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Parnassius apollo nevadensis: Identification of recent population structure and source – sink dynamics

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Abstract

Population persistence depends in many cases on gene flow between local populations. *Parnassius apollo nevadensis* is an endemic subspecies of Apollo butterfly in the Sierra Nevada (southern Spain), whose populations are distributed in discrete patches at altitudes between 1850–2700 m. In this paper, we use 13 microsatellite loci to examine the genetic structure of this *P. apollo* subspecies. We revealed both a strong pattern of isolation by distance (which was stronger when calculated with realistic travel distances that accounted for topography) and source–sink dynamics. The observed population genetic structure is consistent with strongly asymmetrical gene flow, leading to constant directional migration and differential connectivity among the populations. The apparently contradictory results from the clustering algorithms (Structure and Geneland) are also consistent with a recent (<100 ya) reduction in the distribution range. The results point to global warming as a possible cause of this reduction, as in other populations of this species. We identify some natural and anthropogenic barriers to gene flow that may be the cause of the recent population structure and source–sink dynamics.

Introduction

Connectivity between separate patches across a species' distribution can be very important for the persistence of populations in the landscape, since it facilitates gene flow and the recolonization of available habitats (Fischer and Lindenmayer 2007; Buchalski et al. 2015). It has been demonstrated by theoretical models and field studies that the maintenance of genetic diversity and population viability is critically dependent on gene flow among local populations (Swindell and Bouzat 2005; Apodaca et al. 2012). When habitat fragmentation compromises gene flow, the viability of the population and individual fitness will be theoretically affected as inbreeding accumulates deleterious mutations (Lynch et al. 1995; Saccheri et al. 1998). Small populations are particularly likely to lose genetic variation by drift, but gene flow counteracts genetic drift and spreads potentially adaptive gene, so maintaining local genetic variation (Frankham et al. 2002; Segelbacher et al. 2010). Maintaining this genetic diversity means that evolutionary potential is sustained and is fundamental to the long-term survival and recovery of species (Frankham 2005).

Butterflies are known for being very sensitive to changes in their environment, and their populations have already been shown to be vulnerable to climatic change (Parmesan et al. 1999; Roy and Sparks 2000; Wilson et al. 2005; Wilson et al. 2007; Forister et al. 2010; Wilson and Maclean 2011; Radchuk et al. 2013; Descombes et al. 2015; Oliver et al. 2015). The effect of climate change seems to be even stronger in montane taxa, which could face extreme increases in temperature (Nogués-Bravo et al. 2007; Wilson et al. 2007).

Parnassius apollo (Linnaeus, 1758) is a relic of glacial fauna in the Eurasian continent. It is distributed in the Palearctic region, with the exception of North Africa and the Arabian Peninsula. Many subspecies have been described from Spain to southern Fennoscandia and Eastern China (Eisner 1976). Since the first half of the 20th century, extinctions and declines of its populations have been documented in numerous sites (Collins and Morris 1985; van Swaay and Warren 1999; Descimon et al. 2006; van Swaay et al. 2010), despite large-scale conservation efforts (Łozowski et al. 2014; Fred and Brommer 2015); the main causes for this decline seem to be anthropic, such as shepherding, pollution, tourism, collection or habitat loss (Gomariz Cerezo 1993; Habel et al. 2009; Fred and Brommer 2015). Another cause for the decline of *P. apollo* populations could be related to the fact that their populations are locally distributed due to their specific ecological requirements (Fred and Brommer 2005; Fred et al. 2006). The species is consequently very sensitive to habitat alteration and climate change (Ashton et al. 2009; Todisco et al. 2010), and thus a high priority for conservation. Accordingly, *P. apollo* is categorized as vulnerable by the International Union for Conservation of Nature (IUCN) (Baillie et al. 1996), listed in the European Red Data Book (van Swaay and Warren 1999), and in Annex IV of Appendix II of the Habitat Directive of the European Union (EEC 92/43/EWG), is presently included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) lists and is considered to be subject to High climate change Risk (HR) (Settele et al. 2008).

Parnassius apollo populations are particularly small and isolated in the south of Europe, where their distribution is restricted to mountain ranges (Descimon 1995; Todisco et al. 2010). In Spain, 23 subspecies of *P. apollo* have been described (Gómez-Bustillo and Fernández-Rubio 1973) and each one of these subspecies is isolated in a different mountain range. *Parnassius apollo nevadensis* Oberthür, 1891, the object of our study, is endemic to the mountain range of Sierra Nevada (Southern Spain). In addition to the limited distribution range of the *P. apollo* Spanish subspecies, *P. apollo nevadensis* is one of the Spanish subspecies allegedly threatened by excess tourism and habitat (Gomariz Cerezo 1993) and is considered as endangered by the Spanish government (Ministerio de Agricultura Alimentación y Medio Ambiente 2013).

Other Spanish subspecies of *P. apollo* have already shown a rise in their altitudinal range, in response to climate change (Wilson et al. 2005; Ashton et al. 2009) and the high-mountain populations of *P. apollo nevadensis* could be particularly vulnerable to these environmental changes. Their altitudinal range is considered to lie between 1850 and 2500 m (Olivares et al. 2011), but in this work we include samples from individuals captured between 1850 and 2704 metres of altitude, and in recent years the species has regularly been observed at altitudes up to 2700 m (González-Megías et al. 2015). This kind of elevational shift in distribution range can be a problem, as it can reduce the areas available to a species and result in populations becoming smaller and more isolated (Wilson et al. 2005).

In this study we use a set of fast-evolving markers (microsatellites) developed for the species (Mira et al. 2014) to analyse the genetic structure of *Parnassius apollo nevadensis*. With these genetic tools we determine: (i) whether there is a population genetic structure in Sierra Nevada; (ii) the level of gene flow (migration rates) between patches in this mountain range; (iii) standard indices of genetic diversity (including heterozygosity, allelic richness, and effective population size), and the degree of differentiation between patches (or populations). Finally, (iv) we attempt to identify the factors that have shaped that structure (such as distance or barriers to the gene flow) as an approach to define the conservation status of *Parnassius apollo nevadensis*.

Materials and Methods

Sampling

Three hundred and ninety-six *Parnassius apollo nevadensis* individuals were sampled during the summers of 2007–2011. Individuals were caught at 13 different sampling locations (Table 1; Fig. 1), in meadows scattered across the Sierra Nevada. The distance between the sampling locations ranged from 0.54 to 53.41 km. A sampling location included all the sampling points from the same hillside with a continuous presence of butterflies between them. The limits of each locality (in which the samples were assigned) were defined as the area (with butterflies) that was surrounded by zones without butterflies and by a geographical delimitation such as a facing hillside, river, ravine, valley or mountain peak (Fig. 1).

Table 1: Location name, code, sample size and average altitude of the 13 sampling locations.

Loc.	Caballo	Piuca	Chorrillo	Otero	Vacares	Papeles	Mirador	Postero	Hornillo	Chullo	Lagunilla	Almirez	Rayo
Code	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**
N	11	51	53	43	19	45	20	46	31	19	30	22	7
n2007	0	0	0	0	0	0	0	3	0	2	1	0	0
n2008	7	0	0	0	2	26	0	0	0	0	0	0	0
n2009	0	0	18	0	0	0	0	8	0	0	0	12	7
n2010	4	8	22	0	9	19	8	13	0	17	29	0	0
n2011	0	43	13	43	8	0	12	22	31	0	0	9	0
h	2486	2376	2648	2257	2515	2255	2264	2038	2351	2294	2283	2370	2405

N, total number of individuals sampled; n2007 - n2011, number of samples collected for each year stated; h, average altitude (metres above sea level)

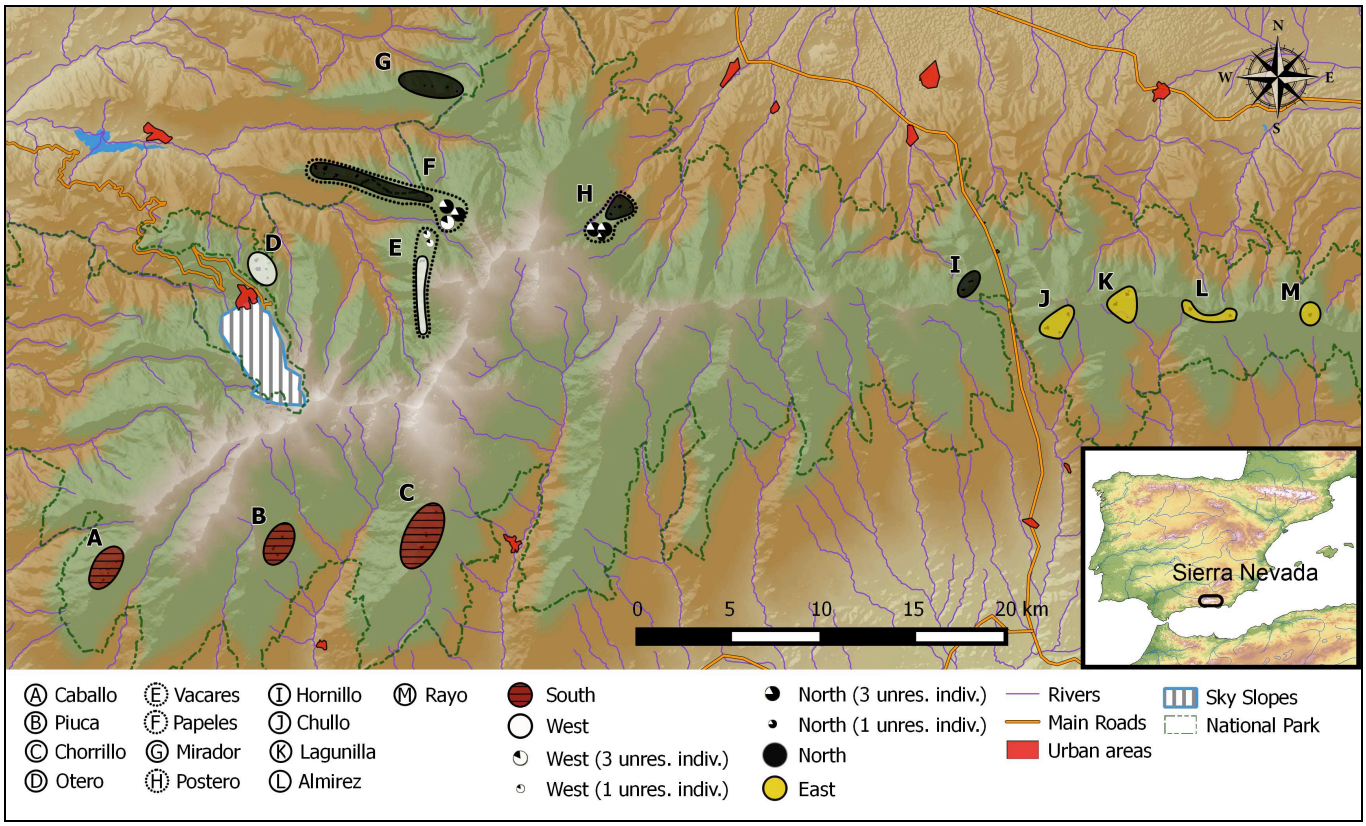

Fig. 1 Map including the location of all the samples clustered in 13 sampling locations. The locations are coded from A to M. A sampling location included all the sampling points from the same hillside with a continuous presence of butterflies between them. The colour of the circles surrounding the samples shows the assignment of the sampling locations to the different clusters defined by GENELAND. The "unresolved" individuals ("unr. indiv.") from the North and West clusters are those that in some of the five best outputs were assigned to North and in some other to West All other samples were consistently assigned to the same population. The green zone marks the altitude used by *P. apollo nevadensis*. The dashed line in dark green marks the limit of the National Park. Red polygons correspond to urbans areas of human populations

All individuals used in this study were captured at the end of their flying period (end of July), when most individuals had presumably already mated and females laid most eggs. The exact point of capture for each individual was recorded using a GPS device (Garmin eTrex); all individuals were transported alive to the lab where they were frozen within a few hours and kept at -20ºC until DNA extraction. All individuals were caught and used to extract DNA under permission of the National Park (Parque Nacional y Natural de Sierra Nevada) and the Consejería de Medio Ambiente (Junta de Andalucía). The sample size (Table 1) was dependant on accessibility to the site and the apparent relative abundance of adults in a meadow. The number of individuals caught per year in each location was never higher than 50; in locations with apparently low densities we caught fewer individuals (e.g. in "Caballo" (A) where we caught just 7 individuals in 2008 and 4 in 2010).

Molecular methods

Genomic DNA was extracted from a single leg of each adult butterfly. DNA extraction was carried out using an ammonium acetate salt precipitation protocol (Nicholls et al. 2000; Richardson et al. 2001).

All the 396 samples were genotyped with 20 polymorphic microsatellite loci developed specifically for *P. apollo nevadensis* (Mira et al. 2014); the amplification and genotyping were performed following Mira et al. (2014).

Microsatellite analyses

All loci were tested for linkage disequilibrium (LD) in each location using GENEPOP on the web v4.2 (Rousset 2008); a sequential Bonferroni correction was applied to LD *P* values (Rice 1989). All loci were tested for deviation from Hardy–Weinberg equilibrium (HWE) for each location using GENEPOP. The frequency of null alleles was estimated for each locus in samples from each sampling location using CERVUS v3.0.3 (Kalinowski et al. 2007) and MICRO-CHECKER 2.2.3 software (van Oosterhout et al. 2004). After these tests, seven loci were dropped as non-suitable (see Results) and the data obtained from the remaining 13 validated of the 20 loci was used for the analyses of population structure.

Genetic Structure

The global and pairwise F_{ST} (See Supplementary Materials and Methods) were estimated in GenAlEx 6.501 (Peakall and Smouse 2006, 2012); the same software was used to perform the AMOVA. All those analyses were performed with 9999 permutations to test for significance (See Supplementary Materials and Methods)

An Isolation by Distance analysis (IbD) was performed to check if genetic distances were related with geographical distances. The degree of correlation between the multilocus pairwise F_{ST} values and the log-transformed geographical distances between locations was tested using a Mantel test in ISOLATION BY DISTANCE WEB SERVICE (IBDWS) 3.23 (Jensen et al. 2005). For these analyses, distances between sites were computed as the projected shortest distance in a straight line calculated in the software SPAGeDi

v1.3 (Hardy and Vekemans 2002). *P. apollo nevadensis* occurs in meadows at heights between 1850 and 2700 metres. Given this, a more realistic "travelling" distance between the sites was estimated, not in a straight line but following the shortest route between sites given their altitudinal locations. These "travelling" routes were drawn "manually" in QGIS 2.10.1 (QGIS Development Team 2015) connecting the locations through the most direct path keeping the line inside the altitude range of the butterflies; this meant avoiding mountain peaks and valleys when necessary. When the only possible way between two locations was through a valley lower than the altitude range of the species, we connected both sides at the narrower point of the valley with a straight line. The IbD analysis to compare genetic distances and log "travelling" distances was performed using a Mantel test in IBDWS.

Clustering Methods

To determine the number of genetic populations, and assign individuals to them, two different Bayesian clustering methods were used (see Supplementary Materials and Methods).

The first method was implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000). The Admixture model with correlated allele frequencies was used as the prior, without any information about sampling locations or origins of samples (See Supplementary Materials and Methods). STRUCTURE was run for *K* values from one to 15. Ten independent runs were conducted for each *K* value. In each one of the 150 runs (15 different *K* values × 10 replications), 1.2 x 10^6 MCMC iterations were analysed after a burn-in of 600 000. After all runs, the values of the estimated natural logarithm of the posterior probability (log-likelihood) were plotted for all 150 runs, to find which *K* obtained the highest likelihood and if there was convergence of the replications for any *K* value. STRUCTURE HARVESTER (Earl 2012) was used to confirm the best *K* value using the Δ*K* method by Evanno et al. (2005), which helps to visualise the rate of change in the log probability of our data between the successive K values.

The second method is based on a spatial model of cluster membership, the Voronoi tesselation model (see Supplementary Materials and Methods). This analysis was executed in the R package GENELAND 4 (Guillot et al. 2005; Guedj and Guillot 2011).

GENELAND was run with the correlated allele frequencies model and the spatial option, including the geographical coordinates for each individual. Twenty independent runs of 1.5 x 10^6 MCMC iterations each were performed; from each run, thinning was set to 500 (one out of every 500 iterations was saved); the maximum number of nuclei was set to 500 and uncertainty on Coordinates to 0.01. The range for likely *K* values was set from 1 to 15, starting at 1. The most likely *K* value was decided according to the median *K* inferred in the models with the highest log posterior density. Finally, another run was set with the same parameters but with the inferred *K* value fixed, to accurately estimate the membership of each of the individuals.

Multivariate Methods

A spatial Principal Component Analysis (sPCA) was performed using the package ADEGENET (Jombart and Ahmed 2011) in RStudio. sPCAs is a multivariate technique with the same characteristics that the PCA but including the geographical information (See Supplementary Material and Methods).

A Neighbourhood by Distance Connection Network (Type = 5) was chosen to model the possible geographical connections between samples. To select the number of principal components to plot, the proportion of the total variance explained was considered, as well as the distribution of eigenvalues in a plot according to their variance and Moran's I (autocorrelation) components (Jombart 2014).

To check for a correlation between alleles and local (negative) eigenvalues, and between alleles and global (positive) eigenvalues, a local and a global test for spatial structures (Jombart et al. 2008) were performed with 9999 permutations to evaluate the existence of a significant correlation between the alleles and the eigenvectors, which would indicate significant genetic structure at either the local or global scale.

Standard Indices of Genetic Diversity

Standard genetic diversity parameters were computed for each population as defined by the clustering software. GenAlEx was used to estimate the observed and expected heterozygosities (H_O and H_E, respectively). As each population included a different number of samples, the Unbiased Expected Heterozygosity (uH_E) (Nei 1978) was also computed, since it allows groups with different sample sizes to be compared. HP-rare 1.1 (Kalinowski 2005) was used to determine Allelic Richness (A_R) and Private Allelic Richness (P_A) per site, using rarefaction. Large samples are expected to have more alleles than small samples; rarefaction is a statistical method that accounts for this effect to produce unbiased estimates of allelic richness.

The level of inbreeding was estimated using the inbreeding coefficient (F_{IS}, Wright 1965). The F_{IS} value for each population was estimated by Fstat 2.9.3.2 (Goudet 1995) after 1040 randomizations. Differences in uHe, A_R, P_A and F_{IS} between populations were analysed using a one-way ANOVA.

Effective population size

The approximate Bayesian computation online service OneSamp (Tallmon et al. 2008) was used to estimate the effective population size (N_e) of each population. OneSamp requires the user to set lower and upper limits for the maximum effective population size computation. Setting large values in the upper limit will extend the computation, so preliminary analyses were done to confirm which upper limit would be sufficient, starting with low numbers (100) and running the simulations separately with higher values (500; 1000, 10000). After the preliminary analyses, the upper limit for the maximum effective population size for each location was set to 700, since this value was higher than any of the obtained estimations of N_e in all the analyses.

Migration and Gene flow

BayesAss 3.0 (Wilson and Rannala 2003) was used to infer the magnitude of recent gene flow between populations. The method uses a genetic assignment method (identifies individuals carrying genotypes that indicate admixture with genotypes from other populations) to estimate the recent migration rate (over the last few generations) with a Bayesian MCMC approach. The program was run 6 times with 6 different seed numbers, and the number of iterations was set at 9,000,000, with an initial burn-in of 3,000,000 and a thinning of 1200 chains.

Bottleneck

BOTTLENECK 1.2.02 (Piry et al. 1999) was used to identify recent drastic changes in the population size of the populations defined by the clustering software. The program was set independently for two different mutation models; a Stepwise Mutation Model (SMM) and a Two-Phase Mutation Model (TPM), as those are the models recommended for microsatellites studies (Piry et al. 1999) (See Supplementary Materials and Methods). The TPM combines the SMM and Infinite Allele Model (IAM) in different variances and proportions. The TPM was set independently with two different values of variance (12 and 31); and for each variance value, three different proportions of SMM (95, 90 or 80 per cent).

The outputs of these models were analysed with a sign test for heterozygote excess and a Wilcoxon test for heterozygote excess or deficiency, to identify the populations that may have been through a genetic bottleneck.

Results

Microsatellite analyses

After preliminary analyses with the 20 microsatellite markers, none of the pairs of loci compared displayed significant linkage disequilibrium (LD) in all 13 different sampling locations ($p > 2.02*10^{-5}$); one pair of loci *Pan03* and *Pan21* displayed LD only in individuals sampled at sites B, D and G, and *Pan03–Pan43* displayed LD when assessed in the individuals collected at sites B and D.

The estimated frequency of null alleles for the loci *Pan37*, *Pan46* and *Pan53* was above 10% in most of the locations. *Pan03*, *Pan21*, *Pan22*, *Pan27*, *Pan37*, *Pan46* and *Pan53* showed evidence of significant deviation from Hardy–Weinberg equilibrium in most of the locations.

We therefore excluded seven loci (*Pan03*, *Pan21*, *Pan22*, *Pan27*, *Pan37*, *Pan46* and *Pan53*), the remaining 13 loci were used for the analyses presented below.

Genetic Structure

The AMOVA shows that 5% of the total genetic variation occurred within sampling locations, while differences among locations explains just 1% of the variation; the remaining variance (94%) is explained by the differences within individuals (See Supplementary Table S2). Global F_{ST} (0.010) shows a low but significant genetic structure (p = 0.0001).

Pairwise F_{ST} values are also low (Table 2), ranging from 0.007 to 0.029 in the significant comparisons (p < 0.05).

A significant positive relationship was found between genetic and geographical distances (slope = 0.0033). The Mantel test calculated in IBDWS indicates a positive correlation between genetic and geographical distances (r = 0.34; p = 0.0036). The analysis using the "travelling" distances shows a higher correlation (slope = 0.0045, Fig. 2), suggesting a larger extent of IbD (r = 0.50; p < 0.0001) and that up to 23% of the variation in pairwise F_{ST} values is explained by geography.

Table 2: "Travelling" distances (see methods) and pairwise F_{ST} values between sampling locations.

Loc.	A	B	C	D	E	F	G	H	I	J	K	L	M
A		7.60	13.94	14.73	19.27	20.68	25.66	27.00	39.40	43.21	45.59	49.89	53.40
B	0.013		6.36	11.79	13.80	16.16	21.10	20.48	31.93	35.65	38.04	42.32	45.82
C	0.004	0.004		13.31	11.93	15.12	19.46	16.28	25.93	29.48	31.88	36.08	39.56
D	0.019*	0.015*	0.013*		6.78	6.46	11.17	15.25	30.35	34.86	37.02	41.64	45.22
E	0.029*	0.015*	0.015*	0.000		3.42	7.57	8.53	23.61	28.16	30.29	34.92	38.49
F	0.022*	0.017*	0.020*	0.013*	0.001		5.02	9.72	25.34	30.00	32.01	36.65	40.20
G	0.029*	0.018*	0.026*	0.018*	0.013*	0.007		9.00	24.11	28.84	30.61	35.20	38.65
H	0.019*	0.008*	0.012*	0.008*	0.000	0.000	0.006		15.65	20.34	22.31	26.95	30.49
I	0.012	0.007*	0.003	0.010*	0.007	0.005	0.017*	0.000		4.73	6.69	11.33	14.89
J	0.016	0.005	0.010*	0.018*	0.011	0.008	0.004	0.004	0.008		2.41	6.81	10.40
K	0.019*	0.007*	0.006	0.018*	0.013*	0.010*	0.010*	0.006	0.005	0.000		4.64	8.21
L	0.018*	0.010*	0.007	0.014*	0.015*	0.009*	0.005	0.006	0.005	0.000	0.000		3.59
M	0.020	0.020	0.011	0.017	0.018	0.012	0.017	0.007	0.006	0.018	0.016	0.007	

Pairwise F_{ST} values below the diagonal ("*" indicates a significant p-value below 0.05); travelling distances between locations (km) above the diagonal. Loc., the names or the locations (codes from A to M) are respectively A: Caballo, B: Piuca, C: Chorrillo, D: Otero, E: Vacares, F: Papeles, G: Mirador, H: Postero, I: Hornillo, J: Chullo, K: Lagunilla, L: Almirez and M: Rayo

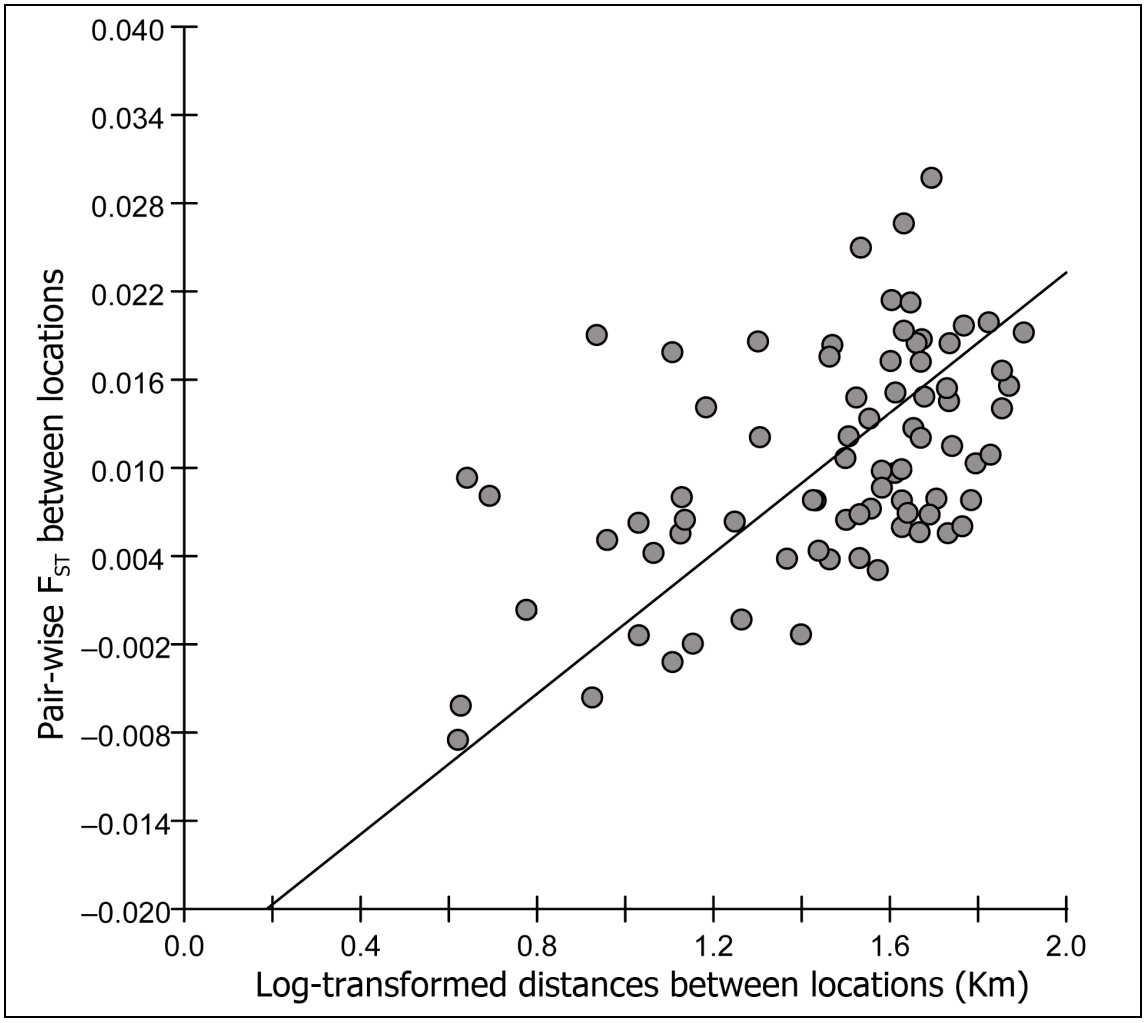

Fig. 2 Correlation between pairwise F_{ST} values and "travelling" geographic distances (see Methods) between locations

Clustering Methods

In the results of the analysis with no prior population information in STRUCTURE, the highest ΔK value is shown for two or fewer clusters. The log probability values converge at their highest value at $K = 1$ (Fig. 3), suggesting the existence of previous extensive gene flow between all the sampling locations and thus a single panmictic population.

In GENELAND, the 20 runs to infer the number of clusters (K) agree in all cases that the preferred number of clusters was equal to four. We set the number of populations to four ($K = 4$) in the subsequent runs to estimate the other parameters. From this second set of runs, we considered the clusters given by the run with the highest log posterior density as four different populations: South, West, North and East (Fig. 1). The first population, South (at the Southwest) includes the sampling locations A, B and C. The second population, West, includes all samples in D and E, and three samples from F (see Fig. 1). The third population, North, includes almost all samples from F (except three samples assigned to West), and includes all samples from G, H and I. The last population, East, include all samples from J, K, L and M.

The population assignment of individuals in the best five runs is concordant in 95.5% of the samples. Eighteen individuals are assigned in some runs to the North cluster and in other runs to West (Fig. 1).

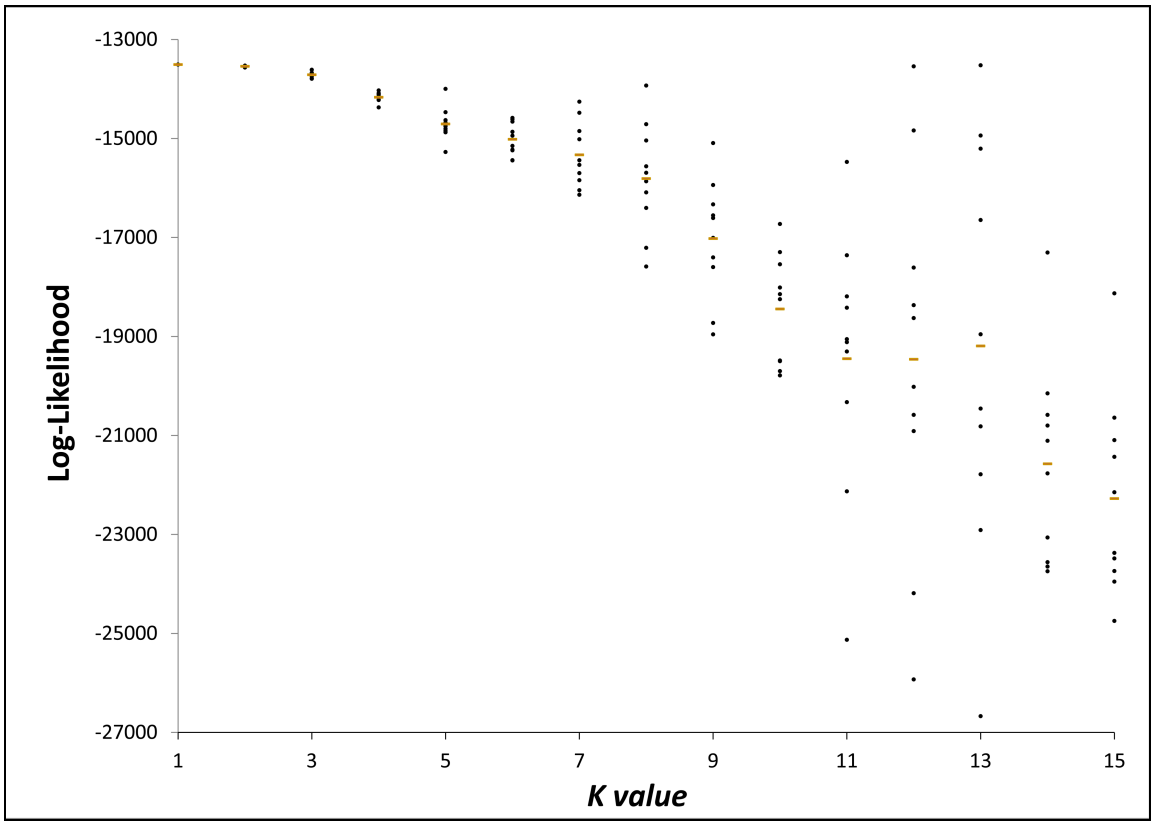

Fig. 3 Log-likelihood for each of the 10 independent runs for each *K* value; the average L(*K*) for each *K* is marked with a yellow line

Multivariate Methods

In the sPCA the components of global structure are more informative than the components of local structure (Fig 4). Two global components appeared to be informative (positive eigenvalues of 0.0215 and 0.0177), while the eigenvalues of the local structure (negative eigenvalues) are much less informative. Confirming this population structure, the global test for spatial structure gives a greater probability for the alternative hypothesis (existence of global structure), this being significant ($p = 0.030$), whereas the local structure was non-significant ($p = 0.415$). The samples are grouped into different zones according to their eigenvalue scores (Fig. 4). Two zones can be detected (with high values of opposite sign): the North zone with the highest positive scores (including F, G and H locations, in blue) and the South zone with the highest negative score values (including A, B and C, striped red). The other zones have neutral values (in white).

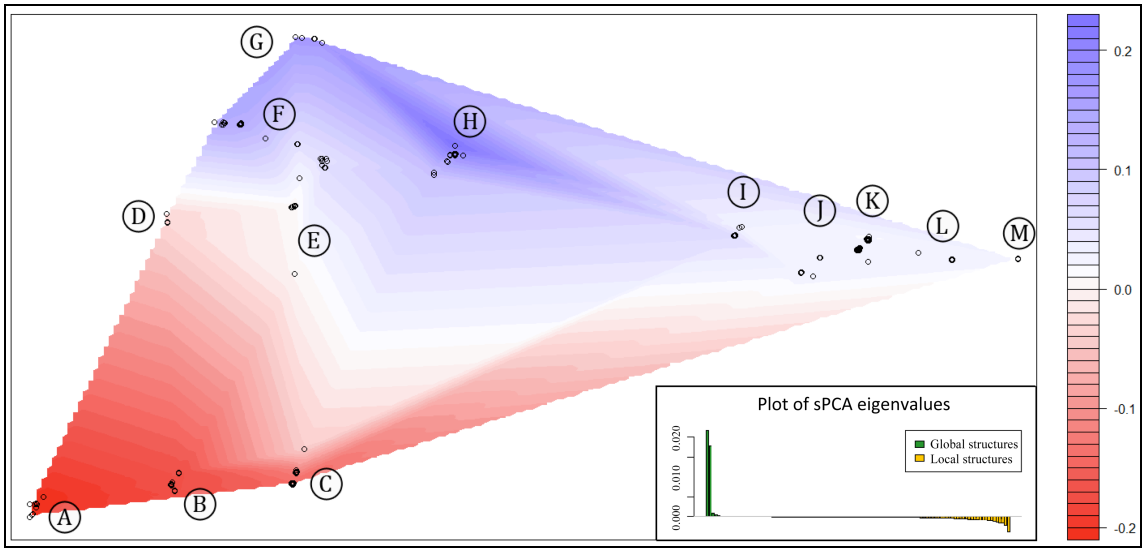

Fig. 4 Heat map of the sPCA results. Small black circles represent samples as they are distributed in the Sierra Nevada; the colours correspond to the first positive eigenvalue score of significant global genetic structure. Highest values are shown in red and the lowest values in blue; the magnitude of the values corresponds to each sample score relative to the genetic structure of the overall sample set. In the box at right bottom are shown the sPCA eigenvalues for the significant positive global structures (green) and the negative local structures (yellow)

Standard Indices of Genetic Diversity

In the four populations identified using GENELAND, the mean values of expected and observed heterozygosities are similar, and similar to the unbiased expected heterozygosity (uH_E). The average H_E ranges from 0.585 (South) to 0.609 (West), uH_E from 0.587 (South) to 0.614 (West) and H_O from 0.556 (East) to 0.597 (West; Table 3). Mean unbiased values of A_R range from 6.49 (South) to 7.77 (East). P_A values ranged from 0.06 (South) to 0.89 (East). All the obtained F_{IS} values are non-significantly different from zero ($p > 0.001$, which is the p-value adjusted for 5% nominal level, provided by FSTAT, Table.3).

The ANOVA does not detect significant differences in H_O, H_E, uH_E or A_R between populations; however, there are significant differences in P_A ($F_{3, 48} = 3.431$; $p = 0.024$) (Supplementary Table S1).

Table 3: Standard indices of allelic diversity for the populations delimited by GENELAND

Population	N	H_O	H_E	uH_E	A_R	P_A	F_{IS}
South	115	0.578±0.059	0.585±0.058	0.587±0.058	6.49	0.06	0.015
West	65	0.597±0.061	0.609±0.052	0.614±0.052	6.81	0.35	0.028
North	139	0.592±0.060	0.606±0.580	0.609±0.058	7.41	0.34	0.028
East	77	0.556±0.058	0.589±0.061	0.593±0.061	7.77	0.89	0.063

Name given to the population, number of samples included (N) and allelic diversity indexes. H_O, mean observed heterozygosity (± SD); H_E, mean expected heterozygosity (± SD); uH_E, unbiased expected heterozygosity (± SD); A_R, allelic richness; P_A, private allele richness; F_{IS}, Inbreeding coefficient

Effective Population Size

The values of N_e obtained from ONESAMP range from 63.1 to 166.6. The credible intervals are large, overlapping in most cases, but we can see differences in some of the populations (Fig 5).

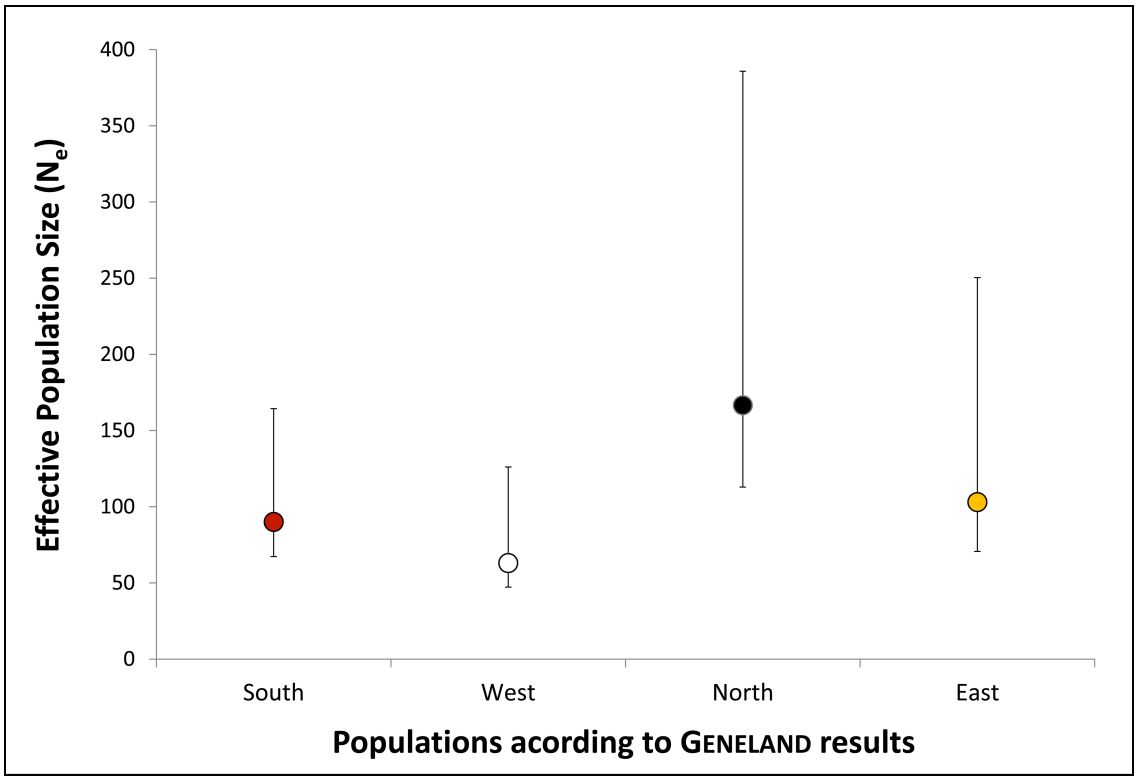

Fig. 5 Mean effective population size (N_e) and 95% credible intervals of the populations defined by GENELAND

Migration and Gene flow

BayesAss results for gene flow over the last few generations were almost identical for the six independent runs. Populations differ greatly in the average estimates of gene flow. Estimates of the mean migration rate (*m*) range from 0.003 to 0.315. According to the 95% confidence intervals, *m* estimates into and out of all populations are indistinguishable from zero, with the exception of migration from the South population into all the other populations, with an overall rate of 0.30 and non-overlapping 95% confidence intervals with all the other migration rates. Therefore we can consider the out-flow of emigrants from South greater than the almost non-existent in-flow of immigrants from the other populations (Fig 6).

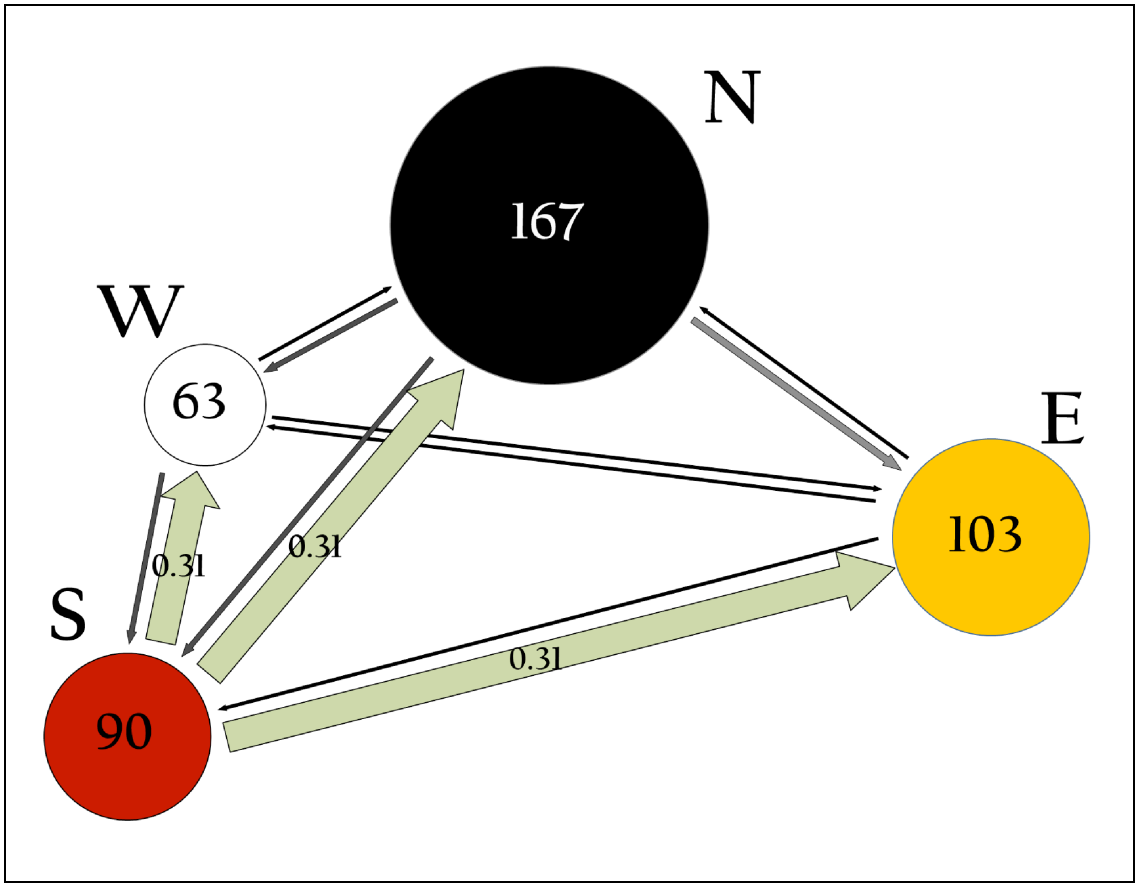

Fig. 6 Metapopulation flow chart displaying four asymmetrically connected populations or demes. The radius of the circles is proportional to the mean effective population size (N_e) of the populations; the arrows between the circles represent the migration rates (m) between the populations. The width of the arrows is proportional to the average migration rate. Unless otherwise indicated, the migration rate that corresponds with the arrows is below 0.013 and the confidence intervals include the "0", therefore are indistinguishable from zero. The three arrows with a higher m value (0.31) have non-overlapping confidence intervals with all the other migration rates for the same deme. The populations or demes of the metapopulation are South (S), West (W), North (N) and East (E)

Bottleneck

The sign test to test for heterozygosity excess in the populations detects a significant recent bottleneck for the SMM in three of the populations: South, North and East, and also with the TPM model in East (with 90 and 95% of SMM) and in North (with 95% of SMM and 12 of variance). According to those results, North and East have suffered a recent reduction in population size. Wilcoxon's Test finds a significant heterozygosity deficiency in all populations for the SMM and in North and East with the TPM model with the highest percentage of SMM (Table 4).

Table 4: Bottleneck results for SMM and six different variations of TPM

		SMM	TPM (Variance: 31)			TPM (Variance: 12)		
Analysis	Pop.	100%	95%	90%	80%	95%	90%	80%
Sign Test	South	0.045*	0.129	0.495	0.501	0.130	0.285	0.493
	West	0.127	0.128	0.133	0.286	0.126	0.127	0.251
	North	0.009**	0.114	0.110	0.259	0.039*	0.112	0.251
	East	0.002**	0.012*	0.046*	0.127	0.002**	0.046*	0.044*
Het. def.	South	0.034*	0.084	0.188	0.294	0.073	0.122	0.249
	West	0.034*	0.095	0.207	0.368	0.064	0.122	0.294
	North	0.001**	0.047*	0.084	0.170	0.020*	0.055	0.108
	East	0.001**	0.004**	0.029*	0.073	0.003**	0.020*	0.034*
Het. excess	South	0.971	0.927	0.830	0.729	0.936	0.892	0.773
	West	0.971	0.916	0.812	0.658	0.945	0.892	0.729
	North	0.999	0.960	0.927	0.847	0.984	0.953	0.905
	East	0.999	0.997	0.976	0.936	0.998	0.984	0.971

The table presents the output of three different tests using the Stepwise Mutation Model (SMM) and a Two-Phase Mutation Model (TPM). Sign test to detect recent bottlenecks; Wilcoxon Test of Heterozygosity deficiency (Het. def.) and Wilcoxon test of Heterozygosity excess (Het. excess). All the analyses were run for the four populations (Pop.): South, West, North and East. *indicates a significant p-value (below 0.05); ** marks p-values below 0.01

Discussion

Genetic structure and gene flow

Our results show the existence of weak but significant genetic structure (F_{ST} = 0.01; p < 0.0001) in *Parnassius apollo nevadensis*. One of the main factors driving the differentiation between populations is a clear and strong process of isolation by distance (r = 0.41). As expected, the correlation is even stronger (r = 0.53) if, instead of the straight line projected distances, we use a more realistic measure of the distance that a butterfly would travel from one location to another (see Methods).

The Bayesian clustering methods are not concordant in relation to the degree of genetic structure of Apollo butterfly populations in the Sierra Nevada. STRUCTURE was not able to delimit different populations, and the AMOVA results also support that the variation is greater inside than between groups. Considering just the AMOVA and STRUCTURE results, we could consider *P. apollo nevadensis* to be one large panmictic population with extensive gene flow between all the locations; this would be the preferred scenario for the conservation of the species. On the other hand, the results from GENELAND, multivariate methods (sPCA), the difference in private alleles between populations and the migration analyses (BayesAss) point towards a more structured and complex situation.

Δ*K* is the most widely method used to estimate the number of clusters, but it can not identify the best *K* when *K*=1 (Evanno et al. 2005), so we confirmed the best *K* by plotting the log likelihood values (Fig. 3) and this confirmed that *K*=1. The Structure bar plot (See Supplementary Figure S1) in which we present

the different sampling locations sorted geographically from West to East reinforces this result (K=1) as the assignment does not show any pattern. It has been recently proven that uneven sampling sizes in STRUCTURE often can lead to wrong inferences on hierarchical structure and downward biased estimates of the number of subpopulations (Puechmaille 2016). Computer simulations of large populations show that recent human barriers to gene flow can require hundreds of generations (one generation equals one year in *P. apollo nevadensis*) to allow genetic differentiation to become sufficiently high to be detected through F-statistics or some Bayesian clustering methods (Gauffre et al. 2008). In these situations GENELAND can be useful, as it has been previously reported that can detect weaker genetic spatial structure than other Bayesian clustering software, and it has been suggested as the preferred method to deal with recent human-induced changes in the landscape (Coulon et al. 2006). Accordingly, the different results could be due to a recent and moderate population differentiation that some analyses are not able to detect. In our case, all the results of GENELAND clearly agreed with the existence of four populations.

It should be noted that in some cases the delimitation of populations by clustering programs can be arbitrary, as a consequence of Isolation by Distance. This has been shown for Structure in continuously distributed populations (Frantz et al. 2003). The strong process of IbD is provably influencing the delimitation of the populations, but we should note that *P. apollo nevadensis* has a patchy distribution and that the subdivisions shown by Geneland are supported by sPCA, a method that makes no previous assumptions of HWE. It shows the existence of structure that clearly separates the locations assigned by GENELAND to the South population (A, B and C) from all the others, these three being the only ones in red in the plot (Fig. 4). A and C also have a non-significant pair-wise F_{ST}, suggesting they form part of the same population or that there is extensive gene flow between them. In all the GENELAND results J, K, L and M are always grouped in East, while A, B and C are always grouped in the South population. This may indicate some kind of recent filter that reduces gene flow from all the populations to South and East, or from these to all the others. In particular, the BayesAss results show that the South population is sending but not receiving migrants from North, East and West, while East, West and North do not exchange migrants at all, just receiving them from the South population. BayesAss analyses show this strong asymmetry in the rate of gene flow between these populations. These results on population structure and clustering analyses suggest that the intensity of gene flow has been changing over the generations. Recent (< 100 ya) differentiation would explain the apparently contradictory results between the analyses (Coulon et al. 2006; Gauffre et al. 2008). At first, and according to the results from STRUCTURE, gene flow between all populations was extensive enough to consider all of them together as a single population; however, during recent years the rate of gene flow has been lower (indistinguishable from zero between some of the populations), with the exception of the inflow of migrants from South to the other populations, which is still high and seems to be the only source of gene flow. The lower and asymmetric gene flow may be due to recent filters or barriers to gene flow (natural or man-made), or could be the consequence of population isolation because of the rise of temperature as a result of climate change: some populations in the highlands may have become isolated and this implies a certain grade of differentiation between populations.

The existence of population structure, despite some gene flow, and the asymmetry of gene flow between populations suggest the existence of metapopulation dynamics in *P. apollo nevadensis* (Howe et al. 1991). The asymmetric gene flow in this case has a source–sink dynamic (Howe et al. 1991), where the South population acts as the source deme in the metapopulation, while the other populations act as a sink (Fig. 6).

Differences in genetic diversity

The values of *P. apollo nevadensis* genetic diversity indices in the different populations are moderately high and there are no significant differences between them. The observed heterozygosity per population has a range of 0.556–0.597, which is slightly higher than in other butterfly species studied using microsatellite markers: Butterflies from non-migratory species considered to be non-endangered showed a range of 0.395–0.484 mean overall observed heterozygosity (Keyghobadi et al. 2002; Fauvelot et al. 2006; Sarhan 2006; Saarinen et al. 2014). In particular, in other *Parnassius sp.* populations studied with microsatellite loci (Meglécz et al. 1998; Keyghobadi et al. 1999, 2002; Petenian et al. 2005), the observed heterozygosity ranges between 0.33–0.68, a range into which our results fit well.

All the sampled populations show private alleles; P_A is inversely related to Nm, with N being the local population size and m the proportion of migrants (Slatkin 1985); this is then indicative of a small or well-structured population with low gene flow. The ANOVA confirmed a significantly lower number of P_A in the South population, concordant with the larger amount of gene flow from South to all the other populations (Fig. 6). The new alleles from the South population are distributed to the sink demes of the metapopulation, while the new alleles in sink populations are kept there, acting as a reservoir and maintaining the genetic variation (Morrissey and de Kerckhove 2009). This agrees with the general conclusion from modelling exercises that alleles from populations with more emigrants than immigrants have a great probability of ending up being present in all the demes of the entire metapopulation (Lundy and Possingham 1998).

Values of N_e are in part dependent on the sample size (England et al. 2006), but effective population sizes seem to be smaller in West and South populations (63 and 90, respectively), even if the South population has the second highest sample size after the North population. This means that the South population has a smaller N_e than expected. South acts as the main source deme for the entire metapopulation and a small population size in this source deme may indicate that it was historically big, but the size is decreasing.

The analysis of past reductions in population size (Bottleneck) detected significant results in most of the populations. The sign test was significant in South, East and North populations, indicating a recent bottleneck event. The strongest evidence of recent changes in population size is found for East and North populations, which show significant results also under the mixed model (TPM). Significant results of the sign test (evidences of bottleneck) are caused by a heterozygosity excess, but instead of heterozygosity excess, the Wilcoxon test, which is less robust to the violation of the assumptions than the sign test, but with more statistical power (Cornuet and Luikart 1996), shows a significant deficiency of heterozygotes

in all populations under the SMM. A recent bottleneck and deficiency of heterozygotes are compatible results in this case, where there is gene flow and where the loci can evolve under SMM. The loci used in this work have perfect dinucleotide repeats (Mira et al. 2014) and thus are expected to evolve mainly according to the SMM (Cornuet and Luikart 1996); it has been found that loci evolving under one step SMM can be in heterozygosity deficiency (instead of in heterozygosity excess) after a bottleneck (Cornuet and Luikart 1996). In addition, we have seen that there is gene flow between South and the other populations; and a population reduction (bottleneck) followed by an event of immigration will increase the proportion of rare alleles (input by migration) in the population, also resulting in a heterozygosity deficiency (Maruyama and Fuerst 1985). Non-random mating could also lead to heterozygosity deficiency, but given that F_{IS} values for the four populations were non-significantly different from zero, we can discard this option.

Factors shaping population structure

It has been seen for mobile species living in recently fragmented habitat that habitat loss after disturbance may lead to local population extinction but may augment genetic diversity in remnant local populations. This increase is due to gene flow by immigrants from disturbed sites (Fauvelot et al. 2006). In this model, known as the Refugee model (Porter 1999), genetic reorganization by movements of refugees (immigrants) causes deviations from genetic equilibrium, increasing genetic variation within the remaining populations and decreasing differentiation among them. According to this, a loss of suitable habitats could explain recent changes in population sizes (bottleneck results), and the magnitude of gene flow observed. This model can similarly explain the high allelic diversity (observed heterozygosity) because of the input of migrants (refugees) from other patches, as well as the heterozygosity deficiency.

In this case, the heterozygosity deficiency may be a product of the substantial input of immigrants from the South population, adding new alleles to the remnant populations. This would support the hypothesis that there has been a population size reduction in the sink populations. It has already been demonstrated for other butterflies species that anthropic disturbance can lead to a reduction in genetic diversity that is, however, maintained by dispersal from other populations (Takami et al. 2004).

The Refugee model is concordant with the life cycle of this species: a habitat that is becoming slowly less suitable for the species would decrease the survival rate. This process may be slow, as it affects the larval and adult phases differently, as they feed on different plants and have different requirements (Olivares et al. 2011; Radchuk et al. 2013). Possible factors that have been found to be the cause of the habitat becoming less suitable for the butterflies are the rise in temperatures and habitat loss through human alterations (Wilson et al. 2005; Wilson et al. 2007; Forister et al. 2010; Oliver et al. 2015)

The minor presence of private alleles and the asymmetric gene flow indicate that this population is almost isolated against the input of migrants, but not for the output of emigrants, which indicates the existence of filters to migration. The South population boundary towards the northeast (where it meets the North population) could be defined by the distance and presence of natural barriers (Fig. 1), which in some cases can be more influential than human disturbance (Leidner and Haddad 2010). In this case, the

natural barriers or filters are the mountain peaks of Sierra Nevada, which define a zone over 3000 metres of altitude where there are no Apollo butterflies, which the butterflies probably cannot cross easily (in white in Fig. 1). If we assume that butterflies do not usually fly over 3000 metres, the only other connection between the South and West populations (specifically with the D location) would be through zones affected by high herbivory, a heavily used road, the buildings of the tourist resort of Pradollano and the ski slopes (Fig. 1) – all of them unsuitable for butterflies. Although the ski station is closed in the summer, the slopes and their surroundings are still used for human activities, and its vegetation and natural cover have been seriously modified. In fact, there are no known localities with adults of *P. apollo* flying in these areas (personal obs.). The West population area is very close to this heavily modified zone (specifically the D location), and it has the smallest N_e. This adds to other work emphasizing the need to take into account the effect of human alterations that can be responsible for small population sizes and smaller N_e by making the habitats less suitable for the butterflies (Kati et al. 2012; Nyafwono et al. 2014).

The factors shaping the differences between East and North are similar: The "I" location is grouped within the North population by GENELAND, despite the fact that it is closer to most of the locations grouped within the East population (Fig. 1). None of the pairwise comparisons of F_{ST} values between I (North) and J, K, L and M (East population) are significantly different from zero, meaning that the separation must be recent (Gauffre et al. 2008). The East population is separated from the other populations by the mountain pass of "Puerto de la Ragua" (2041m), with a heavily used road and some important land transformations in recent years, which could be reducing the pass of migrants and could have disconnected populations on both sides. During our visits to sample butterflies in location I and East population, we never saw butterflies flying in the area surrounding the mountain pass, suggesting a limited use of this area by *P. apollo*. However, 40 years ago Apollo butterflies were abundant here (Gomariz Cerezo 1993; González-Megías et al. 2015).

There seems to be no strong barriers to gene flow between the other populations detected by GENELAND (North and West), apart for one of the rivers of Sierra Nevada, which could act as a filter to gene flow since the stream runs at a lower altitude than those used by the species. The higher connectivity between these locations (D, E, F, G and H) could be the reason why some GENELAND outputs differed in the assignment of some individuals sampled in locations E, F and H ("unresolved" circles in Fig. 1). These 18 individuals were assigned as belonging to either the North or West populations depending on the run. The two clusters were probably better connected in the recent past and gene flow might have been restricted recently by a regression in the distribution area of *P. apollo nevadensis*, as has been reported in other Spanish subspecies of *Parnassius apollo* and attributed to climate change (Ronca 2005; Ashton et al. 2009). For example, *P apollo gadorensis*, the southernmost Spanish subspecies, is now considered extinct (Barea-Azcón et al. 2008) and *P. apollo filabricus* has suffered a severe contraction in its distribution in Sierra de Baza-Filabres (Barea-Azcón et al. 2008; Martínez et al 2016 *in prep*). The loss of suitable habitat has been reported in many other butterfly populations because of climatic change (Wilson et al. 2005; Wilson et al. 2007; Gutiérrez-Illán et al. 2012). Warmer temperatures will move the distribution of butterflies to higher altitudes, which will result in smaller and more isolated areas of distribution, and in a different availability of host plants (Wilson et al. 2005; Ashton et al. 2009).

Conclusions

We have found evidence of significant isolation by distance, and of recent and weak, but still significant, genetic structure that separates the *P. apollo nevadensis* into four populations or demes. Those demes are connected by asymmetric gene flow in a source–sink dynamic. In agreement with the Refugee Model, there is a slightly high heterozygosity, compared with other butterflies, as well as signs of recent population reductions in some populations (North and East populations) and small effective population sizes (in South and West populations) that may be indicative of the fragility of the entire subspecies, because one of the populations with this "fragility" is the South population, which we have identified as the unique source deme of the metapopulation and is the only one with almost no private alleles.

The separation between populations seems to be coincident with some natural and human filters or barriers impeding the passage of dispersers. The presence of human barriers to gene flow and the migration analysis indicate that those barriers are recent. Given this, the next efforts in conservation should focus on removing these barriers or making it possible for individuals to pass through them. One of the most popular landscape strategies for reducing the effects of habitat fragmentation is the conservation or restoration of landscape corridors (Hilty et al. 2006; Milko et al. 2012) and this has been shown to be effective in the case of butterflies (Sutcliffe and Thomas 1996; Haddad 1999).

The next step in this study should be to attempt to identify the causes of these possible reductions in population size, and to try to reveal the causes of the asymmetric gene flow and the differences between populations. All of this may be linked to habitat change or climate change driving the recent reduction in population size, but we still do not know the factors that make habitat unsuitable and prevent passage for butterflies through some locations that are within the altitudes at which they are typically found.

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SUPPLEMENTARY INFORMATION

Article title: Parnassius apollo nevadensis: Identification of recent population structure and source–sink dynamics

Journal name: Conservation Genetics

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Supplementary Materials and Methods

Genetic Structure

The global Fixation Index (F_{ST}) was estimated to detect genetic structure, a significant higher value will mean a more structured population, while lower values will mean a less structured situation, no significant values will mean no population structure, an thus a panmictic population.

The Analysis of Molecular Variance (AMOVA) will allow us to know whether the main source of genetic variation is within individuals, within locations or among locations.

As a measure of genetic distance between each pair of locations, the pairwise F_{ST} values were estimated, to identify which sampling locations are genetically more distant to others.

All of this analyses where computed in GenAlEx, to determine if the estimated values are significant, the program shuffles (randomizes) the samples between locations, and performs the analysis for each shuffle (9999 times in this case), then compares if the obtained values for the given populations are greater than the obtained randomly (Peakall and Smouse 2006, 2012).

Clustering softwares

STRUCTURE and GENELAND are both based on Bayesian statistical frameworks and use Markov Chain Monte Carlo (MCMC) simulations to allow simultaneous estimation of many interdependent parameters in complex models (Clark 2005), but they rely on models with different assumptions and limitations. The methods group individuals in clusters with the minimum possible deviation from Hardy-Weinberg equilibrium (HWE) and minimum levels of linkage disequilibrium (LD). The deviation from HWE and the values of LD would be high if individuals from different randomly mating populations were incorrectly grouped into a single cluster, which then could not be considered a population (Guillot et al. 2009).

STRUCTURE runs MCMC iterations to assign each individual to the most likely cluster relative to the other individuals. For each run we must provide the number of clusters (K) in which Structure must try to group the individuals. The non-spatial model with non-previous assumptions of the membership of the individuals has the implicit assumption that individual cluster membership does not display any particular spatial pattern (Guillot et al. 2009).The Admixture option of this model assumes that each individual draws some fraction of its genome from each of the *K* populations, and that the allele frequencies are likely to be similar due to migration or shared ancestry. The use of the Admixture model with correlated allele frequencies is the recommended for being the most likely scenario in natural open populations (Falush et al. 2003).

GENELAND uses a Voronoi tesselation model that relies on the assumption that the domain occupied by the inferred clusters can be approximated by a small number of polygons or cells (Guillot et al. 2009). This kind of model focuses on the sampling sites and asumes that neighbouring points in the map are more likely to share the same membership than a set of pixels taken at random. Two sampling sites are considered to be neighbours if there is no other sampling site "around a straight line" that joins them (Guillot et al. 2009). Voronoi cells in GENELAND are not associated with individuals, but with 'territories'. Each territory can group several individuals within a single Voronoi cell. The geographic locations of the cells as well as their number are considered as parameters of the model and are estimated using an MCMC algorithm (François and Durand 2010). This component of the model is referred to as Free Voronoi tessellation, as the cells are constructed independently of the sampling sites(Guillot et al. 2009).

The burn (number of discarded iterations) in GENELAND is decided *a posteriori*, by observing the plot of the log likelihood values (corresponding to each chain of MCMC iterations) versus the value of *K*. The left tail of low log-likelihood values, corresponding to the first MCMC iterations before reaching the *plateau*, where the value of *K* is stabilized, are eliminated.

Multivariate methods

Unlike the Bayesian clustering methods, the multivariate techniques make no assumptions regarding HWE or LD that may account for spatial autocorrelation issues such as neighbour mating and sample distribution, and therefore are complementary to Bayesian approaches (Wilson et al. 2015).

Multivariate methods decompose a data table into a new set of uncorrelated (i.e., orthogonal) variables. These variables are the principal components or eigenvectors. The importance of each component is expressed by the variance of its projections or by the proportion of the variance explained (Abdi 2003). The variables that explain the major part of the variance are typically used to plot the data, and the position of the points will help us to understand the relationship between the data. As the new variables are uncorrelated, the axis can be flipped if necessary.

The Principal Component Analysis (PCA) analyzes the data (allelic frequencies) as continuous variables, and uses the Euclidian distances to compute the proximity between them.

The sPCA uses the allelic frequencies and the geographical coordinates to find the population scores that maximize the product of variance and the spatial autocorrelation. Large positive eigenvalues will correspond to global structures (In green, in Fig. 4), while large negative eigenvalues correspond to local structures (In yellow in Fig. 4). Their comparison allows differentiating global structures (patches and clines) from local ones (strong genetic differences between nearby samples in the same zone) (Jombart et al. 2008).

The analysis can be adjusted to different models of geographical connection between samples. For our analyses we choose the Neighbourhood by distance Connection Network (Type=5). In this network, two points are considered connected only if the distance between them is below a certain value. To allow the model to connect all the adjacent or neighbouring locations between them, we considered as neighbours all samples closer than 15.38 Km, as this is the distance between the two closest samples from the two more distant adjacent (or neighbouring) locations.

In some cases, the sPCA eigenvalues may not clearly indicate if the global and/or local structures are significant. However, if a global pattern exists among populations, a large number of alleles are expected to be correlated to at least one of the positive eigenvalues; and respectively if a strongest local pattern exists among individuals (within populations), a large number of alleles is expected to be correlated to at

least one of the negative eigenvalues. The local and a global test for spatial structures (Jombart et al. 2008) checks if that correlation is significantly different from a random sampling, helping us to know if global or the local structure needs to be considered.

In a PCA usually the data is ploted in a scatter plot with one or two axis (corresponding to the eigenvalues explaining the major part of the variance) and the samples are points whose position is relative to the value (score) of that sample for each of the plotted eigenvalues. As the sPCA uses the geographical information, we can see the results in a spatial context to see if there is any geographical pattern. ADEGENET asigns a colour to each point (sample) according to the value (score) of that sample for the eigenvalue explaining the major part of the variance, and then distributes the points according to their geographical coordinates.

Bottleneck

There are three different mutation models used in the software Bottleneck. The Stepwise Mutation Model (SMM), where each mutation creates a new allele by adding a single repeat to the sequence; the Infinite Allele Model (IAM) , in which each mutation can give any different allele independently of the size; and finally the Two-Phase Mutation model (TPM). The TPM is a more complex model that combines the SMM with the IAM. can be set up to combine the SMM and the IAM in different proportions and variances.

We chose two tests to analyse the models; the sign test is more robust than Wilcoxon test, but suffers however from low statistical power (Cornuet and Luikart 1996). The Wilcoxon Test for heterozygote excess or deficiency is considered appropiate for studies with less than 20 microsatellite loci (Piry et al. 1999) and any number of individuals sampled. This test identifies populations with signals of either heterozygosity excess or heterozygosity deficiency. A heterozygosity excess can be observed when there has been a recent population size reduction, because the decrease in allele number (loss of rare alleles) is faster than the reduction in heterozygosity (Maruyama and Fuerst 1985).

Supplementary Tables

Table S1 ANOVA of the private alleles (P_A) for all the populations

	Df	**Sum Squares**	**Mean Square**	**F value**	**p - value**
Population	3	4.692	1.565	3.431	0.024*
Residuals	48	21.897	0.456		

Df = Degrees of freedom, "*" marks a significant value; F value = Critical value of the F statistic in the distribution.

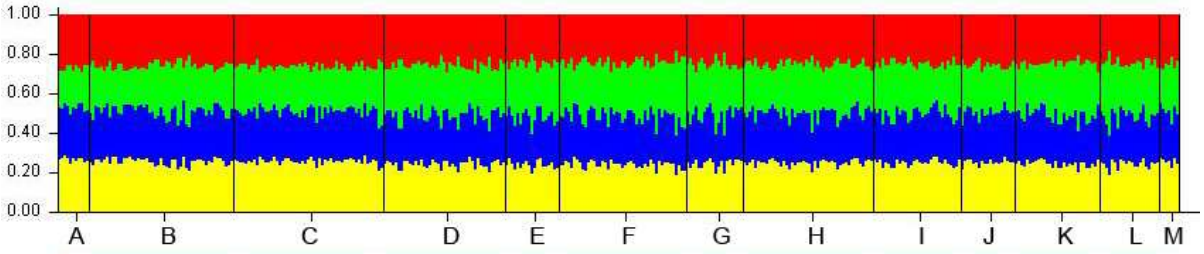
Table S2 Analysis of Molecular Variance

Source	**Df**	**Sum Squares**	**Mean Square**	**Est. Var.**	**Mol. Var**
Among Pops.	12	77.022	6.419	0.039	1%
Among Indiv.	383	1575.704	4.114	0.184	5%
Within Indiv.	396	1483.500	3.746	3.746	94
Total	791	3136.226		3.969	100%

Df = Degrees of freedom; Est. var. = estimated variance; Mol. Var = Percentage of the molecular variance

Supplementary Images

Supplementary figure S1 Bar plot from STRUCTURE for K=4 showing the assignment of all the individuals sorted by sampling locations. The locations are sorted from West to East, the names or the locations (codes from A to M) are respectively A: Caballo, B: Piuca, C: Chorrillo, D: Otero, E: Vacares, F: Papeles, G: Mirador, H: Postero, I: Hornillo, J: Chullo, K: Lagunilla, L: Almirez and M: Rayo



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