

Population genetics of Copper butterflies (*Lycaena* spp.) in the European Alps

by

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from Wismar

Accepted Dissertation thesis for the partial fulfilment of the requirements for a

Doctor of Natural Sciences

Fachbereich 3: Mathematik/Naturwissenschaften

Universität Koblenz-Landau

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Date of the oral examination: 21.05.2021

Chapters I–VI of the thesis are published in peer-reviewed journals as follows:

Chapter I

Trense, D., Reim, E. & Fischer, K. (2019). Characterisation of polymorphic microsatellite loci in three copper butterfly species (*Lycaena* spp.). *Molecular Biology Reports* 46 (6): 6585-6591.

Chapter II

Trense, D., Habel, J. C., Kramp, K., Schmitt, T. & Fischer, K. (2021). Does specialisation affect genetic diversity in (pre-) Alpine populations of four species of Copper butterflies?. *Journal of Insect Conservation* 25 (2): 321-338.

Chapter III

Trense, D., Jager, L. & Fischer, K. (2022). The central Alps comprise a major dispersal barrier between western and eastern populations of two butterfly species. *Journal of Biogeography* 49 (8): 1508-1520.

Chapter IV

Trense, D., Hoffmann, A. A. & Fischer, K. (2021). Large-and small-scale geographic structures affecting genetic patterns across populations of an Alpine butterfly. *Ecology and evolution* 11 (21): 14697-14714.

Chapter V

Trense, D., Schmidt, T. L., Yang, Q., Chung, J., Hoffmann, A. A. & Fischer, K. (2021). Anthropogenic and natural barriers affect genetic connectivity in an Alpine butterfly. *Molecular Ecology* 30 (1): 114-130.

Chapter VI

Trense, D., Habel, J. C., Finger, A. & Fischer, K. (2022). Contrasting genetic responses to habitat fragmentation for two Lycaenid butterfly species. *Insect Conservation and Diversity* 15 (3): 337-347.

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Summary

Population genetics investigates genetic diversity and its changes within and between populations over space and time. Genetic diversity is important for fitness, adaptive capacity, and the survival of populations and is influenced by several factors, such as mutation, selection, genetic drift and gene flow. Copper butterflies (*Lycaena*) are suitable for analysing structures influencing population connectivity as they potentially form more or less closed populations. However, very little is known about their genetic diversity and what influences it. Therefore, this thesis (1) provides newly developed microsatellite markers and uses genetic markers (2) to investigate genetic diversity across four different *Lycaena* species in the European Alps and to determine (3) which geographic and species-specific factors influence population structure, (4) which large- and small-scale structures impact the population structure, (5) how natural and anthropogenic structures influence the population structure within an Alpine valley, and (6) whether and how genetic diversity changes over time. It was shown that the postglacial relict species *L. helle* has a relatively high genetic diversity compared to the other three species investigated. This suggests that *L. helle* is still able to adapt to environmental changes. Low genetic diversity was found in *L. tityrus subalpinus*, although high gene flow was found within one population of this species. High mountain ridges and large river valleys can act as dispersal barriers for Copper butterflies and thus have an impact on population structures. Here, dispersal ability as a species-specific factor also plays an important role, as some barriers are less likely to affect the population structure in the more mobile species *L. virgaureae*. Furthermore, forests, ravines and roads, but not small rivers, represent dispersal barriers for *L. tityrus subalpinus* within an Alpine valley. Finally, over ten years, the genetic diversity of *L. hippothoe eurydame* has decreased, whereas that of *L. helle* has remained stable. Against the backdrop of increasing global changes, it is important to understand the genomic underpinning of population structure and adaptation as well as to investigate and monitor whether populations are able to adapt to changing environmental conditions.

Zusammenfassung

Populationsgenetik untersucht die genetische Diversität und dessen Änderungen innerhalb und zwischen Populationen über Raum und Zeit. Genetische Diversität ist entscheidend für die Fitness, Anpassungsfähigkeit und das Überleben einer Population und kann von verschiedenen Faktoren wie Mutation, Selektion, genetischer Drift und Genfluss beeinflusst werden. Feuerfalter (*Lycaena*) sind repräsentativ für die Analyse von Strukturen, die die Konnektivität von Populationen beeinflussen, da sie potenziell mehr oder weniger geschlossene Populationen bilden. Allerdings ist nur sehr wenig über deren genetische Vielfalt und wodurch diese beeinflusst wird bekannt. Diese Arbeit (1) stellt neu entwickelte Mikrosatelliten-Marker zur Verfügung und untersucht anhand von genetischen Markern (2) die genetische Diversität von vier verschiedenen *Lycaena* Arten in den Europäischen Alpen, (3) welche geographischen und artspezifischen Faktoren und (4) welche groß- und kleinräumigen Strukturen einen Einfluss auf die Populationsstruktur haben, (5) den Einfluss von natürlichen und anthropogenen Strukturen innerhalb eines alpinen Tals und (6) ob und wie sich die genetische Diversität über Zeit verändert. Die Ergebnisse zeigen, dass die postglaziale Reliktart, *L. helle* im Vergleich zu den anderen drei untersuchten Arten eine relativ hohe genetische Diversität besitzt. Dies deutet darauf hin, dass *L. helle* in der Lage ist, sich an Umweltveränderungen anzupassen. Eine geringe genetische Diversität wurde in *L. tityrus subalpinus* festgestellt, obwohl innerhalb einer Population dieser Art ein hoher Genfluss gefunden wurde. Hohe Gebirgskämme und große Flusstäler können für Feuerfalter eine Ausbreitungsbarriere darstellen und somit einen Einfluss auf die Populationsstrukturen haben. Hierbei spielt die Ausbreitungsfähigkeit als artspezifischer Faktor eine Rolle, da einige dieser Barrieren die Populationsstruktur der mobileren Art, *L. virgaureae* weniger stark beeinflussten. Des Weiteren stellten Wälder, Schluchten und Straßen, aber keine kleinen Flüsse Ausbreitungsbarrieren für *L. tityrus subalpinus* innerhalb eines alpinen Tals dar. Schließlich verringerte sich die genetische Diversität über zehn Jahre in *L. hippothoe eurydame*, blieb aber stabil in *L. helle*. Angesichts zunehmender globaler Veränderungen ist es wichtig, die genomischen Grundlagen der Populationsstrukturen und Anpassung zu verstehen und außerdem zu untersuchen und beobachten, ob Populationen in der Lage sind, sich an veränderte Umweltbedingungen anzupassen.

General introduction

To understand evolutionary processes, it is important to have a thorough understanding of population genetics (Hedrick 2011). Population genetics examines genetic diversity within and between populations and includes investigating changes in the frequencies of alleles (variation of a gene) or genes in populations over space and time (Clark 2001; Templeton 2006; Nève 2009). Genetic diversity is the variety of DNA in different individuals of a species or population (Frankham et al. 2002). The source of genetic diversity is mutations (Frankham et al. 2002). The dynamics of genetic diversity over population history (time) can be explained as changes in the allele frequencies caused by genetic drift and selection (Gillespie 2004; Templeton 2006; Hedrick 2011). Additionally, genetic diversity can be influenced by geographic distribution (space), which can be caused by the subdivision of a population by geographic and/or mating barriers or by the diffusion of populations through gene flow caused by migration or dispersal (Gillespie 2004; Templeton 2006; Hedrick 2011).

The importance of genetic diversity

Genetic diversity is considered to be of crucial importance for population fitness, survival of species, and adaptive capacity (Reed and Frankham 2003; Ellegren and Galtier 2016; Ørsted et al. 2019). Adaptive capacity is the potential of a species or population to tolerate or adapt to environmental and climate change; it varies among species and populations (Dawson et al. 2011; Hoffmann and Sgrò 2011; Ofori et al. 2017). High levels of genetic diversity in a population often indicate increased fitness and therefore higher probability of survival and a higher adaptive capacity (Booy et al. 2000; Reed and Frankham 2003; Vellend and Geber 2005). In contrast, low genetic diversity may result in reduced reproductive performance and lower stress resistance.

This can decrease the adaptive capacity of a species, which thus faces a higher risk of extinction (Saccheri et al. 1998; Spielman et al. 2004; Frankham 2005a,b; Willi et al. 2006; Kahilainen et al. 2014). Reduced genetic diversity may stem from a small population size, which potentially leads to inbreeding and hence inbreeding depression (reduced fitness due to inbreeding), which is likely to further reduce the population size (Keller and Waller 2002; Day et al. 2003; Willi et al. 2006; Bijlsma and Loeschcke 2012; Hoffmann et al. 2020). Threatened species often occur in small and isolated populations; hence a loss of genetic diversity in these populations may reduce their adaptive capacity in comparison to widespread generalist species with large population sizes, which are described in **chapter two** (Spielman et al. 2004; Willi et al. 2006; Habel et al. 2013).

Factors affecting genetic diversity

Genetic diversity can be affected by several factors interacting in a complex dynamic way, including mutation, selection, founder and bottleneck events, genetic drift, inbreeding, and gene flow (Hughes et al. 2008; Ellegren and Galtier 2016). With each generation, new alleles can arise by mutation, thus increasing genetic diversity (Eyre-Walker and Keightley 2007; Ellegren and Galtier 2016). Selection may either maintain genetic diversity by purging deleterious mutations or decrease it by promoting specific alleles (Reed and Frankham 2003; Ellegren and Galtier 2016). A reduction or loss of genetic diversity, specifically heterozygosity, occurs in almost all founder events due to a strong genetic drift, which is the random fluctuation of allele frequency across generations (Charlesworth 2009; Ellegren and Galtier 2016; Ørsted et al. 2019). In contrast, inbreeding is a stochastic process, which reduces heterozygosity if closely related individuals mate (Frankham 1995; Day et al. 2003; Bijlsma and Loeschcke 2012). Gene flow may compensate for the effects of inbreeding and genetic drift by

exchanging genes between populations, but it is affected by geographic distance and isolation of populations (Bohonak 1999; Frankham 2010; Kahilainen et al. 2014).

Factors affecting population structures

Environmental and species-specific factors are important for understanding the spatial patterns of genetic diversity and population structure (Banks et al. 2013; Vernesi et al. 2016; Després et al. 2019). On a spatial scale, the heterogeneity of environments is the principal factor influencing the patterns of populations and their genetic diversity. It can be further divided into large- and small-scale structures, which are reported on **chapters three** and **four** (Manel et al. 2003; Manel and Holderegger 2013; Yang et al. 2017). The impacts of large- and small-scale barriers are often species-specific (**chapter three**); they depend on the size and extent of (un-)suitable habitats as well as on the ability of a species to adapt (Hewitt 1999; Keyghobadi 2007; Sheth et al. 2020). Large-scale structures comprise oceans, deserts, and mountain ranges, for instance, the European Alps and the Pyrenees formed geographic barriers after the Last Glacial Maximum (Hewitt 1996, 1999; Taberlet et al. 1998). Small-scale structures include both natural barriers, such as lakes, rivers, forests, mountain ridges, and gorges and anthropogenic barriers, such as human settlements, agriculture, railways, and roads (e.g. Storfer et al. 2010; Heidinger et al. 2013; Miles et al. 2019; Sheth et al. 2020). On a finer scale, i.e. even within populations (**chapter five**), natural and anthropogenic structures can fragment habitats and may thus hamper dispersal (e.g. Riley et al. 2006; Fraser et al. 2011; Muñoz-Mendoza et al. 2017; Schmidt et al. 2018). Furthermore, these structures can force organisms to disperse across unfavourable areas in order to locate suitable habitats, which increases time, energy, and survival costs (Bonte et al. 2012; Schloss et al. 2012; Baguette et al. 2013).

Dispersal is defined as any movement to suitable habitat patches, potentially resulting in gene flow (Dieckmann et al. 1999; Bowler and Benton 2005), and it reflects a major part of a species' ability to respond to environmental change (Senner et al. 2018). It comprises three stages: departure from the current patch, inter-patch movement, and settlement in a new patch (Bowler and Benton 2005; Clobert et al. 2009). Dispersal allows connectivity among populations, which reduces genetic differentiation and therefore has an impact on population dynamics and structure (Dieckmann et al. 1999; Bowler and Benton 2005; Willi et al. 2006).

Many alpine environments are shaped by natural habitats and structured by landscapes with various natural barriers and are therefore suitable locations for investigating the impact of barriers on genetic structures of populations and the dynamics of genetic diversity in a mostly intact area (Leidner and Haddad 2010; Getzner et al. 2016). Furthermore, alpine systems are characterised by high landscape heterogeneity, steep ecological gradients, and the high vulnerability of alpine species due to global climate change (Engler et al. 2011; Schmitt et al. 2014; Cortés and Wheeler 2018). However, changes arising from land-use by humans, such as the intensification of agriculture and increasing anthropogenic barriers, are also occurring in alpine environments (Tasser et al. 2005; Mottet et al. 2006). In this thesis, I also address the effects of these land-use changes on Alpine systems (**chapters two – six**).

Long- and short-term dynamics

On a temporal scale, past climates have the most influence on the current distributions of species, e.g. oscillations of cold and warm periods in the Pleistocene in Europe (Hewitt 1996, 1999; Schmitt et al. 2006). This resulted in latitudinal and altitudinal range shifts. During cold periods, species were restricted to southern refuges, and they

expanded northwards in warm periods, see **chapters three** and **four** (Hewitt 1996; Taberlet et al. 1998; Després et al. 2019). Short-term effects, such as overexploitation, habitat fragmentation and destruction as well as a decline or loss of population abundance caused by human-induced changes in land-use and by the climate can lead to genetic erosion and a loss of genetic diversity (Dirzo et al. 2014; Miraldo et al. 2016; Hoban et al. 2020). These two factors may undermine population fitness, the potential to adapt to future environments, and the survival of a species (Reed and Frankham 2003; Frankham 2005b; Hoban et al. 2020). Therefore, monitoring changes in genetic diversity in populations over time (**chapter six**) can provide valuable information, e.g. for species conservation management.

Genetic markers

Gene flow, genetic differentiation, genetic diversity, and relatedness can be estimated in and among populations using genetic markers (Bohonak 1999; Ouborg et al. 1999). In this thesis, the focus will be on three genetic markers: nuclear gene sequences, microsatellites – also named short tandem repeats (STRs) or simple sequence repeats (SSRs) – and single nucleotide polymorphisms (SNPs).

Gene sequences are relatively conserved, evolve slowly, and may be under selection (e.g. Ballard and Whitlock 2004; Hoffmann and Daborn 2007; Jasso-Martínez et al. 2018). Hence, they are less suitable for revealing recent gene flow and population genetic structures. However, they are useful when analysing the origin of populations and past colonisation routes, e.g. during and after the last glacial period (e.g. Seddon et al. 2001; Pecsénye et al. 2018). Furthermore, there are two different types of genetic diversity: adaptive and neutral. Neutral genes have almost no or no effect on fitness, while adaptive genes have an effect on fitness, meaning neutral genetic diversity does not necessarily equate with adaptive genetic diversity (Holderegger et al. 2006;

Whitlock 2014; Ellegren and Galtier 2016). This seems to be of importance when trying to assess the adaptive potential of populations as a response to environmental and climate-related change (Hoffmann and Daborn 2007; Reusch and Wood 2007; Hoffmann and Willi 2008; Donihue and Lambert 2015). In **chapter two**, I used nuclear gene sequences and microsatellite markers.

A microsatellite is a specific repetitive DNA motif with a length of two to six base pairs, which can be amplified by a polymerase chain reaction (PCR) with labeled primers (Litt and Luty 1989). Microsatellites are locus-specific, highly polymorphic, and codominant. In addition, only a small amount of DNA is needed for a PCR (Queller et al. 1993; Jarne and Lagoda 1996). Furthermore, they are suitable for revealing recent gene flow and population genetic structures (Ellegren 2004). The development of microsatellites is described in **chapter one**, and **chapters two, three** and **six** report on their use. However, microsatellites are taxon-specific and very expensive to develop, and null alleles can occur (Queller et al. 1993; Jarne and Lagoda 1996; Putman and Carbone 2014). A null allele is any allele that consistently fails to amplify in a PCR reaction because a variation in the flanking region of the primer prevents the primer from annealing to the template DNA (Dakin and Avise 2004; Chapuis and Estoup 2007). The presence of null alleles is relatively high in some species. For example, in butterflies, there are multiple copies of microsatellite loci with highly similar flanking regions (Meglecz et al. 2004). In population genetic studies, microsatellites have been the marker of choice but, due to the development and use of SNPs as genetic markers, SNPs are replacing microsatellites (Morin et al. 2004; Liu et al. 2005; Putman and Carbone 2014).

An SNP is a single base variation in a DNA sequence, in which typically two different nucleotides can be found (Collins et al. 1998). SNPs as genetic markers are highly stable due to their low mutation rate, have a high frequency in the genome, usually do not need any information about the target regions, and can detect outlier loci even in non-model organisms (Brookes 1999; Butler et al. 2007; Helyar et al. 2011). Outlier SNPs or loci are loci with significantly lower or higher genetic differentiation between populations than expected under neutrality and are potentially under selection (Feng et al. 2015). They can provide insight into the genetic basis of local adaptation to specific environmental variables (Ahrens et al. 2018), such as altitude (**chapters four and five**). However, the sequencing methods for SNPs are time-consuming and cost-intensive, high-quality DNA is required, a high number of SNPs are needed, and large data sets have to be handled (Butler et al. 2007; Helyar et al. 2011; Flanagan and Jones 2019). This relatively new genetic marker is used in landscape genetics to analyse gene flow, dispersal, and population structures on a temporal scale as well as on large and small spatial scales (**chapter four**) and on a fine spatial scale (**chapter five**) (Nève 2009; Segelbacher et al. 2010; Fountain et al. 2018).

The European Alps as a field lab for population genetics

The European Alps are the highest and most extensive mountain range system in Europe, and their species composition has been influenced by dynamics since the Last Glacial Maximum (Taberlet et al. 1998; Hewitt 1999). They currently harbour hybrid zones and endemic species across heterogeneous environments, including unsuitable and suitable habitats in close proximity (Martin et al. 2002; Hewitt 2004; Dirnböck et al. 2011; Dagnino et al. 2020). Due to their heterogenic structure and complex geography, the European Alps are a suitable area for investigating population genetic structures and the dynamics of genetic diversity. Additionally, Alpine species seem to be

particularly vulnerable to global climate change compared to other species (Engler et al. 2011; Schmitt et al. 2014). Furthermore, the adaptation of species can be expected in Alpine environments due to the steep gradients in altitude, temperature, oxygen concentration, and ultraviolet radiation (Collinge et al. 2006; Karl et al. 2008a; Cheviron and Brumfield 2012; Polato et al. 2017; Montero-Mendieta et al. 2019).

Study organisms

Butterflies (Lepidoptera: Rhopalocera) are suitable model organisms for analysing the impact of climate and habitat change as they are sensitive environmental indicators (Parmesan et al. 1999; Warren et al. 2001; Mair et al. 2012). Within the butterfly family Lycaenidae, the genus *Lycaena* (Copper butterfly) is ecologically well characterised and its biology is particularly well understood, including its life history evolution, habitat use and preference, (mating) behaviour, the interactions of butterfly larva and host plant, stress biology, and local adaptation (e.g. Fischer et al. 1999; Fischer and Fiedler 2001c; Karl et al. 2008a; Fischer and Karl 2010; Strausz et al. 2012; Haaland 2015; Klockmann et al. 2016; Klockmann and Fischer 2017). However, less is known about the population genetic structure of Copper butterflies. In this thesis, four species of the genus *Lycaena*, which probably have limited dispersal ability, were investigated (Figure 1). Mark-recapture studies have revealed that these species originated from more or less closed populations with individuals moving mainly within patches. They therefore may occur genetically in strong population structures (Fischer et al. 1999; Fischer and Fiedler 2001c; Ricketts 2001; Settele et al. 2008; Finger et al. 2009). This makes the four *Lycaena* species suitable organisms for analysing factors that influence population genetic structures. Nevertheless, although they belong to the same genus, thus suggesting that their genetic constitution is relatively similar, these four species differ regarding their level of threat and, at least to some extent, regarding their

population connectivity (Tolman and Lewington 2008; Settele et al. 2008; Klockmann et al. 2016; Klockmann and Fischer 2017). *Lycaena helle* (Denis & Schiffermüller, 1775) probably shows the lowest dispersal ability and, thus, population connectivity, as indicated by ecological and genetic data (Fischer et al. 1999; Bauerfeind et al. 2009; Habel et al. 2010). The three other species' preferences of habitat and patterns of distribution indicate a higher dispersal ability, specifically for *L. virgaureae* (L., 1758), which is possibly the most mobile species (Ebert and Rennwald 1991; Settele et al. 2008; Barua et al. 2011; Kudrna et al. 2015).



Figure 1 Pictures of the four investigated Copper butterflies: upper left *Lycaena helle*, upper right *L. hippothoe*, lower left *L. tityrus*, and lower right *L. virgaureae* (all pictures: © Klaus Fischer).

Lycaena helle, the Violet Copper, has a largely boreal distribution, ranging from Western Europe to Scandinavia and Northern Asia (Kudrna et al. 2015). In Central Europe, it is a postglacial relict species with only a few isolated relict populations (Habel et al. 2010, 2011). It inhabits mires, spring habitats, swampy grasslands, and moist meadows (Fischer et al. 1999; Settele et al. 2008; Finger et al. 2009). The species has one to two generations per year and hibernates as pupa (Tolman and Lewington 2008; Settele et al. 2008). In Central Europe, the larva feeds on a single plant, *Bistortia officinalis* Delabre (Polygonaceae) (Ebert and Rennwald 1991; Fischer et al. 1999). The species is declining within its European range and is therefore listed in the EU Habitat Directive (van Swaay and Warren 1999; Settele et al. 2008; Habel et al. 2014b).

I focussed on the Alpine populations or subspecies of the following three *Lycaena* species:

Lycaena hippothoe (L., 1761), the Purple-edged Copper, is a widespread temperate-zone butterfly, ranging from northern Spain in the West throughout most of the northern Palearctic area eastwards to the easternmost parts of Siberia and China (Ebert and Rennwald 1991; Tuzov 2000). Within Central Europe, two subspecies are found: *Lycaena hippothoe euridice* (Rottemburg, 1775), which occurs throughout most parts of the species' range in Central Europe, and the Alpine subspecies *Lycaena hippothoe eurydame* (Hofmannsegg, 1806), which is confined to the higher elevations of the Alps (Ebert and Rennwald 1991; Tolman and Lewington 2008). Its habitats include unimproved grassland and meadows (Ebert and Rennwald 1991; Settele et al. 2008). The Alpine subspecies has one generation per year and hibernates as half-grown larva (Fischer and Fiedler 2001b; Tolman and Lewington 2008). The larva feeds mainly on *Rumex acetosa* L. (Polygonaceae) and *R. scutatus* L. (Ebert and Rennwald 1991; Westenberger 2005).

Lycaena tityrus (Poda, 1761), the Sooty Copper, is a widespread temperate-zone butterfly, and its distribution ranges from Western Europe to Central Asia (Ebert and Rennwald 1991). Within Europe, two subspecies are known: *Lycaena tityrus tityrus* (Poda, 1761), which occurs throughout most parts of the range, and the alpine form *Lycaena tityrus subalpinus* (Speyer, 1851), which can be found only in the Alps and some other mountain ranges (Tolman and Lewington 2008). This species occurs on moist, dry meadows, and in open woodland (Brunzel et al. 2008; Settele et al. 2008). *Lycaena tityrus subalpinus* has one generation per year and hibernates as half-grown larva (Fischer and Fiedler 2000; Tolman and Lewington 2008). The larva feeds mainly on *R. acetosa* but also utilises some congeneric plant species, such as *R. acetosella* L. and *R. scutatus* (Ebert and Rennwald 1991; Tolman and Lewington 2008; Settele et al. 2008).

Lycaena virgaureae, the Scarce Copper, is a butterfly with a widespread temperate-zone distribution, ranging from the northern Iberian Peninsula and southern France across Central Europe to Central Asia and Mongolia (Bräu et al. 2013). It inhabits different kinds of unimproved grassland, such as moist to semi-dry, nutrient-poor and often acidic meadows, forest edges, and clearings (Ebert and Rennwald 1991; Bräu et al. 2013). This species has one generation per year and hibernates as eggs (Tolman and Lewington 2008). The larva feeds on *R. acetosa* and *R. scutatus* (Ebert and Rennwald 1991; Westenberger 2005; Bräu et al. 2013). The populations of *Lycaena virgaureae* have declined substantially in Central Europe but the species is still relatively common and widespread in the Alps (Bräu et al. 2013; Haaland 2015).

To study genetic diversity and differentiation, microsatellite markers were developed for three *Lycaena* species: *L. hippothoe*, *L. tityrus*, and *L. virgaureae*:

Chapter I: Development of microsatellites

After verifying the microsatellite markers, in the **second chapter**, I tested whether genetic diversity is reduced in a postglacial relict species with isolated populations. Lower genetic diversity limits the adaptive capacity of a relict species compared to more widely distributed species such as *L. h. eurydame*, *L. t. subalpinus*, and *L. virgaureae*:

Chapter II: Genetic diversity across species

The *Lycaena* populations seem to be structured within the European Alps. To compare two different species within this area may help to determine whether barriers are common in both species and identify which species-specific factors impact their genetic structure and diversity. Therefore, the hypothesis set out in the **third chapter** is that geographic but also species-specific features affect the population genetic structures of *L. h. eurydame* and *L. virgaureae*:

Chapter III: Geographic and species-specific factors affect population genetic structures

In the **fourth chapter**, I report on how genome-wide SNPs were used to more precisely characterise large- and small-scale geographic features and their influences on the population genetic structure. Therefore, I hypothesized that mountain ridges, river

valleys, and postglacial range expansions affect the population structure of *L. t. subalpinus*, and populations from different altitudes show signatures of local adaptation based on outlier loci:

Chapter IV: Effects of large- and small-scale structures on population genetic structures

Because SNPs provide a fine-scale resolution, in the **fifth chapter**, I analysed the population genetic structure of *L. t. subalpinus* in one valley in the European Alps. I hypothesized that small-scale barriers constrain dispersal, affecting the genetic structure within populations and that individuals from different altitudes adapt to local conditions:

Chapter V: Fine-scale genetic structures in an Alpine valley

Natural and anthropogenic barriers can fragment habitats of species. This is important as anthropogenic barriers are increasing in the Alpine region, e.g. due to intensified agriculture (e.g. Tasser et al. 2005). Against the backdrop of these trends, in the **sixth chapter**, I described my hypothesis that the genetic diversity of *L. helle* and *L. h. eurydame* is decreasing and their genetic differentiation is increasing over recent years:

Chapter VI: Genetic diversity over time

Each of the six studies is described in an individual chapter, including an abstract, introduction, material and methods, results, and discussion section. The manuscripts

of the chapters have been either submitted, accepted or published in peer-reviewed journals.

Chapter I Development of microsatellites

Characterisation of polymorphic microsatellite loci in three Copper butterfly species
(*Lycaena* spp.)

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Published in: *Molecular Biology Reports* (2019) 46: 6585–6591

Note by the author:

This chapter is based on the following journal publication. Due to copyright issues, the text of the chapter was replaced by the reference information. Thus, the interested reader is kindly asked to read the published paper via the following reference:

Trense, D., Reim, E. & Fischer, K. (2019). Characterisation of polymorphic microsatellite loci in three copper butterfly species (*Lycaena* spp.). *Molecular Biology Reports* 46 (6): 6585-6591.

DOI: 10.1007/s11033-019-05056-3

Abstract

The genus *Lycaena* is widely used for studying life history evolution, local adaptation, stress biology, and behaviour. Furthermore, several species are currently declining and thus of conservation concern. In order to provide the molecular basis for population genetics and conservation biology, we report the development of 36 microsatellite markers for *Lycaena* spp.. Loci were screened in 21 individuals each per species using individuals from Greifswald, north-eastern Germany (*L. tityrus*) or the Italian and Austrian Alps (*L. hippothoe* and *L. virgaureae*). Ten, 16, and ten polymorphic microsatellite loci are characterized in *L. tityrus*, *L. hippothoe*, and *L. virgaureae*, respectively. Allele numbers per locus ranged from three to 20 and expected heterozygosity from 0.37 to 0.94. Nineteen out of the 36 loci were successfully cross-amplified in at least one other taxon, resulting in a total of 13 loci for *L. tityrus tityrus*, 14 for *L. tityrus subalpinus*, 20 for *L. hippothoe*, and 18 for *L. virgaureae*. These markers will be useful for addressing population genetic issues in *L. tityrus*, *L. hippothoe*, and *L. virgaureae*, and potentially other Copper butterflies.

Keywords cross-species amplification, Lepidoptera, Lycaenidae, NGS microsatellite, SSR, STR

Chapter II: Genetic diversity across species

Does specialisation affect genetic diversity in (pre-)Alpine populations of four species of Copper butterflies?

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Published in: *Journal of Insect Conservation* (2021) 25: 321-338

Note by the author:

This chapter is based on the following journal publication. Due to copyright issues, the text of the chapter was replaced by the reference information. Thus, the interested reader is kindly asked to read the published paper via the following reference:

Trense, D., Habel, J. C., Kramp, K., Schmitt, T. & Fischer, K. (2021). Does specialisation affect genetic diversity in (pre-) Alpine populations of four species of Copper butterflies?. *Journal of Insect Conservation* 25 (2): 321-338.

DOI: 10.1007/s10841-021-00302-1

Abstract

Genetic diversity is of crucial importance for population fitness and the potential of populations to adapt to environmental change. Population-level genetic diversity is expected to be reduced in specialists having small and isolated populations. We analysed genetic structure and diversity in (pre-)Alpine populations of four Copper butterfly species, differing in the degree of habitat specialisation (*Lycaena helle* > *L. hippothoe* > *L. virgaureae* > *L. tityrus*). Despite substantial variation among genetic markers and molecular indices, genetic diversity and thus evolutionary potential tended to be highest in *L. hippothoe* and lowest in *L. tityrus*. Microsatellite analyses revealed that genetic diversity tended to be higher in *L. hippothoe* and *L. virgaureae* than in *L. helle* and *L. tityrus*. MDH sequencing indicated only few differences among species, while GAPDH sequencing showed higher genetic diversity in *L. hippothoe* and *L. tityrus* than in *L. virgaureae* and *L. helle*. These results do not match our *a priori* predictions of a high genetic diversity in *L. tityrus* and a particularly low one in the glacial relict *L. helle*. In *L. helle*, populations showed strong genetic differentiation but without clear

spatial structure, while the other species showed genetic differentiation among clusters according to cardinal directions. We conclude that (1) genetic diversity and therefore evolutionary potential may not be generally reduced in rare relict species and that (2) it is more meaningful to focus on specific populations of concern to assess genetic vulnerability to environmental change.

Keywords conservation genetics, genetic differentiation, glacial relict species, global change, *Lycaena*, population genetic structure

Chapter III: Geographic and species-specific factors affect population genetic structures

The central Alps comprise a major dispersal barrier between western and eastern populations of two butterfly species

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Published in: *Journal of Biogeography* (2022) 49: 1508-1520

Note by the author:

This chapter is based on the following journal publication. Due to copyright issues, the text of the chapter was replaced by the reference information. Thus, the interested reader is kindly asked to read the published paper via the following reference:

Trense, D., Jager, L. & Fischer, K. (2022). The central Alps comprise a major dispersal barrier between western and eastern populations of two butterfly species. *Journal of Biogeography* 49 (8): 1508-1520.

DOI: 10.1111/jbi.14397

Abstract

Aim: Environmental and species-specific factors shape spatial patterns in genetic diversity and population structure. Comparing different species within the same area helps to disentangle more general from species-specific factors affecting such geographic patterns. Here, we examined genetic diversity and population structuring through geographic features in two alpine butterfly species.

Location: European Alps.

Taxon: Copper butterflies (*Lycaena* spp.).

Methods: We used 14 and nine microsatellite markers to analyse the genetic diversity and structure of 21 *Lycaena hippothoe* and 14 *L. virgaureae* populations, respectively.

Results: We found a higher genetic diversity and a more pronounced population structure in *L. hippothoe* than in *L. virgaureae*. Both species displayed a major genetic barrier in the central Alps. Western and eastern *L. hippothoe* populations but central *L. virgaureae* populations showed the highest genetic diversity.

Main Conclusions: The population genetic structures of both Copper butterflies seemed to be strongly affected by population history and demography. Patterns indicate for both species a western and an eastern glacial refuge. The high genetic diversity and pronounced population structure found in *L. hippothoe* seems to be related to a low dispersal ability and closed populations with high local abundances as opposed to *L. virgaureae*. The higher dispersal of the latter likely caused hybridization in the central alpine contact zone boosting genetic diversity, which was not the case in *L. hippothoe*. These findings suggest that different conservation strategies are needed for these closely related species.

Keywords dispersal ability, genetic diversity, *Lycaena*, microsatellites, population genetics, population structure

Chapter IV: Effects of large- and small-scale structures on population genetic structures

Large- and small-scale geographic structures affect genetic patterns across populations of an Alpine butterfly

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Published in: *Ecology and Evolution* (2021) 11: 14697-14714

Note by the author:

This chapter is based on the following journal publication. Due to copyright issues, the text of the chapter was replaced by the reference information. Thus, the interested reader is kindly asked to read the published paper via the following reference:

Trense, D., Hoffmann, A. A. & Fischer, K. (2021). Large-and small-scale geographic structures affecting genetic patterns across populations of an Alpine butterfly. *Ecology and evolution* 11 (21): 14697-14714.

DOI: 10.1002/ece3.8157

Abstract

Aim: Understanding factors influencing patterns of genetic diversity is of particular importance in the current era of global climate change and habitat loss. These factors include the evolutionary history and range dynamics of a species as well as the spatial heterogeneity in the environment it currently occupies. Here, we investigate to what extent populations of the Sooty Copper butterfly (*Lycaena tityrus*) are structured by mountain ridges, large river valleys, and post-glacial range expansions in a topographically complex Alpine environment. Additionally, we explore if different evolutionary lineages are present within this species, and search for signatures of local adaptation to different altitudes.

Location: European Alps.

Methods: We analysed population structure and genetic diversity across the European Alps, using genome-wide SNPs identified through RADseq.

Results: We found substantial genetic differentiation within the Alps caused by high mountain ridges and large river valleys. A strong reduction of genetic diversity from

west to east suggests that the Alps were recolonized from a southwestern refuge after the last glacial period. We detected 40 outlier loci likely under altitudinal selection, including several loci related to membranes and cellular processes.

Main conclusion: We suggest that efforts to preserve alpine *L. tityrus* should focus on the genetically diverse populations in the western Alps, and that the dolomite populations should be treated as genetically distinct management units. This study demonstrates the usefulness of SNP-based approaches for understanding patterns of genetic diversity, gene flow and selection in a region that is expected to be particularly vulnerable to climate change.

Keywords barrier to dispersal, evolutionary significant unit, genetic differentiation, genetic diversity, glacial refuges, SNP outlier loci

Chapter V: Fine-scale genetic structures in an Alpine valley

Anthropogenic and natural barriers affect genetic connectivity in an Alpine butterfly

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Published in: *Molecular Ecology* (2021) 30: 114-130

Note by the author:

This chapter is based on the following journal publication. Due to copyright issues, the text of the chapter was replaced by the reference information. Thus, the interested reader is kindly asked to read the published paper via the following reference:

Trense, D., Schmidt, T. L., Yang, Q., Chung, J., Hoffmann, A. A. & Fischer, K. (2021). Anthropogenic and natural barriers affect genetic connectivity in an Alpine butterfly. *Molecular Ecology* 30 (1): 114-130.

DOI: 10.1111/mec.15707

Abstract

Dispersal is a key biological process serving several functions including connectivity among populations. Habitat fragmentation caused by natural or anthropogenic structures may hamper dispersal, thereby disrupting genetic connectivity. Investigating factors affecting dispersal and gene flow is important in the current era of anthropogenic global change, as dispersal comprises a vital part of a species' resilience to environmental change. Using fine-scale landscape genomics, we investigate gene flow and genetic structure of the Sooty Copper butterfly (*Lycaena tityrus*) in the Alpine Ötz valley system in Austria. We show surprisingly high levels of gene flow in *L. tityrus* across the region. Nevertheless, ravines, forests, and roads had effects on genetic structure, while rivers did not. The latter is surprising as roads and rivers have a similar width and run largely in parallel in our study area, pointing towards a higher impact of anthropogenic compared with natural linear structures. Additionally, we detected eleven loci potentially under thermal selection, including ones related to membranes, metabolism, and immune function. This study demonstrates the

usefulness of molecular approaches in obtaining estimates of dispersal and population processes in the wild. Our results suggest that, despite high gene flow in the Alpine valley system investigated, *L. tityrus* nevertheless seems to be vulnerable to anthropogenically-driven habitat fragmentation. With anthropogenic rather than natural linear structures affecting gene flow, this may have important consequences for the persistence of species such as the butterfly studied here in altered landscapes.

Keywords ddRAD, gene flow, habitat fragmentation, landscape genetics, SNP outlier loci, thermal adaptation

Chapter VI: Genetic diversity over time

Contrasting genetic responses to habitat fragmentation for two Lycaenid butterfly species

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Published in: *Insect Conservation and Distribution* (2022) 15 : 337-347

Note by the author:

This chapter is based on the following journal publication. Due to copyright issues, the text of the chapter was replaced by the reference information. Thus, the interested reader is kindly asked to read the published paper via the following reference:

Trense, D., Habel, J. C., Finger, A. & Fischer, K. (2022). Contrasting genetic responses to habitat fragmentation for two Lycaenid butterfly species. *Insect Conservation and Diversity* 15 (3): 337-347.

DOI: 10.1111/icad.12556

Abstract

1. The global loss of biodiversity is one of the most urgent challenges in contemporary conservation biology.
2. Apart from species declines and lowered abundances, the loss of their genetic diversity is equally concerning as it may undermine their fitness and potential to adapt to future environmental change.
3. We compared genetic diversity of historical and recent pre-(alpine) populations of two butterfly species, *Lycaena helle* and *L. hippothoe*, over a period of about ten years.
4. Using microsatellite markers, we found no changes over time in *L. helle*, while genetic diversity decreased, and differentiation increased in *L. hippothoe*.
5. *Lycaena helle* inhabits peat bogs and fallows with populations being typically isolated, while *L. hippothoe* occurs in population networks on hay meadows, with the latter being strongly exposed to agricultural intensification.

6. We conclude that currently *L. hippothoe* populations are strongly declining due to land use change, resulting in genetic erosion potentially due to the collapse of population networks.

Keywords agricultural intensification, biodiversity loss, comparative genetics, genetic differentiation, genetic diversity, glacial relict, *Lycaena* butterflies, microsatellite marker, population networks, population structure

General discussion

Development of microsatellites

Microsatellite loci can be used as genetic markers, which allow investigation of the population genetic structure and the genetic diversity of species (Estoup and Angers 1998). Therefore, 16, ten, and ten microsatellites were developed and characterised for *L. hippothoe eurydame*, *L. tityrus*, and *L. virgaureae*, respectively.

Twenty-six of these 36 microsatellite loci deviated significantly from the Hardy-Weinberg equilibrium (HWE). Such a deviation could be due to several reasons: heterozygosity deficiency, inbreeding, missing data, population substructure, selection, and a small sample size (Ewers-Saucedo et al. 2016). In this case, the most likely explanations are the small sample size of 21 individuals used to test the microsatellites in each species and the heterozygosity deficiency, which can be caused by the occurrence of null alleles (Brookfield 1996; Meglecz et al. 2004). Usually, null alleles are caused by dissimilarities in the flanking regions of the PCR primers (Meglecz et al. 2004; Chapuis and Estoup 2007). In addition, a common phenomenon in Lepidoptera is the presence of multiple copies of microsatellite loci with highly similar flanking regions (Meglecz et al. 2004; Zhang 2004). This may reduce the utility of these markers and potentially affect the results of studies that use these microsatellites. However, these microsatellites are essential for analysing the population genetic structure and diversity in *Lycaena* butterflies.

Genetic diversity across species

The **second chapter** outlined my investigation of genetic diversity and, hence, the putative adaptive potential of a postglacial relict species *L. helle* in comparison to more widely distributed species with lower habitat specialisation such as *L. hippothoe eurydame*, *L. tityrus subalpinus*, and *L. virgaureae*. The genetic diversity was

comparatively high in three species: *L. h. eurydame*, which displayed the highest genetic diversity, followed by *L. helle* and *L. virgaureae* (Figure 2). The fourth species, *L. t. subalpinus* showed the lowest genetic diversity. *Lycaena hippothoe eurydame* exhibited a high genetic diversity, even though this species has a comparably high habitat specificity and its population has declined severely throughout Central Europe (Settele et al. 2008; Binot-Hafke et al. 2011). This is probably related to its large population sizes. A similarly high level of genetic diversity was found in *L. virgaureae*, which exhibited less habitat specificity in comparison to *L. h. eurydame*. In both species, I found a rather strong divergence among eastern and western populations. My detailed investigation of this divergence was described in **chapter three**.

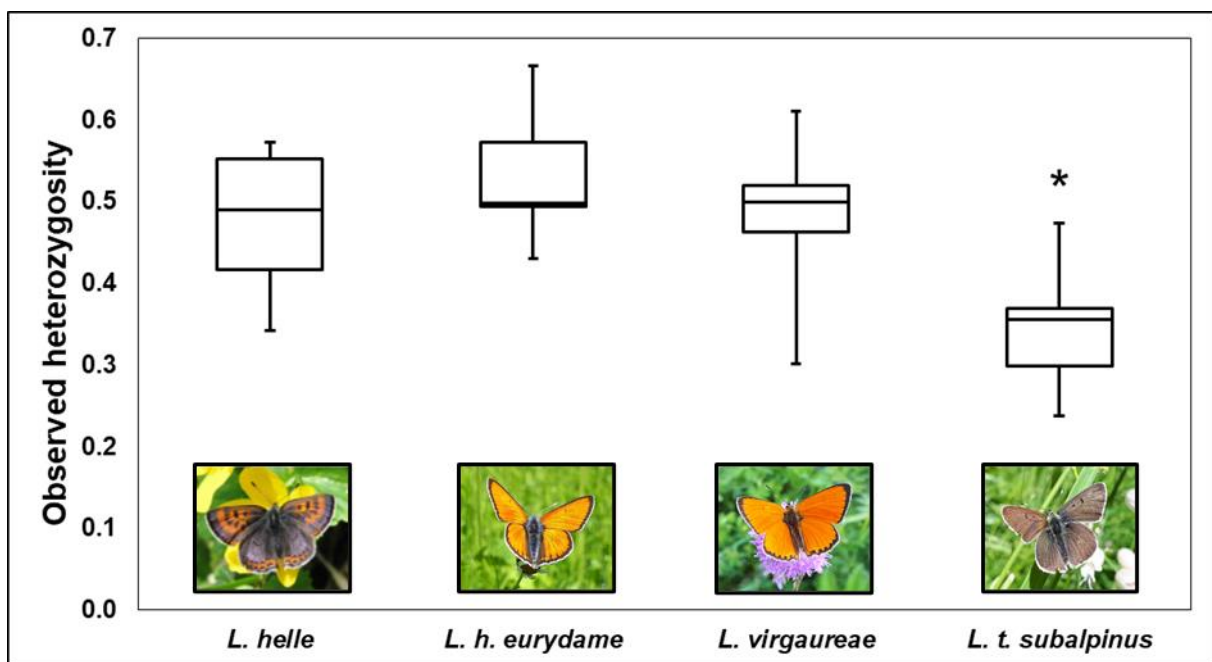


Figure 2 Boxplots of observed heterozygosity based on five microsatellite loci in eleven populations of *Lycaena helle*, *L. h. eurydame*, *L. virgaureae*, and *L. t. subalpinus*. The boxes represent 50% of the data, indicated by the first quartile (lowest line), median quartile (middle line), and third quartile (upper line). The whiskers show the ranges of observed heterozygosity. The asterisk indicates that the observed heterozygosity of *L. t. subalpinus* is significantly lower than that of the other three species.

Although *L. helle* is a postglacial relict species with high habitat specificity (Habel et al. 2010), which contradicts my hypothesis, it exhibited high genetic diversity. This might be caused by higher levels of gene flow in the past and high local population abundance (Finger et al. 2009). As high genetic diversity is expected to be advantageous for evolutionary potential and fitness (Booy et al. 2000; Reed and Frankham 2003; Bijlsma and Loeschcke 2012; Ørsted et al. 2019), *L. helle* is probably still able to adapt to changes in the environment (Finger et al. 2009). Furthermore, I detected a much more pronounced genetic structure in *L. helle* than in the three other species, even when the populations occur in close proximity. This potentially indicates limited gene flow and a strongly reduced dispersal ability of this species (Fischer et al. 1999; Guschanski et al. 2007; Stoeckle et al. 2017; Goudarzi et al. 2019).

However, contrary to my expectations, *L. t. subalpinus* was found to exhibit low genetic diversity in comparison to the three other species. I assume that *L. t. subalpinus* has suffered population losses due to agricultural intensification, characterised by too frequent mowing and eutrophication of many meadows in the Alps (Cizek et al. 2012; Grübler et al. 2015). These land-use changes may affect *L. h. eurydame* and *L. virgaureae* equally, but probably *L. t. subalpinus* occurs in panmictic populations in the Alps, and thus needs gene flow to maintain its genetic diversity. Its rather low genetic diversity is of concern and could negatively affect its evolutionary potential in the future (Karl et al. 2008a).

These findings indicate that glacial relict species or other vulnerable species are not necessarily threatened by reduced genetic diversity. Furthermore, focusing on specific populations seems to be more informative than trying to assess species-level diversity, as each population is influenced by its specific history.

Geographic and species-specific factors affect population genetic structures

In the **third chapter**, I examined the geographic and species-specific factors that affect the population genetic structures of *L. h. eurydame* and *L. virgaureae* in the European Alps. I found a higher genetic diversity and a more pronounced population structure in *L. h. eurydame* than in *L. virgaureae*. The genetic diversity of *L. h. eurydame* populations seems to be influenced by high mountain ridges and large river valleys. This is probably the result of the lower dispersal capacity of *L. h. eurydame*. This species has been found to occur in closed populations with, at least in part, large population sizes (Fischer 1998; Fischer and Fiedler 2001a). In contrast, *L. virgaureae* seems to occur in more open population structures with lower population sizes (Chuluunbaatar 2004; Barua et al. 2011).

Furthermore, the pronounced genetic structure of *L. h. eurydame* may also be influenced by more recent environmental changes such as habitat fragmentation caused by the agricultural intensification of alpine habitats (Mottet et al. 2006; Locatelli et al. 2017). This may indicate that *L. h. eurydame* is more vulnerable to land-use change than *L. virgaureae*, a phenomenon which might correlate with the differences in their dispersal ability.

For both species, the high and often glaciated ridges of the central Alps comprised a dispersal barrier between the western and eastern populations (Figure 3). This suggests that the central Alps are colonised from eastern and western glacial refuges. In other studies, potential refuges were detected in Western and Eastern Europe (Hewitt 1999, 2004; Schmitt 2007, 2009). Within this dispersal barrier in the central Alps, genetic diversity was low in *L. h. eurydame*, but high in *L. virgaureae*, suggesting different species-specific features. Here, the most likely feature seems to be dispersal

ability. The higher dispersal of *L. virgaureae* probably caused hybridization, thus boosting the genetic diversity of this more mobile species (Schneider et al. 2003). Similar findings on hybridization zones have been found in studies of other species (e.g. Petit et al. 2003; Knopp and Merilä 2009). The overall high genetic diversity and pronounced population structure found in *L. h. eurydame* seem to be related to its low dispersal ability and the fact that its populations are closed with high local abundances (Fischer 1998; Fischer and Fiedler 2001a).

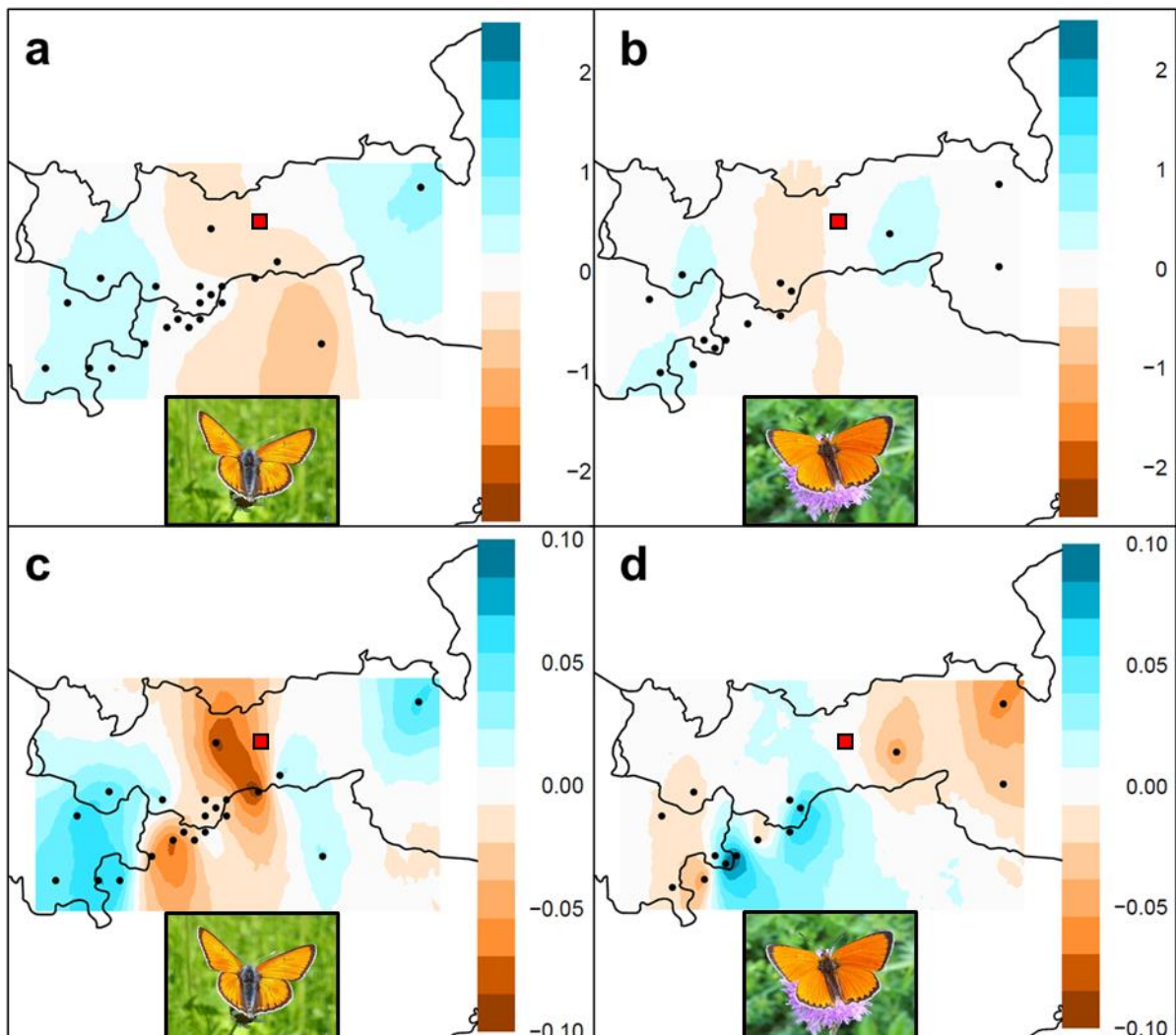


Figure 3 Estimated effective migration surface (EEMS) plots for the posterior mean of the effective migration surface based on 21 populations of *Lycaena hippothoe eurydame* (a) and 14 populations of *L. virgaureae* (b), and the effective diversity surface of *L. h. eurydame* (c) and *L. virgaureae* (e). The colours blue, white, and orange

indicate areas of high dispersal, isolation by distance, and low dispersal (dispersal barrier), respectively (a, b). The relative effective genetic diversity ranges from high in blue to low in orange/brown (c, d). Black dots and lines show the population locations and the national borders, respectively. The red square represents the city of Innsbruck.

In summary, the structure of both species are influenced by the central Alps. However, the population genetic structures and diversity patterns of the species differ as a result of species-specific differences in demography and dispersal ability. *Lycaena hippothoe eurydame* may reach high population densities but has a poor dispersal ability. *Lycaena virgaureae*, in contrast, does not seem to be limited regarding dispersal, but shows low genetic diversity.

Effects of large- and small-scale structures on population genetic structures

In **chapter three**, I presented results that led me to conclude that high mountain ridges and large river valleys affect the structure of populations of *Lycaena* butterflies in the European Alps. Next, in **chapter four**, I provided further details about the effects of large- and small-scale structures on the population genetic structure of *L. t. subalpinus*. I found substantial genetic differentiation of *L. t. subalpinus* in the Alps, caused by large river valleys and high mountain ridges. The river valleys of Rienz and Inn seem to separate the *L. t. subalpinus* populations in the European Alps. This finding is in line with other studies which discovered that large rivers affected the genetic patterns of insect populations (Mardulyn 2001; Schmitt et al. 2007; Cupedo and Doorenweerd 2020). Mountains above 2 500 m a.s.l. in the whole study area, especially when covered by glaciers, do not constitute suitable habitats for *L. t. subalpinus* and thus act as a dispersal barrier for this species. Again, this is in line with other studies on insects which have found evidence that various mountain ranges have a barrier effect on

dispersal, thus affecting gene flow among populations (Britten et al. 1995; Hewitt 1996; Després et al. 2019; Jaffé et al. 2019).

In two *L. t. subalpinus* populations, four putative half-sibling pairs were found, suggesting occasional long-range dispersal, although *Lycaena* species are predicted to be the least mobile butterfly species (Ricketts 2001). These two populations were sampled in the Dolomites and are clearly genetically separated from the other populations. Although these populations seem to be the closest to an Italian refuge, they exhibited low genetic diversity. This is not consistent with the general assumption that genetic diversity decreases from the core of a species' range to its edge (Brown et al. 1995; Eckert et al. 2008). Thus, the Dolomite populations were probably founded by another, more distant refuge, explaining their low genetic diversity, the high similarity between the two Dolomite populations and their strong separation from all other populations. Additionally, the genetic diversity in the whole study area decreased from west to east, suggesting the high importance of the southwestern Alps. I suppose that there is a glacial refuge present in the southwestern Alps, as *L. t. subalpinus* possibly recolonised the Alps after the last glacial period. The importance of the southwestern Alps was also found for other Alpine species (Schönswetter et al. 2005; Schmitt 2009; Louy et al. 2014).

I detected 15 loci that could be assigned to membrane transport or proteins, suggesting selection on membrane features in *L. t. subalpinus* along the altitudinal gradient. This is probably related to membrane fluidity, which may affect cold tolerance in ectotherms (Hazel 1995; Ohtsu et al. 1998; Hochachka and Somero 2002). Furthermore, five other loci were detected as potential outlier loci in *L. t. subalpinus*, which are involved in several cellular processes. In line with my findings, studies on other insects have

detected some of these loci as potential outlier loci in relation to altitude (Waldvogel et al. 2018; Montero-Mendieta et al. 2019; Jackson et al. 2020).

Finally, this chapter showed the impact of local geographic features, such as high mountain ridges and large river valleys, acting as dispersal barriers and thus affecting the spatial genetic diversity and structure of *L. t. subalpinus*, along with that of postglacial range expansions. Moreover, *L. t. subalpinus* populations from different altitudes adapted to local conditions, suggesting an increased cold tolerance in populations exposed to higher altitudes.

Fine-scale genetic structures in an Alpine valley

On the fine-scale, within the Ötz valley, I detected surprisingly high levels of gene flow in *L. t. subalpinus*, but also genetic structuring across natural (altitude, forests, and ravines) and anthropogenic (roads) structures, which apparently acted as a dispersal barrier (Figure 4). Specifically, I found that forests, ravines, and roads limited dispersal, while rivers did not. Other studies on insects using different genetic markers have also found that forests and roads may serve as barriers to dispersal (Schmitt et al. 2000; Keller and Largiadèr 2003; Heidinger et al. 2013).

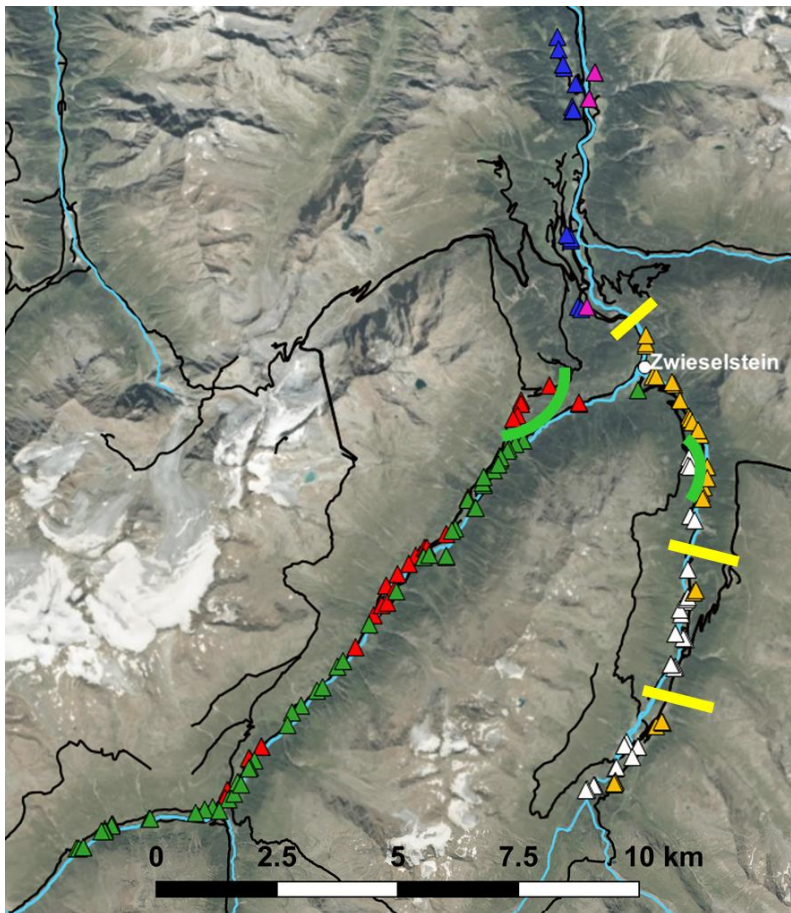


Figure 4 The study site within the Ötz valley system on an aerial map. The triangles show the location of individuals and the white dot illustrates the village Zwieselstein. The yellow lines indicate ravines, and the green half-circles indicate steep wooded slopes, putatively comprising barriers to dispersal. The blue lines indicate rivers and the black lines represent roads. Blue, red, and white triangles represent individuals on the right side of the road, and violet, green, and orange triangles represent individuals on the left roadside. The underlying river and road networks were taken from Austria's 'Roads' and 'Waterways' shapefiles, available at MapCruzin.com. The map was generated with QGIS version 3.14 (www.qgis.org).

Surprisingly, rivers did not have a comparable effect, although rivers and roads run largely in parallel and have roughly the same width in this Alpine valley. Either traffic and associated roadkills affect the gene flow across roads, or *L. t. subalpinus* is less reluctant to cross familiar natural structures (rivers) than novel structures (roads). Several studies have shown that roads may act as a barrier to dispersal for insects and

that small insects are overrepresented in roadkill (Soluk et al. 2011; Skórka et al. 2013; Muñoz et al. 2015). I assume that roads act as a barrier due to roadkill and thus constitute a major threat to *L. t. subalpinus* in the Ötz valley. Therefore, anthropogenically driven habitat fragmentation seems to be a greater threat than natural barriers, such as rivers, to genetic connectivity for *L. t. subalpinus* in this Alpine valley.

Five membrane-related proteins were detected along the altitudinal gradient, similar to the findings presented in **chapter four**. Another protein is involved in metabolism and may indicate climatic adaptations in *L. t. subalpinus*. Metabolic cold adaptation, with increasing metabolism under thermally challenging conditions, is widespread in ectotherms (Addo-Bediako et al. 2002; Shik et al. 2019). The gene product of prophenoloxidase inhibits proteins and recognizes bacterial and fungal components and is, therefore, an important part of insect immune defence (Söderhäll and Cerenius 1998). This suggests that prophenoloxidase and, thus, the immune defence of *L. t. subalpinus* have an impact on local adaptation to different altitudes.

In summary, natural and anthropogenic small-scale barriers in the Ötz valley constrain dispersal and therefore can affect the genetic structures of *L. t. subalpinus* populations. Furthermore, individuals of *L. t. subalpinus* from different altitudes adapt to local conditions, suggesting temperature to be a selective agent for this species, which is expected to be related to altitudinal variation.

Genetic diversity over time

In the last chapter, I compared the genetic diversity and differentiation of historical and recent (pre-)Alpine populations of two *Lycaena* butterflies over about ten years. In

contrast to my a priori hypothesis and due to their isolated populations, the genetic diversity of the postglacial relict species *L. helle* did not decrease over time, but showed stable genetic diversity and differentiation. *Lycaena helle* occurs in relatively large populations, which I assume buffers genetic drift and inbreeding (Fischer et al. 1999; Finger et al. 2009; Bauerfeind et al. 2009). In addition, a reason for this species' stable genetic diversity over years could be that the investigated populations occur in peat bogs or wetland fallows and are thus less likely to be exposed to the effects of agricultural intensification.

In contrast, I found decreased genetic diversity and increased genetic differentiation of *L. h. eurydame* over a time period of ten years, suggesting a substantial drop in population size. This pattern could be driven by habitat fragmentation, intensive management as well as more frequent and early mowing of hay meadows (Cizek et al. 2012; Krämer et al. 2012; Gruebler et al. 2015). The occurrence of *L. h. eurydame* is strongly associated with hay meadows in the European Alps (Ebert and Rennwald 1991), and more frequent mowing can cause direct mortality or interfere with the successful larval and pupal development of *L. h. eurydame*. This supports the findings of other studies on butterflies, which have shown that they respond negatively to early and frequent mowing (Johst et al. 2006; Cizek et al. 2012).

The conclusions made in this chapter reveal that two closely related species have shown different genetic responses over time. These differences among the species may arise from different population structures. *Lycaena hippothoe eurydame* certainly occurs in population networks which rely on gene flow to maintain genetic diversity, while *L. helle* occurs in disjunctive and isolated populations, which may be able to maintain genetic diversity without gene flow (Habel and Schmitt 2012). I assume that

each species has a different genetic background, is exposed to different challenges arising from habitat fragmentation or anthropogenic land-use, and has a different potential to adapt to the changing environment (Reusch and Wood 2007; Hoffmann and Sgrò 2011; Engler et al. 2014). However, the decreased genetic diversity of *L. h. eurydame* is of concern as it could reduce the ability of the species to adapt to further environmental changes.

Conclusions and limitations

This thesis provided new insights into the population structure and genetic diversity of four *Lycaena* species in the European Alps. (1) New microsatellite markers were developed. (2) A first comparative overview of the genetic structure and diversity of four *Lycaena* species in the European Alps was provided. (3) A relatively high genetic diversity of the postglacial relict species *L. helle* and a low genetic diversity of *L. t. subalpinus* were found. (4) High mountain ridges and large river valleys were identified as dispersal barriers for *Lycaena* populations. (5) Forests, ravines, and roads, but not small rivers, were identified as dispersal barriers for an *L. t. subalpinus* population in an Alpine valley. (6) High gene flow was found in *L. t. subalpinus*. (7) *Lycaena* populations and individuals from different altitudes were found to adapt to local conditions. (8) Genetic diversity decreased in *L. h. eurydame* populations, but remained stable in *L. helle* populations.

The results may be confounded by the occurrence of null alleles in the studies in which microsatellites loci were used as genetic markers, as presented in **chapters two, three, and six**. Hence, the datasets should be interpreted with caution. However, null alleles should not hamper the comparative studies of genetic diversity either over time or between species, as past and recent datasets or species were probably affected by null alleles to a similar extent.

The finding that the postglacial relict species *L. helle* is unlikely to be threatened by genetic erosion provides evidence for the ability of *L. helle* to adapt to environmental change. However, more insights into the adaptive potential of this species are needed. Future studies should involve more genetic markers or genome-wide SNPs to show whether this high genetic diversity persists in *L. helle* or whether studying five microsatellites may not have yielded sufficient evidence to make a statement about its overall genetic diversity.

The decreasing genetic diversity of *L. h. eurydame* over ten years and the low genetic diversity of *L. t. subalpinus* are of concern. These decreasing trends in these populations should be observed in order to monitor future developments. Moreover, to address the decrease in their genetic diversity, appropriate conservation strategies should be developed for both species. Furthermore, *L. t. subalpinus* seems to be vulnerable to anthropogenic habitat fragmentation, because gene flow is more affected by anthropogenic rather than natural linear barriers. One possible explanation is that *L. t. subalpinus* is less reluctant to cross familiar natural structures (rivers) than novel structures (roads), which could be tested in future studies by investigating edge-following and edge-crossing behaviour. This behaviour might have important consequences for the persistence of species such as *L. tityrus* in altered landscapes. The result that *L. t. subalpinus* populations from different altitudes adapt to local conditions suggests an increased cold tolerance in populations exposed to higher altitudes. This conclusion needs further investigation because only a few outlier loci were assigned to a putative function. This is probably related to a missing annotated genome of *L. tityrus*, because an available annotated genome would clearly improve the detection of loci with putative functions under selection.

Increasing global changes, including climate warming (e.g. Shukla et al. 2019), land-use change (e.g. Nilsson et al. 2013), and resulting habitat fragmentation (e.g.

Selwood et al. 2015), have been shown to be even more severe in alpine habitats (e.g. Brunetti et al. 2009). Against this backdrop, it is important to understand the genomic underpinning of adaptation and to investigate and monitor whether populations are able to adapt to changing environmental conditions (Reusch and Wood 2007; Pauls et al. 2013).

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Acknowledgments

First, I want to thank my advisor Professor Klaus Fischer for guiding and encouraging me throughout my PhD. He assisted me in collecting samples, statistical analyses, and manuscript writing. I am very grateful for his speed in replying to emails and correcting papers!

I want to thank Professor Ary Hoffmann for being my second referee, teaching me a new method in his lab, his valuable comments on projects, and for constructive criticisms on two of my drafts, as well as for a warm welcome in his working group. From the latter, I especially want to thank Nicholas Bell, Jessica Chung, Nancy Endersby-Harshman, Thomas Schmidt, and Qiong Yang.

Thanks go further to Professor Ulrich Sinsch for a valuable experience when leading the labor practical course together, and his encouragement for my work.

Further, I thank Dagmar Savelsberg for her helpful manner, answers to any questions, and always having an open ear for me.

Thanks go to Brigitte Nillow-Lange for her willingness to help and organisation of the lab course.

Melina Frenzel, Alena Hantzschmann, Jacqueline Hilgendorf, Domenica Kaiser, Jonas Köhler, Tobias Mohr, Ange Raharivololiana, Wolfram Remmers, Ronny Richter, Tamara Rischen, and Valentina Zizzari, I am grateful for the wonderful time that I had with you in Koblenz and at the university.

I thank the students Laura Jager, Michela Audisio, and Nico Weingart for their lab assistance.

My parents, my sister (Dominik Ceballos Contreras), Susanne Grützmacher, Zuhul Gültekin, Nedyá Yazar, and Michele Zuther - thank you so much for always being by my side during this sometimes very difficult time, for listening to my problems at any time, and for always giving me hope. I will never forget that :)

Many thanks to Martha Maria Sander for the helpful discussions about my thesis and helpful comments on it.

Thanks to Dominik Ceballos Contreras for reading my thesis and searching for formatting errors :)

We are grateful to the governments of Bavaria and Rhineland-Palatinate for the sampling permits and Austria, Italy, and Switzerland for not demanding such permission.

I thank Amanda Habbershaw for the correction of my English.