Population genetics of the endangered obligatorily myrmecophilous butterfly Phengaris (=Maculinea) arion in two areas of its European range

Genetic structure of the endangered obligatorily myrmecophilous butterfly *Phengaris (Maculinea) arion* in two remote areas of its European distribution range

Running head: Genetic structure of *Phengaris arion*

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Abstract

1. The socially parasitic *Phengaris arion* is one of the most threatened butterfly species in Europe. Using 12 microsatellite loci, we studied the genetic structure of 14 ecologically diverse populations (285 individuals) originating from two distinct areas of the European distribution range (Poland and Italy).

2. Italian populations were more differentiated (F_{ST}: 0.124) than Polish populations (0.073). However, in contrast to the results of previous studies concerning *COI* (mtDNA) and *EF-1α* (nucDNA) genes, indices of genetic variability were higher in Poland. Within-population genetic variability in Italy decreased southwards, whereas in Poland no similar gradient of changes was detected.

3. Analysis with STRUCTURE suggested that all Polish populations and two of the most northern Italian populations had the highest probabilities of ancestry from the same genetic clade, while the five remaining Italian populations formed a second group. This may indicate a common evolutionary history for populations inhabiting the northern slopes of the

Alps and Central Europe. The alternative hypothesis, concordant with a previous study, is that the alpine populations are localized in a mixed zone of different post glacial colonization routes. When analyses were performed for each country separately, the identified genetic clades predominantly reflected the species' geographical distribution.

4. The pattern of ecological variation did not influence genetic structure and no grounds for separation of subspecies (*P. arion ligurica* and *P. arion obscura*) were found. Our results are therefore in agreement with conclusions inferred from other studies carried out in Europe on *P. arion*, including those using allozymes.

Key words. conservation genetics, habitat fragmentation, Italy, microsatellites, Poland, post-glacial colonization routes

Introduction

The genetic structure of any given species is shaped by many factors, e.g. biogeographic history, dispersal abilities, dietary specialization and availability of potential biotopes within the landscape. Sedentary and local specialists are usually more prone to genetic drift and show a higher level of within-population differentiation than common and abundant generalists. Numerous examples of this general pattern can be found among European butterflies, which are traditionally a relatively well studied group of insects (e.g. Nève, 2009 and references therein; Sigaard *et al*., 2008; Habel *et al*., 2009; Vandewoestijne & Van Dyck, 2010; Sielezniew, *et al*., 2012). Many research activities are focused on species which are endangered on a local or global scale, and therefore of high concern to conservationists. One of the most intensively studied genera are *Phengaris (Maculinea)* butterflies (Lycaenidae), which could be considered as icons of world insect conservation (Thomas & Settele, 2004; Settele *et al*., 2005; Settele & Kühn, 2009). They are a highly demanding group because of their complicated life history, which requires two different types of resources for completion of their metamorphosis. Caterpillars develop initially on specific host plants (depending on species: *Thymus* or *Origanum, Gentiana* and *Sanguisorba*) then continue and complete their development inside the nests of specific red ants (*Myrmica)* as social parasites feeding on the hosts' brood (Thomas, 1995).

According to recent evaluations using IUCN criteria, the most threatened *Phengaris* species (and simultaneously one of the most endangered butterflies in Europe) is the Large Blue *P. arion* (van Swaay *et al*., 2010). Although this butterfly is relatively widespread and

still present in many European countries, it is in continuous decline almost everywhere (Van Swaay & Warren, 1999). The UK is an important exception, but the population is growing there as the result of successful reintroductions following habitat restoration, as well as a conservation management programme (Thomas *et al*., 2009)

P. arion is considered not only as a charismatic conservation target but also as an umbrella for many other species associated with xerothermal grasslands and traditional land use (Spitzer *et al*., 2009; Thomas *et al*., 2009; Casacci *et al.,* 2011). Complicating the conservation of the species is the fact that *P. arion* is relatively diverse as far as biotope use is concerned and shows considerable morphological variation, which is suspected to be an adaptation to local climatic conditions (Sielezniew & Dziekańska, 2011). Moreover, across Europe the butterfly inhabits a variety of xerothermic habitats ranging from lowland forest-steppes (related mainly to pine forests) to alpine meadows (Thomas, 1996; Sielezniew *et al*., 2010a; Casacci *et al*., 2011; Bereczki *et al*., 2011).

The relative rarity of *P. arion* may be to some extent explained by recent data from Poland suggesting that the butterfly, at least in some regions, requires large patches of biotopes since its potential host ants usually occur in low densities (Sielezniew *et al*., 2010a). Moreover, the survival rate of caterpillars during their parasitic phase of development is relatively low. *P. arion* is a less effective social parasite than the "cuckoo" species *P. alcon* (including the controversial taxa *P. 'rebeli'*), and potential host ants of *P. arion* form generally smaller colonies than hosts of *P. teleius* and *P. nausithous,* whose caterpillars are also predacious (Thomas & Elmes, 1998). As a consequence, *P. arion* is rather sparse on many sites (Sielezniew *et al*., 2010a) and probably only the high dispersal distances inferred from genetic data obtained during studies in Sweden (Ugelvig *et al*., 2012) prevent the species from many regional extinctions. Swedish populations simultaneously show a relatively high level of differentiation compared to those studied in Poland (Sielezniew & Rutkowski, 2012). Ugelvig *et al*. (2012) explain this finding by the better connectivity (in terms of suitable biotopes) of Polish populations.

Confirming this interpretation, recent studies by Patricelli *et al*. (2013) with application of markers used mostly in phylogeographical studies (mitochondrial gene *COI* and nuclear gene *EF-1α*), show that Polish populations are also less differentiated than those sampled in Italy (a highly fragmented habitat). In this work it is also suggested that the observed pattern of genetic variation for *P. arion* may be explained by the rear edge hypothesis (Hampe & Petit, 2005). Populations from Italy represent the low-latitude limits of

a species' distribution and this is possibly one reason why they show higher differentiation compared to those occurring in a continuous range, i.e. in Poland.

In the present paper we studied Polish and Italian populations using microsatellites which are neutral, hyper variable and therefore sensitive markers. They are nowadays widely applied in molecular ecology and are especially useful for studies on rare species (Selkoe & Toonen, 2006). However butterfly research is faced with constant problems related to the identification of usable microsatellite loci, probably because of the structure of the Lepidoptera genome (Nève, 2009). Fortunately newly developed techniques seem to at least partially overcome these difficulties, and as far as *P. arion* is concerned the number of available polymorphic loci has recently been increased (Ugelvig *et al*., 2012), making this tool more powerful. We applied these markers to investigate and compare the genetic structure of populations not only inhabiting different parts of the European range but also as related to different biotopes, hence fully representing the ecological and morphological variation of the butterfly across the continent.

Material and Methods

Sample collection - Poland

We used DNA isolated from the legs or thoraxes of specimens previously collected for earlier microsatellite analyses, which were based on a lower number of loci (Rutkowski *et al*., 2009, Sielezniew & Rutkowski, 2012). A total of 144 samples, originating from seven Polish populations, (15 to 26 individuals per population, sampled between 2007 and 2008) were analysed. The distances between the Polish populations ranged from a minimum of 8 km (KLU and SRO) to a maximum of 462 km (SOW and SRO). The localities can be divided into two types: xerothermic grasslands on southerly exposed slopes with *Thymus pulegioides* (KLU and SROM), and dry grasslands (often clearings in pine forests) on sandy flat areas where *T. serpyllum* was used as a larval food plant (all other localities) (Table 1, Fig. 1).

Sample collection - Italy

We used DNA extracts from samples collected for earlier studies (Patricelli *et al*., 2013). In the 2009 and 2010 seasons, a total of 141 *M. arion* adults were captured, and a middle left leg was removed from each individual. We analysed seven populations (14 to 25 samples each), separated by distances that varied from a minimum of 58 km (between LOA and CUN) to a maximum of 736 km between VFE and AUR (Table 1, Fig 1). Sampling sites

can be divided into three categories: (i) woodland clearings on upland localities where *M. a. ligurica* (a putative subspecies, Wagner, 1904), oviposit to *Origanum vulgare* (CUN, LOA, BDR); (ii) mountain pastures in the Alps where *M. a. obscura* (a putative subspecies, Christoph, 1878), is dependent on *Thymus* spp. (VFE, CDF); and finally (iii) clearings and pastures in the Apennines where *M. a. arion* uses *Thymus* spp. (AUR, CET) (Table 1, Fig. 1).

Amplification of microsatellite markers

We analysed 12 microsatellite loci in a total number of 285 individuals of *Phengaris arion*. We used five loci cross-amplified from *P. naustihous* and *P. alcon* (Zeisset *et al*., 2005), applying PCR conditions described in previous papers (Rutkowski *et al*., 2009; Sielezniew & Rutkowski, 2012). Additionally, we amplified seven loci developed specifically for the species by ECOGENICS GmbH (Zürich, Switzerland) and described by Ugelvig *et al*. (2011): Macari02, Macari05, Macari16, Macari18, Macari19, Macari22 and Macari23. Loci Macari02, Macari05, Macari19 and Macari23 were amplified in a single multiplex reaction. The reaction mix contained 1,5µl of primer mix ('forward' and 'reverse' for each loci 2pmol/µl); 7.5µl PCR MasterMix (QIAGEN); 2.7 µl of PCR-graded water, and 3–5 µl template DNA. The reaction conditions were as follows: 15 min. in 95°C; 39 cycles: 30 s in 94°C, 90 s in 57°C; 90 s in 72°C; one cycle: 30 s in 94°C, 90 s in 57°C; 10 min. in 72°C. 'Forward' primers were labelled with one of the WellRead labels (Sigma-Aldrich): Dye2; Dye3; Dye4. Loci Macari16, Macari18 and Macari22 were amplified in the independent PCRs. The reaction mix (25 µl) contained 5–6 µl of DNA extract, 12.5 µl REDTaq PCR ReadyMix (Sigma), 7.5 µl of water and 10 pmol of each primer. The PCR reaction was performed under the following conditions – initial denaturation: 94°C in 3 min.; 30 cycles: 94°C in 45 s; 55°C in 45 s; 72° C in 45 s; final elongation: 72°C in 5 min. Negative PCR controls were always included for each set of reactions. No amplification product was found in any negative controls after electrophoresis in agarose gels and analysis in an automatic sequencer.

The length of the amplified fragments was estimated using a CEQ8000 Beckman Coulter automatic sequencer. Data were analysed using Beckman Coulter Fragment Analysis Software.

Statistical analysis

The polymorphism of microsatellite loci was estimated on two levels. Firstly, we assessed allelic diversity (*A*), allelic richness (*R*; Petit *et al*., 1998), mean number of private

alleles (*P*), private allelic richness (R_P), effective number of alleles (*Ne*), observed heterozygosity (H_O) and unbiased expected heterozygosity (H_E) (Nei & Roychoudhury, 1974) separately for Poland and Italy. A fixation index (F_{IS}) was calculated for each country and its significance was tested under a randomization procedure and Bonferroni correction for multiple comparison. These analyses were performed using GenAlEx version 6.0 (Paekal & Smouse, 2001), FSTAT version 2.9.3 (Goudet, 2001) and HP-RARE (Kalinowski, 2005). Genotypic linkage disequilibrium between all pairs of loci, as well as a probability test for deviation from the Hardy-Weinberg equilibrium was evaluated using Genepop, Web version 4.0.10 (Raymond & Rousset, 1995; Rousset, 2008). Analyses were then performed for each of the populations.

Genetic differentiation between populations was estimated using F_{ST}. Overall F_{ST} (Weir & Cockerham, 1984) for Poland and Italy and pairwise F_{ST} among all populations studied were obtained with FSTAT. The 95% confidence intervals for overall F_{ST} were also estimated in FSTAT. Additionally, as pairwise F_{ST} strongly depends on the level of heterozygosity within populations (Heller & Siegismund. 2009; Meirmans & Hedrick, 2011) we calculated a standardized measure of genetic differentiation F'_{ST} (Hedrick 2005). The F'_{ST} was calculated by dividing pairwise F_{ST} by the maximum value obtained using RecodeData v.0.1 (Meirmans, 2006).

The significance of differences between mean values of allelic richness (calculated across separate populations within each of the countries), F_{IS}, F_{ST}, and observed and expected heterozygosity and relatedness (calculated using estimator equivalent [Queller & Goodnight's, 1989]) in both Poland and Italy were tested using the permutation procedure as implemented by FSTAT.

The Bayesian-clustering method (STRUCTURE version 2; Pritchard *et al*., 2000) was used to examine how well the predefined "populations" corresponded to genetic groups (K). STRUCTURE was run 15 times for each user-defined K (1–14), with an initial burn-in of 50. 000, and 100. 000 iterations of the total data set. The admixture model of ancestry and the correlated model of allele frequencies were used. Sampling location was not used as prior information. Next, we examined ΔK statistics that identify the largest change in the estimates of *K* produced by STRUCTURE, as ΔK may provide a more realistic estimation of *K* than those based on likelihood (Evanno *et al*., 2005).

To visualize the STRUCTURE results we used STRUCTURE HARVESTER (Earl & vonHoldt 2012). Then, we applied CLUMPP (Jakobsson & Rosenberg, 2007) to average the

multiple runs given by STRUCTURE and correct for label switching. The output from CLUMPP was visualized with DISTRUCT v 1.1 (Rosenberg, 2004) to display the results.

Analysis with STRUCTURE was performed for all 14 populations, as well as separately for Poland and Italy.

RESULTS

Genetic diversity

We successfully amplified 12 microsatellite loci in all analysed samples. No significant linkage disequilibrium among loci was found. Only one Polish population was in HWE, and three from Italy. Significant F_{IS} value, indicating heterozygote deficiency was found in three Polish and two Italian populations (Table 2). Overall F_{IS} value was nearly twice as large in Italy as in Poland.

All indices of genetic variability were higher in Poland (Table 2). Among the studied populations the highest genetic variability was found in HUT and SUK, the smallest in KLU (in terms of number of alleles and private alleles) and ORC (in terms of heterozygosity measures). In Italy, the highest mean number of alleles was found in CDF and VFE, and allelic richness was the highest in LOA. The highest observed heterozygosity was also found in this population. However VFE also had the highest number of private alleles and effective number of alleles. The smallest number of alleles and heterozygosity was found in the CUN population, whereas AUR presented the smallest number of private alleles (Table 2).

Comparison between the two groups of populations (Poland and Italy) indicated a significantly higher allelic richness and observed heterozygosity in Poland than Italy (R = 6.858 and 5.061 respectively, $P = 0.019$, $H_O = 0.676$ and 0.609 respectively, $P = 0.042$, two-sided test, 1000 permutations), whereas F_{ST} and relatedness coefficient were significantly higher in Italy than Poland ($F_{ST} = 0.124$ and 0.073 respectively, $P = 0.040$; *Relat*. = 0.201 and 0.127 respectively, $P = 0.047$; one-sided test, 1000 permutations). We found no significant differences in F_{IS} or expected heterozygosity.

Genetic population structure

Both measures of genetic differentiation (F_{ST} and F'_{ST}) indicated similar patterns of differentiation (Table 3). In Italy F_{ST} was generally higher than 0.1 (16 among 21

comparisons), reaching the highest value for the AUR-CUN pairwise comparison. In Poland, a F_{ST} higher than 0.1 was only found for the majority of comparisons with KLU and ORCH-SRO pairwise comparison (4 among 21 comparisons). Similarly, F'_{ST} was generally lower than 0.35 in Poland (16 among 21 comparisons) compared to only six among 21 comparisons in Italy. Both measures indicated the smallest genetic differentiation between SUK and HUT in Poland and CDF and VFE in Italy.

Among the Polish and Italian populations we found a generally higher level of differentiation (0.068–0.215) than within each country (0.044–0.144 and 0.060–0.213 respectively). Only in seven out of 49 comparisons was F_{ST} lower than 0.1, and in four out of 49 comparisons F'_{ST} was lower than 0.35 (Table 3). The highest differentiation was found for comparisons of CUN and ORC (F_{ST}) and BDR and ORC (F'_{ST}), the smallest for comparisons of SUK and CDF (F_{ST}) and PIA and VFE (F'_{ST}). Generally, populations LOA, CDF and VFE were less differentiated from Polish populations than from the four remaining Italian populations (AUR, CET, BDR and CUN).

Analysis with STRUCTURE suggested the presence of two genetic groups (Fig. 2A and B). All populations from Poland had their highest proportions of ancestry from group I (Fig 3, green bars). In Italy populations AUR, CET, BDR, LOA and CUN had the highest proportions of ancestry from group II (Fig 3, red bars), whereas individuals from CDF and VFE had mixed ancestry from two genetic groups, with a slightly higher proportion of individuals originating from group I.

Analysis with STRUCTURE performed for each country indicated the presence of two genetic groups in Poland (Fig 4A and B.), and separated KLU and SRO from other Polish populations (Fig. 5), with some individuals from SRO of mixed origin. In Italy, we found three genetic groups (Fig. 6A and B). Populations AUR, CET and BDR had the highest proportions of ancestry from group I (Fig. 7, red bars); CUN from group II (green bars) and CDF and VFE from group III (blue bars). Individuals from LOA had mixed origins, the majority having the highest proportion of ancestry from group II.

Discussion

Genetic diversity

We found that genetic variability of the analysed microsatellite loci was generally higher in Poland than in Italy. This result is not in accordance with the 'southern-richness, northern-purity' pattern detected in Europe for many species, consisting in the reduced genetic

variability of northern populations as compared to those located farther south (Hewitt 1996; 1999; 2000). For example, a significant decline in the number of alleles from southern to northern populations was observed for the lycaenid butterfly *Polyommatus coridon* by Schmitt *et al*. (2002). This pattern was also confirmed when the Apennine Peninsula was compared with more northerly situated regions for some vertebrates (e.g. Stefani *et al*., 2012; Vences *et al*., 2013). It is believed that populations in Northern Italy (e.g. the Alpine populations from our study) were established as a result of recent and rapid colonization of these regions from refugium in Calabria (Canestrelli *et al*., 2006). Simultaneously, rapid expansion reduced the genetic variation of northern populations, due to serial founder events (Hewitt 1996; 1999).

In the present studies, the indicators of genetic variability almost linearly decreased southward, reaching their lowest values in the population from the Aurunci Mountains, i.e. the southernmost population of all those analyzed, and the highest in the Pennine Alps. However, it is important to note that most of our sampling sites in Italy were distributed in northern parts of the country and the more southern regions of the Apennine peninsula (e.g. Calabria), bearing potentially the highest genetic diversity (e.g. 'southern richness - northern purity' within the Apennine Peninsula; Canestrelli *et al*., 2006; 2008), were not studied (see Fig 1.). To confirm that genetic variability in Italian *P. arion* decreases southward, an analysis of populations from more southerly located sites is required. Indeed, the appropriate sampling scheme seems to be crucial in disentangling the evolutionary forces shaping genetic variability in Apennine refugium (Canestrelli *et al*., 2006; 2008).

Hence, we could compare two groups of populations with reduced genetic variability according to the expectation of post-glacial colonization from southern refugees. Polish populations presently possess significantly higher genetic variability, due to better connections among patches of habitat, while in Italy (northern and central parts) genetic variability has been continuously decreased by a higher level of habitat fragmentation. Polish populations of *P. arion* inhabit mostly lowland regions where clear barriers between sites are often lacking, i.e. on a local scale it is sometimes difficult even to determinate borders between populations. Such circumstances favour gene flow and maintenance of high genetic variability. In contrast, in Italy a higher level of habitat fragmentation has been observed due to topography and climatic conditions, probably reducing allelic richness due to genetic drift in isolated populations and overall heterozygosity due to the Wahlund effect.

Another explanation for the observed pattern of genetic variability could be the fact that the highest within-population diversity is expected for the continuous range of the species

(especially the admixture zone) and a lower diversity is expected for rear and leading edges (Hampe & Petit, 2005). Comparison of our data with the genetic variability of Scandinavian *P. arion* populations (Ugelvig *et al*., 2011; 2012) indicated that allelic richness was no higher than 5.0 for any of the Swedish populations, and in the present studies only two Italian populations had a richness lower than this level. In contrast, for all Polish populations allelic richness was above 6.0, with the exception of the most isolated KLU population. Hence, we could explain the obtained results as a rather typical pattern: higher genetic variability in the continuous range of the species (Poland) rather than in leading (Sweden) and rear (Italy) edges. Also, it is important to note that our research sampling could be biased towards relatively strong populations because of the availability of material, since *P. arion* occurs in low densities. Large populations tend to have a higher level of genetic diversity than small populations as was shown, e.g. for endangered xerothermophilous skipper *Thymelicus acteon* (Louy *et al*., 2007). However Habel *et al*. (2010), studying another endangered lycaenid species, *Lycaena helle,* using microsatellites, also found that genetic diversity (especially allelic richness) in Scandinavia is lower than in Poland. The reason for the low diversity of Scandinavian populations may be also related to their current isolation. Cassel-Lundhagen (2010) reports reduced diversity for nymphalid *Coenonympha arcania* in Scandinavia, which shows a pattern of distribution in Europe similar to that of *P. arion.*

Allelic richness and expected heterozygosity in our studies were relatively high in comparison with the results of microsatellite analyses concerning other *Phengaris* species e.g. *P. nausitho*us in southwestern Germany (3.7-5.5 and 0.41-0.62) (Anton et al., 2007) as well as *P. alcon* in Poland and Lithuania (1.6-5.9 and 0.13-0.61) (Sielezniew et al., 2012). Bereczki et al. (2011) suggest, based on comparative allozyme analyses, that the relatively high level of genetic diversity of *P. arion* observed in Central Europe results from their dispersal abilities, which are higher than those of other *Phengaris* species.

Interestingly, two Italian localities sampled for our analyses (CUN and VFE) were almost simultaneously subjected to extensive metapopulation studies (Bonelli et al, 2013). Their results indicate significantly higher mortality rates during dispersal and simultaneously significantly reduced dispersal distances in CUN, where we detected the lowest indices of genetic variability. Genetic diversity of populations is considered an essential factor for their fitness, as genetic impoverishment can be reflected in reduced adaptability and therefore higher rates of decrease (Saccheri *et al*., 1998; Nieminen *et al*. 2001; Schmitt & Hewitt 2004; Vandewoestijne *et al*. 2008).

Moreover, Polish and Italian populations of *P. arion* generally seem to be less specialized (Sielezniew *et al*. 2010a; b; Casacci *et al*. 2011) than Swedish populations, which are considered to be more specific towards their host ants (i.e. *M. sabuleti*). These populations even served as donors for the reintroduction of *P. arion* in Britain, where extinction of the butterfly was attributed to the decline of *M. sabuleti* (Thomas *et al*., 2009). Perhaps the higher host-ant specificity observed in the case of *P. arion* in NE Europe results from genetic drift. We found in our earlier studies that the only *P. arion* population in Poland for whom a significant specialization level in host ant specificity was detected also showed the greatest genetic impoverishment (Sielezniew & Rutkowski, 2012).

The results obtained from microsatellites are not concordant with the previous studies concerning the same sampling area when different markers were used. Almost no polymorphism in COI (mtDNA) was detected in Poland, while as many as 11 haplotypes were recorded for Italy. Moreover, , within-population diversity levels for the EF-1α (nucDNA) gene were comparable in both countries (Patricelli et al., 2013). Indeed, levels of within-population diversity could differ significantly when their estimates were performed with different molecular markers. The results of the simulation study by Mariette *et al.* (2002) suggest that low correlations may be expected, for example when comparing large populations with a high gene flow among them, or populations with high heterogeneity within the genome, or when performing estimates with a limited number of markers; or when comparing recently created populations. It is not excluded that the pattern observed for COI (Patricelli et al., 2013) differs because this marker, used mostly in phylogenetic and phylogeographical studies, retains older patterns of genetic diversity distribution, i.e. without detectable effects of strong human impact on *P. arion* habitats, while microsatellites due to higher polymorphism, a faster mutation rate and fast lineage sorting give us the opportunity to see the pattern shaped by more recent habitat fragmentation, resulting from anthropogenic pressure.

Moreover, during fast range expansion, the variation in mtDNA can be reduced more quickly due to its fourfold lower effective population size compared to nuclear markers, e.g. microsatellites (Meiklejohn et al., 2007; Galtier et al., 2009). Other explanations may be regionally acting 'selection sweeps' (Ballard &Whitlock, 2004) and *Wolbachia* infection, which seems to be common as far as *P. arion* is concerned (Bereczki et al., 2013; Patricelli etal., 2013). Hence, we can speculate that variation in mtDNA was rapidly eliminated during the post-glacial expansion into northern Europe, whereas mtDNA polymorphism was still maintained in southern refuges. On the other hand, nuclear variation was not so quickly or

significantly reduced during expansion, and because of good connections among populations, it still persists in Poland. A similar pattern was detected, for example, in *Rana dalmatina* (Vences *et al*., 2013).

Genetic population structure

The F_{ST} and relatedness coefficient was shown to be significantly higher in Italy (0.124 and 0.201 respectively) than in Poland (0.073 and 0.127), i.e. the pattern was similar to the one found in aforementioned studies concerning *COI* and *EF-1α* (Patricelli *et al*., 2013). Therefore our new data seems to support the rear edge hypothesis of Hampe & Petit (2005), as populations from the low-latitude limits of a species' distribution (Italy) are more differentiated than those from a continuous range (Poland). According to this hypothesis an increase in differentiation accompanied by a decrease of within-population diversity is also typical for leading edge areas. For *P. arion* in Europe, this area is probably represented by Scandinavia, and interestingly data from Ugelvig *et al*. (2012) perfectly match this pattern. Overall genetic differentiation in Sweden (F_{ST} = 0.23) was much higher when compared both to Poland (0.073) and Italy (0.124). However, it is important to note that biogeographic history is not the only factor shaping population structure. In mountainous areas, e.g. the Alps and the Apennines, there are more barriers for specialized xerothermophilous species than in the lowlands, and they are more distinct. For alpine butterflies, reduced connectivity of habitats resulting from, e.g. more forest cover at high altitudes, may lead to a greater genetic differentiation among populations (Keyghobadi *et al*., 2005).

However, we found in Poland that even flat areas covered by relatively open fen vegetation may effectively hamper gene flow (Sielezniew & Rutkowski, 2012). The extended set of microsatellite markers analysed in the present study indicated that only one Polish population (KLU) was in HWE, and simultaneously the lowest number of private alleles was detected there. This population inhabited xerothermal grasslands on southern slopes with distinct boundaries, i.e. different exposures and/or forest and agricultural land. On the contrary, three populations, i.e. PIA, ORC and HUT were shown to have a significant deficiency of heterozygotes, although they were clearly not isolated by unpleasant biotopes. They thrived in the landscape with other local populations, which suggests the possibility of the Wahlund effect. In Italy, only LOA, BDR and AUR were in HWE, while significant F_{IS} values were recorded for two alpine populations (CDF and VFE). This again could result from the Wahlund effect rather than being a symptom of inbreeding depression, as in both localities

sampling was performed at metapopulation level in more than one discrete patch of habitat supporting metapopulation systems.

Besides KLU, a low number of private alleles was recorded for SOW (Poland) and AUR (Italy). Private alleles, i.e. those present in a single population, are eliminated by genetic drift but could also be uncommon when gene flow is high. As far as AUR is concerned the latter explanation is not possible as this population was far away from other sampling sites and also showed a relatively low level of genetic variation. The SOW population was more diverse but was in decline and moreover inhabited a former military area neighbouring a big city. Hence in the past this population could have been affected by serious fluctuations in abundance, due to habitat destruction, leading to the elimination of some rare alleles. This could suggest that despite a lower overall level of genetic differentiation in Poland than in Italy, in the former region populations of *P. arion* are more stretched out in a continuous environment, exhibiting a slightly marked small-scale genetic structure, while in Italy particular locations are more compact/dense but significantly differentiated from other such locations due to reduced gene flow.

The more pronounced genetic structure of *P. arion* in the Italian populations may also be interlinked with the history of the species during Plio-Pleistocene glaciations. It was shown that within the Apennine refugium many species underwent climate-linked cycles of fragmentation and allopatric divergence (a multiple-refugia scenario, e.g. Canestrelli *et al*.; 2008; Canestrelli & Nascetti, 2008). This process is still detectable in populations as a significant geographical distribution of genetic variation (e.g. Canestrelli *et al*., 2012). Indeed, analysis of our data with STRUCTURE suggested a separation of the Italian populations into three groups, reflecting mostly geographical distribution, with two Alpine populations assigned to a single genetic clade. In Poland, two groups only were observed, with two mountainous populations (KLU and SRO) forming a single clade, while the KLU population was more uniform than SRO. In studies concerning a higher number of sites but lower number of loci the same KLU population is even more distinct (Sielezniew & Rutkowski, 2012). The recent bottleneck is again the most probable explanation of this pattern.

Analysis with STRUCTURE, performed for the total data set, suggested the presence of two genetic groups with one group consisting of all Polish populations and two consisting of the northern most Italian populations from the Alps, while the five remaining Italian populations were assigned to the second group. This might suggest a common evolutionary history for populations inhabiting the northern slopes of the Alps and Central Europe, e.g. colonization from the Iberian peninsula as inferred from phylogeografical studies of another

butterfly, *Melanargia galathea* (Habel *et al*., 2005), and other animals, for example the frog *Rana temporaria* (Stefani *et al*., 2013). On the other hand, taking into consideration the previously detected differentiation in *COI* and *EF-1α* (Patricelli *et al*., 2013) we could rather hypothesise that alpine populations are localized in a mixed zone of different colonization routes, i.e. those populations were genetically enriched by specimens from other parts of Europe (e.g. glacial refuges on the Iberian peninsula or in Eastern Europe) during post glacial expansion. The relatively high heterozygosity observed for Alpine populations in Italy is typical for areas of admixture from colonization originating in different refugia (Nève & Verlaque, 2010). Hence, the third diverse LOA population may have a mixed origin from both northern and southern parts of Italy.

Finally, we think that our studies do not provide enough evidence concerning the genetic distinctness of Italian populations classified as *P. arion ligurica* and *P. a. obscura*. Populations using *Origanum vulgare* were not assigned to a distinct group in STRUCTURE analyses, and alpine populations (*P. a. obscura*) were assigned to the same clade as nominotypic populations sampled in Poland. The pattern of ecological variation recorded in both countries was not reflected in previous studies using different markers (Patricelli *et al*., 2013) either. Therefore, our findings are consistent with those of Bereczki *et al*. (2011, 2013), who examined populations from the Carpathian Basin using allozymes and mtDNA markers and concluded that there are no grounds for the separation of subspecies as far as *P. arion* is concerned. However, although genetic differentiation at neutral markers does not indicate the existence of any obvious evolutionary significant units sensu Moritz (1994), in the conservation of *P. arion* we should take ecological data into consideration, i.e. follow the suggestion of Crandall et al. (2000) that the categorization of population distinctiveness should be broader.

Acknowledgements

We thank the Italian Ministry for the Environment (prot. DPN-20-0023821) and the Polish Ministry of the Environment, who issued the relevant permissions for our studies. We thank Dr. Gabriele Fiumi, Prof. Giuseppe Manganelli and Dr. Guido Volpe for the Italian population selections. This research was funded within the project CLIMIT (Climate Change Impacts on Insects and their Mitigation; Settele & Kühn, 2009; Thomas, Simcox & Clarke, 2009) funded by DLR-BMBF (Germany), NERC and DEFRA (UK), ANR (France), Formas (Sweden), and Swedish EPA (Sweden) through the FP6 BiodivERsA Eranet. The Italian

Ministry of University and Research (MIUR) “A multitaxa approach to study the impact of climate change on the biodiversity of Italian ecosystems” project and the Polish Ministry of Science and Higher Education (grant no. 2 P04G 024 30) and BST-108 (University of Bialystok) also provided funding for this research.

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Table 1. Information on sampling locations of *Maculinea arion* studied in Italy and Poland. a.s.l. – above sea level. LHP – larval host plant.

Locality		Region	Coordinates	Elevation (a.s.l.)	LHP
Code	**Name**				
Poland					
PIA	Piaski	Narew Valley	53º13'N/22º45'E	105 m	*T. serpyllum*
SOW	Sowlany	Podlasie	53º09'N/23º15'E	160 m	*T. serpyllum*
ORC	Orchówek	Polesie	51º31'N/23º35'E	150 m	*T. serpyllum*
SUK	Suków	Kielce Upland	50º47'N/20º42'E	250 m	*T. serpyllum*
HUT	Hutki-Kanki	Kraków-Częstochowa Upland	50º24'N/19º30'E	360 m	*T. serpyllum*
KLU	Kluszkowce	Gorce Mts.	49º27'N/20º19'E	730 m	*T. pulegioides*
SRO	Sromowce	Pieniny Mts.	49º24'N/20º24'E	530 m	*T. pulegioides*
Italy					
VFE	Val Ferret	Aosta Valley	45º50'N/6º59'E	1636 m	*T. pulegioides*
CDF	Colle Delle Finestre	Piedmont	45º04'N/7º03'E	2185 m	*Thymus* sp.
LOA	Loazzolo	Piedmont	44º39'N/8º14'E	356 m	*O. vulgare*
CUN	Cuneo	Piedmont	44º25'N/7º35'E	443 m	*O. vulgare*
BDR	Bagno di Romagna	Emilia Romagna	43º50'N/11º53E'	600 m	*O. vulgare*
CET	Mt Cetona	Tuscany	42º56'N/11º52'E	1006 m	*Thymus* sp.
AUR	Mt Aurunci	Lazio	41º18'N/13º38'E	1218 m	*Thymus* sp.

Table 2. Summary of mean genetic variability indices in 12 microsatellite loci of *Phengaris arion* from 14 localities (n = 285). A – number of alleles; R – allelic richness; P - private alleles; R_P - private allelic richness; Ne - effective number of alleles; H_O – heterozygosity observed; H_E – heterozygosity expected; *HWE* – P-values for HWE exact test for heterozygote deficiency/excess; F_{IS} – fixation index (* – values significant after Bonferroni correction, 3360 randomization, adjusted P-value = 0.0003).

Site	**N**	**A**	**R**	**P**	**R_P**	**Ne**	**Ho**	**He**	**HWE**	**F_{IS}**
PIA	15	7.33	6.94	0.33	0.41	4.22	0.611	0.710	<0.001	0.173*
SOW	26	8.00	6.71	0.17	0.15	4.58	0.689	0.716	<0.001	0.057
ORC	18	6.92	6.12	0.42	0.46	3.51	0.569	0.656	<0.001	0.160*
SUK	20	9.42	7.80	0.58	0.61	5.67	0.679	0.765	<0.001	0.109*
HUT	25	9.25	8.12	0.58	0.56	5.59	0.750	0.781	0.014	0.065
KLU	22	6.00	5.38	0.08	0.13	3.73	0.686	0.692	ns	0.032
SRO	18	7.75	6.95	0.50	0.48	4.40	0.694	0.714	0.015	0.056
VFE	24	7.17	6.26	0.83	0.72	4.36	0.599	0.734	<0.001	0.205*
CDF	21	7.17	6.35	0.33	0.31	4.31	0.566	0.726	<0.001	0.244*
LOA	17	6.92	6.33	0.33	0.31	3.93	0.708	0.713	ns	0.038
CUN	25	5.00	4.49	0.42	0.37	2.96	0.558	0.622	<0.001	0.123
BDR	14	5.83	5.71	0.58	0.65	3.09	0.612	0.647	ns	0.091
CET	21	5.75	5.13	0.42	0.36	3.34	0.647	0.669	0.0423	0.057
AUR	19	5.50	4.99	0.17	0.16	3.23	0.605	0.632	ns	0.069
Poland	144	16.67	16.42	5.08	5.01	6.46	0.676	0.788	<0.001	0.146*
Italy	141	15.50	15.37	3.92	3.96	5.48	0.609	0.780	<0.001	0.222*

Table 3. Genetic differentiation among 14 populations of *Phengaris arion* from Poland and Italy. Above diagonal - F_{ST} sensu Weir and Cockerham (1984), below F'_{ST} (Hedrick 2005). Populations from different countries are separated by a dashed line. All values were significant after Bonferroni correction (1820 randomizations, adjusted P-value = 0.00054)

	PIA	SOW	ORC	SUK	HUT	KLU	SRO	VFE	CDF	LOA	CUN	BDR	CET	AUR
PIA		0.048	0.055	0.054	0.054	0.118	0.094	0.074	0.093	0.119	0.198	0.149	0.134	0.148
SOW	0.181		0.044	0.046	0.046	0.110	0.082	0.094	0.117	0.124	0.185	0.164	0.149	0.179
ORC	0.188	0.149		0.051	0.062	0.140	0.144	0.127	0.140	0.142	0.215	0.208	0.181	0.207
SUK	0.236	0.195	0.198		0.028	0.084	0.067	0.076	0.068	0.070	0.151	0.142	0.120	0.134
HUT	0.227	0.190	0.230	0.134		0.087	0.066	0.102	0.088	0.100	0.164	0.144	0.125	0.146
KLU	0.423	0.392	0.457	0.343	0.343		0.079	0.130	0.117	0.097	0.193	0.184	0.166	0.162
SRO	0.358	0.308	0.490	0.291	0.274	0.283		0.107	0.107	0.104	0.180	0.159	0.146	0.148
VFE	0.291	0.362	0.450	0.342	0.438	0.482	0.418		0.060	0.087	0.137	0.120	0.133	0.122
CDF	0.365	0.447	0.490	0.304	0.376	0.429	0.416	0.241		0.073	0.130	0.111	0.132	0.120
LOA	0.451	0.464	0.484	0.303	0.418	0.346	0.392	0.341	0.285		0.097	0.104	0.112	0.086
CUN	0.622	0.584	0.623	0.532	0.562	0.588	0.568	0.447	0.420	0.304		0.156	0.196	0.213
BDR	0.507	0.556	0.642	0.551	0.538	0.598	0.541	0.423	0.387	0.352	0.450		0.121	0.135
CET	0.462	0.512	0.568	0.470	0.471	0.548	0.503	0.474	0.466	0.385	0.577	0.378		0.113
AUR	0.481	0.581	0.616	0.492	0.520	0.507	0.480	0.411	0.399	0.280	0.596	0.397	0.339	
	PIA	SOW	ORC	SUK	HUT	KLU	SRO	VFE	CDF	LOA	CUN	BDR	CET	AUR

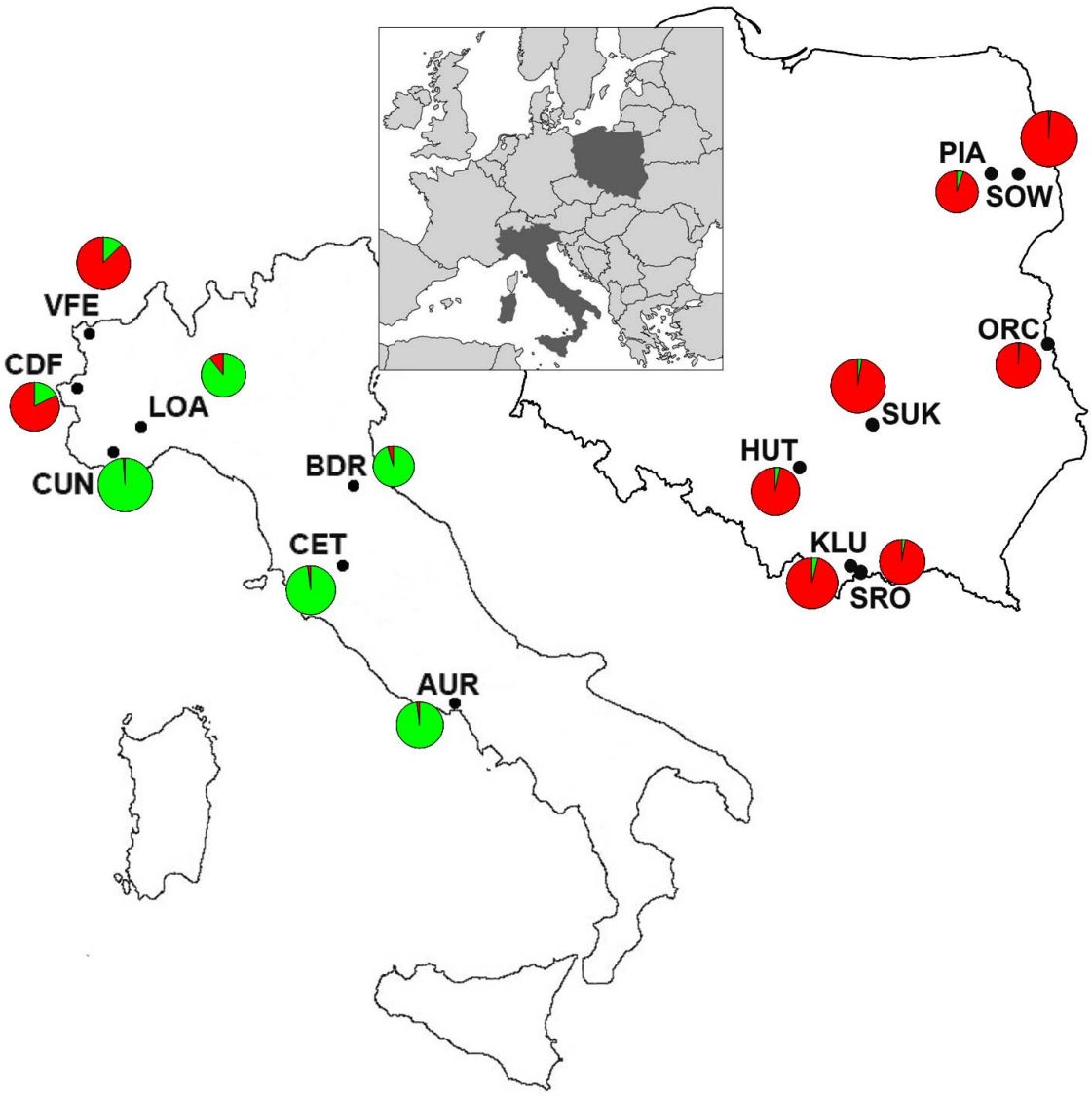

Fig. 1. Pattern of microsatellite variation in populations of *Phengaris arion* sampled in Poland and Italy (for full site names and other details see Tab. 1). Pie charts for each population represent the proportion of individuals assigned to each of the two clusters within the population. Sizes of circles are proportional to the number of individuals analyzed.

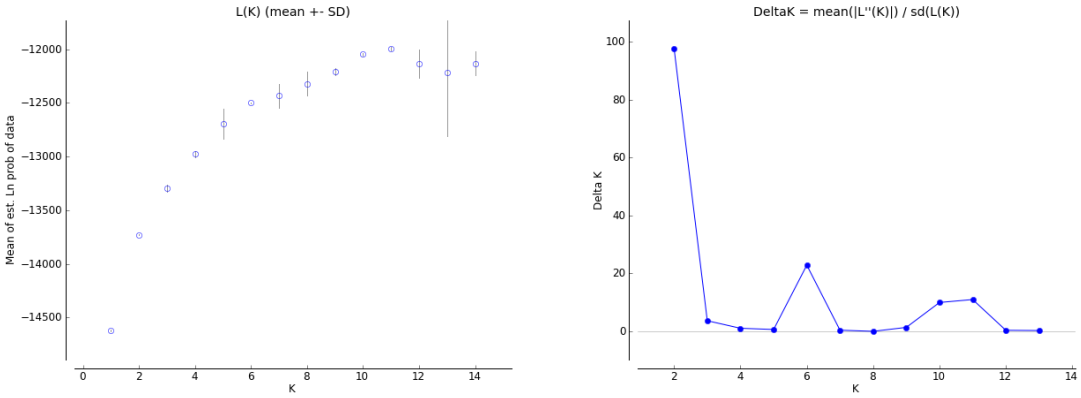

Fig. 2. Estimated likelihoods, ln P(D), of each number of inferred genetic clusters (left) and the corresponding *ΔK* curves as a function of *K* (right) for the pooled sample of *P. arion* from Italy and Poland.

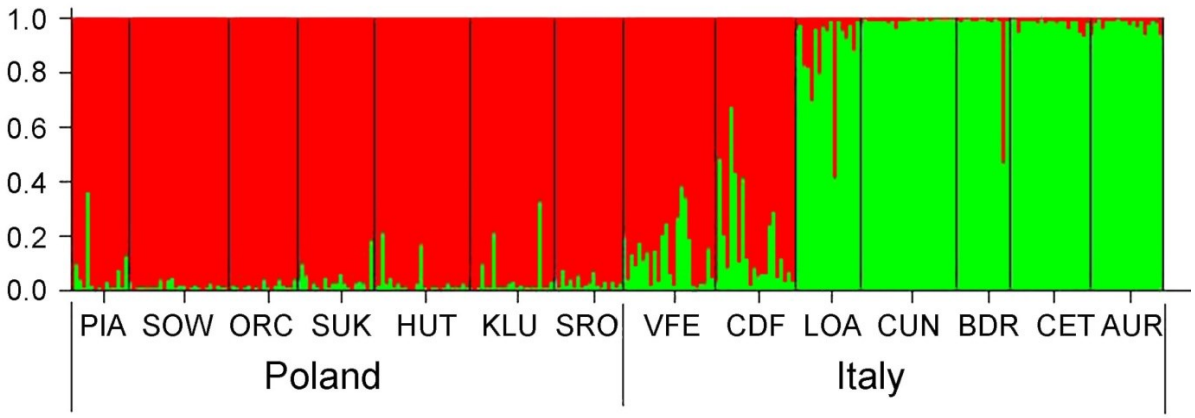

Fig. 3. Bayesian assignment of individuals in the pooled sample of *P. arion* from Poland and Italy to two genetic groups. Each bar represents the estimated posterior probability of each individual butterfly belonging to each of the three inferred clusters. Solid black lines define the boundaries between the populations used in the analysis.

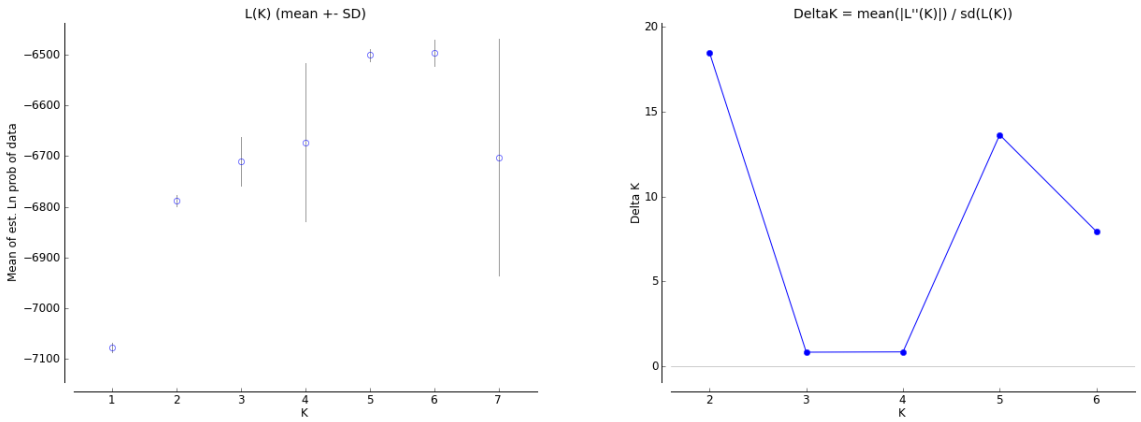

Fig. 4. Estimated likelihoods, ln P(D), of each number of inferred genetic clusters (left) and the corresponding *ΔK* curves as a function of *K* (right) for the pooled sample of *P. arion* from Poland.

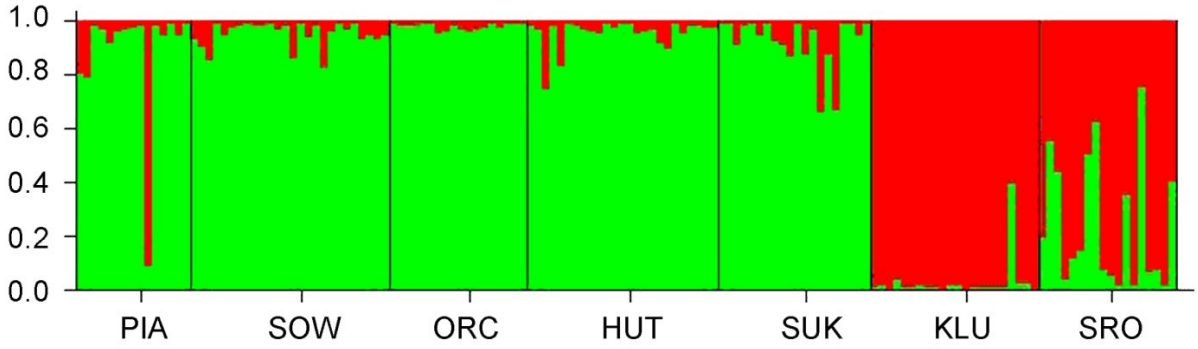

Fig. 5. Bayesian assignment of individuals of *P. arion* from Poland to two genetic groups. Each bar represents the estimated posterior probability of each individual butterfly belonging to each of the three inferred clusters. Solid black lines define the boundaries between the populations used in the analysis.

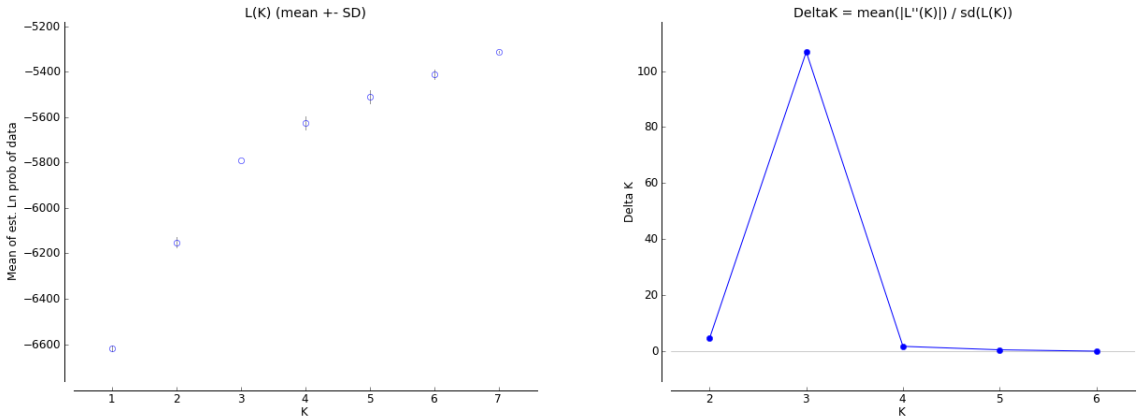

Fig. 6. Estimated likelihoods, ln P(D), of each number of inferred genetic clusters (left) and the corresponding *ΔK* curves as a function of *K* (right) for the pooled sample of *P. arion* from Italy.

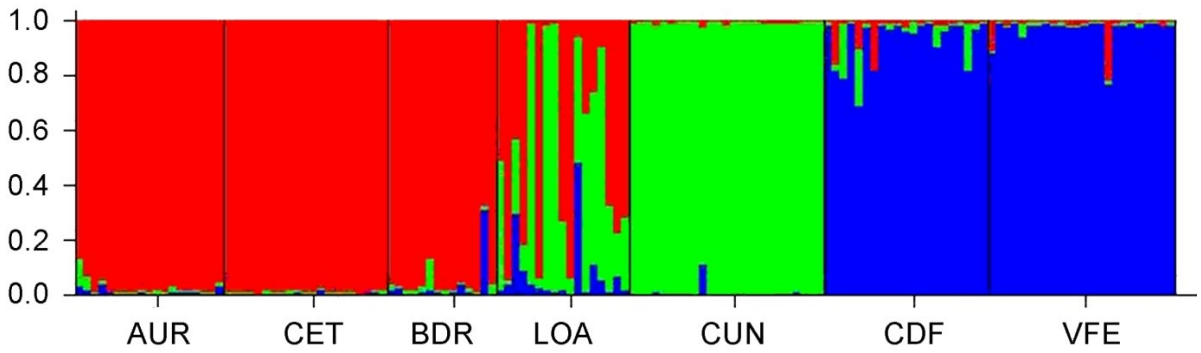

Fig. 7. Bayesian assignment of individuals of *P. arion* from Italy to two genetic groups. Each bar represents the estimated posterior probability of each individual butterfly belonging to each of the three inferred clusters. Solid black lines define the boundaries between the populations used in the analysis.