Universidade de Aveiro Departamento de Biologia

Ano 2012

Cecília do Carmo Diogo Fernandes

Mobilidade de borboletas: estudos genéticos e de marcação e recaptura

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada – Ramo de Ecologia Biodiversidade e Gestão de Ecossistemas, realizada sob a orientação científica da Professor Doutor Thomas Schmitt, Professor Catedrático do Departamento de Biogeografia da Universidade de Trier e co-orientação da Professora Doutora Ana Maria de Jesus Rodrigues, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

o júri

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palavras-chave microssatélites, marcação e recapture, mobilidade, borboletas

resumo

A dispersão biológica das espécies sempre foi um tema importante em vários estudos em ecologia de populações. Sabe-se que diferentes espécies de borboletas apresentam diferentes tipos de comportamentos: migratório ou sedentário. São conhecidos vários estudos genéticos que dizem respeito à estrutura populacional e aos problemas de isolamento. No entanto, são geralmente desconhecidos estudos de comportamento de dispersão das espécies para a análise populacional. Seleccionaram-se, por isso, dois métodos distintos, microssatélites e marcação e recaptura, para caracterizar a mobilidade de borboletas.

Para o método genético foram selecionadas 16 populações (634 indivíduos) de *Brenthis ino* (Rottemburg, 1775), recolhidas em Rheinland-Pfalz, Alemanha e Alsace, França, a fim de identificar a estrutura genética das populações em estudo. Através da análise por microssatélites de onze loci polimórficos, foi possível identificar a sua variabilidade e a estrutura genética entre populações. A diferenciação genética entre as populações (F_{ST} = 0.040) foi significativa. A média da heterozigosidade observada e o erro padrão foi de 0.64±0.013, enquanto a heterozigosidade esperada foi 0.73±0.008. Oito dos onze loci estavam em equilíbrio de Hardy-Weinberg, mas a presença de alelos nulos é provável para três loci. Foi encontrado um sistema de isolamento por distância, que é significativo ao ponto de explicar quase 42% da diversidade genética entre as populações. Nenhum sistema de isolamento por distância foi encontrado nas montanhas de Hunsrück, indicando que ocorre um grande fluxo genético entre populações da região.

O segundo método utilizado foi o de marcação e recaptura. Em Trier, Rheinland-Pfalz, Alemanha, foram marcados 1.210 indivíduos de cinco espécies diferentes, A*nthocharis cardamines*, *Pieris napi*, *Pieris rapae*, *Leptidea reali* e *Araschnia levana*, a fim de examinar o comportamento de dispersão. A baixa taxa de recaptura indicou que se está perante grandes populações de *Pieris napi; Pieris rapae* e *Leptidea reali*. A função exponencial negativa (NEF) mostrou ser o melhor modelo para prever os movimentos de longa distância dos indivíduos por captura/recaptura para as espécies *Pieris napi, Pieris rapae* e *Leptidea reali*. Os resultados desta previsão indicam que estas três espécies poderão voar longas distâncias, apresentando uma grande mobilidade.

Os resultados obtidos para B. ino são importantes uma vez que esta apresenta um estatuto vulnerável em Rheinland-Pflaz. Programas de monitorização poderão ser aplicados para as cinco espécies do estudo de marcação e recaptura para se caracterizar as tendências populacionais de mobilidade.

keywords microsatellites, mark-release recapture, mobility, butterfly

abstract Biological dispersal has always been an important topic in several studies in population ecology. It is known that different butterfly species present a sedentary or migratory behaviour. While the genetic analysis intends to respond to population structure and isolation issues that are mostly well studied, the effects of different dispersal behaviours of species are widely unknown. Therefore, we selected two different methods, a genetic and an ecological, to characterise butterfly mobility. For the genetic method we selected 16 populations (634 individuals) of *Brenthis ino* (Rottemburg, 1775), collected in Rhineland-Palatinate, Germany and Alsace, France in order to identify the genetic structure of the study populations. Through analysing eleven polymorphic microsatellite loci, we could identify the genetic variability and structure among populations.

The genetic differentiations among populations (F_{ST} = 0.040) was highly significant. The mean value of observed heterozygosity and the standard error was 0.64±0.013, while the one of the expected heterozygosity was 0.73±0.008. Eight of the eleven loci were in Hardy-Weinberg equilibrium, but presence of null alleles is likely for three loci. A system of isolation-by-distance was found and it explains almost 42% of the genetic differentiation among populations. No system of isolation-by-distance was found in the Hunsrück mountains leading to a large gene-flow among populations occurring in this region.

The mark-release recapture was the second method used. In Trier, Rhineland-Palatinate, Germany, we marked 1.210 individuals of five different species, A*nthocharis cardamines*, *Pieris napi*, *Pieris rapae, Leptidea reali* and *Araschnia levana* in order to examine their dispersal behaviour. The Negative Exponential Function (NEF) was the best model to predict long distance movements of the capture/recapture individuals for the species *Pieris rapae*, *Pieris napi* and *Lepdtidea reali*. The results of this prediction show that they can move large distances, therefore, we can assume that these three species have a large mobility.

The results obtained for the genetic structure seems to guarantee a genetic long-term survival for most of our 16 populations of *B. ino*. This is an important result once it has a vulnerable status for Rheinland-Pflaz. All five species have a least concern status for the same region, although conservative measures should not be forgotten. Butterfly monitoring programs are an option that describes large-scale population trends.

abbreviations

a.s.l - above sea level

DNA - Deoxyribonucleic acid

IUCN - International Union for Conservation of Nature

Km - kilometres

LC - Least concern

m - metres

mm - millimetres

MRR - Mark-release recapture

NT - Not Threaten

PCR - Polymerase Chain Reaction

RL - Reference Locality (plural RLS – Reference Locality Systems)

“Nem tudo o que pode ser contado conta, e nem tudo o que conta pode ser contado.”

Albert Einstein

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Preface

For an easy comprehension and organization of the two methods that we selected for the analyses of butterfly mobility, the introduction was separated into four main parts. The first part gives an overview on the concept of metapopulations, the different factors that can affect them and why this concept is so important for ecological studies. In the second part we described the ecology of the study species. Once we choose two different analyses for our study, representing the third part based on population genetics, we described the use of the microsatellites method. For population ecology, we used the mark-release-recapture method, representing the fourth and last part of the introduction.

1. Introduction

1.1 Metapopulation Ecology – an overview

According to Levins (1970), a metapopulation is a population consisting of many local populations. Later, Harrison (1994) identified several different types of metapopulations, e.g., the classical metapopulations (Levins, 1970), mainland-island metapopulations (Mac Arthur and Wilson, 1967), or the non-equilibrium population (Harrison, 1991, 1994). Furthermore, Hanski (1998) described two different terms for metapopulations, the first one is “metapopulation” that has been used for any spatially structured population, while the second term is “metapopulation dynamics”, used to refer to any population dynamics involving spatial patterns.

The metapopulation described by Levins (1970) assumed that all local populations have a finite probability of extinction, and Hanski (1999) added that, in a long-term, survival of a species is at the regional or metapopulation level. The survival probability of such a population network is determined by many factors like the ratio of habitat edge to interior (Chen et al., 1995; Radeloff et al., 2000), the isolation of habitat fragments (Collinge, 2000), patch area (Kruess and Tscharntke, 2000), patch quality (Dennis and Eales, 1997; Kuussaari et al.,2000; Hanski and Singer, 2001), microclimate (Braman et al., 2000) and the type of matrix between patches (Maes et al., 2004). These factors are

responsible for the abundance of organisms in a landscape and therefore, influence the turn-over equilibrium of colonizations, extinctions and recolonizations.

Since the 90´s, several theoretical and experimental studies have analysed the effects of fragmentation and habitat size on the survival probability of populations (Peacock and Smith, 1997; Hanski, 1999; Knutsen et al., 2000), leading to a reduced gene-flow and thus increasing genetic drift with the subsequent loss of genetic diversity (Holzhauer et al., 2005), often correlated with severe reductions of the fitness of the individuals (Taylor et al., 1993; Frankham et al., 2002; Hansson and Westerberg, 2002; Reed and Frankham, 2003).

Concluding, we can describe the concept of metapopulation ecology as the regional assemblages of many organisms guaranteeing the long-term survival of a species depending on a shifting balance between local extinctions and re-colonisations in a network of more or less interconnected local habitats (Hanski, 1991, 1999; Hanski and Gyllenberg, 1993; Rockwood, 2006; Habel and Schmitt, 2009).

1.2 Description of study species

In this study, we handled six species of butterflies. *Brenthis ino* for the microsatellite analyses, and the five species - A*nthocharis cardamines; Pieris napi; Pieris rapae; Leptidea reali* and *Araschnia levana* - for the mark-released-recaptured study.

1.2.1 Brenthis ino Rottemburg, 1775

The butterfly family Nymphalidae includes the genus *Brenthis*, and is represented by the following three species in Europe: *Brenthis daphne* (Denis and Schiffermüller, 1775), *Brenthis hecate* (Denis and Schiffermüller, 1775) and *Brenthis ino* (Rottemburg, 1775), in Germany only *B. daphne a*nd *B. ino* (Figure 1) occur, according to Tolman and Lewington (1997).

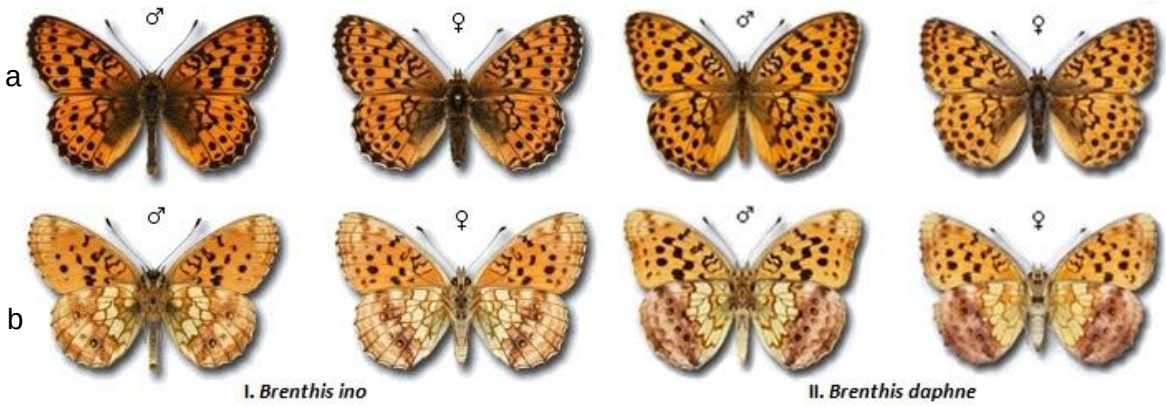


Figure 1 - Forewing and hindwing a) upper- and b) under-sides of I. *Brenthis ino* and II. *Brenthis daphne*, male and female, respectively (Adapted from Lepidoptera.pl)

The average forewing length of adult male butterflies is 17 to 20 mm in *B. ino* (Higgins and Riley, 1970). *B. daphne* and *B. ino* are morphologically very similar and the main distinguishing feature of these two species is in the hind-wing underside base of the cell s4 (adjacent to the cell-end), which is wholly yellow and visible as a discrete, rectilinear spot separating the cell from the dark postdiscal area (Mihoci and Šašić, 2005).

Furthermore, Matsuoka et al. (1983) investigated the allozyme data of *B. ino* and *B. daphne* which showed lack of genetic differentiation between them, indicating that their divergence might be a relatively recent event in evolutionary history. *B. daphne* and *B. ino* are both Palaearctic species. *B. ino* or the common name Lesser Marbled Fritillary has an extensive range and can be found almost all over Europe including major parts of northern Europe (Danish mainland, Norway, Sweden and Finland) (http://www.faunaeur.org/), all the way until the Ussuri region, North China and Japan (Tolman and Lewington, 1997). Although, this species has disappeared in the Netherlands through habitat loss, probably land drainage (Chinery, 1989). *B. ino* is also not present in some South European countries like in the northern and middle part of Italy and southern part of Spain and in Portugal is described as one of the rarest species (Maravalhas et al., 2004). In Central and West Europe, the typical biotopes include humid to wet grasslands, riverine marshes, bogs, clearings in wet forests, mountain valleys and subalpine tall herb formations (Zimmermann et al., 2005).

The Lesser Marbled Fritillary is a univoltine butterfly. According to Lepidopterologen-Arbeitsgruppe (1987), adults fly from mid-June to the end of July and until August at the highest altitudes. Males are generally patrolling to search for females, while females spend most of their time flying and searching for nectar. The nectar plants most used by males are *Cirsium* spp. and *Centaurea jacea* L. and by females are *Sanguisorba officinalis* L. and *Knautia arvensis* L. Coult. (Zimmermann et al., 2005). Mostly females lay

eggs singly at the underside of plant leaves and larvae live solitarily. The information in literature about host plant use is incongruous. The most frequently reported plant species is *Filipendula ulmaria*, but also *Sanguisorba officinalis* and *Rubus* spp., but some authors also mention *Aruncus vulgaris*, *Potentilla palustris*, *Sanguisorba minor*, and a few other plant species (e.g., Hrubý, 1964; Henriksen and Kreutzer, 1982; SBN, 1987; Ebert and Rennwald, 1991; Lepidoptera Specialist Group, 1991; Tolman and Lewington, 1997; Settele et al., 1999; Agnes, 2000; Sawchik et al., 2003). Thus, it seems that *B. ino* is associated with various Rosaceae, perhaps with a trophic range varying with geographic locality (Zimmermann et al., 2005). The imagines show a preference for violet flowering nectar plants like knapweeds (*Centaurea* spec.) and thistles (*Cirsium* spec.) (Ebert and Rennwald, 1991).

The conservation status of *B. ino* is controversially discussed: while some authors (e.g. Zimmermann et al., 2005) argue for increasing populations, its habitats (wet grasslands) are declining in many parts of Europe (Gibbs, 2000; Brinson and Malvárez, 2002; Öckinger et al., 2006). Most importantly, the drainage of wetlands and the conversion into arable fields are responsible for the decline of suitable habitats, especially in Central Europe. However, *Brenthis ino* is a rare example of a successful wetland butterfly (Zimmermann et al., 2005). Van Swaay and Warren (1999) reported that the Lesser Marbled Fritillary populations are increased in countries like Hungary, Slovenia, and Croatia, as well as in Luxembourg and the Czech Republic. However *B. ino* has decreasing in Austria, where a decrease of 75-100% has been observed over the last 25 years, and a decrease of 15-25% as been reported in Germany, Denmark and Romania (Swaay and Warren, 1999), as well as in Bulgaria (Ganev, 1985) and Serbia (Jakšić, 2003).

According to the IUCN 2010 classification, the Lesser Marbled Fritillary has been categorised as least concern (LC) in Europe and specifically as not threatened (NT) in Germany (Van Swaay and Warren, 1999). According to Schmidt (2010) in the region of Rheinland-Pfalz, *B. ino* has a vulnerable status.

1.2.2 Anthocharis cardamines Linnaeus, 1758

Anthocharis cardamines belongs to the family Pieridae. This species is widespread from the Iberian Peninsula through nearly all Europe, except in the northernmost parts of Scandinavia, and extends through temperate Asia to China (Tshikolovets, 2011; Kudrna et al., 2011). *Anthocharis cardamines* can be found in a variety of moderately damp meadows, woodland margins, rides and clearings (Buszko and Mastowski, 2008), open

grassy slopes, steppes, rivers valleys, roadsides and gardens (Tshikolovets, 2011). During the dispersal period, butterflies also visit dry open habitats (Buszko and Mastowski, 2008).

The Orange-tip butterfly is a univoltine butterfly. According to Courtney and Duggan (1987) and Asher et al. (2001), adults normally fly between mid-April and mid-June, they may also be seen as early as mid-March in southern countries, and occasionally until August in mountains (Tshikolovets, 2003; 2011).

Males have vivid orange tips, whereas female have no orange coloration and are predominantly white on the uppersides. On the underside wings, they have a mottled pattern of yellow-greenish and black scales (Figure 2) (Dempster, 1997; Asher et al., 2001). Females lay eggs at the base of flower heads on plants growing in the full sun. It is unusual that more than one egg is laid on a single flower head (Asher et al., 2001). The pupae overwinter in tall vegetation close to the larval food plants (Tshikolovets, 2003).

Figure 2 - Forewing and hindwing upper- and under-sides of *Anthochardis cardamines*, female and male, respectively (Adapted from Lepidoptera.pl)

The average forewing length of adult male butterflies is 33-48 mm and 29-49 mm in females (Tshikolovets, 2011). As host main plants, several crucifers are used, especially *Cardamine pratensis*. Other plants like *Alliaria petiolata*, *Sisymbrium officinalis, Barbarea vulgaris, Brassicae rapa, Sinapis arvensis, Cardamine amara, Arabis hirsutam, Lunaria annua* and *Hesperis matronalis* can also be used (Wiklung and Åhrberg, 1979, Asher et al., 2001). The plant species *Allaria officinalis, Arabis turrita, A. alpine, Biscutella mollis, Brassicae campestris, Cardaminopsis arenosa, Hesperis lacinata* and *Isatis tinctoria* have been also described as food plants for *Anthocharis cardamines* (Tshikolovets, 2003; 2011).

Orange-tip butterfly have a European status as NT and with a low butterfly conservation priority (Swaay and Warren, 1999). According to IUCN 2010, it is classified in Europe as LC. This status also applies to the region of Rheinland-Pfalz (Schmidt, 2010).

1.2.3 Pieris rapae Linnaeus, 1758

Pieris rapae belongs to the family Pieridae. This species has a range from North Africa and Macaronesia through nearly all Europe and temperate Asia to Japan (Kudrna et al., 2011). It was introduced in the 19th century and now is a resident and widespread species in North America and Australia (Baker, 1978; Asher et al., 2001; Tshikolovets, 2011). As an ubiquitous species, it inhabits open habitats on farmland and woodland (Buszko and Mastowski, 2008), these habitats include cultivated land from sea level up to 3000 m (Tshikolovets, 2011), gardens, hedgerows and wood edges where wild crucifers occur (Asher et al., 2001). The species, sometimes is regarded a pest in gardens and cabbage fields.

The Small White is a multivoltine butterfly from February to October (November) in more southern regions (Asher et al., 2001; Tshikolovets, 2003; 2011), but it's a univoltine butterfly, from May – August, in the North and in high mountains. Imagos fly throughout the year in the extreme south of Europe and the Canary Islands (Tshikolovets, 2003; 2011).

Males have one black spot on top of the upperside forewings, whereas females present two black spots, and usually have a more pallid colour (Figure 3). The bottle-shaped eggs are laid only in warm weather, singly on the underside of leaves of host-plants, although there may be several eggs on each plant (Courtney, 1986; Asher et al., 2001). Pupation takes place in a variety of situations: the spring generation may remain on the foodplant or nearby vegetation, while the overwintering pupae are generally attached to a surface of a wall, post or tree trunk (Courtney, 1986; Asher et al., 2001).

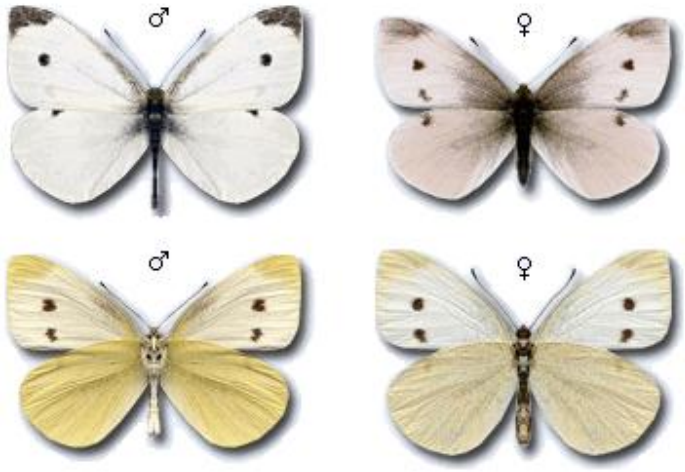


Figure 3 - Forewing and hindwing upper- and under-sides of *Pieris rapae*, male and female, respectively (Adapted from Lepidoptera.pl)

The wingspan of males and females are from 39-50 mm (Tshikolovets, 2011). The main host-plants are cultivated Brassicaceae, specially cabbages and *Tropaeoleum majus*. The species *Brassica oleracea, Sinapis arvensis, Sisymbrium officinale, Alliaria petiolata, Lepidium draba, Reseda lutea* are used to a lesser extent (Asher et al., 2001) and many species of Brassicaceae, Resedaceae, Tropaeoloceae and Capparaceae, can also be used (Tshikolovets, 2011).

The Small White is considered of low conservation priority and presents a European status as not threatherned (NT) (Swaay and Warren, 1999). The IUCN 2010 refers to this butterfly as LC in Europe, and according to Schmidt (2010), this species has also a LC status in the region of Rheinland-Pfalz.

1.2.4 Pieris napi Linnaeus, 1758

Pieris napi belong to the family Pieridae. This species occurs in the temperate and subtropical parts of the Paleartic (Tshikolovets, 2011), so it is distributed from North-West Africa through Europe, throughout the temperate parts of North Asia eastwards to Japan (Kudrna et al., 2011). According to Asher et al. (2001), this species also occurs in North America and has a stable range in most of the European countries. It inhabits a variety of open habitats and open woodlands (Buszko and Mastowski, 2008; Tshikolovets, 2003; 2011). Adults occur widely but tend to gathers in damp, lush vegetation where their foodplants are found, especially in hedgerows, ditches, bank rivers, lakes and ponds, damp meadows and moorland, woodland rides and edges (Asher et al., 2001; Tshikolovets, 2003; 2011). Butterflies can be observed in cultivated areas from sea level up to 2000 m or more (Tshikolovets, 2011).

The Green-veined White presents a voltinism that depends on the locality and altitude. Therefore, it is univoltine from June to July in colder climates, represented by higher latitudes and altitudes, and is bivoltine or trivoltine from March to November in the south (Courtney, 1986; Tshikolovets, 2003; Tshikolovets, 2011; Asher et al., 2001).

Males have one black spot on top of the upperside forewings, whereas females present two black spots. At the underside hindwings, both have veins distinct by dark streaks (Figure 4). Females usually deposit their eggs on small plants, laying them singly on the undersides of leaves. The pupae, in the northern populations remain in well-hidden positions, among the surrounding vegetation while, in the southern populations, the second or the third generation overwinters as pupae (Dennis, 1985; Asher et al., 2001).

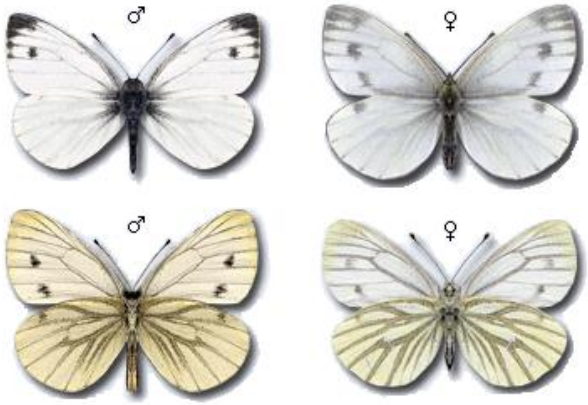


Figure 4 - Forewing and hindwing upper- and under-sides of *Pieris napi*, male and female, respectively (Adapted from Lepidoptera.pl)

The average forewing length of adult male butterflies is 34-50 mm, while females show an average length of 32-48 mm (Tshikolovets, 2011). The main host plants are normally wild crucifers such as *Alliaria petiolata, Cardamine pratensis, C. amara, Sisymbrium officinale, Rorippa nasturtium-aquaticum, Sinapis arvensis, Brassica oleracea* and *Raphanus raphanistrum.* Occasionally they can also be found in cultivated crucifers as *Tropaeolum majus* (Asher et al., 2001). Tshikolovets (2011) even add *Arabis turrita, A. sagittata, Brassica campestris* and *Cardamine palustris* to the list of species.

The Green-veined White is presently of low butterfly conservation priority and it ranks with a European status as NT (Swaay and Warren, 1999). According to IUCN 2010, it is rated in Europe as LC, and also considered LC by Schmidt (2010) for the region of Rheinland-Pfalz.

Agreeing with the comment made by Tshikolovets (2011), the geographical variations and relationships between plain and mountain, northern and southern populations are not well understood and consequently require further study.

1.2.5 Leptidea reali Reissinger, 1989

Leptidea reali belongs to the family Pieridae. In some countries, it overlaps with a very similar species, *Leptidea sinapis* (Asher et al., 2001; Buszko and Mastowski, 2008; Tshikolovets, 2011; Kudrna et al., 2011). In recent years, it was reported from almost all European countries, except Great Britain and northernmost Scandinavia, Caucasus and Transcaucasia, but its true distribution requires further clarification (Tshikolovets, 2011). The species inhabits dry or wet half-open woodland clearings and margins (Buszko and Mastowski, 2008), open and bushy flowery meadows, margins of cultivated areas, sometimes also open grassy areas up to 2000 m (Tshikolovets, 2011).

Reál's Wood White is univoltine from end of May until August in the North and mountains, bivoltine from May to June and July to August in the central countries of its distribution, and is trivoltine from Mach to October in the South (Tshikolovets, 2011).

Identification of the two Wood Whites requires examination of genitalia. Both species have rounded white wings, with dark wing tips on the forewing on the upperside, present in males, while females have more pallid coloration or even do not have the dark wing tips (Figure 5). On the underside of hindwing, they are usually greenish with grey markings (Asher et al., 2001; Tshikolovets, 2011). The eggs are similar in both species and are laid singly on the upper parts of foodplants. Identical with *L. sinapis,* this butterfly overwinters in the pupae (Asher et al., 2001; Tshikolovets, 2011).

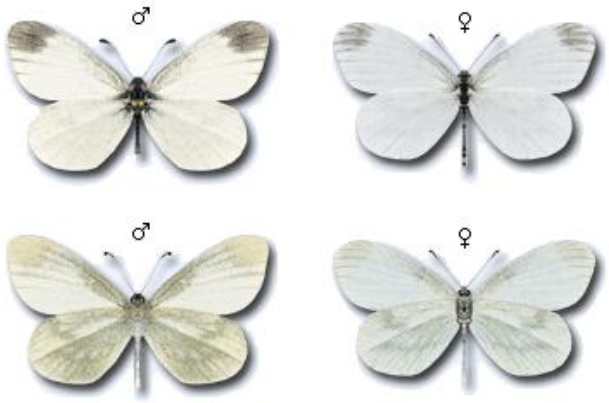


Figure 5 - Forewing and hindwing upper- and under-sides of *Leptidea reali*, male and female, respectively (Adapted from Lepidoptera.pl)

The wingspan of males is from 32 to 41 mm and the females are slightly bigger from 33 to 43 mm (Tshikolovets, 2011). The main host-plant is *Lathyrus pratensis* (Tshikolovets, 2011).

According to IUCN 2010, Reál's wood white is rated in Europe as LC and is also described as a LC status butterfly by Schmidt (2010) for the region of Rheinland-Pfalz.

1.2.6 Araschnia levana Linnaeus, 1758

Araschnia levana belongs to the family Nymphalidae and presents a range from the Pyrenees to the Ural Mountains, through most of Europe (absent in the North and most of the Mediterranean region) and across the woodland belt of temperate Asia eastwards to Japan (Kudrna et al., 2011), but also in the Caucasus and Transcaucasia (Tshikolovets, 2003; Tshikolovets, 2011). It inhabits meadows and various open places with nettles in light or mixed woodland, bushy margins, grassy gorges and small rivers and clearings in

damp broad-leaved forests (Asher et al., 2001; Tshikolovets, 2003; 2011). It is also found in neglected parks and ruderal habitats (Buszko and Mastowski, 2008).

Araschnia levana on the one hand is a univoltine butterfly at higher altitudes and in northern areas, from June to early August, but on the other hand generally bivoltine in warmer regions from late April until May and late June to August (Asher et al., 2001). Occasionally it is trivoltine in the southern areas and in particularly warm years (Fric and Konvička, 2000; Tshikolovets, 2003, 2011). When presenting bivoltine phenology, the brood named *levana* (Linnaeus, 1758) and *prorsa* (Linnaeus, 1758) represent the spring and summer broods, respectively. When occurring in a trivoltine phenology, the third brood named *porima* (Ochsenheimer, 1808) (Tshikolovets, 2003; 2011).

The Map, the common name for *Araschnia levana*, demonstrates a big different appearance between the adult butterflies of the first and the second generation (Asher et al., 2001). The forewings of the first brood have a reddish-orange background colour with a black standard pattern and black margins, while the second brood is black, with one intermittently white list, followed for orange lists and with white markings in the margins of the hindwings (Figure 6), the third brood have brownish background with listed orange colour. On the undersides, the wings have a colourful pattern in males and a bit different one in females.

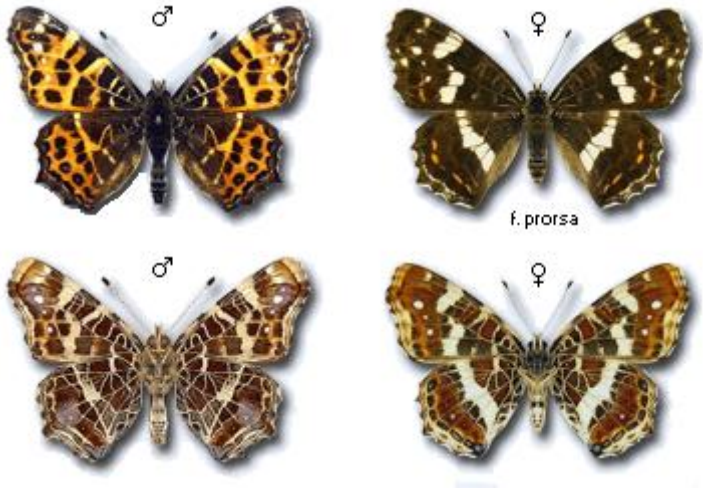


Figure 6 - Forewing and hindwing upper- and under-sides of *Araschnia levana*, male and female, respectively, with the identification of the form prorsa, the summer generation (second brood). (Adapted from Lepidoptera.pl)

Eggs are laid in a long string that resemble nettle flowers, and are fixed to the undersides of the leaves of its foodplants (Asher et al., 2001). The Map butterfly overwinters in the pupal stage (Asher et al., 2001; Tshikolovets, 2011). The average forewing length of the adult butterflies is from 28 to 39 mm (Tshikolovets, 2003; 2011).

The main host plants are *Urtica dioica* and in rare cases *U. urens* (Asher et al., 2001; Tshikolovets, 2003; 2011).

According to IUCN 2010, it has the status of LC in Europe. The Butterfly conservation priority is not assessed (Swaay and Warren, 1999), and it also considered with the status LC by Schmidt (2010) in the region of Rheinland-Pfalz.

1.3 Why Microsatellites?

The recent and strong decline of biodiversity caused by anthropogenic impacts is a phenomenon of world-wide implications (Ostfeld and Logiudice, 2003; O'Connor and Crowe, 2005; Brooks et al., 2006; Junker and Schmitt, 2010). This deterioration of biodiversity is not only occurring in the observed global biodiversity hotspots like tropical rainforests and coral reefs, but the European continent is also suffering a high rate of biodiversity loss (Greuter, 1994; Thomas et al., 2004; Schmitt and Rákosy, 2007). Consequently, member states of the European Union established the NATURA 2000 program aimed to counteract the biological depletion of Europe and to conserve the emblematic diversity in all of its regions (Commision of the European Communities, 2002; Mehtälä and Vuorisalo, 2007). To reach this goal, a high number of animal and plant species along with their specific habitats are strictly protected and listed in several Annexes of the Habitat Directive (Kudrna, 2000).

The increasing destruction, degradation and fragmentation of habitats are the main reasons for such a strong species loss during the last few decades (e.g. Abbitt et al., 2000; Fahrig, 2003; Henle et al., 2004). According to Kraus et al. (2004), the habitat fragmentation is one of the major threats to biodiversity leading to the extinction of species (Reed, 2004). The two main components of habitat fragmentation, reduced fragment size and reduced connectivity, produce different population effects (Krebs, 2001).

This process of habitat fragmentation creates dispersal barriers, leads to isolation of populations (Gerlach and Muslof, 2000), and results in a decrease of genetic variation within populations and an increase of genetic differentiation among populations (Frankham et al., 2002; Marsh et al., 2008). Consequently, increased risks of inbreeding, resulting in negative effects on the respective populations and also increase the probability of population extinctions. Hence, smaller habitats cause smaller populations and increased isolation leading to reduced colonisation rates, hereby enhancing the risk of extinction (Rosenzweig, 1995). Therefore, the genetic diversity of populations decreases

with increasing habitat fragmentation (e.g. Young et al., 1996; Buza et al., 2000; Pedersen and Loeschcke, 2001; Keller and Largiadèr, 2003; Williams et al., 2003).

Beside actual anthropogenic habitat fragmentation, species are often naturally isolated because of biotic and abiotic (geological and microclimatical) conditions and evoke long term species specific abundance patterns (Habel et al., 2009b). Consequently, it is important that the population genetic studies take the different factors like the historical level of isolation and recent differentiations among populations into account (e.g. Bermingham and Avise, 1986; Cunningham and Moritz, 1998).

The decrease of genetic diversity, used as an indicator of inbreeding, will increase the extinction risk of populations due to the decline in fitness of individuals (Saccheri et. al., 1998; Reed and Frankham, 2003). Therefore, conservation biologists have to pay attention on the effects of habitat fragmentation on genetic diversity (Krauss et al., 2004). Typically, conservation genetic studies have analysed fragmentation effects by documenting patterns of genetic differentiation among populations and the differing levels of genetic diversity of these populations (Harrison and Hastings, 1996; Oostermeijer et al., 1996; Young et al., 1996, Habel et al., 2009b).

Due to the decline of its habitats in Central Europe, we selected the butterfly species *Brenthis ino* that might be affected by anthropogenic landscape changes. Therefore, the knowledge of the genetic structure, diversity and differentiation of populations as well as gene-flow among populations can be helpful for understanding the effects of increasing landscape fragmentation. Thus, we analysed fifteen populations situated in the region of Rheinland-Pfalz (south-western Germany) and one in the region Alsace (north-eastern France) to address these questions. The use of microsatellites as analytical tool is explained by their high variability making them useful for the detection of genetic diversity and differentiation in isolated and fragmented populations, since they show more often polymorphisms than other molecular markers (Selkoe and Toonen, 2006). Thus, microsatellites will be suitable to answer our population genetic questions (Frankham et al., 2002). To our knowledge, no microsatellite primers have been available for the Lesser Marbled Fritillary prior to this study (Molecular Ecology Resources Primer Development et al., 2012).

In the near future, the increasing fragmentation of the landscape and the progressive habitat loss caused e.g. by the intensification of agriculture and infrastructural developments may therefore become the most important issues for conservation biology

in these areas (Junker and Schmitt, 2010). Hence, it will be of essential importance to obtain knowledge about specific indicator organisms to analyse their potential to adapt to these new circumstances and to minimize the negative consequences for the biodiversity as a whole. This may help to avoid similar developments as those being responsible for the critical situation of many species groups in Central Europe (Junker and Schmitt, 2010).

1.4 Biology of Dispersal

The biology of dispersal is essential to many areas of ecology and evolutionary biology, from issues of population regulation, through community dynamics, to gene-flow and speciation (Clobert et al., 2001; Bullock et al., 2002; Bowler and Benton, 2005; Kokko and Lopez-Sepulcre, 200). Furthermore, in the perspective of habitat loss, fragmentation and global climate change, understanding dispersal is crucial. According to Stevens et al. (2010), the ecological and evolutionary functioning of natural populations affected by habitat fragmentation, alterations of their climatic envelopes, or a mixture of both, depends on (i) the availability of functionally connected networks of habitats, and (ii) sufficient dispersal ability of the affected species to track these changes. Dispersal also drives the spatial and temporal redistribution of genotypes being inseparable from the evolution of life-history traits (Ronce, 2007).

It is important to identify the three different types of movements (mobility, dispersal and migration - Figure 7.) that are presented by butterflies (Stevens et al., 2010). For example, dispersal can be defined as the spreading of individuals away from each other (Begon et al., 2006), or as the movement of an organism away from its birth place or from centres of population density (Ricklefs and Miller, 1999). The term ‘mobility’ is often used to described types of butterfly movement, including foraging movements, vagrancy or migration propensity. However, in turn the term ‘migration’ normally refers to directional and periodically reversed mass movements (even if these movements are not performed by the same individual) (Stevens et al., 2010). Commonly, dispersal studies are separated according to their methodology into direct and indirect investigations. On the one hand, direct investigations take into consideration mark-release-recapture (MRR) or point-release experiments, the dynamics of patch colonisation and extinctions, data on range expansions, occupancy of islands, or results obtained from cage experiments. On the other hand, indirect methods rely on the description of the distribution of genetic diversity

among local populations from which gene-flow and inter-population genetic distances are inferred (Stevens et al., 2010).

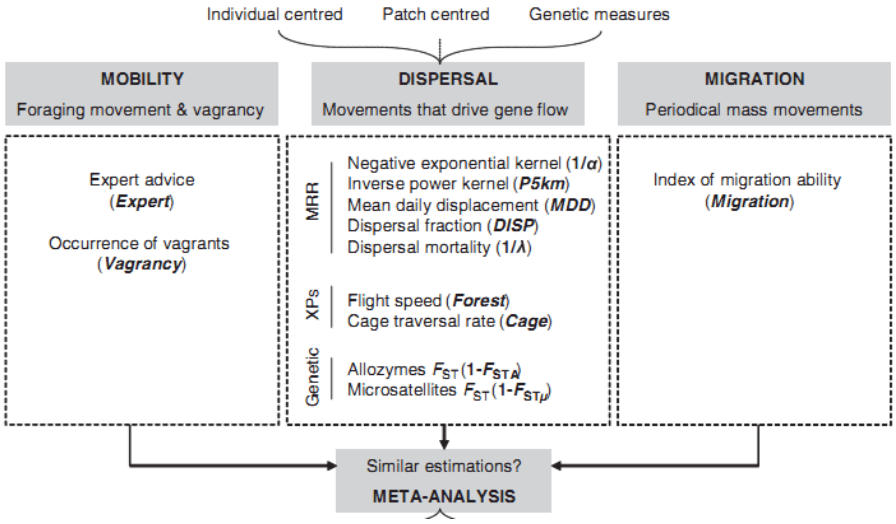


Figure 7 - Schematic representation of the meta-analysis according to Stevens et al. (2010) on dispersal, mobility and migration in butterflies. MRR = mark-release-recapture studies; XPs = experimental studies; F_{ST} = measure of genetic differentiation among populations: F_{STA} from allozymes, $F_{STμ}$ from microsatellites, are some of the studies that can provide an understanding on butterfly mobility

According to Stevens et al. (2010), butterflies have long been recognized as ideal models for the study of fragmented populations for two main reasons. First, the specialization makes the habitats relatively easy to map in heterogeneous landscapes for most species (Baguette and Mennechez, 2004), and second, the natural history of most species is well known (e.g. Dennis, 1992; Ehrlich and Hanski, 2004; Dover and Settele, 2009; Boggs, 2009). The amount of literature presenting butterflies as models for different types of studies is vast. They have been used as models for studies focusing either on molecular adaptation leading to energetic optimization (Watt and Boggs, 2003), or on orientation processes (e.g. Rutowski, 2003). Even more, they are commonly used as biological models in integrated studies of dispersal (Hanski and Kuussaari, 1995; Ehrlich and Hanski, 2004; Hovestadt and Nieminen, 2009) and several recent reviews report on butterfly dispersal in the context of climate change (Dennis, 1992; Parmesan et al., 1999; Hill, Thomas and Huntley, 1999; Hill et al., 2002; Nilsson et al., 2008; Settele et al., 2008; Poyry et al., 2009), habitat fragmentation (Heikkinen et al., 2005; Dover and Settele, 2009) and habitat deterioration (Ockinger et al., 2006).

Adult butterflies usually move for foraging, egg laying or looking for mates, however, there is not so much literature describing how the nature of movements affect the distribution of butterflies at scales beyond that of a single habitat patch. It is expected that different species will show different ways of such movements (Norberg et al., 2002).

According to the same author, some species may be more willing to cross the patch borders into unsuitable habitat and thereby have a greater chance of finding neighbouring patches and utilizing a larger habitat area. Consequently, much species will have a more extensive local distribution than less explorative species. A large variation in mobility is found among adults of different butterfly species (Norberg et al., 2002). Some migratory species, for instance, can move several dozens of kilometres a day (Baker, 1978) while other species rarely move more than a few metres (e.g., Thomas, 1985; Thomas and Harrison, 1992). Apart from true migratory behaviour, considerable variation in mobility exists among species, both in terms of distance moved and in the frequency of inter-patch movements (e.g., Shreeve, 1995). This last type of mobility can be described as a result of habitat exploration (also referred to as ranging, Dingle, 1996).

According to Ehrlich (1961), some unsuitable areas (not only depending on geography, but also on habitat characteristics) occasionally are hard to pass through and a butterfly may “decide” to stay within a certain area; this can be explain by the term ‘intrinsic barriers’ affecting dispersal. Later, Gilbert and Singer (1973) affirm that such intrinsic barriers can be seen by different ways between populations of the same species.

Studies on mobility in butterflies are often based on mark–release–recapture methods. According to Norberg et al. (2002) not knowing what happened to butterflies that were never recaptured is a possible disadvantage of this method, and when the recapture frequency is low, it can become particularly troublesome.

For all this, we can affirm that dispersal behaviour (i.e. movements of individuals from their place of birth to another one; Nathan, 2003; Trakhtenbrot, et al., 2005) is becoming a more and more significant key to the correct comprehension of the life-history for many species, especially when the main subjects are foraging and reproduction. This movement of individuals will lead to gene-flow among populations, and that is why dispersal is vital for the connectivity of populations, resulting in different consequences for the fitness of the individuals on population dynamics and on population genetics. Furthermore, dispersal allows adaptation to the changing environmental conditions (that actually are happening so fast) and influences the distribution and abundance of species in different types of habitats. However, dispersal is a very sensitive and complex ecological process, which is influenced by intraspecific densities (Lidicker, 1975), habitat quality (Wolf and Lidicker, 1980; Lurtz et al., 1997), landscape structure (Hill et al., 1996; Bennett, 1999; Poethke and Hovestadt, 2002), resource allocation in the surrounding landscape (Root and Kareiva, 1984; Munguria et al.1997) and climatic conditions (Parmesan, 1996). Therefore, Sutherland and Dolman (1994) emphasize the great importance of behaviour that can

leads to a better understanding of all dispersal processes that do occur in some groups of animals.

According to Baguette (2003), the long-term survival of butterflies in fragmented landscapes will depend on different ecological key-factors enabling the persistence of stable metapopulation systems. Therefore, we selected a study site in south-western Germany, and established a transect that as predominant habitats consists of vineyards and grasslands. The study site was located in the Avelertal, a valley in Trier. We performed a mark-release-recapture study almost over the entire flight period of five species: *Anthocharis cardamines*, *Pieris napi*, *Pieris rapae*, *Leptidea reali* and *Araschnia levana* to set a deeper understanding of the ecology and the adult behaviour of these species. Dispersal distances define the spatial scale of the population and determine the range of recolonisation after extinction events (Hanski ,1998; 2004) and that is why they will have a central importance in this study. Finally, and although these species do not specifically need a conservation programme on this area, we will try to identify some general conservation applications to the five study species.

1.5 Objectives

Our main aim was to identify mobility differences among populations in different areas using two different types of approaches, microsatellites and mark-release recapture. Therefore we selected study places where the populations of the target species, *Brenthis ino*, A*nthocharis cardamines*, *Pieris napi*, *Pieris rapae*, *Leptidea reali* and *Araschnia levana* were well represented as well as was/were their host plant(s).

1.5.1 Microsatellites

The aim of this part of our study was to determine the genetic structure and genetic diversity of *Brenthis ino* in sixteen populations, distributed in Rhineland-Palatinate (Germany) and Alsace (France). We intend to answer the following questions:

i) Has *Brenthis ino* significant genetic structure among the studied populations?

ii) If so, how is the genetic differentiation at an interregional level among the five different areas?

iii) Has a system of isolation-by-distance established in this species?

iv) Does the *Brenthis ino* population from Ballon d'Alsace (France) show reduced genetic variability?

v) Do populations from the Hunsrück mountains represent one major genetic unit without further substructures?

vi) What are the conservation consequences of these results?

1.5.2 Mark Released Recapture

The main aim of this part of the study was to investigate:

i) Resource use strategies, the influence of behaviour on movement and dispersal patterns within a mostly homogenous habitat for species with a little known mobility potential.

ii) We also try to compare the tendency towards habitat exploration among these species that differ in their distribution patterns.

iii) Furthermore, we intend to examine the dispersal behaviour by applying the best prediction model for long distance movements of individuals, once this may directly influence the functioning of the population system.

2. Materials and methods

2.1 Study region and study sites

2.1.1 Microsatellites analysis

The 16 populations of our study are located in the western part or the region Rheinland-Pfalz in Germany, in different locations, Westerwald, Pfalz, Eifel and Hunsrück, only one populations was from Ballon d'Alsace, identified as an outgroup (Figure 8). This last population is from the region Alsace in north-eastern France, close to the border with Baden-Württemberg, Germany. The samples were collected in the summer of 2010 and 2011 and were immediately stored in liquid nitrogen. This study sites were chosen because (i) pre-studies indicated suitable *Brenthis ino* populations and (ii) they all presented a high abundance of the larval food plant *Filipendula ulmaria.*

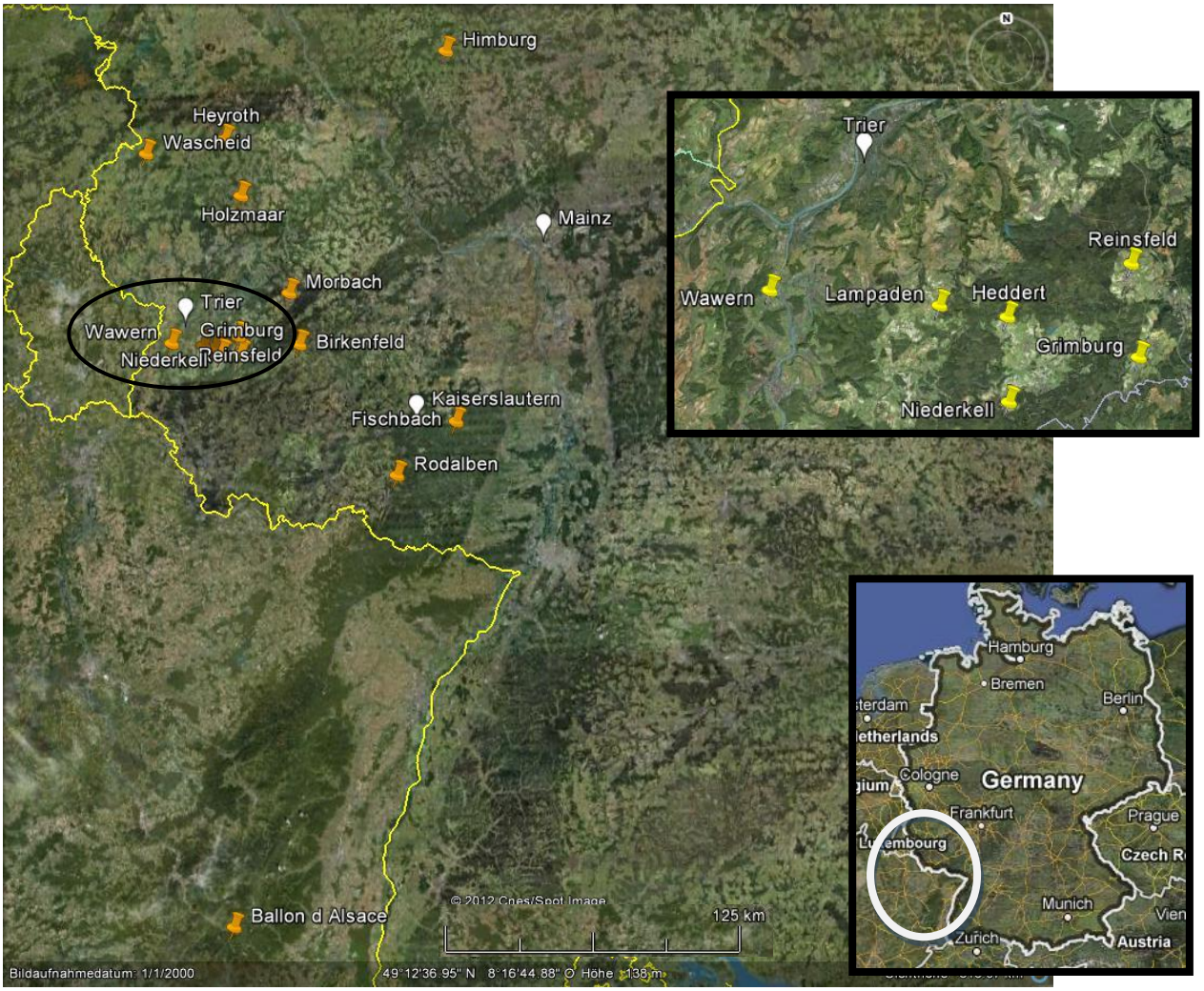


Figure 8 - Geographical location of the study populations. All populations are marked in yellow in the map, Morbach 1 and Morbach 2 are marked as Morbach in the map due to their geographical proximity. The main cities, Trier, Mainz and Kaiserslautern from the region Rheinland-Pfalz, Germany, are marked in white. ® Google Earth 2012

2.1.2 Mark Release Recapture analysis

The study area for the MRR is located in the valley named Avelertal in Trier (Rhineland-Palatinate, Germany: 49°45'N, 6°40'E) at an altitude between 100 and 150 m a.s.l. situated between the city centre and the Trier University Campus (Figure 9).

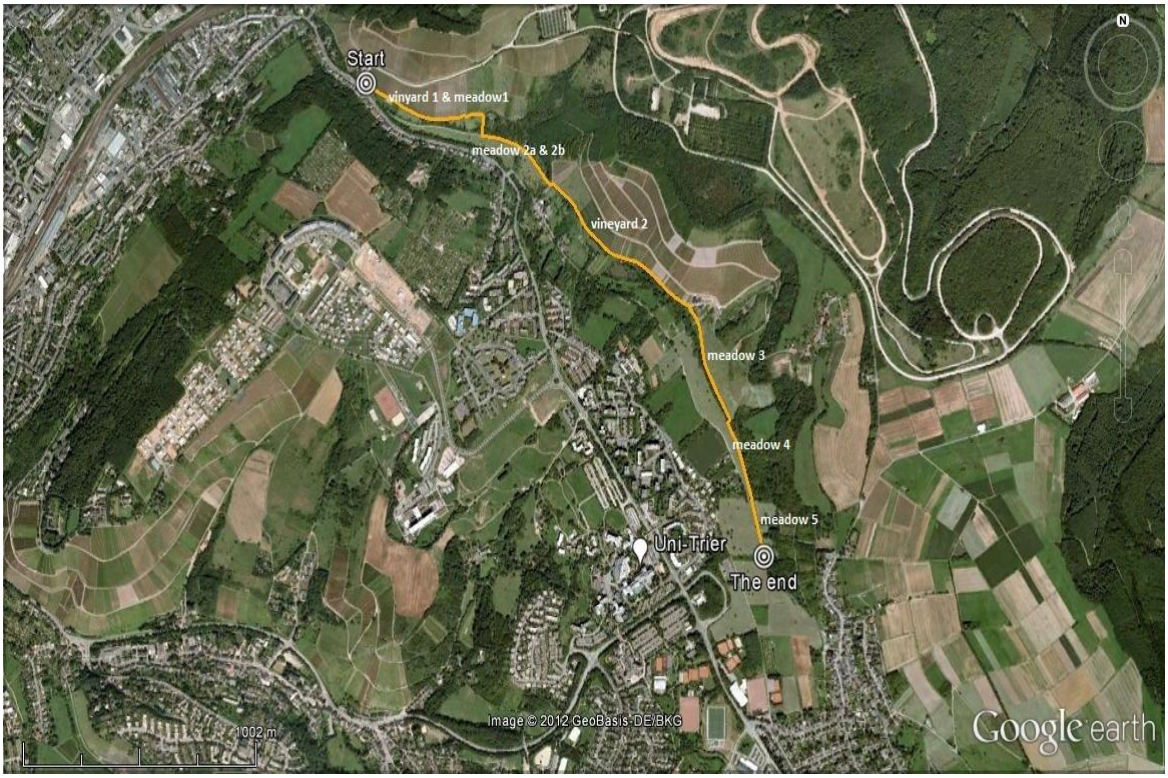


Figure 9 - Map of the mark-release-recapture study site in Avelertal - Trier, Rhineland-Palatinate, Germany. The transect path is marked in yellow. ® Google Earth 2012

The transect's main habitats are vineyards surrounded and interspersed by meadows. The main plants are *Vitis vinifera*, *Trifolium pratense*, *Trifolium repens*, *Clinopodium vulgare*, *Echium vulgare*, *Lythrum salicaria* and some trees for example *Fagus sylvatica*, *Acer pseudoplatanus*, *Acer platanoides*, *Acer campestris* and *Corylus avellana.* For an easier comprehension, the 4 km transect was divided into seven sections (seen Figure 10) depending on different characteristic like different habitats and contact with different anthropogenic factors.

Figure 10 - The seven different sections of the Avelertal transect. ® Fernandes, C.

This study area was chosen because (i) a pre-studies indicated a suitable population for all species, A*nthocharis cardamines*, *Pieris napi*, *Pieris rapae*, *Leptidea reali* and *Araschnia levana* in the MRR study and (ii) the abundant presence of all of their host-plant.

2.2 Sampling and genetic analysis

2.2.1 Microsatellites analysis

The samples were collected in July 2010 and 2011 (Table 1), and stored in liquid nitrogen. DNA was extracted from thorax muscle tissue using the Qiagen DNeasy™ Tissue Extraction Kit (QUIAGEN, Hilden, Germany 2004) following the manufacturer's protocol. The gut tissue inside the chitin was used and it was digested during two hours with the "Proteinase K" at 56°C. After incubation, the DNA was purified with different washing buffers and then eluted as recommended in 100 µl ddH_20 (double distilled water).

The resulting DNA was stored in a freezer at -20°C.

Table 1. Sampling localities and their regions as well as the number of sampled *Brenthis ino* individuals

Population	*Location (Lat./Long.)*	*Number of Individuals*
Alsace, France		
Ballon d'Alsace	47°49'N, 6°50E	40
Westerwald		
Himburg	50°34'N, 7°53'E	40
Eifel		
Heyroth	50°17'N, 6°48'E	40
Holzmaar	50°07'N, 6°52'E	29
Wascheid	50°15'N, 6°24'E	42
Pfalz		
Fischbach	49°24'N, 7°55'E	40
Rodalben	49°14'N, 7°38'E	43
Hunsrück		
Birkenfeld	49°39'N, 7°09'E	40
Grimburg	49°37'N, 6°53'E	40
Heddert	49°38'N, 6°45'E	40
Lampaden	49°38'N, 6°42'E	40
Morbach 1	49°48'N, 7°07'E	40
Morbach 2	49°48'N, 7°07'E	40
Niederkell	49°35'N, 6°42'E	40
Reinsfeld	49°40´N, 6°52'E	40
Wawern	49°39'N, 6°32'E	40
Total	-	**634**

We first tested unlabeled primer pairs in three individuals using gradient PCRs on a Multigene^{TM} OptiMax Thermal Cycler (Labnet) to search for the best annealing temperature. The concentrations for a 10 µl PCR reaction were: 4.4 µl 5PRIME HotMasterMix (0.2 U Taq polymerase, 45 mM KCl, 2.5 mM Mg^{2+}, 200 µM of each dNTP), 5.7 µl H_2O_{bidest}, 0.0625 pmol forward and reverse primer and 10-20 ng template DNA. PCR conditions were as follows: initial denaturation of 2 min at 94°C followed by 33 cycles (denaturation: 30 sec at 94°C, annealing: 30 sec with a gradient of 50-60°C, elongation: 60 sec at 72°C) and final elongation of 10 min at 60°C. For all eleven primer pairs, we achieved reliable PCR conditions, which were combined in four multiplex PCR reactions (Table 2). For each multiplex reaction, we prepared 10 µM primer stock solutions (forward and reverse primers of each locus included).

The multiplex stock solutions contained 4 µl of each primer (see Table 2). Water was added up to 100 µl total volume. For each reaction, we used 1.1 µl of the primer mixture, 5.5 µl Type-it 2x Multiplex PCR Master Mix (Qiagen) and 0.7 µl template DNA. PCR conditions were as follows: initial denaturation of 5 min at 95°C followed by 33 cycles with denaturation of 30 sec at 95°C, annealing of 90 sec at primer-specific temperatures (Table 2), elongation of 30 sec at 72°C and terminated with an elongation of 20 min at 60°C. MegaBACE Fragment Profiler 1.2 (Amersham Biosciences) was used for scoring.

Table 2. Characterization of eleven polymorphic microsatellite primers for *Brenthis ino* with GenBank accession numbers; locus name; repeat motif; primer sequence of forward (F) and reverse (R) primer; fluorescence dye name (Tag); multiplex reaction (MPR); allele size range and annealing temperature (T_a)

Accession no.	*Locus*	*Repeat motif*	*Primer Sequence (5'-3')*	*Tag*	*MPR*	*Allele size range (bp)*	T_a
Q688092	Bi3	$(AC)_{11}$	F:GAGATGATACTCTTACACTGCT R:ATAGTATGTTTGTTATTTCATGGTG	FAM	1	255-287	53°C
JQ688093	Bi8	$(AC)_{13}$	F:ATTTGTAACGCGTCTTCCCAC R:GATTGACGACTAGAACTGGC	HEX	1	359-415	53°C
JQ688094	Bi19	$(AC)_{11}(AT)_7$	F:TCCTTTGGATCTTCTTAGCCGA R:ATGTGTATTTGTCTAGTCTCATTG	TAMRA	2	234-308	53°C
JQ688095	Bi24	$(AC)_{18}$	F:GTTGACTTTCGACCGCATAC RAAGACGCACACGCGCACT	HEX	2	205-225	53°C
JQ688096	Bi29	$(AC)_9$	F:TAAGCCTCAACCTGGTGCTG R:CACGAATGTTTGTACTCCAGTC	HEX	3	274-292	55°C
JQ688098	Bi33	$(TA)_4(TG)_{14}$	F:TTTTATAGAACCAAGACCACGTC R:CTACTAATTCACAGGTTGCTAC	FAM	2	280-290	53°C
JQ688099	Bi36	$(AC)_{23}$	F:CGAATCTCGTCATAGACTGAAG R: ACAATGGCTACGATGATACTGC	TAMRA	4	271-309	55°C
JQ688100	Bi38	$(AC)_{11}$	F: AAGGAGTCATTCGACCGCGA R:CACCGTTAGCGCTATCGAG:	FAM	4	319-325	55°C
JQ688101	Bi39	$(TG)_8$	F:AGTTGTTAAAGAACGGCAAGTATG R:TATTCTCACTTCGCTCGGATG	HEX	4	304-312	55°C
JQ688102	Bi41	$(AT)_{21}(GT)_{10}$	F:ACAATGCGTCTCCTAGACCG R:ACTGGAGTACAAACATTCATGC	HEX	2	331-403	53°C
JQ688103	Bi44	$(CT)_{11}$	F:AATCGAATGAGCCCAAACTCG R:TACCCTTGCTTCGCTCGTG	FAM	3	182-200	55°C

2.2.1.1 Statistic Microsatellites

To prevent possible miscoding of results like possible scoring errors (e.g. stutter bands, large allele dropout or null alleles) (cf. Selkoe and Toonen, 2006), our data was analysed with the program Micro-Checker 2.2.1 (van Oosterhout et al., 2004). Allelic richness and

the test for linkage disequilibrium were calculated using FSTAT 2.9.3.2 (Goudet, 1995). Allelic richness was preferred over allele diversity (mean number of alleles per locus) as it takes into account differences in sample size among sample location.

Hierarchical genetic variance analyses (AMOVA), number of alleles, observed and expected heterozygosities, tests on Hardy Weinberg equilibrium (HWE) and F Statistics (F_{ST}) were calculated using the GenAlEx 6.4 software (Peakall and Smouse, 2006).

HWE can be calculated by:

$$p2+2pq+q2 = 1$$

with p and q being the allele frequencies from the different regarded alleles. Concerning this context, it is important to know that the observed heterozygosity (H_o) is the real heterozygosity of all individuals over all samples. The expected heterozygosity (H_e) equates to the possibility that two random samples have got different alleles. H_e is calculated from the observed allele frequencies with the expression:

$$H_e = 1-\Sigma x^2_i \text{ (Grauer and Li, 2000)}$$

with x_i being the frequency of the allele i.

AMOVAs were calculated using conventional F-statistics based on allele frequencies and the infinite alleles model (IAM) (Slatkin, 1995). This approached included three hierarchical levels: among populations, among individuals within populations and within individuals. Pairwise differentiation between populations was tested for with pairwise F_{ST} using the GenAlEx 6.4 software (Peakall and Smouse, 2006). With the same software, isolation by distance was tested. We tested by means of a Mantel test to infer about the correlations between the geographical distances and the genetic distances (pairwise F_{ST} values) (Nei, 1978) for (i) all populations and (ii) combining only the populations of the Hunsrück region.

Assignment tests for all individuals were done with the program STRUCTURE 2.2 (Falush et al., 2003). This software was used to infer the most probable number of genetic clusters without *a priori* definition of populations. We used the batch run function to carry out a total of 100 runs – ten each for one to ten clusters, i.e. K=1 to K=10. The repetitions were run to see if there were deviations among the different runs for a fixed K and to calculate means and standard deviations. Each burn-in and simulation length was 100,000 and 1,000,000, respectively. Since the log probability values for different K values have been shown to be little reliable in some cases, we used the more refined and *ad hoc* statistic ΔK based on the rate of changing the probability of data between successive K values (Evanno et al., 2005), which has been shown to better unveil the correct number of

genetic clusters, to infer the most likely number of groups (Finger et al., 2009). ΔK is express as

$$\Delta K = (|mL(K+1) - 2mL(K) + mL(K-1)| / sdL(K)$$

with L(K) being the logarithm of the probability that K is the correct number of clusters, m the mean and sd the standard deviation.

The output file of STRUCTURE was analyzed on-line with STRUCTURE HARVESTER (Web version: v0.6.92 March 2012), which is a program for visualizing STRUCTURE outputs and implementing the Evanno method (Earl and vonHoldt, 2012).

2.2.2 Mark Release Recapture analysis

The samples were collected between 16th of May and 18th of August 2012. On each day with suitable weather condition (less than 50% clouds, temperatures ≥ 20°C, weak or moderate wind), one or two persons passed once through the whole study area (11:00 a.m. – 6:00 p.m) and netted all available individuals for *Anthocharis cardamines*, *Pieris rapae*, *Pieris napi*, *Leptidea reali* and *Araschnia levana*. Each captured individual was marked with an individual code at the underside of the wings using a waterproof pen (Steadtler, Lumicolor S). The code consisted of one letter (A-Z) for the capture day and a respective running number. The following information was noted before release additionally to the individual code: weather, sex, GPS (eXplorist reciver 100) data of the capture point (Magellan Meridian Platinum, measuring accuracy: <3 m), vegetation type at capture point, exact time of capture, wing wear (1–4 scale: with 1 being fresh and 4 being heavily damaged cf. Munguira et al., 1997; Zimmermann et al., 2005), and the behaviour prior to capture (i.e. flying, fighting, mating, feeding, resting).

2.2.2.1 Mobility

The detailed GPS data set of capture/recapture events obtained in this MRR study was used to analyse the movement behaviour of *Anthocharis cardamines*, *Pieris rapae*, *Pieris napi*, *Leptidea reali* and *Araschnia levana*. We calculated the total distances between all capture/recapture events to get insights into the sedentariness of the individuals. These distances were used to fit our data to two different mathematical models commonly applied to find the best prediction of rare long distance movements (Baguette, 2003; Kuras et al., 2003; Fric and Konvicka, 2007): the negative exponential function (NEF) and the inverse power function (IPF). We calculated the inverse cumulative proportion of individuals moving certain distance classes while each distance class represented a 50 m interval. These data were fitted to the NEF and the IPF function separately for each

species using linear regression analyses. Additionally, we performed the same analyses with 100 m intervals separately for each species to exclude artefacts based on the selected interval size. For the NEF, the relative proportion of individuals moving to distance D is

$$I_{NEF} = ae^{-kD}, \text{ respective } \ln I = \ln a - kD.$$

The parameter *a* represents a scaling constant while *k* is the dispersal constant describing the shape of the exponential curve. Under the IPF, the proportion *I* is expressed as

$$I_{IPF} = cD^{-n}, \text{ respective } \ln I = \ln c - n (\ln D),$$

where *c* is a scaling constant and *n* a variable describing the effect of the distance on the dispersal (Baguette, 2003). We applied *F*-statistics (SPSS 10.1, curve estimation) to determine the significance of the curve fitting for the NEF and the IPF. Comparing the results of both analyses, we used the best-fit-model to predict the proportion of individuals moving to distances beyond those covered by our MRR study.

3. Results

3.1 Microsatellites

All analysed populations were strongly affected by the presence of excessive numbers of homozygotes. This indicates the existence of null alleles, but three loci (Bi8, Bi 19 and Bi 41) were particularly affected as revealed with Micro-checker. The analysis with GenAlEx showed that all the eleven loci were polymorphic for all 16 populations. The eleven loci yielded between four and 21 alleles (for details see ANNEXE I - Table I. 1).

Allelic richness was calculated with the software FSTAT, and was based on the minimum sample size of 26 diploid individuals. Allelic richness was lowest for the locus Bi38 of population Himburg with a value of 3.0 alleles, and the maximum value was observed in the locus Bi19 of the population Niederkell with a value of 19.49 alleles. The locus Bi38 was also the one that presented the lowest allelic richness over all studied populations (ANNEXE I - Table I. 1).

The numbers of alleles, expected heterozygosity, observed heterozygosity and fixation index (F), over populations for each locus are represented in Table 3. The results showed that Bi19 had 15.81±1.41 alleles being the locus with the higher mean number or alleles. The loci Bi38 and Bi39 presented the mean number of alleles 4.13±0.13 and 4.13±0.25 respectively, thus representing the lowest allele numbers of all analysed loci. The relation

between the observed (H_o) and the expected (H_e) heterozygosity showed that loci Bi24, Bi29 and Bi38 had similar mean values; the locus Bi3 showed only a small difference between H_o and H_e. All remaining loci had high differences between H_o and H_e.

Table 3. Mean values and standard errors (SE) of the parameters of genetic diversity: number of alleles (Na), expected heterozygosity (H_e), observed heterozygosity (H_o) and fixation index (F), over all populations for each locus analysed for 16 populations of *Brenthis ino* from Rhineland-Palatine and Alsace

	Bi3	*Bi8*	*Bi19*	*Bi24*	*Bi29*	*Bi33*	*Bi36*	*Bi38*	*Bi39*	*Bi41*	*Bi44*
Na	10.69	14.19	15.81	6.94	6.31	6.94	6.81	4.13	4.13	12.81	7.88
SE	0.53	0.94	1.41	0.36	0.22	0.34	0.42	0.13	0.25	0.79	0.43
H_e	0.83	0.85	0.72	0.71	0.73	0.71	0.65	0.53	0.72	0.80	0.72
SE	0.01	0.01	0.03	0.02	0.01	0.01	0.01	0.02	0.01	0.02	0.02
H_o	0.82	0.38	0.61	0.71	0.73	0.57	0.58	0.53	0.54	0.47	0.53
SE	0.02	0.04	0.06	0.02	0.03	0.03	0.03	0.03	0.03	0.04	0.07
F	0.01	0.55	0.17	0.00	0.00	0.18	0.11	0.01	0.25	0.41	0.29
SE	0.02	0.05	0.05	0.03	0.03	0.05	0.05	0.03	0.03	0.05	0.08

The locus specific fixation indices showed that the loci Bi24 and Bi29 had a mean value of zero, reflecting a complete panmixis among populations. The loci Bi3 (0.01±0.02) and Bi38 (0.01±0.03) presented mean values close to zero, leading to the same assumption. All of the remains loci showed a variation of mean values from 0.11±0.05 to 0.55±0.05.

Significant deviations from Hardy Weinberg Equilibrium (HWE) due to a heterozygote deficit were detected in the loci Bi41, Bi 8, and Bi19, respectively; Bi41 showed the most significant deviation, followed by Bi8 and finally by Bi19; all the other loci showed no significant deviation from the HWE and all populations did not show a significant deviation from HWE.

The overall F_{ST} value was 0.040 ($p < 0.001$), meaning that 4% of total molecular variance was on the level among populations; 21% was found among individuals and 75% was found within individuals (Figure 11). The pairwise population analysis with 999 permutations showed that Himburg had the maximum mean value of 0.060 for genetic distances, when compared to all populations, followed by Ballon d'Alsace that presents a mean value of 0.058, and Heyroth with a mean value of 0.040. The remaining populations present genetic distances ranging from 0.027 to 0.039, where the population Wawern (0.026) showed the smallest genetic distances (ANNEXE I - Table I. 2).

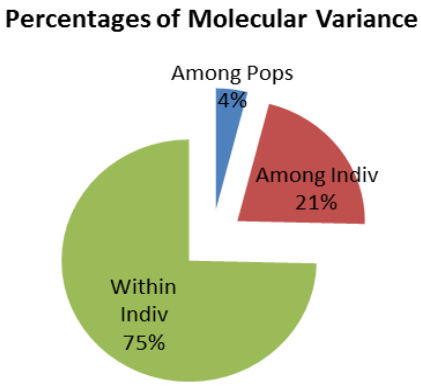


Figure 11 - FSTAT results of Analysis of Molecular Variance ($p < 0.001$) of 16 *Brenthis ino* populations from Rhineland-Palatinate and Alsace based on the analysis of eleven microsatellite loci

The results for the parameters of genetic diversity, Na, H_o, H_e and fixation index, over loci for each Population can be seen in Table 4. The population from Niederkell had the highest mean value for the number of alleles (Na = 11.82±1.99 SE). The relation between the observed (H_o) and the expected (H_e) heterozygosity showed a big difference where the mean value of the observed heterozygosity was always lower than the expected heterozygosity, leading, once again, to the assumption of discrepancy forces like inbreeding. Only the populations Niederkell and Reinsfeld had a small difference between the values of H_o and H_e. The fixation value (F) had a mean value above zero in all populations, the population Heddert had the lowest value of 0.03±0.05.

Table 4. Mean values and standard error (SE) of the parameters of genetic diversity: number of alleles (Na), expected heterozygosity (H_e), observed heterozygosity (H_o) and fixation index (F), over all loci for each population of *Brenthis ino* from Rhineland-Palatinate and Alsace

Pop		*Na*	H_o	H_e	*F*
Ballon d'Alsace	Mean±SE	7.27±0.68	0.65±0.05	0.74±0.02	0.12±0.07
Birkenfeld	Mean±SE	9.82±1.71	0.48±0.06	0.71±0.04	0.30±0.08
Fischbach	Mean±SE	9.73±1.73	0.48±0.06	0.74±0.03	0.33±0.08
Grimburg	Mean±SE	7.910.79	0.61±0.04	0.70±0.03	0.13±0.06
Heddert	Mean±SE	7.64±0.66	0.69±0.04	0.72±0.03	0.03±0.05
Heyroth	Mean±SE	10.00±1.73	0.54±0.07	0.74±0.03	0.27±0.09
Himburg	Mean±SE	8.73±1.44	0.69±0.04	0.73±0.03	0.05±0.06
Holzmaar	Mean±SE	8.55±1.11	0.49±0.08	0.73±0.04	0.32±0.10
Lampaden	Mean±SE	10.18±1.57	0.64±0.05	0.75±0.04	0.13±0.06
Morbach 1	Mean±SE	7.82±0.95	0.55±0.06	0.67±0.04	0.17±0.08
Morbach 2	Mean±SE	8.55±0.84	0.56±0.05	0.72±0.02	0.21±0.07
Niederkell	Mean±SE	11.82±1.99	0.70±0.06	0.75±0.03	0.07±0.07
Reinsfeld	Mean±SE	7.00±0.57	0.63±0.05	0.69±0.04	0.09±0.05
Rodalben	Mean±SE	9.55±1.62	0.50±0.06	0.70±0.03	0.29±0.08
Wascheid	Mean±SE	10.55±1.62	0.55±0.07	0.75±0.03	0.27±0.08
Wawern	Mean±SE	8.55±0.86	0.66±0.05	0.75±0.03	0.11±0.07

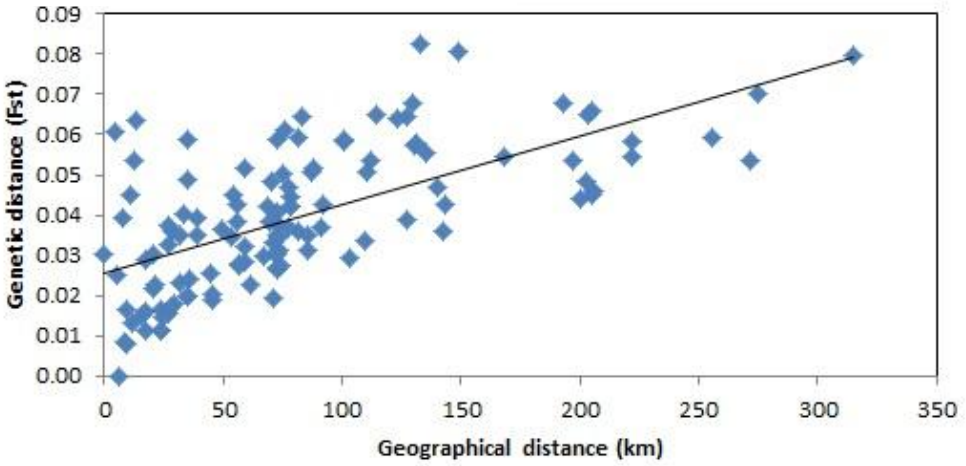
The results of population assignment revealed that 20% of the individuals assembled to other populations and 80% of the overall value assembled to itself population as it is summarised in the Table 5. These “misassigned” individuals might represent immigrants from the other population, or the descendants of such immigrants. Himburg had only one individual assembled to “other” populations, and Niederkell was the population that showed more “misassigned” individuals, a total of 17.

Table 5. Summary of population assignment outcomes to 'self' or 'other' population, with a zero frequency of 0.01, observed in our study of *Brenthis ino* from Rhineland-Palatinate and Alsace

Population	*Self Pop.*	*Other Pop*
Ballon d'Alsace	36	4
Birkenfeld	30	10
Fischbach	33	7
Grimburg	28	12
Heddert	31	9
Heyroth	36	4
Himburg	39	1
Holzmaar	25	4
Lampaden	32	8
Morbach 1	30	10
Morbach 2	32	8
Niederkell	23	17
Reinsfeld	30	10
Rodalben	37	6
Wascheid	32	10
Wawern	31	9
Total	505	129
Percentage	80%	20%

Using the values of the F-statistic and the geographic coordinates, we correlated geographic distance and the respective genetic distance among populations. A first correlation was done for all 16 studied populations (Figure 12a). We found a highly significant correlation indicating isolation-by-distance. In a second correlation, we restricted our samples to the ones samples in the Hunsrück region (Figure 12b), where no isolation-by-distance was found.

a) All populations of *Brenthis ino*



b) Hunsrük populations of *Brenthis*

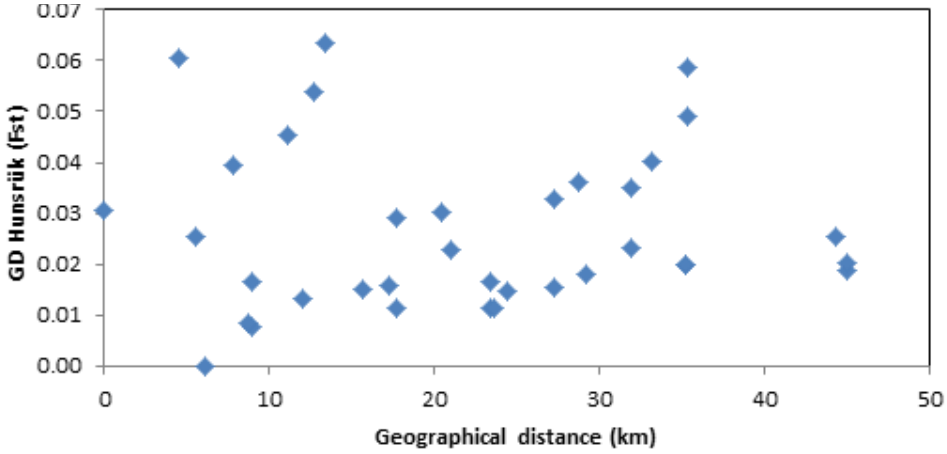


Figure 12 - Correlation between the geographical distances and the respective genetic distances (Nei, 1978) of *Brenthis ino* populations. (a) Correlation between all the populations of the study (r^2=0.4197; Mantel test: p ≥ 0.001), and (b) correlation including only the populations of Hunsrück mountains (r^2=2e^{-05}; Mantel test: p ≥ 0.474)

3.1.1 Population Genetic Structure

Bayesian structure analysis of *Brenthis ino* individuals of 16 populations were performed in the software STRUCTURE. We used the pooled data for microsatellites to infer the genetic clustering of the populations. The highest marginal likelihood (corresponding to maximizing the posterior probability) was obtained for seven clusters (Figure 13).

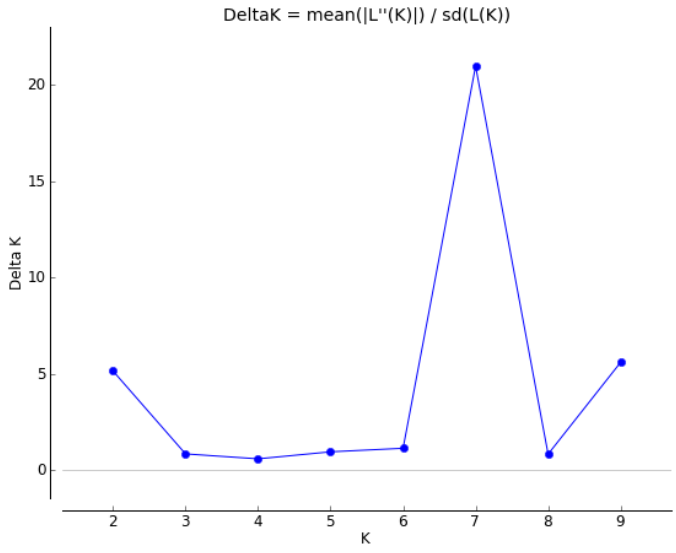


Figure 13 - Graphical representation of the relationship between Delta K (ΔK) and the numbers of clusters (K) based on STRUCTURE analysis of eleven microsatellite loci of 16 *Brenthis ino* populations from Rhineland-Palatinate and Alsace

The stability of this solution was investigated by running the stochastic estimation many times and observing where the algorithm converged. The solution with k = 7 was obtained in most cases (see Table 6).

Table 6. Results of the STRUCTURE analysis of *Brenthis ino* populations from Rhineland-Palatinate and Alsace. Ln(Pr) is the natural logarithm of the probability calculated by the Structure software that K is the correct number of populations. SD is the standard deviation calculated from ten independent runs. The *ad hoc* statistic ΔK is not applicable for K=1, and from the equation given in the methods section it is obvious that it cannot be calculated for the highest K either (because data for K+1 are needed)

K	*mean LN (Pr) ± SD*	*ΔK*
1	-26489.29 ±1.44	-
2	-25989.05 ±1116.07	5.15
3	-26087.42 ±746.58	0.85
4	-25547.50 ±849.23	0.59
5	-25515.99 ±691.08	0.96
6	-26149.84 ±1682.91.09	1.14
7	-24854.27 ±78.51	20.97
8	-25205.80 ±1267.27	0.84
9	-24489.75 ±123.61	5.60
10	-24466.86 ±30.22	-

The results of the barplot can be seen in Figure 14. If the population has a large amount of different colours, it shows that it has large gene pool.

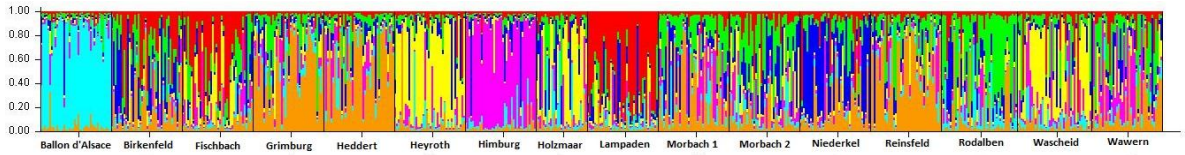


Figure 14 - Bayesian stucture analysis of *Brenthis ino* with the STRUCTURE software (Pritchard et al. 2000). Analysis performed for all individuals in all populations with K=7

3.2 Mark Release Recapture

We marked 1.210 individuals, of which eight butterflies were from *Anthocharis cardamines,* 854 from *Pieris rapae*, 279 from *Pieris napi*, 63 from *Leptidea reali* and six from *Araschnia levana* (Table 7). The longest move was observed for the species *Pieris rapae* and it was almost about 2km. We obtained recaptures only for three species. No recaptures were obtained for the species *Anthocharis cardamines* and *Araschnia levana*. We registered 141 recaptures with 117 recaptures of 77 individuals of *Pieris rapae*, 18

recaptures of 15 individuals of *Pieris napi* and six recaptures of five individuals of *Leptidea reali.*

Table 7. Results of the mark-release-recapture study of *Anthocharis cardamines*, *Pieris rapae*, *Pieris napi*, *Leptidea reali* and *Araschnia levana* in Avelertal, Trier, Germany, separately for each species: number and portion of marked and recaptured individuals, longest distance moved and maximum residence time

Species	*Marked individuals*	*Recaptured individuals*	*Recapture events*	*Recapture ratio (%)*	*Longest move (m)*	*Maximum residence (days)*
A. cardamines	8	0	0	0	na	na
P. rapae	854	77	117	9.02	1932	24
P. napi	279	15	18	5.38	1193	18
L. reali	63	5	6	7.94	459	14
A. levana	6	0	0	0	na	na

na = not applicable

In the species *Pieris rapae*, one male individual, survived for at least 24 days, and another different individual was recaptured after 20 days. It was not possible to estimate demography and population size for any of the species due to the low recapture frequencies.

3.2.1 Mobility

We obtained no registers on the mobility of the species *Anthocharis cardamines* and *Araschnia levana.* However, the recaptured individuals of the species *Pieris rapae*, *Pieris napi* and *Leptidea reali* presented a high level of mobility. Figure 15 shows the presentation of the mobility of the recaptures individuals of *Pieris rapae*. Most of the individuals moved at least 100 meters or more. The majority of the individuals moved in the range from 100 to 400 metres. The longest observed distance was almost 2 km (Table 7) and was travelled by a male individual.

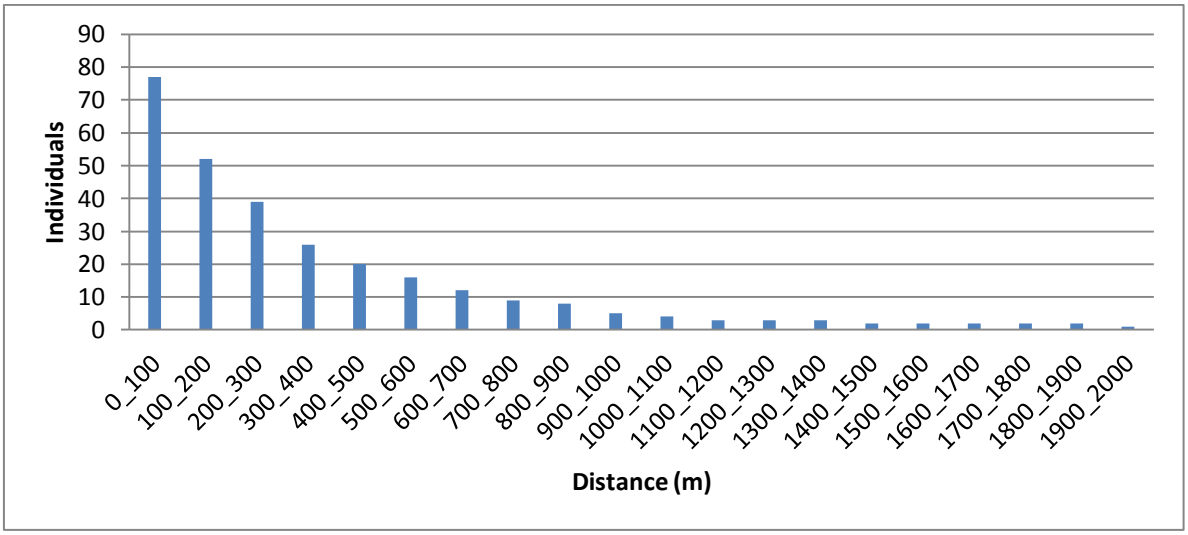


Figure 15 - Number of recaptured individuals of *Pieris rapae* in dependence of the distance moved between capture and following recapture, based on GPS data

According to the Figure 16 for *Pieris napi*, the longest move was from 1193 metres travelled by one individual, and about half of the individuals moved more than 200 metres between recapture events.

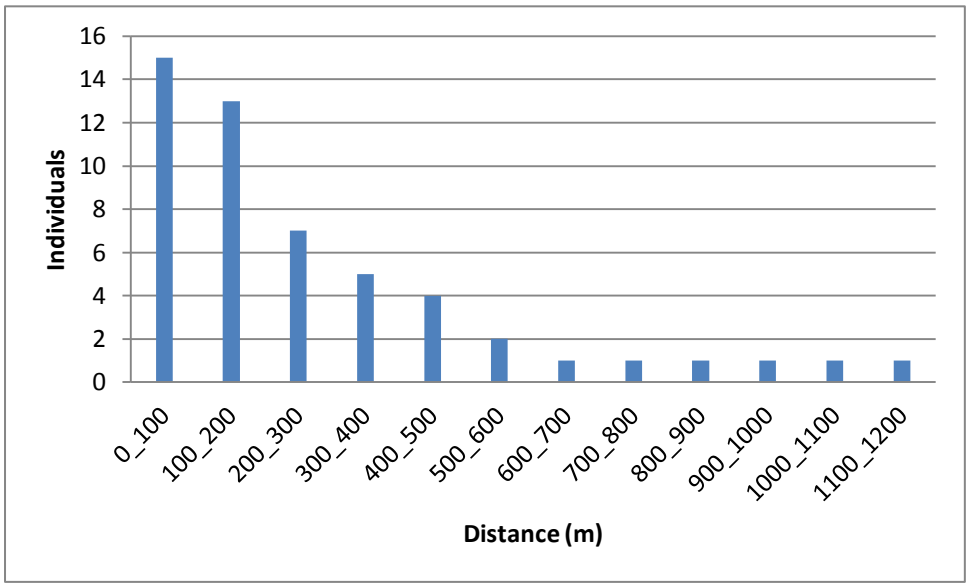


Figure 16 - Number of recaptured individuals of *Pieris napi* in dependence of the distance moved between capture and following recapture, based on GPS data

As seen from Figure 17, *Leptidea reali* apparently moved less than the two other species. All of the recapture individuals did not move for more than 500 metres, and one of the recaptured female only moved 45 meters. Due to the low recapture frequency the comparison against the two other species is problematic and we cannot say that *Leptidea reali* is per se less mobile than the two other species as no statistical test can be applied.

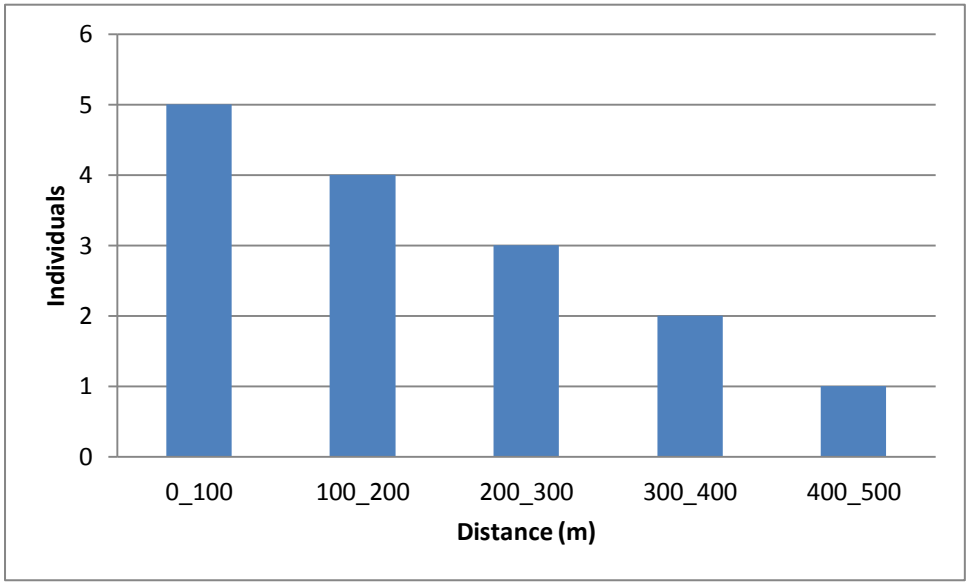


Figure 17 - Number of recaptured individuals of *Leptidea reali* in dependence of the distance moved between capture and following recapture, based on GPS data

The results of fitting the inverse cumulative portions values of the individuals moving a certain distance classes to the negative exponential function (NEF) and the inverse power function (IPF) based on 50 meters and 100 meters intervals are shown in Table 8 and in Table 9 respectively.

Table 8. Results of the fitting of the inverse cumulative proportions of individuals moving certain distance classes to the negative exponential function (NEF) and the inverse power function (IPF): formulas, stability indices and F-statistic for the species *Pieris napi* and *Pieris rapae*. The estimated proportion of the individuals (I) is calculated for distance classes (D) of 50 m intervals

Species	*Funtion*	*Formula*	*Stability index (R^2)*	*F-statistic for fitting*
Pieris napi	NEF	$I = 15.81\ (\pm 1.935)\ e^{-0.157\ (\pm 0.010)\ D}$	0.93	F=260.619 df=19; p<0.0001
	IPF	$I = 18.549\ (\pm 2.481)\ D^{-1.352\ (\pm 0.684)}$	0.75	F=55.917 df=19; p<0.0001
Pieris rapae	NEF	$I = 49.603(\pm 5.007)\ e^{-0.100(\pm 0.004\ )D}$	0.94	F=527.89 df=37; p<0.0001
	IPF	$I = 97.441(\pm 7.381)D^{-3.202(\pm 1.504)}$	0.83	F=174.26 df=37; p<0.0001

The fit of most of all curves was high significant for both distance classes of 50 and 100 metres, respectively (Table 8 and Table 9).

Table 9. Results of the fitting of the inverse cumulative proportions of individuals moving certain distance classes to the negative exponential function (NEF) and the inverse power function (IPF): formulas, stability indices and F-statistic for the species *Pieris napi*, *Pieris rapae* and *Leptidea reali*. The estimated proportion of the individuals (I) is calculated for distance classes (D) of 100m intervals

Species	*Funtion*	*Formula*	*Stability index (R^2)*	*F-statistic for fitting*
Lepidea reali	NEF	$I = 8.4242\ (\pm 1.488)\ e^{-0.391\ (\pm\ 0.053)\ D}$	0.95	F=13.057 df=3; p<0.036
	IPF	$I = 4.395\ (\pm 1.216)\ D^{-0.993\ (\pm 0.658)}$	0.81	F=53.953 df=3; p<0.005
Pieris napi	NEF	$I = 15.08\ (\pm 3.890)\ e^{-0.278\ (\pm 0.035)\ D}$	0.86	F=62.728 df=10; p<0.0001
	IPF	$I = 17.604\ (\pm 2.061)\ D^{-2.19\ (\pm 0.744)}$	0.88	F=72.981 df=10; p<0.0001
Pieris rapae	NEF	$I = 58.658\ (\pm 8.156)\ e^{-0.209\ (\pm 0.012)\ D}$	0.95	F=324.819 df=18; p<0.001
	IPF	$I = 87.77\ (\pm 4.880)\ D^{-1.388\ (\pm 1.379)}$	0.94	F=323.402 df=18; p<0.001

Comparing the R^2 values in Table 8 and Table 9 suggests better fit of the NEF than the IPF. Following the NEF function for distance classes of 50 metres, the estimate proportion of individuals, for the species *Pieris napi* and *Pieris rapae,* moving different distances are showed in Table 10.

Table 10. The results of individuals and the estimate portion of individuals following the NEF formula for distance classes of 50 meter, for the species *Pieris napi* and *Pieris rapae*

Species	Distance (m)	Individuals	% of Individuals
Pieris napi	500	7.21	48.067
	1000	3.289	21.927
	2000	0.684	4.562
	5000	6.161×10^{-3}	0.041
	10000	2.401×10^{-6}	1.601×10^{-5}
Pieris rapae	500	30.08	39.07
	1000	18.247	23.698
	2000	6.713	8.718
	5000	0.334	0.434
	10000	2.251×10^{-3}	2.924×10^{-3}

For distance classes of 100 meters, for the species *Leptidea reali, Pieris napi* and *Pieris rapae* and following the NEF function, the number of individuals and the estimate proportion of individuals moving different distances are showed in Table 11.

Table 11. The results of individuals and the estimate portion of individuals following the NEF formula for distance classes of 100 meter, for the species *Leptidea reali*, *Pieris napi* and *Pieris rapae*

Species	Distance (m)	Individuals	% of Individuals
Lepidea reali	500	1.193	23.86
	1000	0.169	3.38
	2000	3.383×10^{-3}	0.0676
	5000	2.723×10^{-8}	5.446×10^{-7}
	10000	8.806×10^{-17}	1.761×10^{-15}
Pieris napi	500	3.756	25.04
	1000	0.935	6.24
	2000	0.058	0.387
	5000	1.385×10^{-5}	9.239×10^{-5}
	10000	1.274×10^{-11}	8.490×10^{-11}
Pieris rapae	500	20.629	26.79
	1000	7.255	9.42
	2000	0.438	0.569
	5000	1.698×10^{-3}	2.205×10^{-3}
	10000	4.915×10^{-8}	6.384×10^{-8}

Analysing the NEF formulas for each species comparing the two distance classes, we can affirm that *Pieris napi* showed a better fit for 50 m (R^2= 0.93) than for 100 meters (R^2= 0.86). For the species *Pieris rapae*, the distance classes does not show a difference for the NEF function, and it represents a really good fit in both cases R^2= 0.94, R^2= 0.95, respectively for 50 m and 100 m.

3.2.2 Behaviour

Focusing on the behaviour of the five different species, we can say that “flying” was the most frequently registered behaviour before capture for almost all species (Figure 18). With the number of captured individuals for each species, we can affirm that *Anthocharis cardamines* showed a value of 87.5% of flying individuals, in *Araschnia levana*, the value is about 50.0%. *Leptidea reali* presented a value of 92.8%, *Pieris rapae* of 82.5% and for *Pieris napi* the value of individuals with this type of behaviour is 80.5%. The rarest observed behaviour was “mating” (0.21%) and “heating” (0.21%) and was observed in only one species, *Pieris rapae*. The types of behaviour “fighting” and “feeding” were more frequently observed in three of the species, *Pieris rapae, Pieris napi* and *Leptidea reali.* The behaviour type “resting” was observed in all five species.

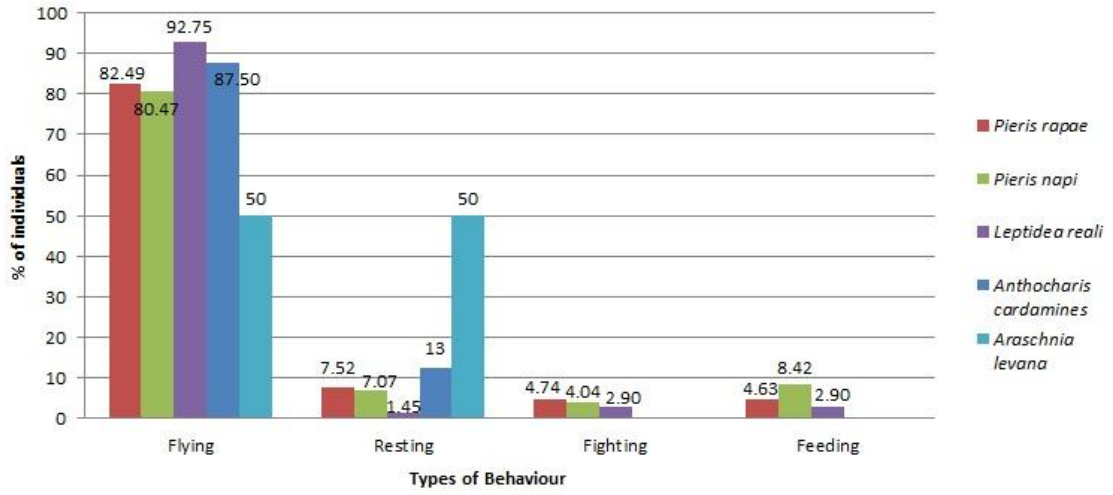


Figure 18 - Percentage of the individuals and their observed behaviour in our mark-release-recapture study for the species *Pieris rapae* (n=854), *Pieris napi* (n=297), *Leptidea reali* (n=69), *Anthocharis cardamines* (n=8) and *Araschnia levana* (n=6)

The degree of wing damage did not show increase for the species *Anthocharis cardamines, Leptidea reali* and *Araschnia levana*, but increased over the flying season for the species *Pieris rapae* and *Pieris napi*.

The part of the transect with the most recapture events was vineyard 2 with 89 events, the wineyard 1 plus the meadow 1 had 27 events, meadow 2a and 3 had 8 events, meadow 2b with 7 events and in meadow 4 was only observed 2 events. On the meadow 5 no recapture event was observed.

With the results of the recaptured individuals for the species *Pieris rapae,* we can analysed more in detail the mobility by days. If we take into account the seven individuals recaptured at least four times or more (Figure 19), we can show that there is no specific pattern involving the movements of the butterflies. For example, in map A of the Figure 19, the distance travelled between 24.06 and 28.06 was 169 m, from 28.06 to 30.06 just 31 m, from 30.05 to 05.07 only 6 m and from 05.07 to 07.07 again 62 m. So, the distance is not influenced by the recapture day. It showed that butterflies can move large distances in a short period of time, or they can just stay in the same place for days.

This type of analysis was not able to be performed for the other four species, because most of the individuals were only recaptured one, two or three times.

Figure 19 - Maps of movements between recapture days for some recaptured individuals of *Pieris rapae*. Each map corresponds to one individual that was recaptured at least four times or more during our study period

3.2.3 Temperature Effects on recapture events

Along our 4 km transect, we exposed three temperature data loggers, Avel 1, Avel 2 and Avel 3 to measure the differences in the temperature along the transect. Hereby, we can correlate temperature to recapture events. In the ANNEXE II - Figure II. 1, Figure II. 2 and Figure II. 3, we can see the three graphs with the variation of the temperatures along the moths May, June, July, August and September of the year 2012.

The first logger, Avel 1, was located in the first part of the transect, vineyard 1 and meadow 1. The values registered were lower compared to the same date in the last years (AccuWeather.com). In the third week of May there is a large drop of the temperature values. Minimum values reached down to nearly -1°C, and maximum were only around 12°C to 14°C. This light frost was the lowest temperate registered for the entire period of our study. In June and July, the temperatures were stable, but still too low if compared with the same period of time of the last years. In the third week of August, we observed a value of 36°C, being the maximum temperature measured by Avel 1. Until the end of August/September, the temperature showed a minimum of 4°C and a maximum of almost 30°C.

The second logger, Avel 2, was located in the central part of the transect, between vineyard 2 and meadow 3. The values registered were similar to the ones registered with Avel 1. The large drop in the values of the temperature also occurred for this data logger, but the temperature did not drop below zero. Between the months June and July, we observe a case of 8°C difference between the values of maximum and minimum observed temperature. In the middle of June, we observed a larger difference between the values of maximum (22°C) and minimum (8°C) showing a difference of 14°C. The maximum temperature, 32°C, was also observed in the middle of August.

The third and last logger, was located in the upper part of the transect, meadow 5 (Figure 20). The same patterns of temperatures were observed for this data logger, the main difference was the minimum and maximum values of temperatures. In June, a large drop of temperature was observed, but only reaching 0°C, and the maximum temperature was about 34°C registered in the middle of August.

All of the temperature data loggers were located with the same shadow/sun cover and all of them were north facing so that the sun never was directly shining on them.

Therefore, the variation of temperatures along the study period is higher the Avel 1 with a maximum observed value of 36°C and a minimum temperature of almost -1°C. Avel 3 showed a maximum value of 34% and a minimum of 0°C, and Avel 2 had a maximum value of 32°C and a minimum of 1 °C. The number of captured butterflies grows as the

temperatures gets higher, but particularly high temperatures, as 36°C, did not infer to a larger amount of captured individuals.

Figure 20 - Temperature data logger, Avel 3, located in the last part of the transect, meadow 5

3.2.4 Effects of vegetation and management

The changes in the vegetation were monitored along the transect. During the mark-release-recapture time, some severe changes occurred in the transect that may have caused some influence on the number of observed and recaptured individuals. The transect main habitats are vineyards and grasslands. In ANNEXE II - Table II. 1, the most common plants in the different sections of the transect are given (determined using Fitter et al., 1986; Rothmaler et al., 1988). Although there were no applications of pesticides in the vineyards, the constantly cutting off the lower vegetation between the vineyards (mostly composed of Gramineae species) showed an effect on the number of observed individuals. In the meadows, the cutting frequency was moderate. Every four weeks, the vegetation was cut off in the meadow, where there was a high number of mostly common herbaceous plants, a lot of which used by the individuals as food source and/or resting spot. After such events, almost no individuals were observed flying in the next days for all species. Not so frequent, but also representing a strong effect on the number of observed butterflies, was pasture with intensive grazing. Occasionally, a large number of sheep was feed on the meadows of the transect, most of their time spending on the meadow 1, 2a and 2b (Figure 21). After this event, no butterflies were observed flying throw that area for at least five or even more days.

Figure 21 - Sheep grazing observed at the meadow 2b, August 14th while waking the transect for the mark-release-recapture study

Meadow 3 was the only one not suffering for any anthropogenic factors. The meadows 4 and 5 (Figure 22) were suffering for any anthropogenic factors constantly.

Figure 22 - Differences observed at meadow 5. In A we see the meadow in 07.07.2012 coved by different flowering plants. In B we see the same meadow in 14.08.2012, ten days after being cut

4. Discussion

According to McNeely et al. (1990) genetic diversity, besides species and ecosystem diversity, is one of the criteria considered worthy of protection by the IUCN. It is expected that genetic diversity contributes to species survival leading to an improved reproductive fitness (Frankham, 2005) and therefore conservation studies should not ignore genetic components (Finger et al., 2009). Actually, genetics are capable to detect population structures, changes in demography on regional and spatial scales, kinship, levels of genetic variability and differentiation assisting enormously in evaluating conservation implications (Finger et al., 2009).

For a better understanding of the discussed results, this last part will be presented in two main parts. First we will discuss our results about the microsatellite analysis. The main objective was to analyse the genetic structure and diversity of *Brenthis ino* in sixteen populations, distributed over five different regions. Secondly, we will discuss the results on the mark-release-recaptured study; the main objective was to identify the movement distances and the behaviour types of five different species: *Anthocharis cardamines*, *Pieris rapae*, *Pieris napi*, *Leptidea reali* and *Araschnia levana*. In the end of each part we comment the conservation implications according to the two approaches of mobility analysis.

4.1 Microsatellites

i) Has *Brenthis ino* a significant genetic structure among populations?

The genetic diversity observed for *Brenthis ino* was not differing compared to the last studies on the same species (Molecular Ecology Resources Primer Development et al., 2012). The mean value and the standard error of observed heterozygosity was 0.641±0.013, while the one of the expected heterozygosity was 0.730±0.008, corresponding to the values presented in the mentioned publication on the same species including a much smaller data set (Molecular Ecology Resources Primer Development et al., 2012). Eight of the eleven loci were in Hardy-Weinberg equilibrium, but presence of null alleles is likely for three loci. Significant departure from Hardy-Weinberg equilibrium was found at loci Bi 41, Bi8 and Bi 19, respectively. All pairwise tests for linkage disequilibrium were not significant. No evidence for stutter bands was detected, but the existence of null alleles was suggested at the following loci: Bi 8, Bi 19 and Bi 41 and the

population Ballon d'Alsace presented a large heterozygote deficit leading to several loci showing evidences for null alleles (Bi 8, Bi 19, Bi 24, Bi 39 and Bi 41).

A common cause of heterozygote deficit is amplification failure of certain alleles at a single locus. Null alleles are those that fail to amplify in a PCR, either because the PCR conditions are not ideal or the primer–binding region contains mutations that inhibit binding. As a result, some heterozygotes are genotyped as homozygotes and a few individuals may fail to amplify any alleles (Selkole and Toonen, 2006). Often, the mutations that cause null alleles will only occur in one or a few populations, as observed in our results, so a heterozygote deficit might not be apparent across all populations.

The presence of a large allele dropout effect in our results may be another explanation for the deficit of heterozygotes in our data set. According to Wattier et al. (1998) large allele dropout is a way that alleles can be missed – the longer allele in a heterozygote does not amplify as well as the shorter one and appears too faint to be detected in the genotype scoring process. This occurs because the replication process in the PCR is more efficient for shorter than longer sequences, and so it will be most pronounced when alleles in a heterozygote are very different in size. A solution for this problem can be re-amplifying individuals homozygous for small alleles and increasing their DNA concentration per sample in the sequencer run (Selkole and Toonen, 2006). Hence, the interpretation of the heterozygosity deficit and deviations from Hardy-Weinberg expectations should be interpreted with caution as null alleles might have pervaded our data set, this has also been described for other studies (e.g. Finger et al., 2009). According to Zhang (2004), the suitability of microsatellites in population genetic studies of butterflies is limited due to low cloning efficiency and lacking specificity due to the similarities in the flanking regions important for the primer annealing.

Therefore, this problem is widely known in microsatellite studies, particularly in lepidopterans (Meglécz and Solignac, 1998; Ji and Zhang, 2004; Meglécz et al., 2007; Habel et al., 2008). However, as the presence of null alleles primarily leads to an underestimation of genetic variability with respect to both allelic diversity and heterozygosity, the comparative values of variability found in this study are valid.

Hierarchical analyses of molecular variance (AMOVA) showed that 75% of the variation was within individuals, leading to the high values of expected heterozygosity. The value of 21% in AMOVA represents the variance among individuals, showing that this percentage is important to understand the differentiation among individuals in all populations, most probably heavily being influences by the observed strong heterozygote deficiency. A value of 4% was representing the molecular variance among populations. This low value shows

that the populations were not strongly isolated among each other. However, the genetic differentiation among population belonging to different regions was clearly observed in the pairwise F_{ST} values, where one of the highest F_{ST} values (0.080) was observed between Ballon d'Alsace and Himburg. This high value was consistent with the large geographic distance between these two sites. The population Himburg was the one showing the highest value for the F_{ST} when compared to the others; and the value 0.082 (between Himburg and Lampaden) was the highest value observed. In this context, it is worth noting that Himburg is the only population representing the region of Westerwald, which is located in the North of Rhineland-Palatinate at the right side of the river Rhine.

Misassignments did occur, in particular for the populations Niederkell (17 individuals) and Grimburg (12 individuals), these two close populations belong to the Hunsrück region, and in some populations (Birkenfeld, Morbach1 and Reinsfeld) located in this regions, we observed values of around 10 individuals assembling to other populations. This might indicate a strong gene-flow among the populations of this region. Otherwise, misassignments may occur due to 1) stochastic processes, 2) survival of old genetic structures of more interconnected populations or 3) recent long-distance dispersal (Finger et al., 2009). Of these, stochastic processes might be the most likely one as the number of loci analyzed (eleven) is relatively low. Therefore, we can affirm the existence of a significant genetic structure between the sixteen studied populations, but not if we look at the regional structures like in the Hunsrück where such a structure is largely missing.

ii) If there is a significant genetic structure, how is the genetic differentiation at an interregional level among the five different areas?

To an easier understanding of the geographic localization of the five analysed regions, we described their distribution in the federal state (Figure 23). Hunsrück and Eifel are located in the northern part of Rhineland-Palatinate, they are at the west bank of the river Rhine, while Westerwald is located at the east bank. The region Pfalz is located in the southern part and is continued in the South in the Vosges Moutains (belonging to France), in whose southern part the population Ballon d'Alsace is located.

Figure 23 – Esquematic location of the study population and their regions for *Brenthis ino*. Adapted from Google Earth 2012

The Bayesian structure analysis clearly reflected seven groups (K=7). Ballon d'Alsace, the French population, that is considered as outgroup, assembles alone in one group. Himburg assembles also as an independent group, representing the region Westerwald These two populations are largely prevented from gene-flow with all the other populations analysed and thus represent the genetically most differentiated regions observed, and they are also the most isolated populations in our study. It would have been interesting to analyse more populations in region Westerwald to compare how this region is actually connected or not with all of the other sampled populations.

Lampaden, is another populations that assembles alone in one group. This population is located in the region Hunsrück, although most of the populations within this region are located at the upland of these mountains, Lampaden is located in a valley between Heddert (F_{st}=0.061) and Wawern (F_{st}=0.045), the F_{st} values reflect that it is not genetically close to any of the surrounding populations. Consequently, the genetic make-up in Lampaden should have evolved differently. One possible explanation this population has evolved laterally due to the geographically situation in the valley and the different patterns in geography (within a river valley delimited by mountains) surrounding it. If we look carefully, it seems that Lampaden is sharing some of its gene-pool with Fischbach (F_{st}=0.043) and Birkenfeld (F_{st}=0.040), as the F_{st} values between populations proved, in part, this assertion. External and unknown factors could explain this genetic connectivity among these three populations.

Birkenfeld and Fischbach are from two different regions and showed a low pairwise F_{st} value of 0.023, supporting an important gene-flow between both populations. Birkenfeld is

still part of the Hunsrück region, but the population is located more to the south-east part of this region. These mountains are surrounded by the rivers Mosel, in the North, Nahe in the South and the Rhine in the East. Fischbach is located in the northern part of the Pfalz region. This region, Pfalz, did not assemble as a specific group, but assemble always with specific populations of the Hunsrück mountains. In this case we might be observing the occurrence of isolation by distance. The population Fischbach also has some genetic similarities with the population Morbach 2 supported by low F_{st} value of 0.030.

The population Morbach 2 assembles in a group formed mainly by populations of the Hunsrück region, Morbach 1, Morbach 2, Wawern, Niederkell and Rodalben from Pfalz. Between Rodalben and all of these populations the F_{st} is about 0.027, a low value that could demonstrate the occurrence of some genetic connection between Rodalben and the populations from another region. The Rodalben population also presents a different genetic pattern, but all of the gene-pool is consistent with the gene-pool present in Morbach 2. In a case like this, where both populations are completely distant geographically one from the other, but genetically close, supported by the low value of F_{st}=0.026, one has to assume a large mobility of the individuals, to lead to a large gene flow between these two populations.

The populations Grimburg, Heddert and Reinsfled, from the Hunsrück mountains also presented the same pattern in gene flow. Grimburg and Reinsfeld even had an F_{st} value of zero, and Heddert and Reinsfeld a value of 0.008 demonstrated how genetically close they were. They are located in the eastern part of the mountains and are geographically close, and they show some genetic similarities with Morbach 1 and Morbach 2, from the latter group. Therefore, we can say that there is a large group existing from the Hunsrück mountains with complete genetic homogenity.

The Eifel region clearly represents a group of its own. This group includes the populations Heyroth, Holzmaar and Wascheid, once again the low values of F_{ST} were consistent with the genetic structure observed.

The region of Pfalz did not assemble in one single group. In fact, there is a large genetic differentiation of the two populations (Fischbach and Rodalben) from the Pfalz region. The populations in the Pfalz region were “recently” colonized (Schulte et al., 2007), and around 100 years ago there were no populations in that area and maybe until now they were not able to stabilize an efficient genetic population pattern. According to all of the geographical barriers, we can affirm that the river Mosel is a barrier that influences the dispersion of *Brenthis ino*, and the effects of the river Rhine on the distribution and the variability of the genetic differentiation should be even larger.

iii) Has a system of isolation-by-distance established in this species?

Two systems of isolation-by-distance were testes for our results. The first correlation included all the sixteen populations and the second correlation was restricted to the populations of the Hunsrück mountains, i.e. the populations Birkenfeld, Grimburg, Heddert, Lampaden, Morbach 1 and Morbach 2, Niederkell, Reinsfeld and Wawern.

In our study, we could find a system of isolation-by-distance occurring among all populations. In Figure 12a, we find a highly significant correlation ($p<0.001$), and the r^2 (0.4197) value is high.

Although the r^2 value presented in our study is high, higher r^2 values for isolation-by-distance have also been reported in other studies, and it seems that the phenomenon of isolation-by-distance tend to be more expressed in Lycaenid butterflies (Schmitt et al., 2004; Finger et al., 2009). For example, in the study of Finger et al. (2009) on *Lycaena helle*, the Mantel test was significant for populations from the Eifel and Ardennes ($r^2=0.75$, $p<0.0001$). The second study, from Schmitt et al. (2003) on *Polyommatus icarus*, also a Lycaenid butterfly, showed again that the Mantel test was significant ($r=0.826$, $p<0.05$). Consequently, enhanced gene-flow might be responsible for the establishment and stabilization of a continental isolation-by-distance system (Wright, 1943) that alone explains 68% of the pan-European differentiation seen in this particular study. Results quite similar to those for *P. icarus* were reported for the generalist green-veined white *Pieris napi meridionalis*, for which as much as 64% of the observed genetic structure could be explained by isolation-by-distance (Geiger and Shapiro, 1992). Therefore, the values seen in our study, lead us to infer that about 42% of the genetic differentiation present for *Brenthis ino* is explained by a system of isolation-by-distance at a regional scale, showing that distance is an important factor for the genetic differentiation among populations of this species at this geographical scale.

Analysing the populations of the Hunsrück mountains, no evidence of isolation-by-distance is revealed. The correlation was not significant and the value of r^2 is extremely low ($r^2=2e^{-0.5}$). These values showed that the populations analysed in the Hunsrück mountains combined with an F_{ST} value of 0.01 for this region clearly underlines strong gene-flow all over this mountain area without any remarkable differentiation among populations. Therefore, we can assume a mostly panmictic system for the Hunsrück mountains, and the absence of larger geographic obstacles between these large Hunsrück populations. Consequently, in the Hunsrück mountains, the genetic diversity in the populations without remarkable differentiation among them is most probably maintained by large and stable populations with continuous gene-flow among them.

iv) Do *Brenthis ino* populations from France show a reduced genetic variability?

The French population, Ballon d'Alsace, showed the same genetic variability compared to the other populations of this study. The parameters number of alleles (Na), expected heterozygosity (H_e) and observed heterozygosity (H_o) did not show a reduced variability for this population. However, the gene pool itself is different when compared to the other populations, as explained by the system of isolation-by-distance. Although in some cases, alleles might be lost through genetic drift and significantly reduce genetic variability in isolated populations, as showed for some relict population of the Apollo butterfly *Parnassius apollo* in central Europe (Habel et al., 2009a), it may be possible that the present genetic status of *Brenthis ino* still reflects a more interconnected distribution pattern of the past, as it was not exclusively restricted to these mountain islands (Ramann, 1880; Möbius, 1905; Urbahn and Urbahn, 1939). Consequently, it is difficult to predict tendencies or future developments of fitness for this specific population, as the same can be affirmed for the population Himburg in the Westerwald. The relatively high F_{ST} value (0.080) between these sites indicates a limited exchange rate and strong genetic differentiation. Other butterfly species like *Thymelicus acteon* (Louy et al., 2007), *Speyeria idalia* (Williams et al., 2003) or *Lycaena helle* (Finger et al., 2009) are also confined to isolated local populations, but still show unexpectedly high genetic diversity.

v) Do populations from the Hunsrück mountains represent a major genetic unit without further substructures?

Due to the strong gene-flow that occurs among all of the populations from the Hunsrück, we can affirm that this region is represented by one major genetic unit without further substructures.

Genetic diversity is known to be an indicator for population fitness (Reed and Frankham, 2003). Accordingly, the high genetic diversity might argue for a high fitness of the populations. As this diversity is similar in the other populations too, these also should be similarly fit. Additionally, the lack of an isolation-by-distance system and generally low genetic diversity let us argue that gene-flow is so high up to 50 km that genetic differentiation is mostly outweighted and not explained by geographic distance. Therefore, we even may say, genetically, of one completely homogenised large Hunsrück population. So, we can conclude that there is a large and stable population with sub-populations in the Hunsrück mountains that inhabit different patches. This overall differentiation pattern, observed in this study, has been found using different statistical approaches adding to this

study credibility, even though, it is observed the presence of null alleles, and that the null alleles might have blurring effects in some calculations, as it was seen by Chapuis and Estoup (2006), in particular for F-statistics. The presence of null alleles, seen in our study, is a constant in this type of studies, and it was also observed in similar recent studies as Finger et al., (2009).

vi) What are the conservation consequences of these results?

According to Schmidt (2010), *Brenthis ino* has a vulnerable status in the region of Rheinland-Pfalz. The genetic structure detected for this species in our study area seems to guarantee the genetic long-term survival for most of our sixteen populations. This observation applies best to the populations from the Hunsrück mountains, due to the missing genetic differentiation among them combined with high genetic diversity of the single populations, thus indicating a strong connectivity all over this area.

It is widely accepted that a species-specific level of genetic diversity is necessary for the viability of its populations (Frankham et al., 2002; Hansson and Westerberg, 2002; Reed and Frankham, 2003; Schmitt and Hewitt, 2004) with many examples offered (e.g., Saccheri et al., 1998; Westermeier et al., 1998; Bryantetal., 1999; Madsen et al., 1999; Meagher, 1999; Rowe et al., 1999; Buza et al., 2000; Luijten et al., 2000; Újvári et al., 2002). Therefore, it is necessary to continue the conservation efforts for the habitats present in the Hunsrück mountains for this species.

For the other populations in other study, low population size and population isolation might lead to inbreeding and later, to extinction (Frankham, 2005). Thus, the most immediate goal in our study should be to increase population sizes and to enhance gene-flow between populations of all regions (Finger et al., 2009). Therefore, it is hard to predict the evolution of the genetic make-up for populations in our study, such as Ballon d' Alsace or Himburg. The survival of each single population might completely dependent on the habitat management, and management mistakes might decrease biodiversity without realistic perspectives of rapid recovery by re-colonisation. A strict conservation of all mountain habitats is therefore necessary for this species' survival and the development of new or the restoration of former *Brenthis ino* locations would further safeguard its populations and the ones of other rare and endangered animal and plant species that live together with this butterfly species. As the lowland populations of *Brenthis ino*, the mountain populations show an important ecological role as an indicator organisms of specific habitat structures, therefore, this data are highly relevant for the conservation of

this species, but also for other species linked to the same habitat, or those who face the same conservations issues. These facts indicate that a more careful analysis of the ecological requirements and population genetic studies are necessary for sustainable conservation of general biodiversity.

4.2 Mark Release Recapture

i) Population densities and habitat conditions

Our mark-release-recapture study revealed large population densities for the species *Pieris rapae*, *Pieris napi* and possibly for *Leptidea reali*. The recapture ratios for all species (9.02%; 5.38% and 7.94%, respectively) was low compared to other studies (Junker and Schmitt, 2010), where values of 29.6% and 25.8% were calculated for both male and female individuals of the species *Euphydrias aurinia beckeri.*

For the species A*nthocharis cardamines* and *Araschnia levana,* the number of captured individuals was so low (8 and 6 individuals, respectively) that none of these individual was recaptured and therefore no estimation could be done about their ecology, population density or behaviour.

In vineyard 2, the 89 events observed in recaptured individuals might be explained in part because of abundance of flowering plants, water, and some shadow places for resting in that part of the transect. The temperature data logger Avel 2 indicated not such a large variance, when compared to the other two, between the maximum and minimum values of temperatures observed in the transect. With some high temperatures along the transect during some days of the study period, the records of Avel 2, and the numbers of recaptured individuals at vineyard 2, possibly indicates that butterflies seems to prefer some hided or shadow parts of the transect.

The main reason for the observed low values in the recapture ratio was because these species are generally very mobile so they simply fly away, and butterflies might have patrolled a large area that surrounds the transect. Another reason, although not so strongly observed like the one presented before, could be seen as a consequence from weather conditions, seen by the high temperature fluctuations registered by the temperature data logger placed in the different locations along the transect (see ANNEXE II). This last reason has more meaning for the small values on captured individuals for the species *Araschnia levana* and A*nthocharis cardamines.*

As it is known, adult butterflies are highly mobile and flight is one of the most essential keys for the maintenance of the existence of populations and the formation of new

colonies (Shreeve, 1981). There are several factors that may affect flight activity. Baker (1969), Gilbert and Singer (1975), and Southwood (1962) suggests that mobility is related to the special characteristics of habitat, and Ehrlich and Gilbert (1973) and Keller et al. (1966) have observed that flight activity may be influenced by the learning of the location of resources. Population density can also influence flight according to Dethier and MacArthur (1964) and Shapiro (1970). Consequently, all of these factors can influence the numbers of captures and recaptures of the study species.

Another possible explanation for the low values in recaptures and captures in some particularly species may be the habitat surrounding of our study area. The continuous presence of anthropogenic factors, such as human activity, as by working with vineyards, or working along the small stream, or even by cut off the meadows, or the grazing done by sheep, in almost all the parts of our transect, may have some influence on such low values.

The example of excessive grazing of the meadows can cause major problems for butterflies, because many species lay their eggs on herbaceous plants that grow around the base of bushes. If they laid their eggs on plants in open areas away from bushes, the resulting caterpillars would perish - either through desiccation in bright sunshine, or because sheep would eat the plants on which they are. Therefore, grassland butterflies will succeed best, both in diversity and abundance, at locations where a well-considered grazing or cutting with a controlled regime will be able to produce a mosaic habitat in which simple meadows, short herbaceous plants and natural vegetation, on which they are, among other, essential elements for the butterfly's survival.

ii) Large Mobility

a) Prediction of long distance movements: NEF versus IPF

We applied the classification of species as sedentary, intermediate or mobile according to Pollard and Yates (1993). This classification has been used successfully in previous studies (e.g., Thomas, 2000). According to Öckinger et al. (2006), the species *P. rapae* is described as mobile and *P. napi* is described by having an intermediate mobility and *Leptidea sinapis*, a closely related species to *Leptidea reali*, is also described as intermediate. Our predictions for NEF and IPF showed that those three species have a mobile behaviour.

According to Junker and Schmitt (2010), the estimation of long distance movements is one central goal in conservation biology. These movement events are of high importance to build functional metapopulation structures and to ensure long-term survival in a

fragmented landscape (Hanski, 1998; Schtickzelle and Baguette, 2003; Baguette and Mennechez, 2004). However, there are no formations of metapopulations of the five study species mentioned in our MRR study. Nonetheless, the effective dispersal behaviour of a species is difficult to determine and may also depend on the habitat quality as well as the spatial scale of the respective study area (Schneider, 2003). Therefore, using predefined models like the negative exponential function (NEF) and the inverse power function (IPF) only yield approximations to predict the probability of long distance movements. In this context, some authors suggest that the IPF in general provides better estimates of the real dispersal capability of a species than the NEF, which may underestimate the probability of long distance movements (Baguette, 2003; Zimmermann et al., 2005). However, the results of our analyses (R^2 values of calculations based on 50 or 100 m intervals) give evidence that the NEF model more accurately describes the movement behaviour of *Pieris rapae, Pieris napi* and *Leptidea reali* in our study site. In one particular case, general predictions for the probability of long–distance movements of *Pieris rapae*, we can argue that about three (2.9) of 100.000 individuals of this species will be able to travel at least a distance of 10 km (see Table 10). Only for *Pieris napi* applying the 100 m distance classes, IPF showed a better fit instead of NEF. Even though, taking into account the NEF formula, we can argue that at least four of 10.000 individuals of *P. napi* will be able to travel a distance of 5 km or more (see Table 10). Our data analyses show the same line of movement for the species *Leptidea reali*. According to the NEF formula, at least six of 10,000 individuals of this species will be able to travel at least a distance of 2 km (Table 11).

b) Emigration versus Dismigration

According to Back et al. (1991), all members of Deutsche Forschungzentrale für Schmetterlingswanderungen - DFZS (1964) presented a list for the classifications of butterflies. In this list, they included *P. rapae* and *P. napi* in the group of “Emigrants”. This group is explained as species that normally migrate within their area of occurrence and do not return to the original areas from which they came. The same authors refers that the potential for migration is present and can in certain populations be induced by external factors. It is more likely that high population density is the decisive factor which causes migration to occur. However, migration is not a prerequisite for the maintenance of their populations because all emigrants are in a position to exist in their birth place at any particular developmental stage. Furthermore, their behaviour in that migration is neither yearly nor periodic.

Our results on the recaptured individuals form the species *P. rapae* and *P. napi* showed a different type of behaviour, being close to the group that Back et al. (1991) described as dismigrants. Dismigrants are those species which are suspected of being migratory, also known as area expanders. This movements leads to a population fluctuations, and hence the population spreads. Their behaviour is described to tend to be of an irregular nature. The breeding areas are left due to various (unknown) factors, and another area is reached without any particular aim. This second type of behaviour, known as dismigrantion, is confirmed for our recaptured species*, Lepidea reali*, *P. rapae* and *P. napi* by the estimations on the values of the NEF and IPF functions.

In accordance with the perdition values of NEF and IPF in our MRR study, we had showed that this species of butterflies could fly long distances; although some studies described this method as not suitable to observe such results. Studies made by Roer (1962) and Knight et al. (1999) argue that direct methods, such as MRR, are poorly suited for detecting long-distance dispersers. Studying successive generations, like our study species *Araschnia levana,* requires more time than studying monovoltine species, as A*nthocharis cardamines.*

Back et al. (1991) even distinguished two forms of migration: the active and the passive migration. The main differences between both are that on the one hand, passive migration is understood as the portion of butterflies or even other insects that moved with the aid of the air. Usually, they do not attempt to resist this movement, even though being able to do so. In this case, the main “force” for passive migration is activated by external factors. On the other hand, active migration is explained as “directed” flight. This flight may be caused by more than one factor (i.e. ecological, climatic or even a factor of genetic nature). In this case of active migration, the target area is prefixed and occasionally this means that the butterflies may have to fly against the wind. These facts lead us to infer that for the three species with obtained recaptures, *L. reali*, .*P rape* and *P. napi*, showed a migration pattern that has to be included in the active forms.

In our results, the largest moved distance was registered for the species *P. rapae*, almost 2 km in our almost 4 km transect. In other studies, this species is described as exploiting rich supplies of their host plants at crop fields in summer (Fric et al., 2006). This species has also been described to behave as classical migrants in parts of their range (Courtney, 1986), and their migrations tend to occur in summer (Asher et al., 2001). The species *Pieris napi* and *Leptidea reali* tend to show the same mobility as seen for *Pieris rapae*. This leads us to affirm that they can be patrolling the entire area of the transect searching for food, water, matting partner and the host plant for laying eggs.

According to A. Shapiro (personal communication seen in Fric et al., 2006), spring individuals of *P. rapae* avoid wind-exposed locations (which might contribute to lower dispersal), whereas summer individuals utilize ascending thermal currents (consistent with a higher wing area and higher dispersal). Certainly, the existence of this pattern does not prove a connection with dispersal, and alternative explanations are possible (Fric et al., 2006). We have to take into account that a species' mobility is not a static trait, but a multiple trait, the components of which may evolve rather rapidly in interaction with the actual spread of resources and other meaningful landscape features (Clobert et al., 2004).

c) Conservation implications

According to Schmidt (2010), the five species in our study area have a LC status in the region of Rheinland-Pfalz. Although this status described for our study species, conservative implications should not be forgotten. The type of habitats in this MRR study is also a habitat for other living beings. Large changes in habitats should be avoided and the species should be in a regular butterfly monitoring program for collecting more data to define carefully the mobility observed of these five species.

Change of land use, which has serious consequences for the conservation of Lepidoptera and other wildlife, and consequent loss of habitats, has led to major declines of Lepidoptera in all European countries (Warren et al., 1993). In Europe, the main threats reported are from agricultural improvements, which affect 90% of the threatened species, building developments (affecting 83%), increasing use of herbicides and pesticides (affecting 80%), and abandonment of agricultural land and changing habitat management (65%). The widespread loss and reduction in size of breeding habitats is affecting 83% of threatened species (van Swaay and Warren, 1999).

A regular monitoring program at sites surrounding the transect can be representative of several different habitat types, such as the programs in Great Britain (Pollard and Yates, 1993) and The Netherlands (van Swaay et al., 1997) could give indications of general, large-scale population trends.

5. Conclusions

In conclusion, we can affirm that a significant genetic structure exists among the populations of *Brenthis ino* analysed in this study. The system of isolation-by-distance, present over all sixteen analyzed populations, might explain part of that differentiation. The French population Ballon d'Alsace and the population Himburg from Westerwald are geographically the most isolated ones, a fact also underlined by their genetic distinctness. Therefore, both show a low gene-flow with the other populations, but not a reduced genetic variability. The Eifel region was clearly identified by the specific genetic pattern observed in the bar plot, and also the large group of the Hunsrück mountains present a diverse genetic group. This group included the populations Lampaden, Grimburg, Heddert, Morbach 1, Morbach 2, Niederkell, Reinsfeld and Wawern. While the population Lampaden was the genetically most distinguished one in this geographical region, Grimburg, Heddert and Reinsfeld showed very close genetic proximity, followed by Niederkell, Morbach 1, Morbach 2, and last Wawern. Probably, gene-flow occurs more strongly among the populations Grimburg, Heddert, Morbach 1, Morbach 2, Reinsfeld, and, maybe, even Rodalben. The population Rodalben was probably the last one to be colonized. If so, we can suggest that in our system the recent colonization (over the past 100 years or so) was made from the North to the South. Thus, the conservation effort may take the genetic differentiation into account, and the mountain habitats of this butterfly should be preserved.

With our MRR, we could conclude that the species *Pieris rapae*, *Pieris napi* and *Leptidea reali* show a large range of mobility, explained by the estimation calculations for the NEF and the IPF functions, and possibly also have a large population density due to the low values of the recapture ratio. The recapture event of the species *P. rapae* indicated that they can patrol the entire area of the transect searching for food, water, mating partner and the host plant for egg laying.

Both of the conclusions on the two analytical methods demonstrate the great importance of population genetics and behavioural studies in conservation ecology.

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ANNEXES

ANNEXE I – Microsatellites analysis for *Brenthis ino*

Table I. 1. Five parameters of genetic diversity for all populations and loci for *Brenthis ino*. Allelic richness (AR), Number of alleles (Na), expected Heterozygosity (H_e), observed Heterozygosity (H_o) and Fixation index (F)

Pop		*Bi3*	*Bi8*	*Bi19*	*Bi24*	*Bi29*	*Bi33*	*Bi36*	*Bi38*	*Bi39*	*Bi41*	*Bi44*
Ballon d'Alsace	**AR**	8.30	9.92	8.71	5.96	6.00	7.15	4.96	4.00	5.72	7.48	5.88
	Na	9	11	10	6	6	8	5	4	6.	9	6
	H_e	0.82	0.82	0.72	0.80	0.80	0.73	0.67	0.61	0.69	0.71	0.76
	H_o	0.88	0.51	0.30	0.63	0.85	0.70	0.72	0.68	0.50	0.50	0.85
	F	-0.06	0.38	0.58	0.22	-0.06	0.04	-0.08	-0.13	0.27	0.29	-0.12
Birkenfeld	**AR**	9.28	15.72	15.98	4.53	5.83	5.67	7.62	3.96	5.41	13.27	10.05
	Na	11	21	19	5	7	6	8	4	6	13	8
	H_e	0.83	0.89	0.82	0.56	0.66	0.74	0.70	0.40	0.69	0.88	0.59
	H_o	0.80	0.30	0.73	0.55	0.60	0.54	0.53	0.35	0.43	0.33	0.18
	F	0.04	0.66	0.11	0.02	0.10	0.27	0.25	0.12	0.39	0.62	0.70
Fischbach	**AR**	9.15	13.95	16.65	6.67	3.65	7.55	6.38	4.00	4.61	10.51	13.59
	Na	11	16	20	8	4	8	7	4	5	18	6
	H_e	0.83	0.86	0.80	0.82	0.60	0.79	0.66	0.57	0.68	0.84	0.67
	H_o	0.80	0.28	0.59	0.72	0.43	0.44	0.49	0.58	0.48	0.36	0.18
	F	0.04	0.68	0.26	0.12	0.29	0.45	0.26	-0.02	0.30	0.57	0.74
Grimburg	**AR**	8.62	10.54	10.29	5.62	5.64	5.64	5.49	4.00	6.60	9.69	8.00
	Na	9	11	12	6	6	6	6	4	7	11	9
	H_e	0.85	0.83	0.59	0.68	0.72	0.69	0.63	0.57	0.73	0.78	0.69
	H_o	0.83	0.46	0.41	0.75	0.83	0.60	0.55	0.63	0.60	0.57	0.45
	F	0.03	0.45	0.30	-0.11	-0.15	0.13	0.13	-0.09	0.18	0.27	0.35
Heddert	**AR**	8.84	10.55	9.79	6.59	5.92	6.26	5.26	3.99	5.95	7.67	7.06
	Na	9	11	11	7	6	7	6	4	6	9	8
	H_e	0.86	0.87	0.61	0.72	0.76	0.71	0.61	0.50	0.75	0.73	0.77
	H_o	0.88	0.63	0.44	0.83	0.88	0.78	0.65	0.55	0.65	0.73	0.65
	F	-0.02	0.28	0.29	-0.15	-0.16	-0.08	-0.06	-0.10	0.13	0.01	0.15
Heyroth	**AR**	7.64	14.58	16.48	7.62	5.64	4.65	4.56	3.97	6.85	11.70	10.82
	Na	12	16	21	10	6	5	5	4	7	17	7.
	H_e	0.80	0.87	0.82	0.83	0.75	0.63	0.54	0.58	0.77	0.79	0.79
	H_o	0.73	0.08	0.82	0.83	0.55	0.50	0.32	0.64	0.49	0.33	0.63
	F	0.10	0.91	0.01	0.00	0.26	0.20	0.41	-0.11	0.37	0.59	0.21
Himburg	**AR**	7.62	10.08	17.55	5.53	5.90	5.30	6.82	3.00	6.96	10.33	8.63
	Na	8	11	21	6	6	6	7	3	7	12	9
	H_e	0.63	0.81	0.90	0.70	0.76	0.65	0.71	0.60	0.82	0.64	0.83
	H_o	0.75	0.38	0.85	0.73	0.76	0.59	0.74	0.63	0.78	0.53	0.85
	F	-0.19	0.54	0.06	-0.03	0.00	0.10	-0.05	-0.04	0.05	0.18	-0.01
Holzmaar	**AR**	6.99	16.41	15.15	5.89	6.00	6.93	6.85	4.96	6.92	13.00	14.84
	Na	7	14	16	6	6	7	7	5	7	12	7
	H_o	0.76	0.29	0.76	0.86	0.82	0.39	0.50	0.26	0.36	0.19	0.21
	H_e	0.80	0.85	0.83	0.73	0.77	0.68	0.66	0.35	0.75	0.82	0.77
	F	0.05	0.67	0.09	-0.19	-0.07	0.43	0.25	0.26	0.52	0.77	0.73
Lampaden	**AR**	11.88	16.27	15.25	5.80	5.87	7.95	7.15	3.98	5.81	15.82	10.75
	Na	14	18	18	6	6	9	6	4	6	15	10
	H_e	0.86	0.88	0.80	0.71	0.71	0.77	0.75	0.38	0.75	0.88	0.79
	H_o	0.85	0.49	0.68	0.68	0.76	0.79	0.59	0.39	0.53	0.42	0.84
	F	0.01	0.45	0.14	0.03	-0.08	-0.03	0.20	-0.04	0.30	0.52	-0.07
Morbach 1	**AR**	10.17	11.07	6.28	4.87	6.94	5.86	4.60	3.88	6.60	11.55	6.87
	Na	11	13	7	5	7	6	5	4	7	13	8

	H_e	0.85	0.81	0.59	0.63	0.78	0.58	0.59	0.48	0.72	0.83	0.52
	H_o	0.78	0.33	0.31	0.68	0.83	0.56	0.58	0.53	0.68	0.51	0.28
	F	0.09	0.59	0.48	-0.07	-0.06	0.03	0.03	-0.08	0.07	0.38	0.47
Morbach 2	**AR**	9.18	14.97	12.01	7.00	5.89	5.67	5.75	4.37	4.64	10.30	14.88
	Na	10	12	14	8	7	6	7	5	6	10	9
	H_e	0.82	0.78	0.71	0.75	0.69	0.72	0.58	0.58	0.71	0.75	0.83
	H_o	0.73	0.23	0.73	0.72	0.72	0.49	0.48	0.58	0.43	0.56	0.54
	F	0.11	0.71	-0.02	0.04	-0.03	0.32	0.18	0.01	0.40	0.25	0.35
Niederkell	**AR**	12.43	13.95	19.49	4.56	6.83	6.89	6.83	4.00	5.94	17.69	10.10
	Na	14	14	27	6	8	10	9	4	7	19	12
	H_e	0.87	0.85	0.82	0.57	0.76	0.76	0.72	0.58	0.66	0.90	0.78
	H_o	0.90	0.45	0.92	0.56	0.75	0.62	0.83	0.63	0.45	0.56	1.00
	F	-0.03	0.47	-0.12	0.01	0.02	0.19	-0.14	-0.08	0.32	0.37	-0.28
Reinsfeld	**AR**	8.95	9.45	7.05	6.26	6.64	5.30	4.68	3.88	4.96	8.02	6.53
	Na	9	10	8	7	7	6	5	4	5	9	7
	H_e	0.85	0.84	0.45	0.70	0.76	0.64	0.58	0.51	0.71	0.79	0.72
	H_o	0.88	0.68	0.29	0.75	0.83	0.73	0.55	0.48	0.58	0.53	0.65
	F	-0.03	0.19	0.36	-0.07	-0.09	-0.13	0.04	0.06	0.18	0.34	0.10
Rodalben	**AR**	10.03	17.97	13.13	5.79	5.75	6.23	5.57	4.63	6.34	11.06	11.40
	Na	13	21	17	8	6	7	6	5	6	11	5
	H_e	0.84	0.90	0.63	0.67	0.63	0.75	0.63	0.68	0.65	0.79	0.55
	H_o	0.88	0.28	0.40	0.58	0.57	0.60	0.56	0.59	0.51	0.38	0.12
	F	-0.05	0.69	0.37	0.13	0.09	0.21	0.11	0.14	0.21	0.52	0.79
Wascheid	**AR**	10.89	16.16	14.29	7.17	5.62	5.62	8.98	3.63	7.19	11.67	10.60
	Na	13	18	21	9	6.	6	10	4	8	14	7
	H_e	0.86	0.86	0.82	0.78	0.76	0.71	0.71	0.55	0.75	0.75	0.73
	H_o	0.83	0.21	0.86	0.67	0.74	0.43	0.40	0.39	0.64	0.29	0.62
	F	0.04	0.75	-0.05	0.14	0.02	0.40	0.43	0.29	0.14	0.62	0.15
Wawern	**AR**	10.43	9.29	9.31	7.16	6.61	7.87	8.34	3.88	4.00	11.97	7.45
	Na	11	10	11	8	7	8	10	4	4	13	8
	H_e	0.86	0.82	0.68	0.76	0.77	0.75	0.67	0.54	0.72	0.87	0.77
	H_o	0.90	0.46	0.75	0.78	0.85	0.46	0.75	0.58	0.58	0.69	0.43
	F	-0.04	0.44	-0.11	-0.02	-0.11	0.39	-0.13	-0.06	0.21	0.20	0.45

Table I. 2. Pairwise FST values (p < 0.001) of all populations analysed of Brenthis ino, provided by GenAlEx. Each number correspond to the same population

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*
Ballon d'Alsace (1)	0.000															
Birkenfeld (2)	0.066	0.000														
Fischbach (3)	0.068	0.023	0.000													
Grimburg (4)	0.044	0.030	0.045	0.000												
Heddert (5)	0.048	0.036	0.051	0.008	0.000											
Heyroth (6)	0.070	0.061	0.039	0.050	0.041	0.000										
Himburg (7)	0.080	0.065	0.068	0.064	0.057	0.064	0.000									
Holzmaar(8)	0.059	0.039	0.034	0.043	0.035	0.022	0.052	0.000								
Lampaden (9)	0.065	0.040	0.043	0.063	0.061	0.059	0.082	0.045	0.000							
Morbach 1 (10)	0.059	0.011	0.038	0.015	0.023	0.052	0.058	0.039	0.059	0.000						
Morbach 2 (11)	0.055	0.029	0.030	0.033	0.035	0.029	0.059	0.035	0.049	0.030	0.000					
Niederkell (12)	0.053	0.018	0.031	0.016	0.025	0.042	0.055	0.032	0.040	0.020	0.020	0.000				
Reinsfeld (13)	0.046	0.023	0.036	0.000	0.008	0.038	0.064	0.036	0.054	0.011	0.016	0.013	0.000			
Rodalben (14)	0.054	0.027	0.037	0.042	0.047	0.058	0.081	0.054	0.059	0.027	0.026	0.027	0.031	0.000		
Wascheid (15)	0.054	0.035	0.043	0.042	0.035	0.017	0.051	0.024	0.048	0.033	0.019	0.037	0.030	0.036	0.000	
Wawern (16)	0.045	0.025	0.029	0.015	0.015	0.034	0.047	0.028	0.045	0.019	0.020	0.016	0.011	0.037	0.030	0.000

ANNEXE II - Mark release recapture for the species *Anthocharis cardamines; Pieris napi; Pieris rapae; Leptidea reali* and *Araschnia levana*

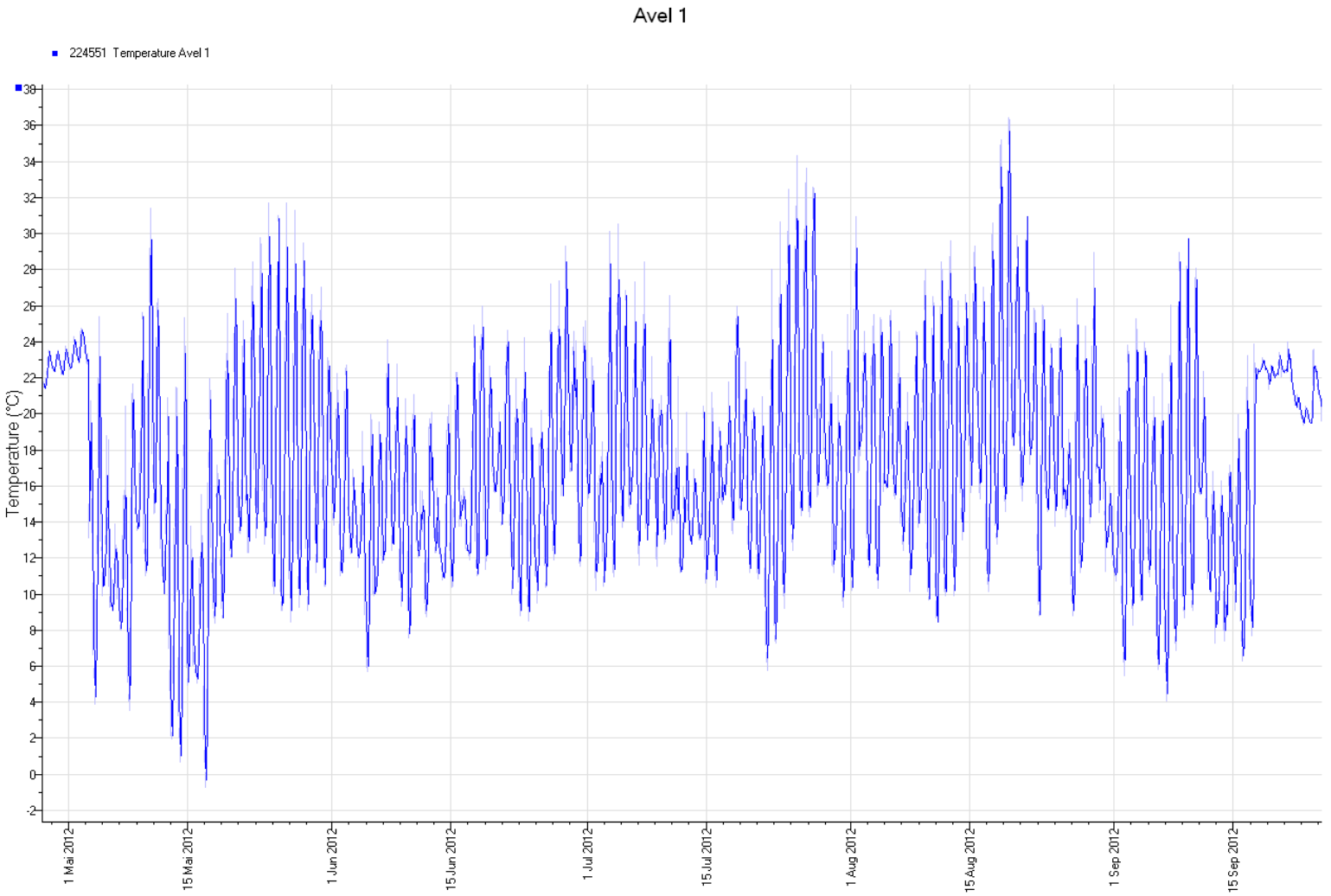

Figure II. 1 – Graphic resulting on the measured temperatures of the data loggers replaced in the beginning of the transect – vineyard 1 and meadow 1

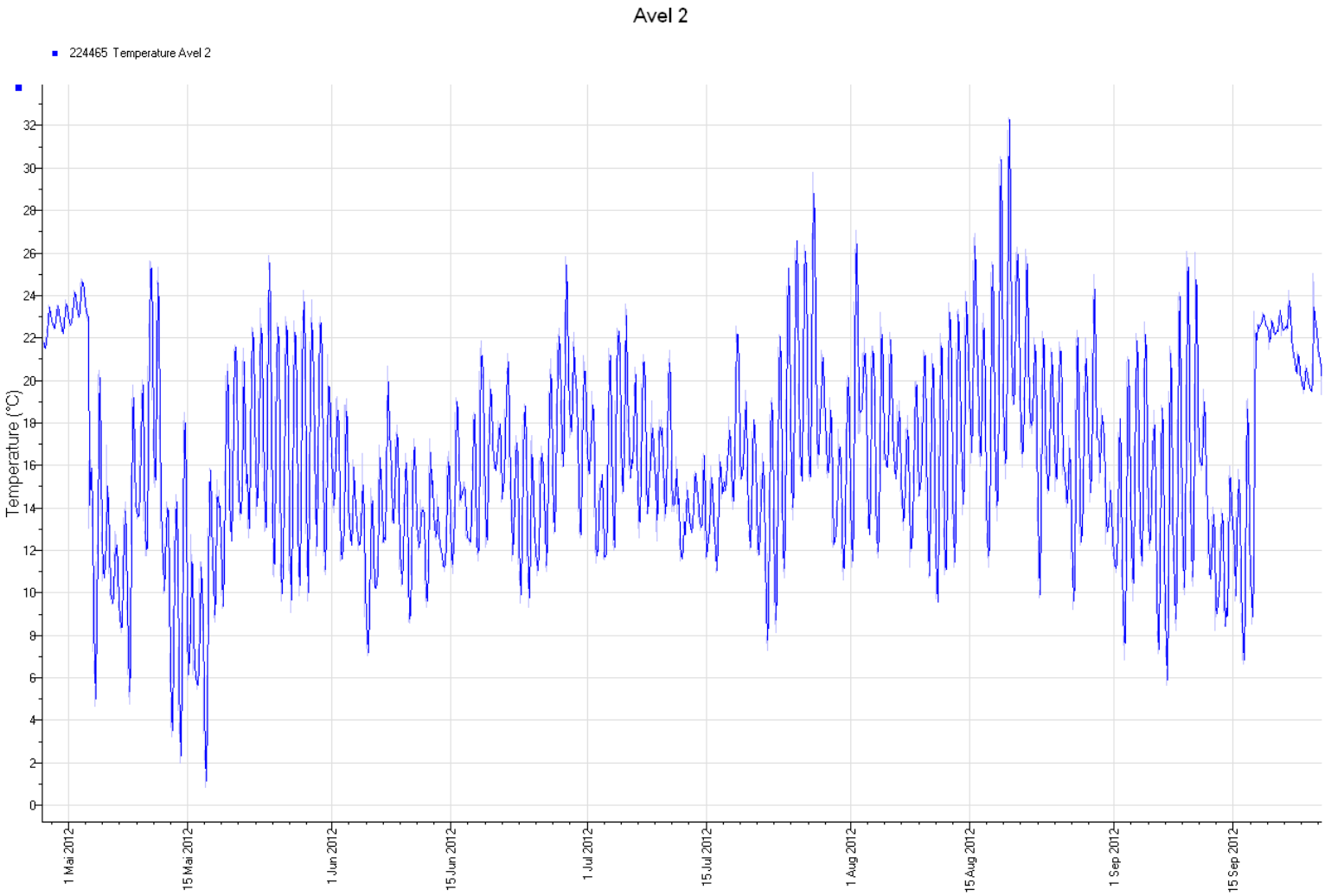

Figure II. 2 – Graphic resulting on the measured temperatures of the data loggers replaced in the middle part of the transect – end of vineyard 2

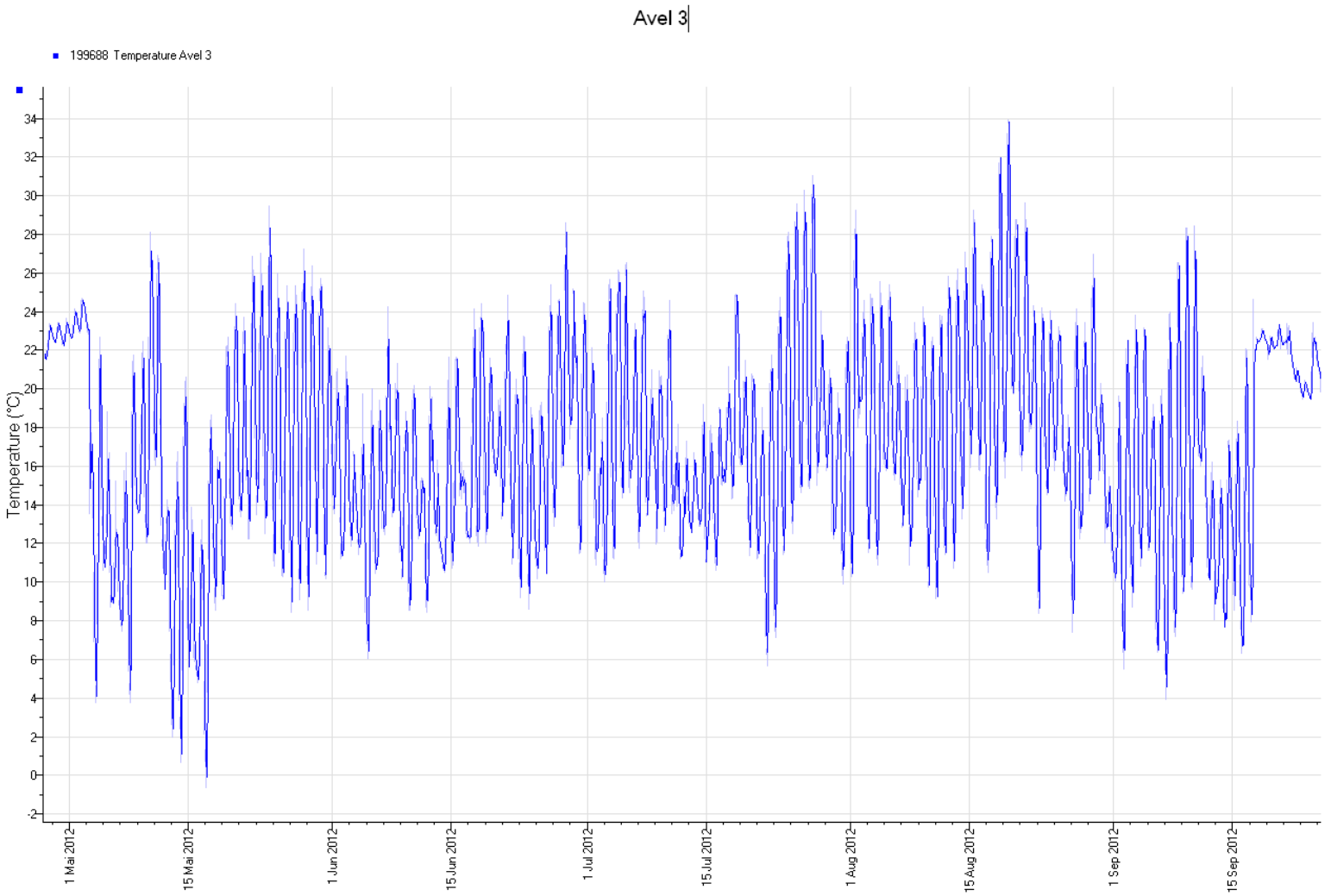

Figure II. 3 – Graphic resulting on the measured temperatures of the data loggers replaced in the last part of the transect – meadow 5

Table II. 1. List of plant species, only the most common and observed along the transect

Location	*Family Species*	*Plant species*
All parts of the transect	Dipsacaceae	*Dipsacus sylvestris*
All parts of the transect	Asteraceae	*Senecio jacobaea*
All parts of the transect	Lamiaceae	*Clinopodium vulgare*
All parts of the transect	Rosaceae	*Rubus sp.*
All parts of the transect	Boraginaceae	*Echium vulgare*
All parts of the transect	Asteraceae	*Centaurea nigra*
All parts of the transect	Asteraceae	*Achillea millefolium*
All parts of the transect	Salicaceae	*Salix* sp.
All parts of the transect	Leguminosae or Fabaceae	*Trifolium pratense*
All parts of the transect	Leguminosae or Fabaceae	*Trifolium repens*
All parts of the transect	Urticaceae	*Urtica dioica*
All parts of the transect	Papaveraceae	*Papaver* sp.
All parts of the transect	Lythraceae	*Lythrum salicaria*
all meadows	Rosaceae	*Potentilla tabernaemontani*
all meadows	Lamiaceae or Labiatae	*Lamium purpureum*
all meadows	Cornaceae	Cornus sanguinea
vineyard 1	Apiaceae	*Daucus carota*
vineyard 1	Convolvulaceae	*Convolvulus arvensis*
vineyard 1	Convolvulaceae	*Convolvulus cneorum*
vineyard 1	Campanulaceae	*Campanula* sp.
vineyard 1, vineyard 2	Vitaceae	*Vitis vinifera*
vineyard 1, vineyard 2	Asteraceae	*Tripleurospermum perforatum*
vineyard 1, vineyard 2	Asteraceae	*Cirsium arvense*
vineyard 1, vineyard 2	Asteraceae	*Cirsium vulgare*
vineyard 1, vineyard 2	Amaranthaceae	*Amaranthus chlorostachys*
vineyard 1, meadow 1	Euphorbiaceae	*Euphorbia peplus*
vineyard 1, meadow 2	Asteraceae or Compositae	*Lapsana communis*
vineyard 1, meadow 3, meadow 4	Fabaceae	*Lotus corniculatus*
meadow 1	Poaceae	*Lolium perenne*

meadow 1	Poaceae	*Deschampsia cespitosa*
meadow 1	Juncaceae	*Juncus effusus*
meadow 1	Poaceae or Gramineae	*Setaria pumila*
meadow 1, meadow 3	Asteraceae	*Hieracium prenanthoides*
meadow 1, meadow 2a, meadow 2b	Rosaceae	*Filipendula ulmaria*
meadow 2a, meadow 2b	Ranunculaceae	*Ranunculus acris*
meadow 2a, meadow 2b, vineyard 2	Fabaceae or Leguminosae	*Robinia pseudoacacia*
meadow 2b	Cupressaceae	*Thuja plicata*
meadow 2b, vineyard 2	Rosaceae	*Crataegus monogyna*
meadow 2b, vineyard 2	Pinaceae	*Picea abies*
vineyard 2	Asteraceae	*Taraxacum officinale*
vineyard 2	Clusiaceae	*Hyperium perforatum*
vineyard 2	Asteraceae	*Erigeron canadensis*
vineyard 2	Asteraceae or Compositae	*Lactua serriola*
vineyard 2	Brassicaceae or Cruciferae	*Bunias orientalis*
vineyard 2	Onagraceae	*Epilobium parviflorum*
vineyard 2	Asteraceae	*Erigeron canadensis*
vineyard 2	Asteraceae	*Lactuca serriola*
vineyard 2	Solanaceae	*Solanum dulcamara*
vineyard 2	Fagaceae	*Fagus sylvatica*
vineyard 2	Aceraceae	*Acer pseudoplatanus*
vineyard 2	Aceraceae	*Acer platanoides*
vineyard 2	Aceraceae	*Acer campestris*
vineyard 2	Fagaceae	*Quercus* sp.
vineyard 2	Fagaceae	*Quercus robur*
vineyard 2	Fagaceae	*Quercus peraea*
vineyard 2	Betulaceae	*Corylus avellana*
vineyard 2	Adoxaceae	*Sambucus nigra*
vineyard 2	Fabaceae or Leguminosae	*Vicia sepium*
Vineyard 2	Asteraceae or Compositae	*Artemisia vulgaris*
vineyard 2	Brassicaceae	*Brassica oleracea*
vineyard 2	Asteraceae	*Solidago canadensis*

vineyard 2	Rosaceae	*Agrimonia eupatoria*
vineyard 2	Dipsacaceae	*Knautia arvensis*
vineyard 2	Asteraceae	*Matricaria maritima*
vineyard 2	Apiaceae	*Pastinaca sativa*
vineyard 2, meadow 3	Asteraceae	*Tanacetum vulgare*
meadow 3	Asteraceae	*Bellis perennis*
meadow 3	Fabaceae or Leguminosae	*Medicago sativa*
meadow 3	Lamiaceae	*Galeopsis tetrahit*
meadow 3	Lamiaceae	*Origanum vulgare*
meadow 3, meadow 4, meadow 5	Oleaceae	*Fraxinus ornus*
meadow 4	Plantaginaceae	*Plantago major*
meadow 4	Polygonaceae	*Polygonum* sp.
meadow 4	Equisetaceae	*Equisetum pratense*
meadow 4, meadow 5	Plantaginaceae	*Plantago lanceolata*
meadow 4, meadow 5	Rosaceae	*Sanguisorba officinalis*
meadow 5	Ranunculaceae	*Ranunculus flanula*