	42, 43, 44, 45, 46, 47, 48, 49, 55, 56, 65	Meadows, unproductive grassland, scattered forest (forest cover of 20-60%) and shrub encroachments.
Shrubbery	57, 58, 59, 60, 64	Areas with shrub vegetation, hedges, tree clusters.
Forest	50, 51, 52, 53	Forest areas (minimum height of 3m and minimum cover ratio of 60%), afforestation.

Extensively managed grassland and shrubbery are expected to have a positive influence on *M. galathea* whereas intensively managed grassland and forest should have a negative impact on the abundance of this butterfly species. Furthermore, data from Florian Zellweger for each square were used to get abiotic and structural parameters for each BDM-square. Out of the 141 parameters, three parameters were expected to be relevant for *M. galathea*:

Table 4: Data about climate and vegetation structure were derived from Florian Zellweger for the BDM squares of one square kilometer. Two abiotic descriptors are expected to control the flight conditions for butterflies. As result of the thermo-biological demands, weather conditions including temperature, precipitation and wind strongly control the ability of butterflies to fly (Ebert & Rennwald 1991a). A third factor assumed to be relevant for butterflies is the spatial structure of vegetation patches. Habitats with vertical diverse vegetation structure provides valuable microclimatic gradients as well as wind shielding or structures for mating.

	Description
PSUavg	The average monthly precipitation sum (1/10mm / mth) over the summer months June to August (1961-1990), derived from Zimmermann & Kienast (1999).
TSUavg	The mean summer temperature (1/100 °C) from June to August (1961-1990), derived from Zimmermann & Kienast (1999).
$P95_{sd}$	The 95^{th} percentile of vegetation heights (P95) for each raster point (20x20m) within the BDM squares was calculated from Light Detection and Ranging data (LiDAR) and standard deviation was calculated for each BDM square ($P95_{sd}$). The maximum vegetation height was restricted to values between 55 m and 1 m height. The data source and preparation is described in Zellweger et al. (2013).

As flight conditions for butterfly imagines are mainly controlled by temperature, radiation and wind (Ebert & Rennwald 1991a, Courtney & Duggan 1983, Turner et al. 1987), low temperatures and high precipitation during the flight period should have a negative effect on the survival and reproduction success of lepidopteran imagines. As measure for a spatial characteristic, the standard deviation of the 95^{th} percentile of vegetation height was computed for each BDM square

(using LiDAR data analyzed by Florian Zellweger). As P95sd was only computed for vegetation heights larger than one meter, grassland was excluded from this measure. Large values of P95sd are assumed to reflect a diverse (vertical) spatial structure that is connected with wind shielding, reduced mowing intensities at the edges of forests and hedges and diverse microclimatic conditions. Although LiDAR data has successfully been used to predict species diversity of birds (Goetz et al. 2007), arthropods (Vierling et al. 2010) or bats (Jung et al. 2012), the application is restricted to forest areas. Applications for areas with forest and grassland is not straight forward as several different arrangements could result in the same $P95_{sd}$ value. Measures of the edge length could be a more valuable predictor for habitat suitability.

For data exploration, the measure for within population genetic diversity N_r and the seven predictor parameters were plotted against each other using the R function pairs (package graphics) with modifications gained from Florian Zellweger. Furthermore, Pearson's correlation coefficient between each pair of parameter were computed. These comparisons are not only suitable to detect dependencies of rarefied allelic richness but also to detect correlations between predictors. Strobl et al. (2008) detected a bias towards correlated predictor variables when variable importance was measured with random forest approaches. Variable importance was estimated to reduce the number of predictors and avoid over-fitting according to Harrell's rule of one predictor per ten observations at minimum (Harrell et al. 1996). The R-package randomForest (Liaw & Wiener 2002) was used to estimate the importance of each parameter to predict genetic diversity within populations. Two measures of importance were gained with the mean decrease in accuracy (IncNodePurity) and the average decrease in mean squared error MSE (%IncMSE). The two parameters with the highest importance were selected for the model, when both measures of variable importance list them in the first positions. For the scenario expecting habitat quality as main reason for differences in allelic richness between clusters, all locations with at least ten samples from the total set of samples were used. For the scenario assuming demographic effects as main reason for differences in genetic diversity between clusters (see following paragraph), all locations from the North-western cluster with at least six samples (18 locations) were used for data exploration (pairs function and randomForest). For the other clusters respectively 2, 5 and 3 locations with sufficient sample sizes exists and a standardization and inclusion in this analysis would not result in meaningful results.

A multivariate linear model was created using the R function *lm*. As we can expect that the variance of the dependent variable (N_r) increases with increasing diversity (see comparison of N_r between different clusters, Appendix 3), we transformed the N_r data and the habitat parameters using the square root to obtain a constant variance with increasing values. Furthermore, a weighting factor was included as we have different sample sizes for each location and can assume that with decreasing sample size the estimate of the genetic diversity is less accurate. Such a weighting with respect to sample size was conducted before by Saccheri et al. (1998). An alternative weighting could be conducted when microsatellite loci with a higher diversity are expected to be more meaningful than microsatellites with lower number of alleles (Olano-Marin et al. 2011). As population size is not the only controlling factor, it is difficult to separate the effect of suitable habitat on genetic diversity from that of (historical) demographic events and high gene flow from neighboring populations. Two scenarios were assumed that encompass two possible causes separately. In the first scenario, we assume that genetic diversity is solely controlled by habitat size and quality which is also causing differences in genetic diversity between clusters. In the second scenario, differences between clusters are expected to be caused solely by (historic)

demographic events. The parameter cluster identity was included to represent these demographic effects. As we have no data on neighboring populations, we cannot compensate for the confounding effect of gene flow. When more data will be sampled in the future, a random intercept and random slope model could be fitted for each cluster with the sampling year as factor to detect a difference in diversity between years.

Results

Genetic Diversity within Individuals and Populations

The conditions for an analysis of variance (ANOVA) were met for H_o, H_s and N_r. The values of H_o, H_s and N_r within each population of the seven markers are normal distributed according to a Shapiro-Wilk-test, QQ-plots and plots of the density distributions. The Bartlett-test indicates no

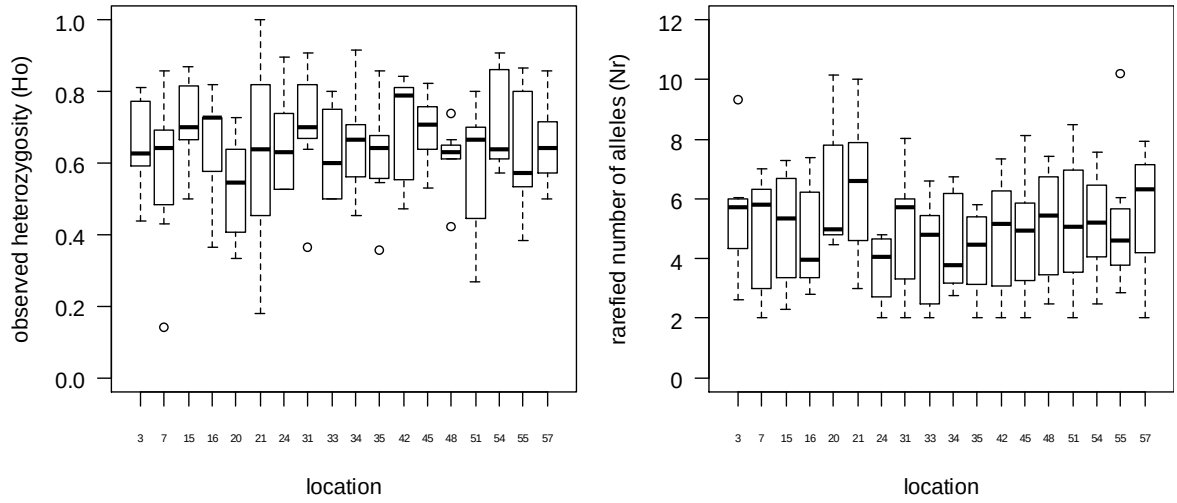

Figure 9: Boxplots of observed heterozygosity (H_o) and rarefied allelic richness (N_r, min.n=18) for the 18 locations with at least ten sampled individuals (represented by the location IDs used during lab work). The different values of H_o and N_r for each location are derived by the seven microsatellite markers.

heterogeneity in variances in H_o, H_s and N_r within locations. The ANOVA indicates no differences in within individual and within population genetic diversity (H_o, H_s and N_r) between the 18 locations with at least ten sampled individuals (Figure 9, Appendix 3). The two measures for

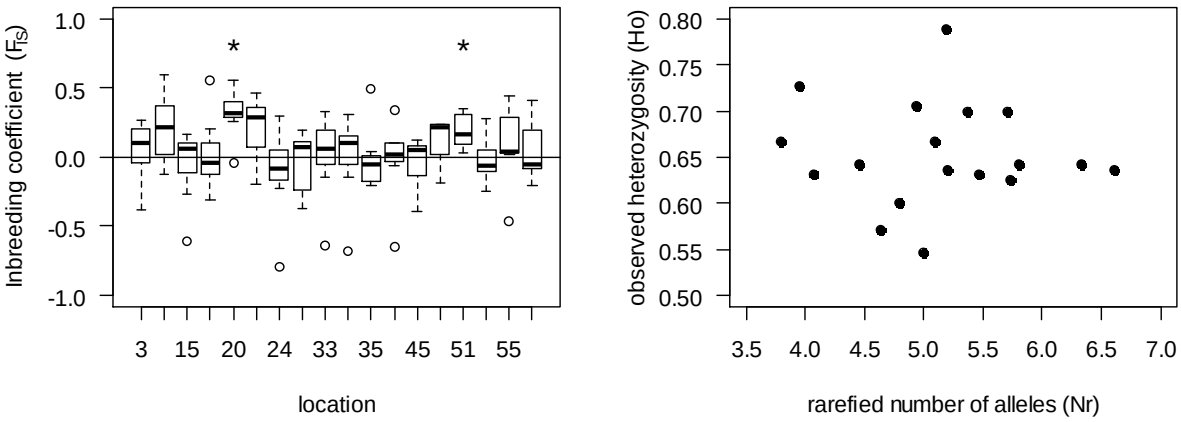

Figure 10: Boxplots of the inbreeding coefficients (F_{IS}) of each marker at each location (left plot). The asterisk (*) indicates significant deviations from zero (see results of a Kruskall-Wallis-test in Appendix 3). No correlation between observed heterozygosity (H_o) and rarefied allelic richness (N_r) was found in this data set (right plot, see also results of a linear regression analysis in Appendix 4).

within population genetic diversity H_s and N_r are positively correlated (df=16, p=0.0003). In contrast, within population genetic diversity (N_r) is not correlated with within individual genetic diversity (H_o) (df=16, p=0.254).

At two locations, the inbreeding coefficient was significantly higher than zero (Figure 10). The two locations with significant positive inbreeding coefficients are positioned near Bourg-en-Lavaux (north of Lake Geneva) and Soglio (Val Bregaglia in canton of Grisons). Although no differences in rarefied allelic richness between locations were detected, the plot of extrapolated

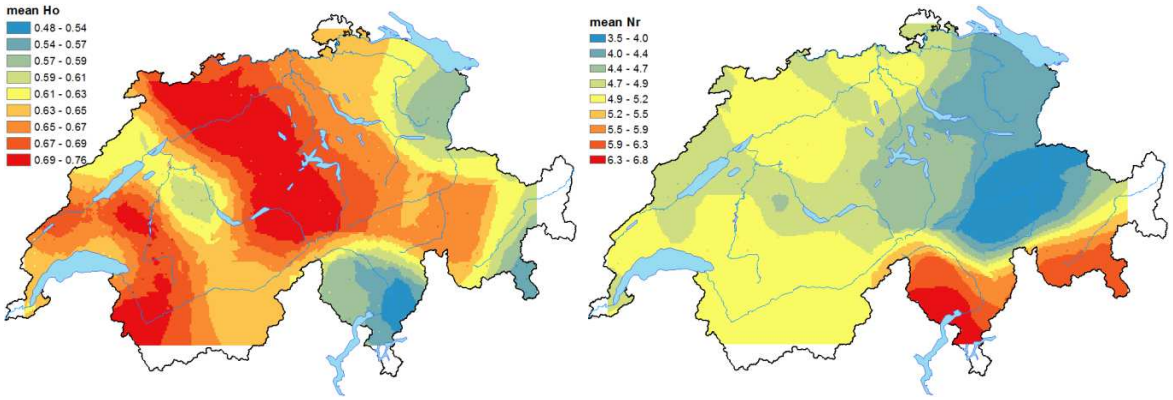

Figure 11: Extrapolated within individual genetic diversity (H_o) and within population genetic diversity (N_r) of *M. galathea* in Switzerland. A moving window approach with a 20 km radius was used to account for low sample sizes at many locations. Diversity values were extrapolated over space using Kriging in ArcGIS 10.2 (ESRI, Redlands, USA). The arithmetic mean over loci was used.

rarefied allelic richness (N_r) indicates strong regional differences in within population genetic diversity of *M. galathea* (Figure 11). Populations of *Melanargia galathea* in North-western Switzerland and the Rhone valley seem to have intermediate values of rarefied allelic richness N_r (4.7-5.2). Low values of extrapolated N_r were detected in the Rhine valley (3.5-4.4) and high values in the Southern Alps (4.9-6.8). This pattern is not found in the extrapolated within

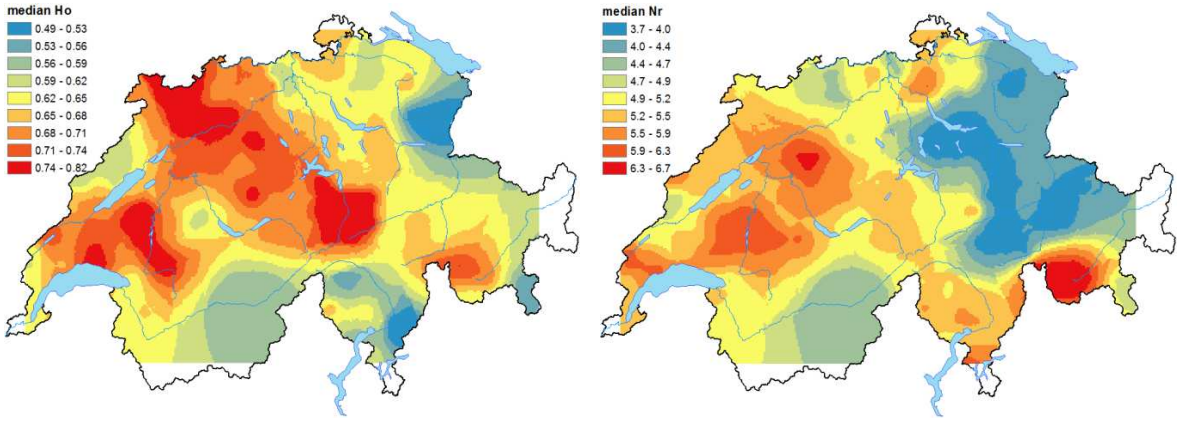

Figure 12: Extrapolated genetic diversity within individuals (H_o) and within populations (N_r) of *M. galathea* in Switzerland using the median to average over loci. A moving window approach with a 20 km radius was used and diversity values were extrapolated over space using Kriging in ArcGIS 10.2 (ESRI, Redlands, USA).

individual genetic diversity H_o (Figure 11) and individuals living in regions with a high number of alleles are not profiting necessarily from increased H_o. Furthermore, differences exist in genetic diversity patterns when we use the median to calculate measures averaged over loci instead of the arithmetic mean (Figure 12).

Genetic Differentiation between Locations

High values of differentiation could be observed between locations of at least ten sampled individuals across Switzerland with a maximum value of pairwise differentiation between locations of D_{est}=0.44 (Figure 13). A significant positive relationship between pairwise distance and differentiation (p=0.001) as well as between pairwise distance and maximum altitude (p=0.001) were detected using multiple regressions on distance matrices (MRM). Using both variables within a single MRM-model, the maximum altitude has a significant positive effect (p=0.002) whereas the distance between locations has not (p=0.392). The isolation by distance plots show no clear correlation when all locations independently from cluster identity were used (Figure 13; top-left). In contrast, the IBD plot for locations in the North-western cluster indicates a correlation between pairwise distance and differentiation (Figure 13, top-right).

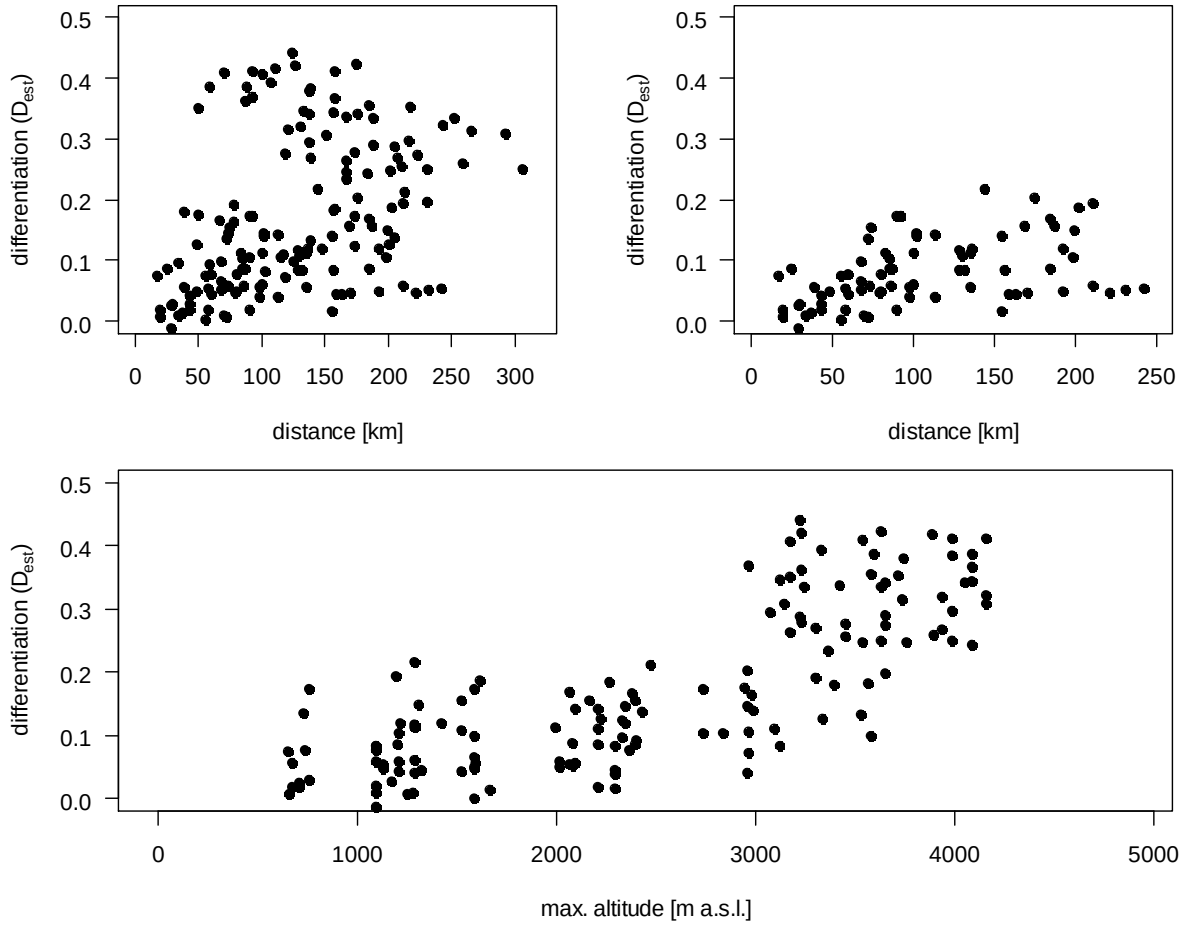

Figure 13: Plots of isolation by distance over all locations with at least ten sampled individuals (top-left), within the North-western cluster (top-right) and isolation by altitude between locations of at least ten sampled individuals (bottom).

A distinct isolation by altitude pattern was detected (Figure 13, bottom). The Mantel correlogram indicates a significant positive correlation of differentiation and maximum altitude up to 2500 m asl (Figure 14). The correlogram of IBD within the North-western cluster indicates significant positive Mantel correlation coefficients up to a distance of 50 km.

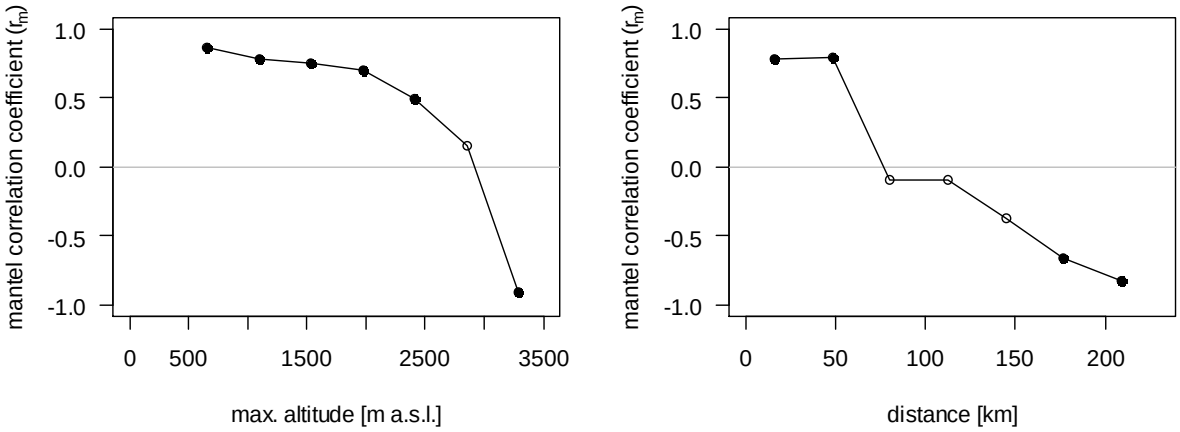

Figure 14: Mantel correlograms of isolation by altitude between all locations of at least ten sampled individuals (left) and isolation by distance between all locations of the North-wester cluster with at least ten sampled individuals (right). Mantel correlation coefficients (r_m) significantly different from zero are pictured with a black fill, whereas r_m are pictured without fill when they were not significantly different from zero.

Genetic Diversity on the Landscape Level

Based on the measures L(K), L'(K) and Delta K, the occurrence of four different clusters (Figure 15) can be expected.

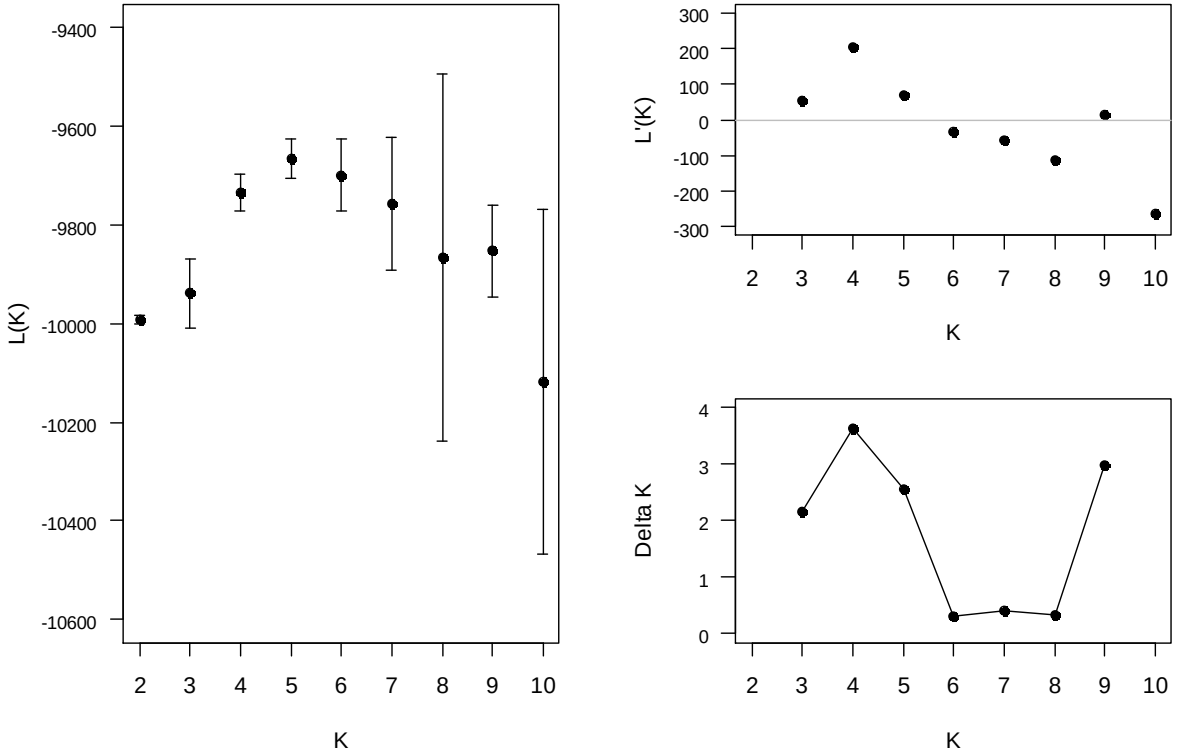

Figure 15: Three measures were derived from STRUCTURE HARVESTER (Earl & vonHoldt 2012) to detect the true number of clusters (K). The average values of log likelihood at each step of the MCMC, *L(K)*, are plotted on the left side. The difference in L(K) of successive K, *L'(K) = L(K)-L(K-1)*, indicates the K after the highest increase in likelihood (top-right). *Delta K* accounts for the standard deviation when Delta K = |L'(K+1)-L'(K)|/sdv(L(K)). The maximum value of Delta K and L'(K) indicate the true number of K.

The four clusters of *M. galathea* in Switzerland are separated spatially into the Rhone valley (canton of Valais), the Rhine Valley (canton of Grisons), the Southern Alps and the remaining North-Western part of Switzerland (Figure 16). The cluster identity correspond well with the pattern of within

Table 5: Pairwise differentiation D_{est} between clusters. All individuals sampled within a cluster were included in this analysis.

	Southern alps cluster	Rhine valley cluster	North-western cluster
Rhone valley cluster	0.2756	0.2948	0.1965
Southern alps cluster		0.3782	0.2547
Rhine valley cluster			0.1310

population genetic diversity N_r (compare Figure 11 and Figure 16). The rarefied allelic richness N_r (min.n=34) is significantly lower in the Rhine valley cluster compared to the Southern alps cluster (Appendix 3). The differentiation between clusters is much larger (average pairwise D_{est} between clusters D_{est}=0.255, see Table 5) than the within cluster differentiation (average pairwise D_{est} between locations of the same cluster D_{est}=0.073).

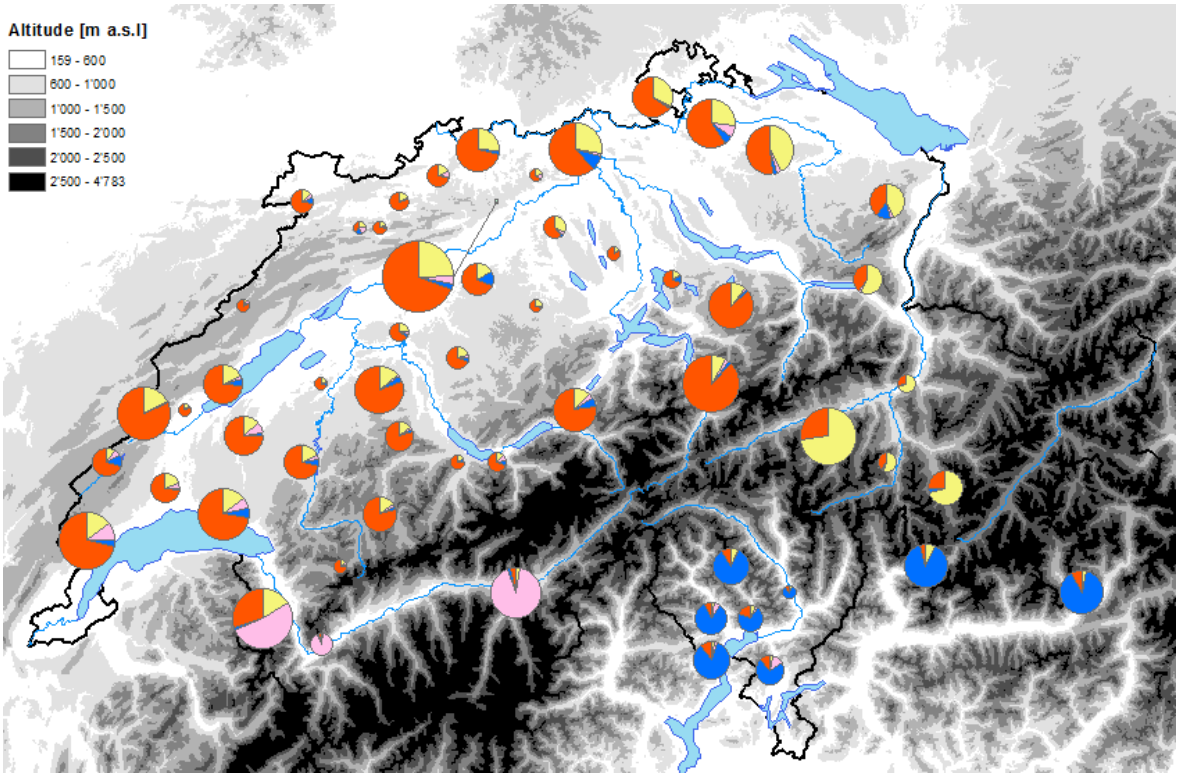

Figure 16: Results of STRUCTURE with K=4. The probabilities belonging to a specific cluster are represented via the pie chart colors. Each cluster has a different color with *blue* for the Southern alps cluster, *pink* for the Rhone valley cluster, *yellow* for the Rhine valley cluster and *orange* for the North-western cluster. The size of the pie charts corresponds to the number of sampled individuals per location.

Several private alleles were found that occur only within a single cluster (51) with 26 private alleles within the North-western cluster, 19 private alleles in the Southern alps cluster, 4 private alleles in the Rhone valley and 2 in the Rhine valley.

Thirty-nine alleles were exclusively occurring at a single location with similar abundances within the single clusters. Surprisingly, the STRUCTURE results for K=2 (Figure 17) assign the locations within the Rhone Valley into the same cluster as the locations of the Southern Alps. This is contrasting to the results of pairwise differentiation between clusters (Table 5) when the Rhone valley cluster is most similar to the North-western cluster.

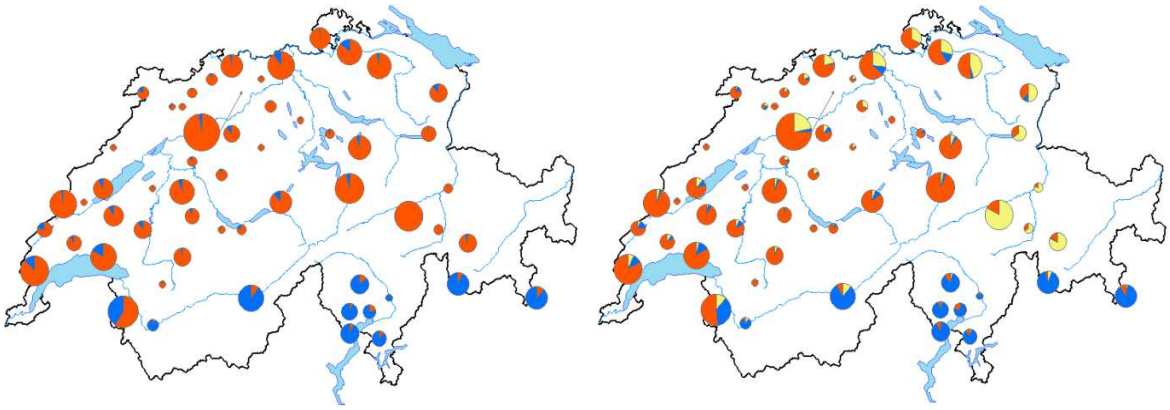

Figure 17: STRCTURE results of *Melanargia galathea* for K=2 (left plot) and K=3 (right plot). The colors represent the cluster identity and the pie size the assignment probabilities to the respective clusters. The size of the pie charts corresponds to the number of individuals sampled at each location.

First Records of *Melanargia galathea* in Switzerland

The data from the Swiss Biological Records Center (CSCF) indicate confirmations of *Melanargia galathea* in Switzerland since 1869 (Figure 18). Early records are mainly available near larger cities (e.g., Geneva, Basel, Zurich or Bellinzona), touristic cities (e.g., Interlaken, Davos or Meiringen) or in areas of interest for nature conservation (e.g., Val Müstair, canton of Grisons). Some regions have been studied much earlier for butterflies (or at least for this species) than others as the areas near Schaffhausen or Geneva, the Rhone Valley and the Canton of Ticino show high densities of early first

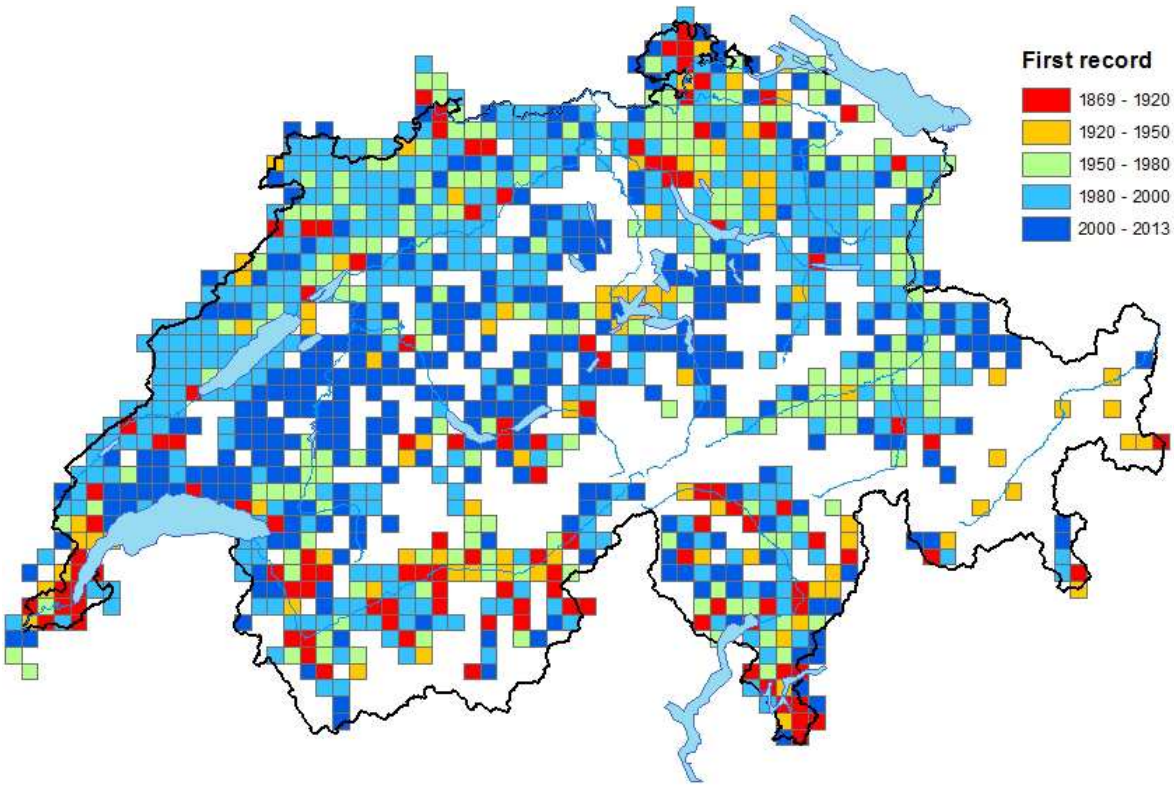

Figure 18: The dates of first record of *M. galathea* in Switzerland extracted from data of the Swiss Record Center CSCF. Data were concatenated to squares of 5x5 km.

records compared to the rest of Switzerland. Every region of Switzerland seem to be colonized by *Melanargia galathea* before the last century as early first records exists everywhere.

Furthermore, it is remarkable that confirmations of *M. galathea* are almost missing in the upper Inn valley (canton of Grisons) with six records before 1950 and one recent record from 2012.

Habitat Suitability Analysis

No significant linear relationship was detected between the population size (estimated by transect counts of the BDM) and within population genetic diversity (see Appendix 4 and Figure 19).

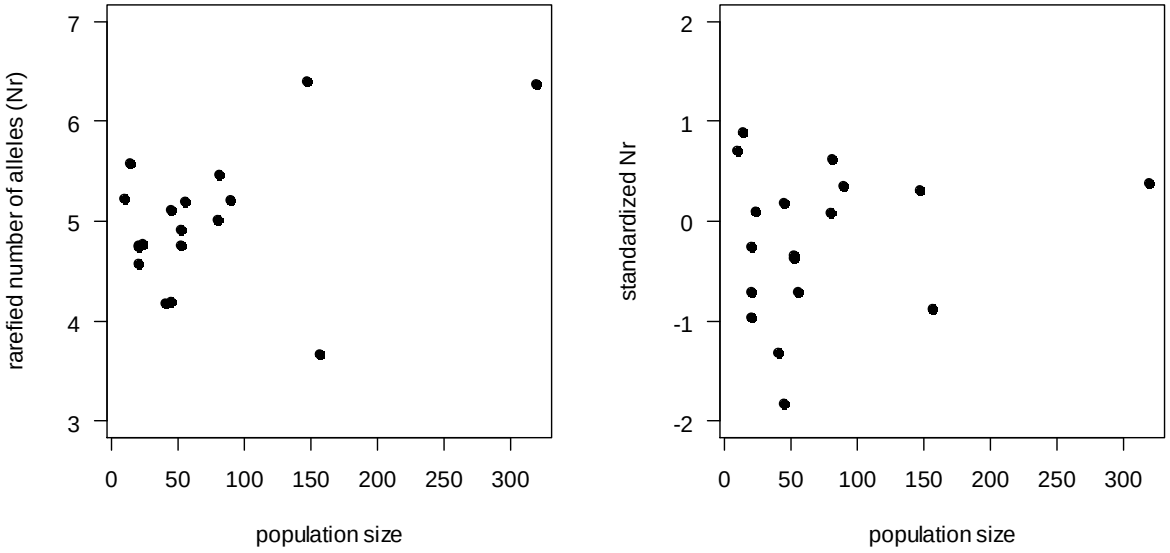

Figure 19: The mean rarefied allelic richness N_r is plotted against population size (estimated by transect counts of the BDM) (left plot). To account of differences between clusters that are independent from population sizes (e.g., founder events, bottlenecks), N_r values were standardized for each cluster (N_r-mean($N_{r\_cluster}$))/sd($N_{r\_cluster}$) (right plot) for all locations with at least six sampled individuals. From these standardized values, the locations with at least ten samples were extracted.

The data exploration using the pairs function showed no distinct correlation between the within population genetic diversity (N_r) and the seven habitat descriptors (Appendix 4). For scenario 1, when all locations with at least ten sampled individuals were included, the maximum Pearson's correlation coefficients was 0.38 showing a weak positive correlation between N_r and the size of shrubbery areas. The second highest correlation coefficient (r_P=0.28) were reached for N_r with forest areas (positive relationship) and extensively managed grassland (negative relationship), which is in contrast to the expected relationship. Strong correlations between predictors were detected between precipitation and temperature (r_P=0.66), temperature and intensively managed grassland (r_P=0.73), forest and intensively managed grassland (r_P=0.63) as well as extensively managed grassland and forest (r_P=0.64). For scenario 2 with locations of the North-western cluster and at least six sampled individuals, no correlation between N_r and predictors were found (r_P<0.25). Similar to scenario 1, several strong correlations between predictors were detected.

This lack of a correlation between a habitat parameter and within population genetic diversity was also shown by the random forest approach. Negative values for the mean percentage of explained variance were computed for both scenarios (Table 6). The percentage of increased mean standard error (%IncMSE) were not higher than 6% in scenario 1 and maximum 10% in scenario 2. The %IncMSE even reached negative values for some parameters. The values of IncNodePurity were not larger than 0.15 for scenario 1 and below 25 for scenario 2. The results between the two measures for variable importance differed except for scenario 2 when extensively managed grassland was listed first for %IncMSE and IncNodePurity.

Table 6: Results of the variable importance estimation using randomForest (Liaw & Wiener 2002) in R 3.1.0. The mean rank in variable importance of the two measures %IncMSE and IncNodePurity was computed for the respective predictor. In scenario 1, the data of genetic diversity (N_r) were used as dependent variable, whereas N_r values were standardized for each cluster in scenario 2.

Scenario 1: Genetic diversity is solely ruled by habitat parameters		Scenario 2: Genetic diversity within clusters is mainly ruled by demographic history	
Variable	**Mean rank in %IncMSE and IncNodePurity**	**Variable**	**Mean rank in %IncMSE and IncNodePurity**
Shrubbery	2	Extensively managed grassland	1
Forest	2	Shrubbery	3.5
Extensively managed grassland	3.5	Temperature	4
Mean of squared residuals	0.02856018		6.255489
Mean % var. explained	-28.54		-17.52

As result, the two predictors shrubbery and forest were selected for regression analysis of scenario 1 and extensively managed grassland for regression analysis of scenario 2. The linear models for scenario 1 and scenario 2 showed no significant effect of the selected habitat parameters on neutral genetic diversity (Table 7). The adjusted R^2 was much lower for scenario 1 compared to scenario 2 which might be a result of over-fitting.

Table 7: Results of the regression analysis to predict neutral genetic diversity within populations by habitat parameters. The lm function (package stats) in R was used. The values were square-root-transformed for a constant variance with increasing values and weighted according to the sample size. Because multiple predictors were tested, the p-values were corrected using the Bonferroni method (p-value*) with 7 and 11 comparisons for scenario 1 and scenario 2.

equation	**df**	**coefficient**	**estim.**	**std. error**	**t-value**	**p-value**	**p-value***	**adj. R^2**
Scenario 1 (differences in genetic diversity between clusters is solely the result of differences in habitat)								
Sqrt(Nr)~ Sqrt(Shrubbery) + Sqrt(Forest)	15	Intercept	1.9959	0.107	18.622	9e-12	6.2e-11	0.16
		Sqrt(Shrubbery)	0.0005	0.000	1.698	0.110	0.771	
		Sqrt(Forest)	0.0002	0.000	1.840	0.086	0.599	
Scenario 2 (differences in genetic diversity between clusters is solely the result of different demographic histories)								
Sqrt(Nr) ~ cluster + Sqrt(Ext. managed grassland)	13	Intercept	2.313	0.067	34.404	4e-14	4.1e-13 *	0.67
		Cluster 2	0.2566	0.089	2.897	0.013	0.137	
		Cluster 3	-0.3280	0.106	- 3.092	0.009	0.094	
		Cluster 4	-0.0615	0.059	- 1.039	0.318	1	
		Sqrt(Ext. Managed grassland)	-0.0001	0.000	- 0.778	0.450	1	

Discussion

The measured values of genetic diversity within individuals (H_o) were comparatively high. As Saccheri et al. (1998) detected a negative fitness impact of H_o values smaller than 0.5, it could be assumed that the fitness of *M. galathea* at the observed locations is not depressed by reduced within individual genetic diversity. As the values of Saccheri et al. (1998) were gained with several allozyme markers together with one microsatellite marker and a direct comparison of heterozygosity values between different marker types is not possible (Powell et al. 1996) such a conclusion cannot be made. Further research would be necessary to quantify the heterozygosity-fitness correlation (HFC) for *M. galathea* with the applied marker set and define the threshold in heterozygosity that is not limiting fitness (Frankham 1995). Although similar levels of H_o were detected for all sampled locations, regions with reduced H_o can be expected in Switzerland and became (partly) visible in the extrapolated H_o values using a moving window approach (Figure 11). When large scale patterns of H_o are no artefacts of extrapolation, a uniform cause of this pattern could be assumed. A relation between within individual (H_o) and within population genetic diversity (N_r) was not detected in our data set and the large scale pattern of N_r and H_o are not overlapping. This indicates the importance of studying H_o and N_r separately. Inbreeding is able to reduce observed heterozygosity when closely related individuals mate while the level of allelic richness is maintained. Causes of inbreeding include small population sizes and isolation. The inbreeding coefficient F_{IS} indicates significant inbreeding within two locations and seem to prove the relevance of this factor, although it is questionable if it could explain the whole H_o pattern over Switzerland. Unconsidered population-substructure can have a distinct effect on expected heterozygosity (Wahlund 1928), might uncouple H_o from H_e and therefore change F_{IS} independently from inbreeding. Although *M. galathea* can be expected to disperse over several kilometers regularly (Baguette et al. 2000), two sub-populations could exist within a single BDM square and the Wahlund-effect might be a relevant factor. Additional deviations from HWE can be created when sex ratio is not even and the mating system does not correspond to random mating. Except for regional outbreaks of *Wolbachia sp.* which could change the sex ratio considerably on large scales (Werren & Windsor 2000), these factors are not assumed to vary between regions.

Although no significant difference in within population genetic diversity could be detected between locations, the extrapolation of N_r indicated some large scale patterns within Switzerland. Population size is one major factor that determines within population diversity (Young et al. 1996) but a correlation was not detected within the data. Such a missing relation might be the result of a wrong estimator of population size. Transect counts of butterflies have been shown to correlate well with real population sizes (Pollard & Yates 1993) but are meaningless when transects are not crossing the whole habitat patch but just a subset. Even when population size has been estimated correctly for a specific year, it might not represent the effective population size when population size fluctuate strongly between years or gene flow from neighboring populations uncouple N_r from population size (Prober & Brown 1994). Despite the missing correlation, a relation between N_r and the size of suitable habitat can be expected when the habitat parameter are able to predict effective population sizes correctly. In our case, no variable of habitat suitability (including habitat types and sizes, abiotic predictors and a measure of vegetation structure) was correlating with within population genetic diversity. This missing link might be again the consequence of the sampling design. The BDM squares were not placed specifically to represent *M. galathea* habitat but were placed randomly. The parameters of habitat type, size and vegetation structure gained

within the BDM square might be not representative for the actually used area of the respective population. Furthermore, the number of locations with enough sampled individuals was low (18 locations with at least ten sampled individuals) and it is questionable if data with a resolution of 100x100 m are useful to detect suitable habitat patches. Ebert & Rennwald (1991a) proposed resolutions of 1x1 m or less for habitat suitability studies of caterpillars. Saccheri et al. (1998) detected habitat patches occupied by the Glanville Fritillary with a size of six square-meters. Although meaningful habitat suitability studies using genetic diversity data seem to be not feasible within the BDM sampling design, a cross validation of species distribution models (e.g., using presence-absence data) could be an option.

The between population genetic diversity revealed isolation by altitude. High levels of differentiation were created (or maintained) by high mountain ranges with maximum altitudes of more than 3000 m asl. As *M. galathea* is assumed to use mountain passes instead of summits to overcome the Alps, the real limiting altitude is much lower and might correspond to the altitudes of the species distribution border (~2500 m asl, see Figure 8). Despite this clear limitation of dispersal, the processes causing this barrier remains unclear. As *M. galathea* is a patrolling species, it can be assumed that males represent the main migrating step in their search for females. This search for female imagines might be concentrated on grassland areas while rocky areas are avoided, but other factors are possible. Distance is predicting differentiation across Switzerland to a lower extent than altitude. Differentiation within the largest cluster in the north-western part of Switzerland revealed an isolation by distance pattern when significant positive Mantel correlation coefficients were measured up to a distance of more than 50 km (Figure 14).

Extrapolated rarefied allelic richness (N_r) over Switzerland corresponds well with the geographic positions of the single clusters and clusters differed significantly in N_r. Either the causes that created the different genetic clusters also established the genetic diversity patterns on the large scale or characteristics within clusters (e.g., population sizes, meta-population structure) caused the differences in diversity. Clusters estimated by Bayesian clustering approaches (in our case STRUCTURE) are representing groups of individuals that are in HWE at unlinked loci (Pritchard et al. 2000). Therefore, clusters consist of regularly interbreeding individuals and are separated from each other by reduced gene flow. Some clusters might have been created during the last glaciation when *M. galathea* had several separated refugia in Southern Europe and Northern Africa when random genetic drift caused differentiation (Widmer & Lexer 2001, Schmitt et al. 2006). Although *M. galathea* has recolonized Europe since last glaciation and ice age lineage are not spatially separated anymore, it can be expected that such ice age lineages are still visible in form of different clusters. In such a case, highest genetic diversity can be found within the areas of former refugia (Habel et al. 2008) and decreases with increasing distance (Taberlet et al. 1998, Widmer & Lexer 2001). When clusters differ in their distance to glacial refugia, differences in genetic diversity can be expected. This might explain the hot spot of genetic diversity (N_r) in the Southern alps cluster compared to the other clusters. As we have no data from Italy and the Balkan region, it is not possible to test if the cluster identity corresponds to glacial lineages. Based on the results of Habel et al. (2005) and Habel et al. (2011), all four clusters within Switzerland are expected to originate from the Italian glacial lineage. This is contrasting to our results as high numbers of private alleles were found in the North-western cluster (26) as well as in the Southern alps cluster (19). More intensive sampling within Switzerland together with an analysis of samples from Italy and the Balkans could provide a more verified conclusion. Further causes of genetic clustering might be isolation by distance (Meirmans 2012, Frantz et al. 2009). In case of

M. galathea, large Alpine valleys (e.g., Rhone valley and Rhine valley) that are surrounded by high mountains hold single clusters that might be created independently from glacial processes. This assumption of isolation as cause of clustering is supported by the low number of private alleles within these two clusters (Provan & Bennett 2008) and the lack of a contact zone between clusters with increased allelic richness (Petit et al. 2003). As result of this isolation, decreased genetic diversity could be expected but seem unrealistic as the size of the Rhone valley's cluster and degree of isolation is similar to the Rhine valley cluster but exhibit a much higher genetic diversity. In contrast, a founder event in connection with isolation could accelerate cluster creation and shape genetic diversity negatively on the long term. First records of *Melanargia galathea* were dated for the beginning of the 20th century for every larger region in Switzerland. On the one hand it can be expected that every region within Switzerland has been colonized by this species more than 100 years ago. On the other hand, it is still possible that the Rhine valley has been colonized much later than the Rhone valley according to the assumed recolonization route of Habel et al. (2005). As the creation of genetic diversity is a slow process, a reduced genetic diversity might be found several hundred years after a founder event. A simulation model was used in the following section to estimate the rate of genetic diversity increases after a founder event. In contrast, special large-scale habitat characteristics that overlay clusters seem to be unrealistic. Population sizes and densities do not seem to be reduced within the Rhine valley cluster compared to the rest of Switzerland. Disturbances might cause population declines (bottlenecks) within areas of several square-kilometers but seem to be unrealistic to occur within an area of thousand square-kilometers (Rhine valley catchment area in Switzerland = 6119 km^2). In contrast to a founder event, such short-term declines in population sizes cannot be detected with data of CSCF. As the causes for genetic clustering in Switzerland remain unclear, it is difficult to define evolutionarily significant units (ESUs). Although differences in allele frequencies are detected, it is questionable if clusters caused by isolation by distance are of the same conservation value as clusters of different glacial lineages. Furthermore, it became obvious that a different number of clusters is obtained when different genetic markers and sampling designs are used. Habel et al. (2011) detected seven clusters for *M. galathea* in Europe and North-Western Africa using allozyme markers (and the software STRUCTURE) with a single cluster in the South-Western Alps. Therefore, the definition of ESUs and conservation units stays a controversial challenge (Ryder 1986, Moritz 1994, Allendorf et al. 2010, Crandall et al. 2000).

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Simulation Models

Introduction

Although comprehensive theory has been developed to analyze and predict genetic processes analytically, most of these models are valid under a set of strict assumptions that are rarely met in natural systems. Random mating with even sex ratios, constant population sizes and uniform gene flow between populations are never met in a natural system. Therefore, most natural, population genetic systems are analytically intractable. During the past decades, simulation models were developed to simulate genetics from the mutation process to population genetic dynamics. These models are highly flexible as every parameter (e.g., marker type, population size, population structure, sex ratio, dispersal rate, selection coefficient) can be chosen individually and changed with proceeding time. This flexibility is resulting in a wide application in the fields of genetic epidemiology, anthropology, evolutionary and population biology and conservation (Hoban et al. 2012, Hoban 2014). In general, two types of simulation models can be distinguished. Forward in time models (also called individual based models) are focused on individuals within populations that pass through a life cycle, differ in their demographic history and can change their locations. Backwards in time models (also referred to as coalescence simulations) consider the genealogy of the sampled DNA and are focusing on genetic lineages (Hoban et al. 2012). The input format is flexible and allows empirical data as well as in silico genetic data. As the output of forward in time simulations has the same format as regular empirical data, every kind of statistical analysis could be conducted to analyze the simulations. The general procedure to create a simulation model includes the definition of a scenario, the determination of the parameter range, choosing the statistics to monitor the outcome, define the number of replicates and the length of each run (Hoban et al. 2012). Simulation models can be used for parameter derivation (e.g., coalescence time), the prediction of future behavior under certain scenarios (e.g., founder events of invasive species), the evaluation of methods and models (e.g., statistics) and to simulate trait evolution (e.g., the evolution of pleiotropy). Puebla et al. (2012) used a coalescence simulation model to infer the reliability of analytically estimated dispersal abilities of coral reef fish species. Daleszczyk & Bunevich (2009) used a forward in time simulation model to estimate the future levels of inbreeding and genetic diversity in the European Bison. As the Białowieża Forest is separated by a border fence between the Polish and the Belarusian part, the scenarios comprise different degrees of relatedness between founders and extents of gene exchange. Fabbri et al. (2007) studied the population genetics of wolves (*Canis lupus*) in the Alps and the Apennines. A simulation model was used to assess the number of individuals that are necessary to explain the current genetic diversity levels after recolonizing the alps more than twenty years ago. Balkenhol et al. (2009) used data gained by simulations to compare the suitability of several statistical methods in landscape genetics including Mantel test and MRM. Guillaume & Otto (2012) studied the evolution of pleiotropy, which describes genes affecting multiple traits of an organism and individual based simulation models were used in addition to analytical approaches.

In case of genetic monitoring programs, simulation models could be useful in several ways. Although Baguette et al. (2000) used a mark–release–recapture approach to estimate the dispersal ability of *M. galathea*, such data are rarely present for other species. A simulation model could be used to test the reliability of the estimated dispersal ability of *M. galathea* based on the

genetic data similar to Puebla et al. (2012) or even estimate the dispersal ability ab initio. Parameter estimation is of special importance when a simulation model for a monitored species has to be developed to predict the future development of genetic diversity or estimate the effect of management actions. Furthermore, simulation models could be useful to estimate the speed of genetic diversity recreation after a founder event and detect the controlling factors. Is it possible that *M. galathea* colonized the Rhine valley more than 100 years ago while the level of genetic diversity is still reduced? In the following section, forward in time simulation models were developed to test their feasibility for two different kinds of questions.

Materials and Methods

A Population Genetic Simulation Model for *M. galathea*

The software NEMO version 2.2.0 (Guillaume & Rougemont 2006) was used to simulate population genetics at neutral microsatellite loci. Successive non-overlapping generations were simulated with a single generation as sequence of mating, dispersal and aging (including population size regulation). As our data on genetic diversity of *M. galathea* are restricted to a single year, it has to be assumed that the measures of diversity and differentiation are at equilibrium within this cluster, therefore are in a mutation drift equilibrium. In case of proceeding monitoring, it could be possible to use older data as input for the simulation model and estimate the parameters that create the more recent data. Single populations were created based on the data of the BDM locations. From the total set of BDM areas with *M. galathea* samples, the locations of the North-western cluster were chosen as we assume that no major barrier exist within this cluster. Population sizes and distances between populations were extracted from BDM data on *M. galathea* collected between 2003 and 2012 (see section The Biodiversity Monitoring Switzerland). As no data on the sex ratios of *M. galathea* within populations exists by now, ♂pop was set to 0.75 assuming a male excess as it has often been observed in insect species (e.g., Keller et al. 2010). As only a couple of individuals were detected in some locations, locations with less than one female were excluded from this set. As it has to be supposed that the transect counts of the BDM might underestimate the population sizes within these populations (when Baguette et al. (2000) detected over 4000 individuals within eight habitat patches in Southern Belgium), we multiplied the transect counts by 2, 5, 10 and 15 to correct for this underestimation. Furthermore, we can expect that our population set is more than incomplete and the majority of populations between the BDM squares could not be detected by the BDM study design. A second scenario of simulations was initiated when additional populations were added six kilometers east of each BDM location into the gaps of the existing BDM grid with the mean population size of 26 individuals (estimated in the North-western cluster). This was done for regions were the BDM sampling grid is not condensed, therefore in all regions of the North-western cluster except the Jura mountains. For a direct comparison of the two scenarios, the BDM population sizes in the second scenario were not multiplied with 2 to reach the same total number of individuals in both scenarios (for 2*transect counts). Each individual was represented as a set of seven diploid loci with a number of 30 alleles at each locus. Mutations at the microsatellite loci were simulated using a stepwise mutation model (SSM) with a constant mutation rate over loci. As the mutation rate has not been measured for our set of markers and considerable differences in this factor can occur (Ellegren 2004, Schlötterer 2000), five different mutation rates (m_1=0.0001, m_2=0.0005,

m_3=0.001, m_4=0.005, m_5=0.01) were simulated to cover the possible range. This approach is additionally valuable to estimate the effect of different mutation rates on diversity and differentiation. Random mating was assumed as mating system with a fixed mean fecundity of a female of 5. As no landscape genetic study has been conducted for *M. galathea*, no assumptions exist on landscape elements that reduce or increase gene flow. Consequently, a homogenous landscape without barriers has to be assumed, thereby distance is the only factor controlling the gene flow between populations. A negative exponential function was chosen to describe the probability of dispersal in dependence of distance. Using a probability for an individual to migrate is beneficial as it induces a population size effect. The higher the population size, the higher the number of individuals that migrate to adjacent populations. Such a negative-exponential relationship and a density dependent dispersal between populations was measured for *M. galathea* by Baguette et al. (2000) using a mark–release–recapture approach (see Figure 6). Based on these assumptions, a function of the exponential power family was used following Austerlitz et al. (2004) with a modification:

$$p(a,b,r,disp) = \frac{b}{2\pi a^2\,\gamma(2/b)}\ exp\left(-\left(\frac{disp \cdot r}{2.5 \cdot a}\right)^b\right) \qquad (5)$$

In formula (5), the *γ* represents the gamma-function and *r* corresponds to the geographic distance between populations. The scale parameter (*a*) and the shape parameter (*b*) were adjusted to obtain a fat-tailed distribution with a maximum probability to migrate of p=0.15 when distance r=0 (a=0.5, b=0.6119472). Furthermore, the term "disp/2.5" was introduced into the formula of Austerlitz et al. (2004) to describe the dispersal ability of a species by the specification of p=0.01 at r=disp. Therefore, a value of disp=15 represents a dispersal curve that reaches a probability to

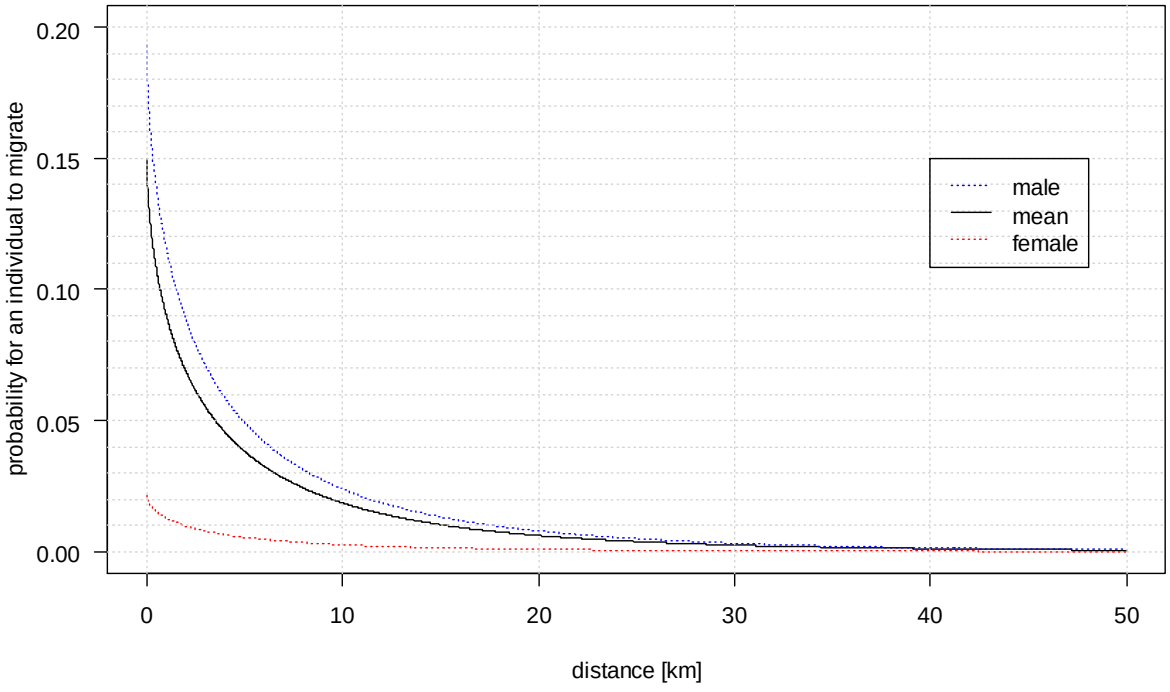

Figure 20: The model of dispersal used for *M. galathea* in dependence of sex ratio within populations and a sex specific dispersal ability. A member of the exponential power family was used with some departures (see formula 5) from Austerlitz et al. (2004). The probability of dispersal is calculated in dependence of the distance to the next patch. A dispersal ability of Disp=15 is plotted as the mean probability to migrate within a population is p=1% at a distance of 15 km. A sex ratio of ♂pop=0.75 and ♀pop=0.25 was assumed was well as a sex specific migration rate of ♂mig=0.9 and ♀mig=0.1 (computed using formula 6 and 7).

migrate of p=0.01 at a distance of 15 km (see Figure 20). To test which dispersal ability of *M. galathea* can explain the observed genetic pattern best, values of $disp_1$=5, $disp_2$=7, $disp_3$=10 and $disp_4$=15 were examined.

As we can expect that males have a higher dispersal ability than females (*M. galathea* is a patrolling species) the proportion of male migrating individuals ♂ *mig* was set to 0.9. As we have assumed sex specific dispersal rates and sex specific individual numbers within populations, it is necessary to calculate different dispersal probabilities for each sex while maintaining the mean dispersal ability of a population. For that purpose, following formulas were used:

$$♀ = \frac{ø}{\frac{♂pop \cdot ♂mig}{(♂mig-1)} + (1-♂pop)} \quad (6)$$

$$♂ = \frac{ø}{\left(♂pop + \left(\frac{(1-♂pop)\cdot(♂mig-1)}{♂mig}\right)\right)} \quad (7)$$

♂ and ♀ correspond to the male and female probability to migrate. The mean probability to migrate is represented as ø and is obtained from (6). ♂ *pop* is specifying the proportion of males within the population and ♂ *mig* represents the proportion of male migrating individuals. Using these functions, dispersal matrices were created to provide the probability for male and female individuals to migrate between each pair of population (see Figure 20). Each simulation run was conducted over 1500 generations, expecting to reach an equilibrium in diversity and differentiation after this time. For each parameter combination, ten replicates were run. An exemplary configuration file can be found in Appendix 5, although this file was used for simulations of the next section. The genotype table of the last generations was exported as FSTAT-file and analyzed using R and the package hierfstat (Goudet 2005). From the total set of simulated populations, the studied locations from 2013 with at least ten sampled individuals were extracted. The rarefied allelic richness was calculated using the function *allelic.richness* with a allele number for rarefaction min.n = 18. Pairwise D_{est} was calculated with the function *basic.stats* for each pair of populations. The difference between the simulated data and the empiric data was calculated to test the suitability of the respective parameters (mean($N_{r\ simulated}$-$N_{r\ measured}$), mean($D_{est\ simulated}$-$D_{est\ measured}$)). The sum of squares was not used as it was important to determine whether our modelled data are larger than the empirical data or smaller.

The Creation of Genetic Diversity and Differentiation

Similar to the previous section, NEMO (version 2.2.0) was used (Guillaume & Rougemont 2006) for forward in time simulations. As we want to estimate if a founder event can cause the observed pattern of within population genetic diversity in the Rhine valley cluster, population sizes and distances between populations were estimated from the BDM data for *M. galathea* in the Rhine valley. All locations with sufficient high population sizes (at least one female) were chosen. Additionally, five populations were added in the Ill valley and near the city Dornbirn to compensate for the missing data in the Austrian part of the Rhine valley. Population sizes of this five additional populations were randomly chosen from the population sizes in the Rhine valley. To estimate the effect of different population sizes, the estimated population sizes from BDM data were multiplied by respectively $disp_1$=2 and $disp_2$=6. A second scenario was established similar to the previous section when additional population were added six kilometers east of each BDM location into the gaps between the regular BDM grid. For a direct comparison of the two

scenarios, the population sizes in the second scenario were multiplied with respectively 1 and 3 to obtain the same total number of individuals present within the cluster. For both scenarios, the initial populations were created with a number of two alleles for each locus and no differentiation between populations (D_{est}=0). To implement these starting populations, a FSTAT-file was created in R and uploaded in NEMO. As the number of alleles in the set of starting populations has to correspond to ntrl_all (that determines the maximum possible number of alleles), an additional population was created that carries the remaining necessary alleles. This additional population was set to have no gene flow with the regular set of populations. The other adjustments of the simulation model correspond to the first model for parameter estimation. Non-overlapping generations are simulated forward in time with a sequence of mating, dispersal and aging (including population size regulation) per generation. Each individual is represented as a set of seven diploid loci. A stepwise mutation model with constant mutation rates across loci of respectively m_1=0.0005, m_2=0.001 and m_3=0.005 was established to estimate the effect of the mutation rate on the creation of diversity and differentiation. The proportion of male migrants was set to ♂ *mig*=0.9 and the proportion of males within populations was set to ♂ *pop*=0.75. The same fat-tailed negative exponential function (5) with a=0.5 and b=0.6119472 was chosen to specify the probability of dispersal in dependence of distance. The sex specific dispersal probabilities were determined using formulas (6) and (7). In contrast to the previous section, dispersal abilities of $disp_1$=2, $disp_2$=6, $disp_3$=10, $disp_4$=14, $disp_5$=20 were simulated to estimate the impact of gene flow on the speed of diversity creation and differentiation. The simulation model was run over 1000 generations with ten replicates for each parameter combination and genotype tables were exported in intervals of 50 years as FSTAT-files (see Appendix 5 for an exemplary

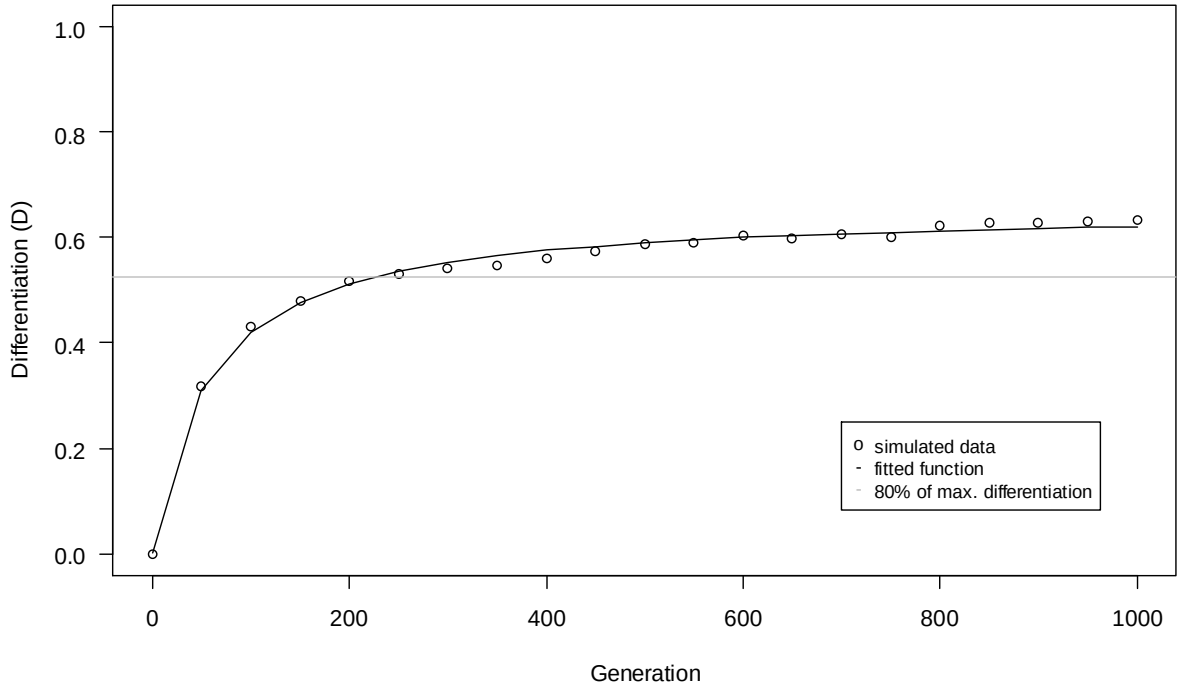

Figure 21: Temporal development of simulated differentiation after a founder event or bottleneck. Locations were designed on the basis of BDM data to represent the Rhine valley cluster. Differentiation was calculated for all populations with a size of at least ten individuals. In that example, dispersal ability was set to disp=2 and the mutation rate was m=0.001. The simulated data averaged over ten replicates are represented with circles. The fitted asymptotic function is displayed as solid black line. The grey line represents 80% of the equilibrium differentiation and the resulting number of generations to reach 80% of max. differentiation #g80 D_{est} = 224 generations.

configuration file for NEMO). From the total set of population, populations with at least ten individuals were extracted and the rarefied allelic richness was calculated using the function

allelic.richness (hierfstat) and min.n=16. As result of long computation times, the differentiation measure D_{est} was calculated as mean over all populations instead of a pairwise D_{est}.

Exploratory simulations gave a hint on an asymptotic development of diversity measures with time (Figure 21). An asymptotic function was fitted on the simulated data using the R function nls (stats) with a least-square approach. The parameters α, β and γ of following functions were estimated:

$$D_{est} = \alpha - \frac{\beta}{Generation+\gamma} \quad (8)$$

$$N_r = \alpha - \frac{\beta}{Generation+\gamma} \quad (9)$$

The parameter α correspond to the position of the asymptote at the y-axis while γ is determining the intercept with the x-axis. β is the determinant of the slope. The parameters α, β and γ were set to be larger than zero. As measures for the speed of diversity recreation, the number of generations (#g) that were necessary to gain 80% of the equilibrium level of diversity (#g80 (N_r) and differentiation (#g80 (D_{est})) were calculated:

$$\#g80\ (D_{est}) = \frac{-\beta}{(0.8*\alpha - \alpha)} - \gamma \quad (10)$$

$$\#g80\ (N_r) = \frac{-\beta}{(\,(0.8*(\alpha-2)+2) - \alpha)} - \gamma \quad (11)$$

The difference between formula (10) and formula (11) is the result of different starting levels with $D_{est}(0) = 0$ and $N_r(0) = 2$.

Results

A Population Genetic Simulation Model for *M. galathea*

Although a wide range of parameters was tested with multiple mutation rates, population sizes and dispersal abilities, it was not possible to reproduce the empirical data of genetic diversity and differentiation at the same time. As the levels of differentiation and diversity are affected contrary by population size and gene flow, the approximation of (simulated) differentiation values to empirical data is resulting in a strong deviation of the simulated diversity values and vice versa.

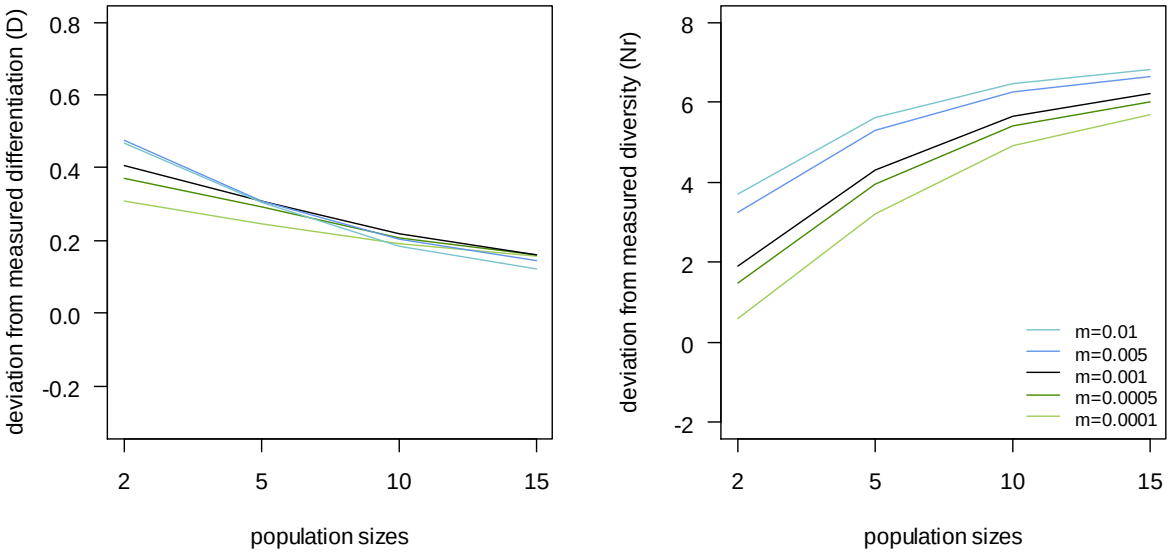

Figure 22: A simulation model was run to reproduce the population genetics of *M. galathea* in the North-western cluster. Population sizes and distances between populations was extracted from BDM data. For a constant dispersal ability of disp=15, different population sizes and mutation rates were simulated. The difference between the simulated and the empirical data is plotted against population sizes. When the simulated data are reflecting the empirical data, the deviation from measured differentiation and diversity is zero. Population sizes represents the multiplicator that was used to increase the transect counts of the BDM.

A negative relationship was observed between differentiation and population size (Figure 23) as well as between differentiation and dispersal ability (Figure 24). As the number of migrants between populations is dependent on population size, gene flow might be the cause of these relations. A positive relation seems to exist between differentiation and the mutation rate, although this relationship was weak for most parameter combinations, not detectable with other parameters and even seems to change into a negative relationship (see Figure 23 and Figure 24). In contrast to differentiation, a positive relationship was observed between allelic richness and population size as well as between genetic diversity and dispersal ability. Similar to differentiation, a positive relationship was observed between diversity and mutation rate. Differences in diversity between mutation rates are much more distinct. The only possible variable that could approximate both kinds of simulated diversity to empirical data at the same time is the mutation rate with mutation rates much lower than 0.0001.

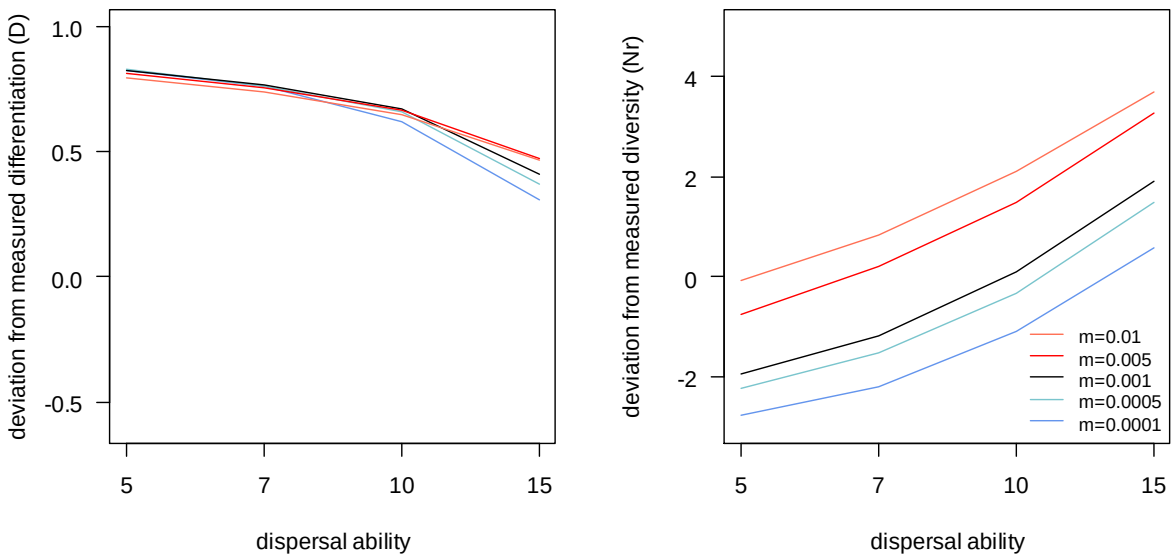

Figure 23: The simulation model was run to reproduce the population genetics of *M. galathea* in the North-western cluster. Population sizes and distances between populations was extracted from BDM data. For a constant population size of 2, different dispersal abilities and mutation rates were simulated. The difference between the simulated and the empirical data is plotted against population sizes. When the simulated data are reflecting the empirical data, the deviation from measured differentiation and diversity is 0. Dispersal ability corresponds to the parameter "disp" used in formula (5) to describe the dispersal ability of *M. galathea*. Mutation rates are pictured in different colors.

Concerning the population structure with twice as much populations and lower mean distances between populations while the total number of individuals was kept constant (see Figure 24), a positive effect could be observed. Adding populations in between does reduce differentiation and increase diversity and might be caused by increased gene flow between populations.

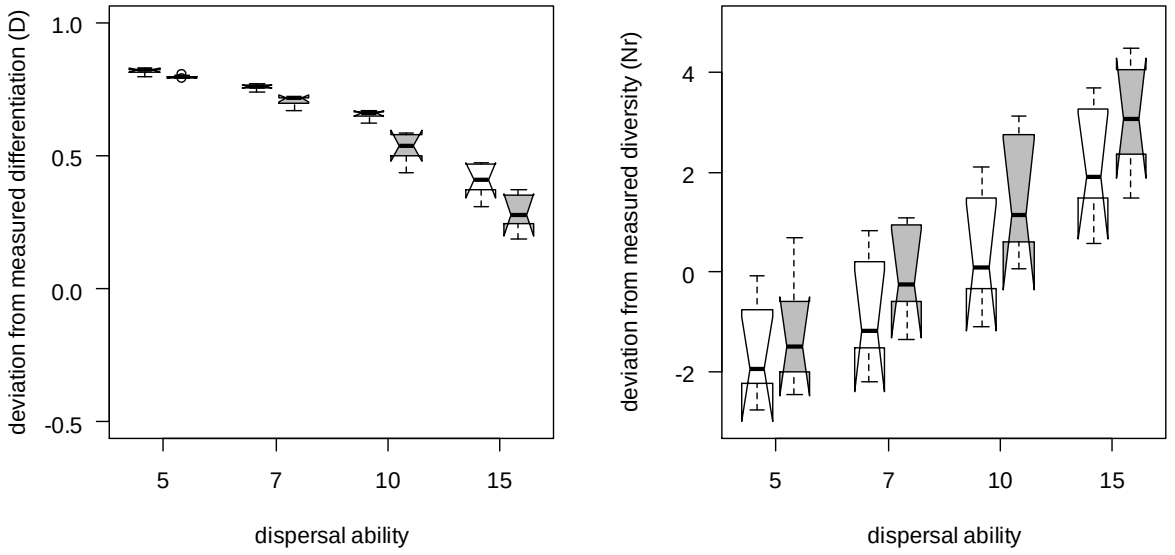

Figure 24: Comparison of the two scenarios with different population structures when the total number of individuals was kept constant. The scenario with additional populations is pictured in grey, while the regular population set is represented with white boxplots. The simulations were conducted for all populations from BDM data with population sizes equals 2 times the transect counts. Different dispersal abilities with disp=5, disp=7, disp=10, disp=15 were simulated. The boxplots show the range of different mutation rates (m_1=0.0001, m_2=0.0005, m_3=0.001, m_4=0.005, m_5=0.01).

The Creation of Genetic Diversity and Differentiation

The number of generation to reach 80% of the equilibrium level of differentiation (#g80 D_{est}) was ranging between less than five generations and 965 generations (mean #g80 D_{est}=445). The number of generations necessary to reach 80 % of the equilibrium level of rarefied allelic richness are ranging between less than one generation and 1191 generations (mean #g80 N_r=763).

Therefore, differentiation reaches equilibrium faster than within population genetic diversity (Kruskal-Wallis-test; chi-squared=24.8623, df=1, p=6.158e-07). There seems to be a positive asymptotic relationship between the difference between the initial level of diversity and the equilibrium level of diversity (deviation in diversity) and #g80 N_r.

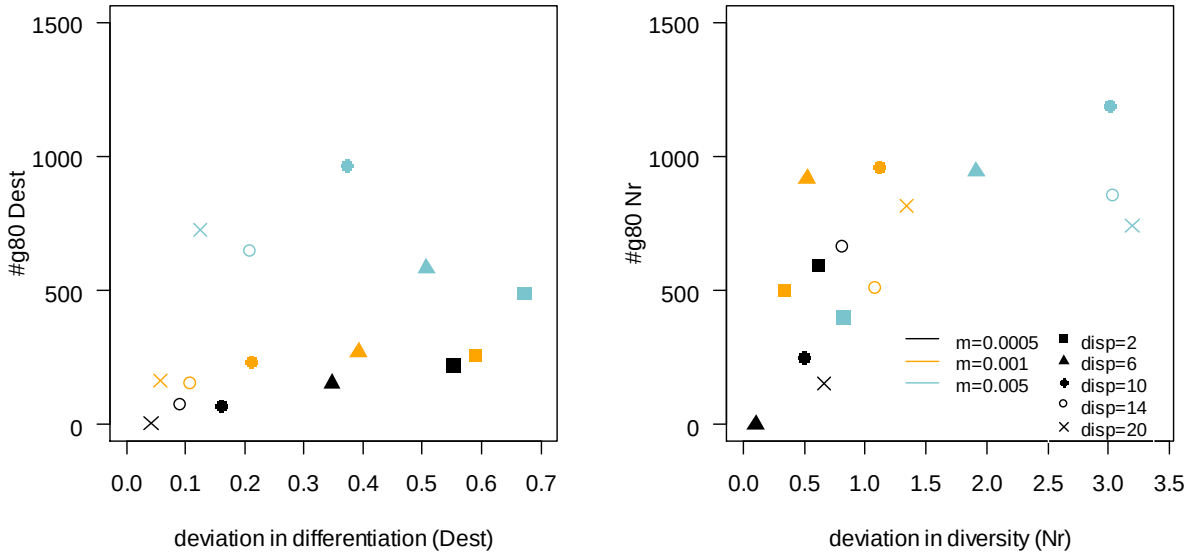

Figure 25: The time to create differentiation and within population genetic diversity is plotted in dependence of gene flow (in form of different dispersal abilities), mutation rate and the difference between the initial level and the equilibrium level of differentiation (deviation in differentiation) and diversity (deviation in diversity). The speed of diversity creation is represented as number generations necessary to reach 80 % of equilibrium diversity. The population set corresponds to the BDM data for *M. galathea* in the Rhine valley cluster. The BDM transect counts were multiplied by 2 (pop=2). The different dispersal abilities (disp) are corresponding to formula (5) and are depicted as different symbols while colors correspond to different mutation rates.

In contrast, #g80 D_{est} was not influenced by the difference between the initial level and the equilibrium level in differentiation (deviation in differentiation). The dispersal ability (and therefore gene flow) had no directed impact on the speed of differentiation and the creation of diversity. In contrast, the mutation rate seems to be the controlling factor of #g80 D_{est}. High mutation rates are connected with a high number of generations to reach equilibrium.

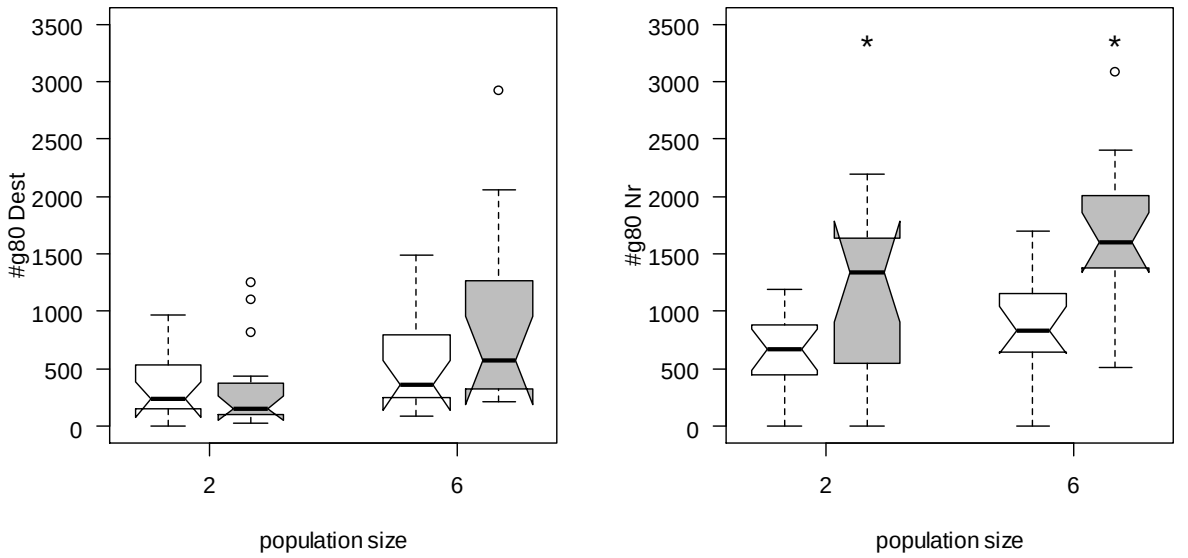

Figure 26: The number of generations to reach 80% of equilibrium differentiation and diversity is plotted in dependence of different population sizes and population structures. Simulations were created to represent the set of *M. galathea* populations in the Rhine valley cluster. Population sizes were extracted from BDM data (2003-2012) and multiplied by respectively 2 and 6. The results for #g80 D_{est} and #g80 N_r for different mutation rates (m_1=0.005, m_2=0.001, m_3=0.0005) and dispersal abilities ($disp_1$=2, $disp_2$=6, $disp_3$=10, $disp_4$=14, $disp_5$=20) are combined in a single boxplot. The grey boxplots represent the set of BDM locations with additional populations in-between, while the total number of individuals was kept constant. The asterisks indicate significant differences between pairs of the same total number of individuals.

Population size seems to have a positive effect on #g80 D_{est} and #g80 N_r, although no significant increase was found for differentiation (Kruskall-Wallis-test; chi-squared=2.4194, df=1, p=0.1198) and diversity (Kruskall-Wallis-test; chi-squared=3.5617 , df=1, p=0.05913). The change in population structure to a more dense population network has a significant positive effect on #g80 N_r for respectively 2*transect count and 6*transect count (Kruskall-Wallis-test; chi-squared=4.2155| 8.7953, df=1, p=0.04006|0.00302) but not on #g80 D_{est} (Kruskall-Wallis-test; chi-squared=0.5269| 1.6004, df=1, p=0.4679|0.2058) (see Figure 26).

Discussion

There are several possible explanations why the population genetics of *M. galathea* could not be reproduced with a simulation model. As was shown in this section, the number of generations to reach an mutation drift equilibrium in genetic diversity and differentiation is high and could range from hundreds to thousands of generations. Therefore, the population genetics of *M. galathea* are expected to have not reached mutation drift equilibrium in the North-western cluster. The BDM study design does not allow to detect all populations in Switzerland or estimate population size correctly. Therefore, the network of *M. galathea* populations in Switzerland remains unknown and the main parameters for a simulation model are not given. The only parameter that could approximate the simulated diversity measures of diversity to the empirical ones is the mutation rate. A mutation rate much lower than m=0.0001 could reduce differentiation as well as allelic richness, but is unrealistic for microsatellite loci (Ellegren 2004). As differentiation seems to reach a mutation drift equilibrium faster than allelic richness, parameter estimation should focus on differentiation instead of diversity. Assuming that our approximation of population sizes and abundance would be correct and at least the measure of differentiation is near a mutation drift equilibrium, a dispersal ability of disp>20 could be deviated from the data. This is much larger than the dispersal ability estimated from the data of Baguette et al. (2000) with disp=0.5 (see Figure 6). The observed drivers of genetic diversity and differentiation are consistent with the expectations. High gene flow in form of a high dispersal ability reduces differentiation but increase the diversity within populations by an increased effective population size. The positive relationship between population size and genetic diversity as well as mutation rate and genetic diversity is known from literature (Frankham 1996, Soulé 1976). The negative impact of population size on differentiation might be the result of the population size dependent gene flow. When additional populations in between were added to simulate a more denser population structure, an increase in diversity and decrease in differentiation was observed. This might be the consequence of an increased gene flow between populations. Due to the high number of controlling factors, it can be expected that the simulation model can be adapted to empirical data with several different parameter combinations simultaneously. It is therefore questionable if it is possible within the BDM monitoring program to accumulate sufficient data about a species to use a simulation model and predict the future development of genetic diversity.

Although it was not possible to approximate a simulation model to the data of *M. galathea*, it becomes clear that the creation of genetic diversity is a slow process. For our set of populations with a sexually reproducing species and non-overlapping generations, the creation of diversity and differentiation is lasting from decades to centuries. This is consistent with the observed pattern of glacial lineages, when reduced genetic diversities has been recorded in Central Europe even thousand years after recolonization (Habel et al. 2008, Widmer & Lexer 2001). Increases and

decreases of diversity and differentiation follow an asymptotic relationship with fast changes at the beginning and slower increases near the equilibrium level. In this study, it has not been tested if an exponential function, as it was found by Frankham (1996), would be more suitable to explain the simulated data. In contrast, this pattern is not exactly matching the results of Austerlitz et al. (1997), when a colonization process was simulated using a stepping stone model and F_{ST} values were reaching a depression after a primary maximum in the beginning. This depression might be the consequence of the colonization process and does not directly correspond to this simulation model with a fixed population system. During the simulations of Austerlitz et al. (1997), time scales of several hundred years to millennia were simulated before equilibria were reached in F_{ST}. Some assumptions could be made about the controlling factors of the rate of diversity loss or creation. Similar to the results of Montgomery et al. (2000) and Frankham (2005), smaller populations were losing or gaining diversity faster than larger populations. In our data, a further relationship was found between the rate of diversity creation and the amount of diversity that has to be created or lost. Therefore, the larger the difference between the initial level of allelic richness and the equilibrium level, the longer was the time to reach equilibrium. This is contrasting to the observation on differentiation, as the rate of differentiation does not depend on the level of differentiation that has to be created. The only factor that seems to be important for the rate of differentiation is the mutation rate when high mutation rates are connected with a high number of generations to reach equilibrium. This result is in contrast to the intuitively expected negative relationship and an examination of the used simulation model seems to be necessary. When this relation would be confirmed by future simulations, some interesting conclusions could be drawn. As mutation rates in coding regions are expected to be smaller than mutation rates at microsatellite loci, it could be assumed that the creation of differentiation relevant for adaptation and evolution could happened faster than observed in this model when selection is weak. This might explain the observation of higher adaptive differentiation (Q_{ST}-values) compared to neutral differentiation (F_{ST}) beside selection (Leinonen et al. 2008).

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General Conclusion & Outlook

How shall we measure genetic diversity? Microsatellite markers are expected to be neutral and represent loci with high mutation rates (Selkoe & Toonen 2006). However, if we want to monitor genetic diversity that is relevant for fitness, adaptation and evolution, neutral genetic diversity is not the primary target of monitoring. On the other hand, a large part of the heritable information is expected to be neutral (Kimura 1984) and alleles may be neutral today but relevant for fitness, adaptation and evolution in future. Furthermore, the quantification of neutral genetic diversity does allow to detect several processes beside selection that shape genetic diversity. As the existing knowledge on adaptive genetic diversity is still limited and relatively costly, large scale monitoring projects of genetic diversity is, for now, restricted to the neutral components for now. In future, it might be affordable to sequence the whole genome of individuals on large scale and study adaptive genetic diversity (Bolliger et al. 2014, Manel & Holderegger 2013). Then, the DNA samples collected today may be of special value in the future. As studies on adaptive genetics are mostly focusing on a set of few model organisms and their close relatives, it could be beneficial to primarily select such species for monitoring programs.

Although the microsatellite marker development has been improved by the usage of next-generation sequencing techniques (Schoebel et al. 2013), differences in the number of successfully developed markers exist between taxa. Transposable elements are expected to create repetitive flanking sequences of microsatellite motifs and, hence, complicate marker development especially in lepidopteran species. Although some aspects of this problem can be handled, the marker development is expected to be more laborious. In case of a multi-species monitoring program, it might be beneficial to refer to species with already developed marker sets or taxa without such problems. An important disadvantage of microsatellites is the missing comparability among loci. As mutation rates vary among and no exact mutation model exists for microsatellite loci, the levels of diversity cannot be compared directly among loci and species. The focus of future research could be laid on techniques and measures to enable cross-species comparisons of genetic diversity.

At which scale do we shall measure genetic diversity? Genetic diversity has to be specified into genetic diversity within individuals, genetic diversity within populations, differentiation between populations and genetic diversity on landscape level with different controlling factors and relevance for species. As result, the target diversity should be defined for each conservation effort precisely. As the heterozygosity-fitness correlation (HFC) has been shown for adaptive as well as neutral marker types (Chapman et al. 2009), a fitness-relevant part of genetic diversity can be quantified. Such reduced within individual genetic diversity is expected to occur in small, isolated populations and could be improved by management efforts (including increased population sizes and gene flow). Nevertheless, areas with the highest within individual genetic diversity do not necessarily correlate with areas of highest number of alleles. Within population genetic diversity is relevant for the fitness of a population, for instance by reducing intraspecific competition and increasing the resistance towards pests and diseases (Karvonen et al. 2012, Reusch et al. 2005). However, empirical evidence is restricted to comparisons between single-genotype populations and multi-genotype populations and, to my knowledge, such an impact on fitness has not been observed at higher levels of allelic richness. It is furthermore difficult to differ between valuable populations and less valuable ones. A population with an average of ten alleles per locus is not

per se of higher value than a population with six alleles per locus or does possess a higher fitness automatically. In such a case, the presence or absence of specific alleles or genotypes may be more relevant than the bare number of alleles or genotypes. As within population genetic diversity is dependent on population size, gene flow from neighboring populations and long-term and large scale demographic processes, it is not always possible or worthwhile to increase this level of diversity. Within a region of low allelic richness, the increase of population size and gene flow is not expected to have a positive effect on within population genetic diversity. Such large-scale patterns of genetic diversity could be detected by a genetic monitoring program within the BDM and could provide valuable knowledge for future management efforts.

A further goal of a genetic monitoring program is the identification of evolutionary significant units (ESUs) that consist of genetically distinct populations (Ryder 1986, Moritz 1994). The identification of ESUs might also be of interest for breeding programs, artificial translocations and the handling of barriers. Although genetic clusters could be identified with microsatellite markers, it is questionable if genetic clusters are identical with ESUs. Such neutrally estimated clusters might not correspond to clusters on the adaptive level or have different histories of origin. For example, is there a difference between clusters that have originated from glacial lineages and clusters created by reduced gene flow? To answer such questions, further research and the application of alternative measures (e.g., phylogenetic trees) is needed. Beside single species genetic diversity, it would be interesting to identify hotspots of genetic diversity for more species on global scale. Are the hotspots of genetic diversity identical to the hotspots of species diversity? Do we have to change the priorities of global nature conservation to include areas with high genetic diversity? For such questions, it would be beneficial to use methods that are directly comparable among species. Using microsatellite markers, the comparisons of allelic richness between markers is questionable as result of different mutation rates. Comparisons between continents with distinct sets of species would not be possible with microsatellite markers.

Beside the possibility to detect spatial patterns and temporal trends of genetic diversity, it is difficult to identify the causes of differences and changes. Possible controlling factors include long-term demographic processes (ranging back to the last glacial period), the species distribution range (see central margin hypothesis), structure and size of populations, dispersal behaviors, connectivity between populations, mating system and locus specific mutation rate. One of the few controlling factors that can be detected with little uncertainty is inbreeding. In contrast, population sizes and positions cannot be estimated detailed enough in every year within every population. The analysis of samples from the whole continent to detect glacial refugia and recolonization routes are not always possible. The creation of genetic diversity is a long process that can last from decades, to centuries and even millennia and genetic diversity and differentiation are rarely in mutation drift equilibrium. Temporal trends of genetic diversity can be expected to occur on the long term from several years to decades. As result of this large set of controlling factors, the limited data on potentially relevant factors and the time lag, it is difficult to detect for example the causal conjunction between landscape fragmentation, habitat loss and genetic differentiation and diversity. More detailed studies on smaller scale would be necessary in case of relevant and interesting trends in genetic diversity.

In addition to analytical approaches, simulation models are a comprehensible and powerful supplement to empirical data analysis and help to understand past and future processes. As result of the high flexibility, almost every scenario and parameter set could be simulated using in silico or empirical genetic data. As for most modelling approaches in ecology, the limiting factor of

simulation models is the amount and quality of available data. Consequently, it is difficult to predict temporal trends and spatial patterns of genetic diversity in detail. Parameter estimation based on genetic data have a reduced informative value. However simulation models are a suitable tool for research and application, especially for basic processes with general validity. This has been shown for the rate of genetic diversity creation. It can be concluded that it can be a long lasting process to increase genetic diversity by enhanced gene flow and population sizes. Therefore, areas or populations with high genetic diversity, as they can be found in the former glacial refugia, are extremely valuable and irreplaceable within short to medium time scales. On the other hand, negative effects on genetic diversity as result of habitat degradation or fragmentation might be visible not immediately but after several years. The creation of a dam could reduce gene flow immediately but negatively impact a fish population decades after its creation.

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Appendix

Appendix 1

PCR protocols

Table 8: Ingredients for a 10 µl PCR approach with M13(-21) label for marker development and the final application of marker D9L2 in the (pseudo-)multiplex 3.

Ingredient	Conc. working solution (µM)	Amount of working solution (µl)	Conc. PCR (µM)
Primer 1 (M13(-21))	5	0.02	0.01
Primer 2	5	0.30	0.15
M13(-21) label (FAM)	5	0.30	0.15
H_2O	-	2.38	-
Qiagen Type-it	2x	5.00	1x
DNA	-	2.00	
	sum	10.00	

Table 9: PCR conditions for marker development with M13(-21) label and final application of marker D9L2. On a Veritiy 96 Well Thermal Cycler, a ramp of 31% (=1.2°C/s) was chosen for the denaturation, annealing and elongation steps (except for the initial denaturation and the final elongation).

Temperature	Time	# cycles
94 °C	5 min	1
94 °C 57 °C 72 °C	30 s 90 s 60 s	30
94°C 53°C 72°C	30 s 90 s 60 s	8
72°C 10°C	30 min ∞	1

Table 10: PCR-mix for Multiplex 1 used for primer application with fluorescently labeled primer.

Ingredient	Conc. working solution (µM)	Amount of working solution (µl)	Conc. PCR (µM)
H2O	-	2.38	-
Qiagen Type-it	2x	5.00	1x
DNA	-	2.00	
C2S5_F	10	0.04	0.04
C2S5_R	10	0.04	0.04
952H_F	10	0.40	0.40
952H_R	10	0.40	0.40
BBJK_F	10	0.05	0.05
BBJK_R	10	0.05	0.05
	sum	10.00	

Table 11: PCR-mix for Multiplex 2 used for primer application with fluorescently labeled primer.

Ingredient	Conc. working solution (µM)	Amount of working solution (µl)	Conc. PCR (µM)
H2O	-	2.38	-
Qiagen Type-it	2x	5.00	1x
DNA	-	2.00	
7QTP_F	10	0.15	0.15
7QTP_R	10	0.15	0.15
522M_F	10	0.40	0.40
522M_R	10	0.40	0.40
48TK_F	10	0.10	0.10
48TK_R	10	0.10	0.10
BS05_F	10	0.15	0.15
BS05_R	10	0.15	0.15
	sum	10.00	

Table 12: PCR-conditions for the application of multiplex 1, 2 and the the single PCR of marker 5LN0. A ramp of 1.2°C/s was chosen for the denaturation, annealing and elongation steps except for the initial denaturation and the final elongation.

Temperature	Time	# cycles
95 °C	5 min	1
95 °C 60 °C 72 °C	30 s 90 s 30 s	30
60°C 10°C	30 min ∞	1

Appendix 2

QQ-plot, density distribution and Shapiro-Wilk test

Rarefied Number of Alleles (N_r)

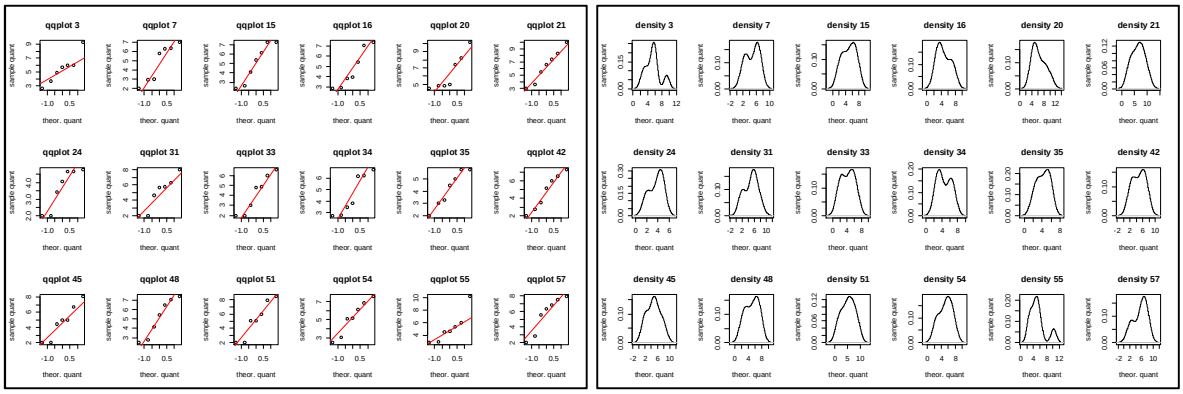

Figure 27: QQ-plot and density distribution of rarefied allelic richness (Nr) averaged over loci within each location.

Table 13: Shapiro-Wilk-test for normal distribution of rarefied number of alleles N_r at each location.

	3	7	15	16	20	21	24	31	33	34	35	42	45	48	51	54	55	57
Test - statistic	0.94	0.85	0.90	0.88	0.84	0.97	0.82	0.91	0.90	0.84	0.92	0.94	0.92	0.91	0.91	0.94	0.85	0.89
p-value	0.62	0.13	0.34	0.21	0.10	0.90	0.07	0.37	0.34	0.10	0.50	0.61	0.49	0.37	0.36	0.68	0.13	0.26

Gene Diversity (Hs)

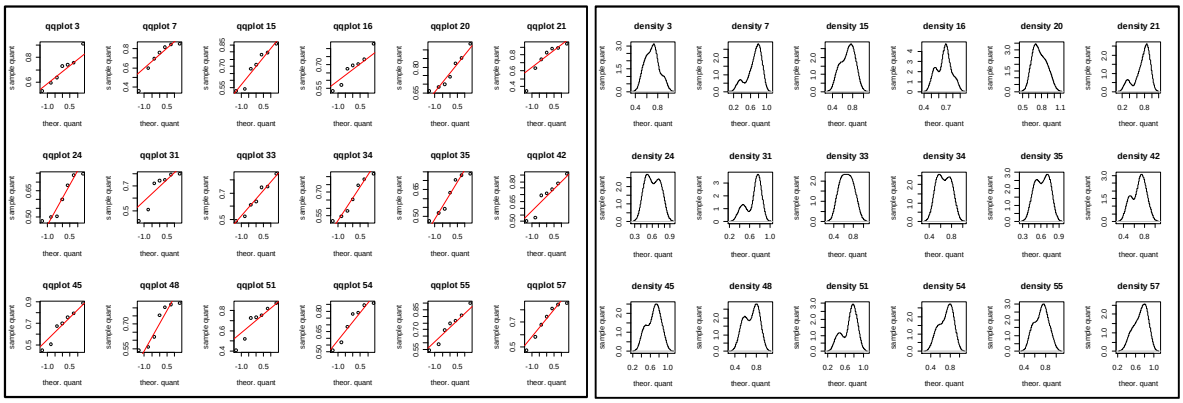

Figure 28: QQ-plot and density distributions of gene diversity (Hs) averaged over loci at each location.

Table 14: Result of the Shapiro-Wilk test for normal distribution of H_s at each location with at least ten sampled individuals.

	3	7	15	16	20	21	24	31	33	34	35	42	45	48	51	54	55	57
Test - statistic	0.96	0.84	0.92	0.96	0.93	0.85	0.86	0.80	0.96	0.92	0.89	0.92	0.95	0.84	0.89	0.90	0.97	0.91
p-value	0.86	0.11	0.46	0.78	0.55	0.11	0.16	0.04 *	0.78	0.49	0.28	0.49	0.73	0.11	0.29	0.31	0.90	0.40

Observed Heterozygosity (Ho)

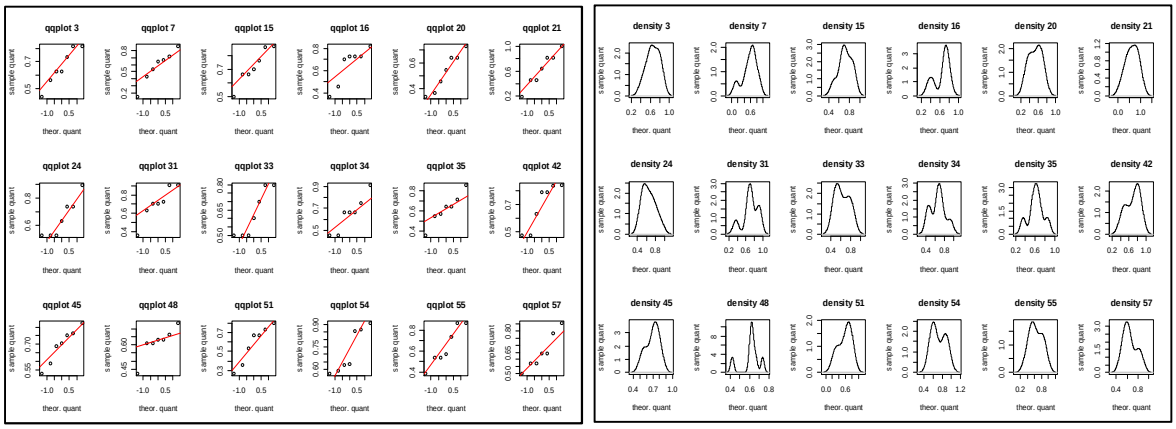

Figure 29: QQ-plot and density distribution of observed heterozygosity (Ho) averaged over loci at each location with at least ten sampled individuals.

Table 15: Result of the Shapiro-Wilk test for normal distribution of H_o at each location with at least ten sampled individuals.

	3	7	15	16	20	21	24	31	33	34	35	42	45	48	51	54	55	57
Test-statistic	0.93	0.94	0.93	0.81	0.94	0.96	0.86	0.89	0.82	0.90	0.97	0.81	0.95	0.84	0.91	0.82	0.90	0.92
SWT – p-value	0.55	0.64	0.51	0.05	0.60	0.81	0.16	0.26	0.06	0.33	0.90	0.05	0.75	0.10	0.42	0.06	0.36	0.47

Inbreeding Coefficient (F_{IS})

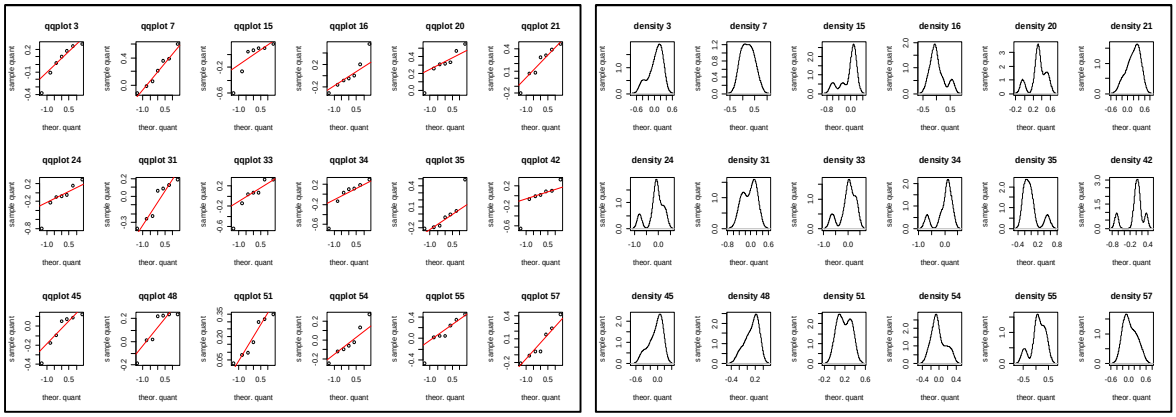

Figure 30: QQ-plots and density distribution plots for F_{IS} at each location with at least ten sampled individuals.

Table 16: Result of the Shapiro-Wilk test for normal distribution at each location with at least ten sampled individuals.

	3	7	15	16	20	21	24	31	33	34	35	42	45	48	51	54	55	57
SWT - statistic	0.90	0.97	0.77	0.92	0.91	0.93	0.89	0.87	0.86	0.83	0.79	0.83	0.87	0.80	0.90	0.95	0.91	0.93
SWT - pvalue	0.33	0.88	0.02 *	0.47	0.40	0.57	0.30	0.20	0.16	0.07	0.03 *	0.07	0.17	0.04 *	0.32	0.69	0.38	0.57

Rarefied Number of Alleles (N_r) within cluster

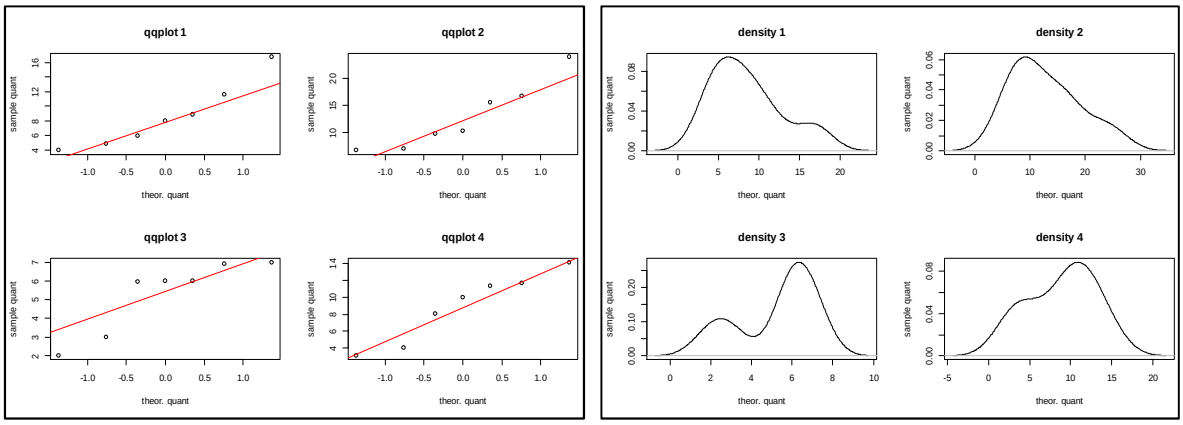

Figure 31: QQ-plots and density distribution plots for N_r in each cluster.

Table 17: Shapiro-Wilk test for normality of N_r-values within each cluster.

	Rhone valley cluster	Southern alps cluster	Rhine valley cluster	North-western cluster
Test - statistic	0.92	0.90	0.80	0.93
p-value	0.45	0.35	0.05*	0.55

Bartlett test for homogeneity of variances

Table 18: Result of a Bartlett-test for homogeneity of variances.

	Df	K squared	p-value
Gene diversity (H_s)	17	7.325	0.979
Observed Heterozygosity (H_o)	17	13.732	0.686
Rarefied number of alleles (N_r)	17	5.795	0.686
Rarefied number of alleles (N_r) within cluster	3	6.391	0.094

Appendix 3

ANOVA and Kruskall-Wallis-test

Table 19: ANOVA of the rarefied number of alleles (N_r) among locations.

	df	Sum Sq	Mean Sq	F value	Pr (>F)
location	17	56.5	3.321	0.769	0.724
residuals	108	466.4	4.318		

Table 20: ANOVA of the observed heterozygosity (H_o) among locations.

	df	Sum Sq	Mean Sq	F value	Pr(>F)
location	17	0.326331	0.019196	0.692224	0.804471
Residuals	108	2.994924	0.027731		

Table 21: ANOVA of gene diversity (H_s) among locations.

	df	Sum Sq	Mean Sq	F value	Pr(>F)
location	17	0.190922	0.011231	0.585315	0.896531
Residuals	108	2.072239	0.019187		

Difference in N_r between clusters

Kruskall-Wallis-test of N_r between clusters

Table 22: Results of a Kruskall-Wallis test for differences in rarefied allelic richness (N_r) between clusters.

chi-squared	df	p-value
7.883	3	0.048*

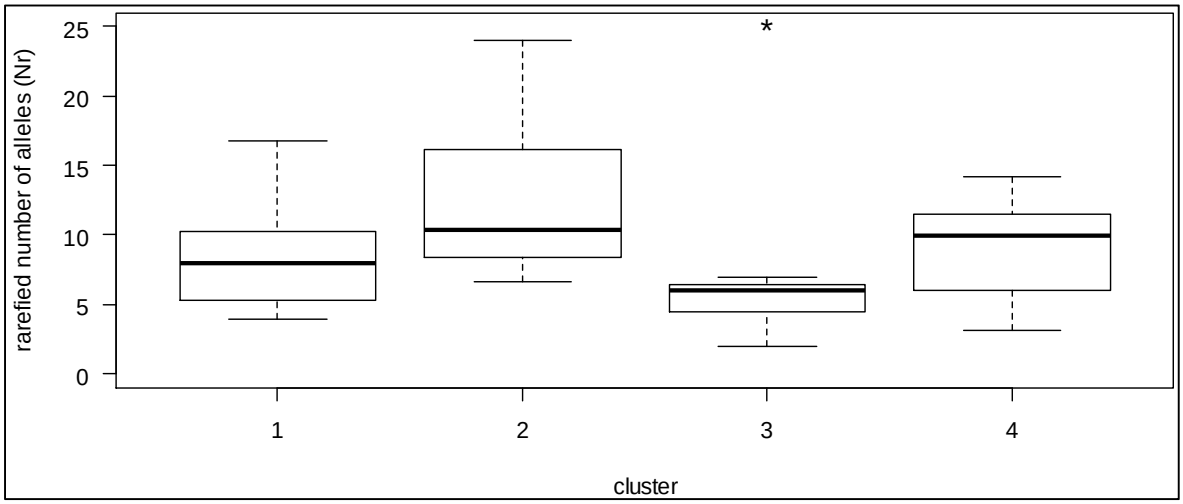

Figure 32: The rarefied allelic richness (Nr) with min.n=68 for each cluster is plotted. Cluster 1 corresponds to the Rhone valley cluster, cluster 2 represents the Southern alps cluster, cluster 3 stands for the Rhine valley cluster and cluster 4 is identical to the North-western cluster.

Inbreeding coefficient (F_{IS})

Table 23: Result of a one-sample Wilcoxon signed-rank test (two-sided) to test for significant deviation of F_{IS}-values from zero.

	3	7	15	16	20	21	24	31	33	34	35	42	45	48	51	54	55	57
statistic	18	24	15	13	27	25	10	10	17	17	9	17	11	25	28	12	21	17
p-value	0.58	0.11	0.94	0.94	0.03 *	0.08	0.58	0.58	0.69	0.69	0.47	0.69	0.69	0.08	0.02 *	0.81	0.30	0.69
lower ci	-0.18	-0.03	-0.28	-0.20	0.14	-0.06	-0.45	-0.30	-0.30	-0.32	-0.19	-0.33	-0.24	-0.08	0.06	-0.17	-0.21	-0.13
upper ci	0.24	0.48	0.13	0.28	0.47	0.39	0.15	0.14	0.33	0.21	0.24	0.21	0.10	0.24	0.32	0.13	0.34	0.29
estimate	0.07	0.21	0.05	-0.03	0.32	0.22	-0.08	-0.07	0.06	0.08	-0.07	0.02	-0.03	0.13	0.19	-0.05	0.13	0.05

Appendix 4

Relationship between rarefied allelic richness and population size

Table 24: Linear model to test for a relationship between the logarithm of the population size and rarefied allelic richness (N_r) using all locations with at least ten sampled individuals (Scenario 1).

	Estimate	Std. Error	t-value	Pr(>|t|)
(Intercept)	1.5523871	0.0430983	36.020	<2e-16 ***
Population size	0.0007011	0.0004247	1.651	0.118

Table 25: Linear model to test for a relationship between population size and rarefied allelic richness (N_r) within the North-Western cluster using all locations with at least six sampled individuals (Scenario 2).

	Estimate	Std. Error	t-value	Pr(>|t|)
(Intercept)	-0.326066	0.248777	-1.311	0.208
Population size	0.001668	0.002451	0.680	0.506

Data exploration using the pairs function in R

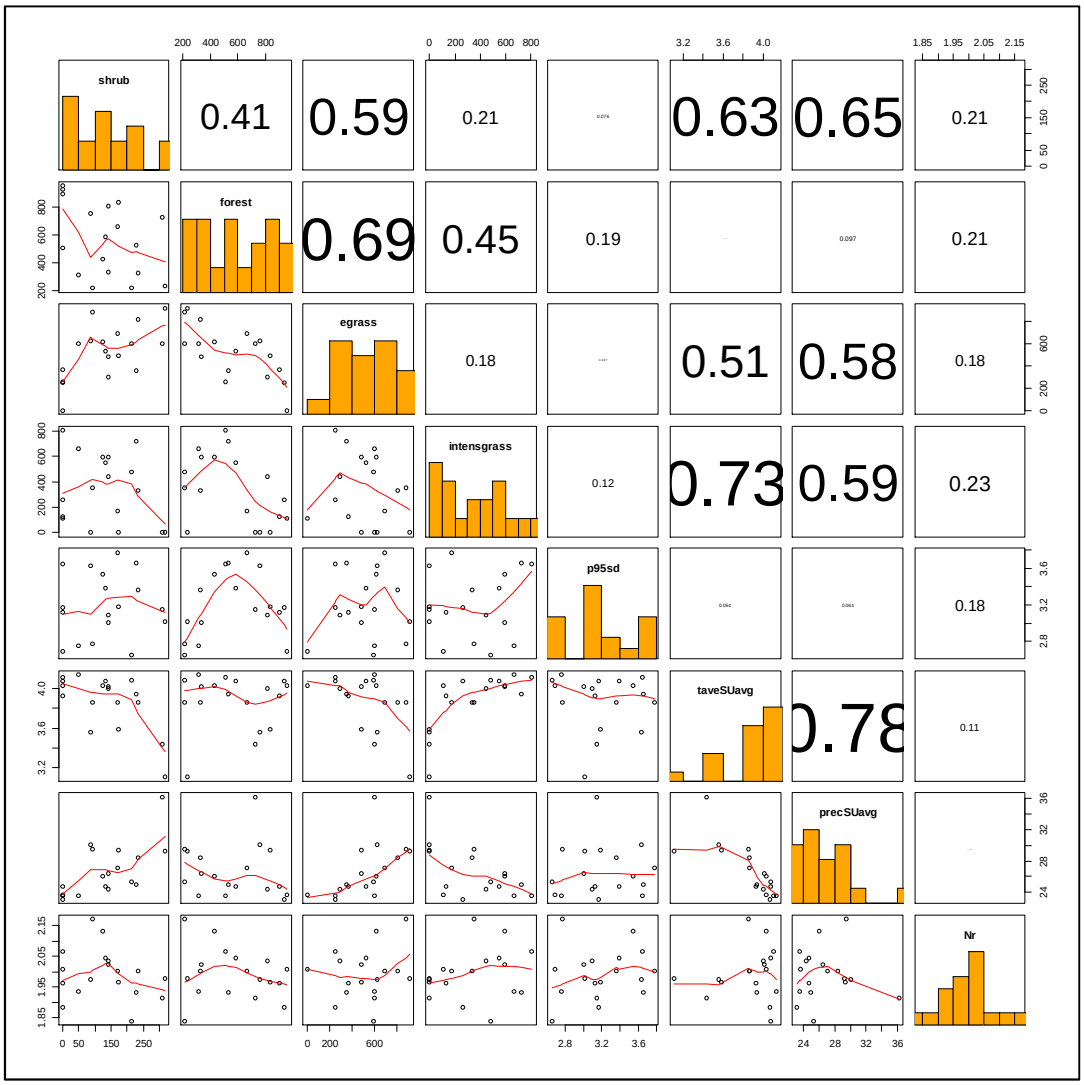

Figure 33: Data exploration using the pairs function. Rarefied allelic richness (Nr) for each plot of the North-western cluster with at least six sampled individuals (Scenario 2) was plotted against the seven habitat predictors. The numbers in the upper triangle represent Pearson's correlation coefficients. Histograms are plotted in the diagonal.

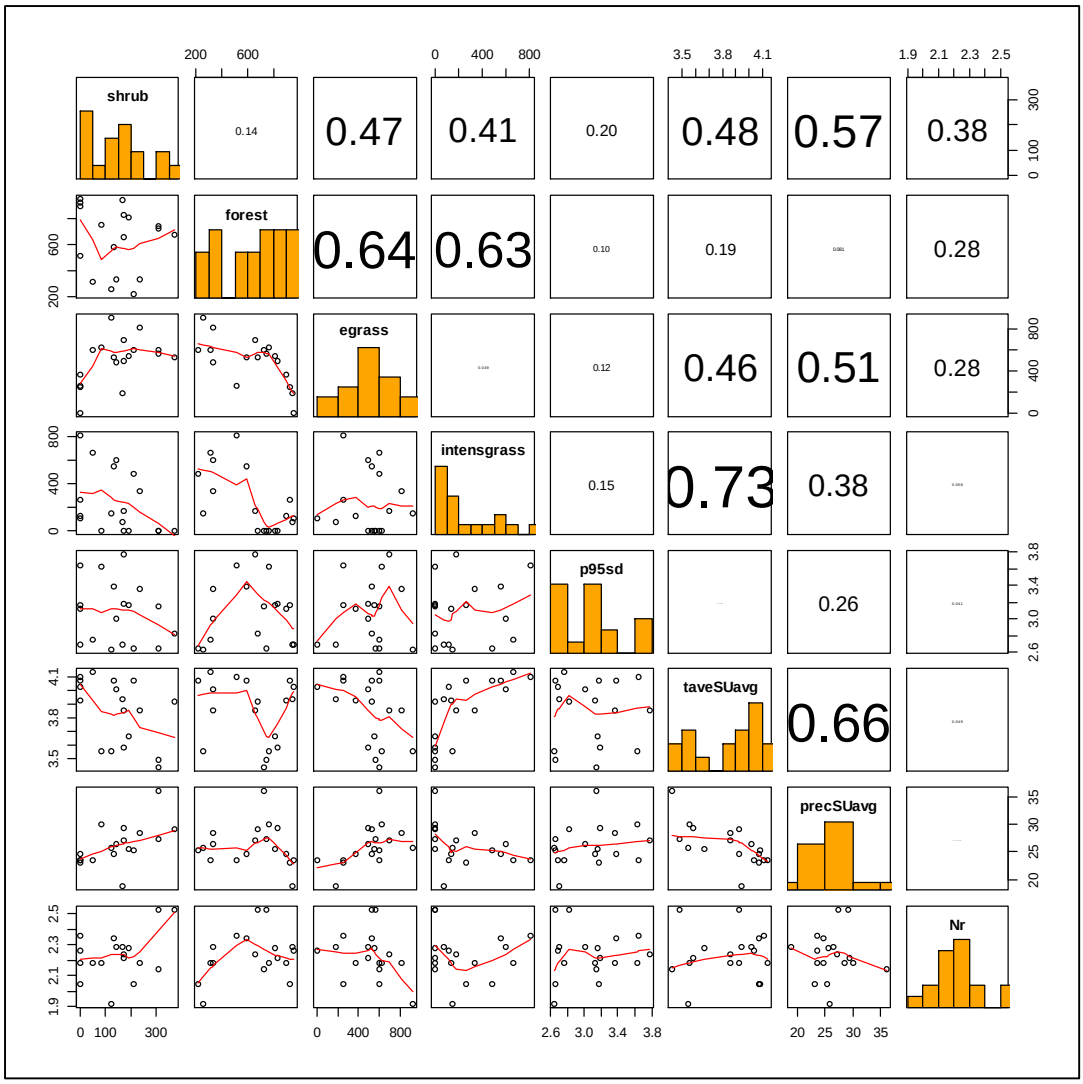

Figure 34: Data exploration using the pairs function. Rarefied allelic richness (N_r) for each plot with at least ten sampled individuals (Scenario 1) was plotted against the seven habitat predictors. The numbers in the upper triangle represent Pearson's correlation coefficients. Histograms are plotted in the diagonal.

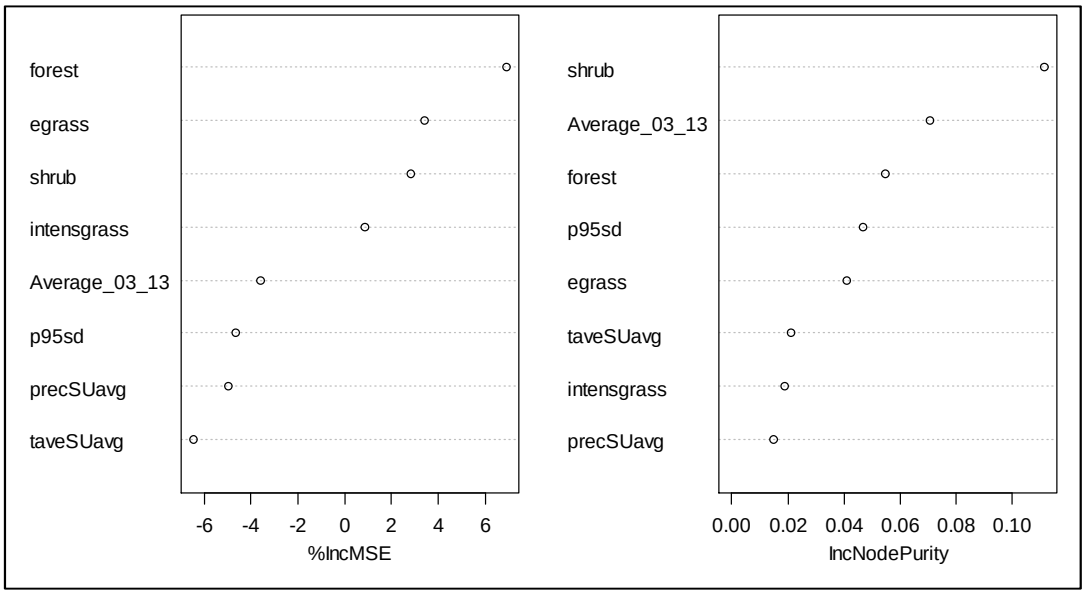

Figure 35: Variable importance estimated by randomForest using the two measures average decrease in mean squared error MSE (%IncMSE) and mean decrease in accuracy (IncNodePurity) and all data from all locations with at least ten sampled individuals (Scenario 1).

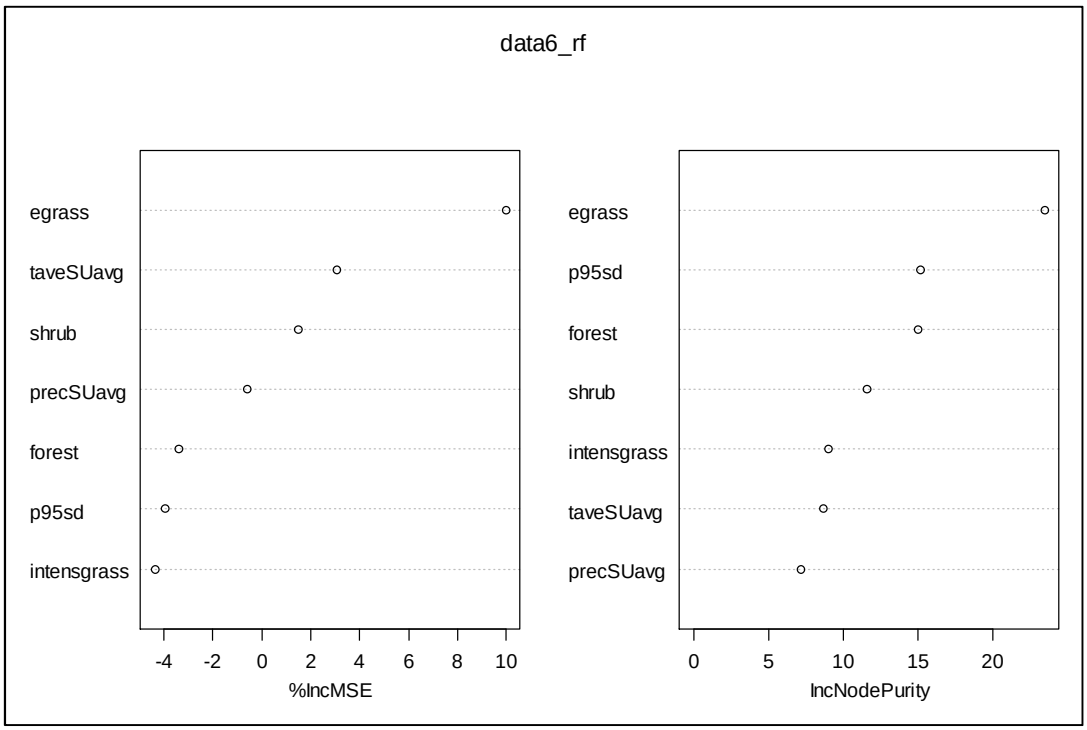

Figure 36: Variable importance estimated by randomForest using the two measures average decrease in mean squared error MSE (%IncMSE) and mean decrease in accuracy (IncNodePurity) and all data from all locations of the North-Western cluster with at least six sampled individuals (Scenario 2).

Appendix 5

Exemplary configuration file for NEMO

```
## NEMO CONFIG FILE
## SIMULATION #### POP2_MIG2 ####

logfile                 logfile.log
run_mode                overwrite
random_seed             486532
filename                %1mrate
replicates              10
generations             1000

## INITIAL POPULATION ##
source_pop              Initial.dat
source_file_type        .dat
source                  preserve
source_fill_age_class   adults

## POPULATION ##
patch_nbfem             { { 5,4,2,4,78,2,32,4,24,20,46,94,4,20,60,7,4,4,4,36,37,2,46,36,20 } }
patch_nbmal             { {
16,7,2,5,236,2,97,4,70,62,139,283,14,61,178,20,12,9,2,108,112,2,139,108, 60 } }

## LIFE CYCLE EVENTS ##
breed                   1       #require adults->offspring
disperse                2       #require offspring
aging                   3       #also performs regulation -> after disp
save_stats              4
save_files              5
store                   6

## MATING SYSTEM ##
mating_system           1
mean_fecundity          15
fecundity               fixed

## NEUTRAL MARKERS ##
ntrl_loci               7
ntrl_all                40
ntrl_mutation_rate      0.0005 0.005 0.001      #seq para
ntrl_recombination_rate 0.5
ntrl_mutation_model     1
ntrl_init_model         1
ntrl_save_genotype      1

## OUTPUT ##
stat                    off.fstat extrate demography
stat_log_time           50
```

```
stat_dir                        data
ntrl_output_logtime             50
# the parameters for the store LCE #
store_generation                50


## DISPERSAL ##
dispersal_matrix_fem            { { 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0}
  {0, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0}
  {0, 0, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0}
  {0, 0, 0, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0}
  {0, 0, 0, 0, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0}
.
.
.
```