Distribución, Abundancia y Estructura genética de *Parnassius apollo* en Sierra Nevada

Distribution, Abundance and Genetic structure of *Parnassius apollo* in Sierra Nevada

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Tesis presentada por Óscar Mira Pérez para optar al grado de Doctor con Mención Internacional en Ciencias Biológicas por la Universidad de Granada.

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A la familia.

A las que me ha aguantado desde el inicio
y las que han ido llegando después.

Incluso a quien no supo quedarse.

“The same thing that your butterflies need [gene flow] is what cultures, ideas and people’s heads need. Isolation and poor diversity is not only bad for butterflies is bad for society and is destroying people lives”

Old computer-repair technician from Syria
- fixing my laptop while he heard me chattering about all my data on it-

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Abstract

ABSTRACT

Butterflies have been traditionally studied due to their sensitivity to climate and even more nowadays because of their vulnerability to climate change. *Parnassius apollo* (Linnaeus, 1758) is a papilionid (Lepidoptera) with a patchy distribution in the Palearctic ; it normally inhabits cold meadows and in the South of its distribution we found it isolated in mountain habitats. As a glacial relict it is even more sensitive to global warming, and in the last decades its populations have shown changes in their distribution, decline in their abundance and even extinction.

The populations of *Parnassius apollo* are locally small, and in the case of the iberian subspecies that inhabit in high mountain habitat, there is a documented elevation in their distribution range as a response to the increase of temperatures, that could be restricting the gene flow between patches.

Small and isolated populations are typically characterized by low genetic diversity, as a result of the combined action of processes such as genetic drift and inbreeding that would deteriorate the evolutionary potential and long-term viability of the species.

The Southernmost populations of this species are *Parnassius apollo gadorensis* Rougeot y Capdeville, 1969; *Parnassius apollo filabricus* Sagarra, 1933; and *Parnassius apollo nevadensis* (Oberthür, 1891) located in different mountains in Andalucía (Spain). The *gadorensis* subspecies was endemic from the Gádor mountain range (sierra de Gádor), and is now considered extinct; there are just two populations of the *filabricus*

subspecies left in sierra de Baza-Filabres; and *Parnassius apollo nevadensis* suffered a recent elevation in their distribution range in Sierra.

Parnassius apollo nevadensis is the perfect example of a glacial relict isolated in high mountain habitat, and a perfect model to study the effects of habitat fragmentation and climate change. In despite of the other subspecies decline we know nothing about their allelic diversity, level of inbreeding of the populations, population size or which variables determine their abundance. In addition, it is important to know more about their reproductive strategies as they are is linked to effective population size, genetic diversity and viability of the populations.

To study this we need highly variable molecular markers. We have developed microsatellite markers for the species as the microsatellite markers previously published did not amplify in samples from Sierra Nevada.

We successfully developed 20 microsatellite markers for *Parnassius apollo nevadensis*. These loci together have a non-exclusion probability of 0.00108 and 0.00001 for the first and second parent respectively.

By using those markers in females and their clutches we identified polyandry in six out of seven analysed females and therefore we can affirm that the *sphragi*s (waxy plug that the male applies to the female after the copula) may not always act as an effective blocking mechanism to avoid other males fertilising the female. Polyandry increases the genetic variance of the offspring, mitigates the effects of endogamy and is sometimes linked to larger clutch sizes.

From the 20 loci described, 13 were in Hardy-Weinberg equilibrium in all the locations analysed of Sierra Nevada. The genotypes of individuals belonging to those 13 locatons show a strong isolation by distance. However according to Structure and the AMOVA (Analysis of Molecular Variance) results there was no genetic structure and all the locations were well connected. On the other hand multivariate analyses (sPCA) and Geneland results allowed us to detect a recent genetic structure.

The 13 locations were grouped in four different subpopulations with a strongly asymmetric gene flow (source-sink) between them. The South subpopulation is the only one receiving significant input of gene flow, while the other three subpopulations act as a sink. We observe signs of recent genetic bottleneck in two of the sink populations and the effective population size is of a few hundred or less reproductive couples per subpopulation.

We carried out a capture-mark-recapture (CMR) experiment to estimate population sizes and survival rate from six of the 13 previous locations. In each location we performed the CMR study in two different areas and estimated the cover and diversity of plants. We perform a Partial Least Squares Regression (PLSR) with the variables of plant cover and diversity and other geographical and climatic variables to determine which variables can predict the population sizes and survival rates of the populations. Our results show a male-biased sex ratio (5 : 1 in average) larger than in other works with species of this genus. In addition we found great differences in population sizes from areas of the same location, suggesting high heterogeneity in small-scale.

The variables relevant to predict abundance are different between sexes; while females' abundance is affected negatively by the proximity to roads and positively by

plant species richness, males abundance is affected positively by the degree of slope and negatively by the presence of plants and bushes. Probably these differences are due to the differences in behaviour, as usually males are more active than females and may need bare-ground patches to increase their temperature. The survival rate is positively correlated with *Sedum sp.* cover (the larval host-plant) and negatively with high minimum temperatures in May and June, which probably are harmful to the larvae.

In the CMR study in the only known population of *P. filabricus* until 2016 we found lower densities of individuals per hectare than in Sierra Nevada; the molecular analyses also show a lower effective population size than *P. apollo nevadensis*. Heterozygosity on the other hand is higher than the estimates from Sierra Nevada; this could imply that the health of the population is better than expected, but can also be an effect of that remnant population acting as a sink of migrants from all the other populations that disappeared in the recent past.

Here we propose the deforestation of some pine trees planted in the 70s in Baza-Filabres that block the pass of dispersers between the studied population and potentially suitable habitats. In Sierra Nevada *Parnassius. apollo nevadensis* does not seem to require urgent management measures but it may be recommended to manage the heterogeneity and plant cover of zones with lower abundance of females to try to improve its situation.

RESUMEN

Los lepidópteros son un grupo tradicionalmente estudiado por su sensibilidad a las condiciones ambientales, y actualmente aún más debido a su sensibilidad al cambio climático. *Parnassius apollo* es un papiliónido (Lepidoptera) que se distribuye de forma parcheada por Eurasia, habitando normalmente praderas frías; pero en el sur de su distribución sus poblaciones se ven especialmente aisladas en lo alto de las montañas. Su condición de especie relíctica glacial la hace aún más sensible al calentamiento global y en las últimas décadas numerosas poblaciones ya han mostrado claros signos de cambios en su distribución, decrecimiento e incluso extinción.

Las poblaciones de *Parnassius apollo* suelen ser localmente pequeñas y en el caso de las subespecies ibéricas que habitan en alta montaña ya se ha visto su sensibilidad a la temperatura reaccionando con un ascenso en altitud como consecuencia, lo que podría estar restringiendo el flujo génico entre poblaciones. Pequeño tamaño poblacional y ausencia de flujo genético suelen conllevar a endogamia y a una pérdida de diversidad genética, lo que puede comprometer el potencial evolutivo de la especie y su viabilidad.

De las tres subespecies más meridionales de esta especie, *Parnassius apollo gadorensis* Rougeot y Capdeville, 1969 (Sierra de Gádor) está considerada extinta; de *Parnassius apollo filabricus* Sagarra, 1933 (Sierra de Baza-Filabres) quedan tan sólo un par de subpoblaciones y en *Parnassius apollo nevadensis* (Oberthür, 1891) se ha registrado un ascenso altitudinal en su distribución en Sierra Nevada.

Parnassius apollo nevadensis es el ejemplo perfecto de relicto glacial aislado en la alta montaña y un modelo ideal para estudiar los efectos de la fragmentación del hábitat y el cambio climático. Pese al claro declive de las otras dos subespecies meridionales no sabemos nada de la diversidad alélica, tamaño poblacional o nivel de endogamia de sus poblaciones en Sierra Nevada. Desconocemos también qué factores determinan el tamaño poblacional en los diferentes parches en los que se encuentra o qué estrategias reproductivas presenta, lo que podría ser muy relevante, ya que tiene repercusión en el tamaño poblacional efectivo, la diversidad genética y la viabilidad de las poblaciones.

Para poder determinar el grado de diversidad alélica y de flujo génico hacen falta marcadores moleculares altamente variables como los marcadores de regiones microsatélites. Por ello en este trabajo decidimos desarrollar nuestros propios marcadores moleculares, ya que se conocían para otras subespecies no funcionaron en nuestras poblaciones.

Hemos desarrollado con éxito 20 marcadores de regiones microsatélites de *Parnassius apollo nevadensis*. Estos marcadores combinados tienen una probabilidad de no excluir (erróneamente) a un posible padre candidato que no esté emparentado de tan solo 0.00108 y habiendo identificado a uno de los progenitores, una probabilidad de no excluir erróneamente al segundo candidato no relacionado de 0.00001.

Usando los marcadores tanto en hembras de *P. apollo nevadensis*, como en sus larvas; encontramos por primera vez evidencias de multi-paternidad en *Parnassius apollo*. Seis de siete hembras grávidas analizadas pusieron algunos huevos cuyas larvas o bien no concordaban con el genotipo del macho con quien fueron encontradas copulando o bien tenían alelos no maternos que pertenecían a más de un macho. Esto demuestra que la

presencia de *sphragis* y el comportamiento de guarda típico de esta especie no garantizan la monopolización de la hembra por parte del macho. Cabe destacar que la poliandria aumenta la variabilidad genética de la descendencia y mitiga los efectos de la endogamia, estableciéndose además en otras especies una relación directa entre la existencia de poliandria y el tamaño de puesta.

De estos 20 marcadores 13 demostraron estar en equilibrio de Hardy-Weinberg en todas las poblaciones de *P. apollo nevadensis* en las que se probaron y fueron usados para analizar el genotipo de individuos de *Parnassius apollo nevadensis* pertenecientes a 13 localidades distintas de Sierra Nevada. Los análisis realizados demuestran un fuerte aislamiento por distancia en las poblaciones de Sierra Nevada. Atendiendo a los resultados del programa STRUCTURE y del Análisis de la varianza molecular (AMOVA) hace centenares de generaciones *Parnassius apollo nevadensis* era una única población panmíctica sin estructuración genética. Pero análisis multivariantes (sPCA) y los resultados de GENELAND que parecen detectar una estructura genética más reciente y lo que muestran que nuestras 13 localidades estarían agrupadas en cuatro subpoblaciones distintas.

El flujo genético entre las cuatro subpoblaciones es asimétrico formando una relación fuente –sumidero en la que una subpoblación (de la cara sur de Sierra Nevada) parece ser la única con una salida significativa de individuos hacia las otras subpoblaciones y apenas ninguna entrada. Observamos indicios de reducciones recientes de tamaño poblacional en algunas de las poblaciones y las estimas de tamaño poblacional efectivo equivaldrían a tan solo unos cientos de parejas reproductoras por subpoblación.

Para obtener una estima actual del tamaño poblacional y tasa de supervivencia realizamos un estudio de captura-recaptura en de 6 de las 13 anteriores localizaciones de Sierra Nevada. En cada localidad realizamos dicho estudio en dos áreas, estimando además el grado de cobertura y diversidad vegetal de estas. Para averiguar qué variables se correlacionan con la abundancia y supervivencia de *P. apollo nevadensis* realizamos un análisis de regresión de mínimos cuadrados parciales (PLSR) con las variables de cobertura y diversidad de plantas y otras variables geográficas y climáticas. Los resultados muestran una gran diferencia de abundancia entre machos y hembras, teniendo una sex ratio media de 5: 1, mucho mayor que la de otros trabajos. Además vemos que las mayores diferencias en tamaño poblacional se dan entre áreas de la misma localización indicando una clara heterogeneidad a pequeña escala.

Las variables relevantes para predecir la abundancia de cada sexo son diferentes, mientras que la abundancia de hembras se ve afectada negativamente por la proximidad a carreteras y positivamente por la mayor riqueza de plantas; los machos se ven afectados positivamente por la pendiente del terreno y negativamente por la presencia de arbustos y plantas con flor.

Probablemente las diferencias encontradas entre machos y hembras se deban a que los machos son más activos que las hembras y necesitan zonas más despejadas y con mayor insolación para elevar su temperatura corporal. Por otra parte la supervivencia está correlacionada positivamente con la cobertura de *Sedum sp.* (la planta de la que se alimentan las lavas) y de plantas con flor, y negativamente con las temperaturas mínimas de mayo y junio; suponiendo que temperaturas mínimas más altas durante el estadio larval perjudicarían a su supervivencia.

Realizamos también un estudio de marcaje-recaptura en la que se consideraba la única población remanente de *Parnassius apollo filabricus* hasta 2016. Además en dos de los años, al final de la época de vuelo se tomaron muestras para analizar su diversidad alélica, heterozigosidad y tamaño efectivo poblacional. Las estimas de tamaño poblacional efectivo y de tamaño poblacional son bajas y no se pueden establecer diferencias entre años. La densidad de individuos por hectárea muestreada parece ser mucho menor que la de la *P. apolo nevadensis* justificando que sea considerada una especie en riesgo de extinción ya sea por factores genéticos o por eventos estocásticos. Los valores de diversidad alélica por el contrario son mayores que Sierra Nevada, lo que podría deberse a una buena salud de esta población o a que al ser esta en una de las únicas subpoblaciones que sobreviven de *P. apollo filabricus*, podría haber actuado de sumidero de inmigrantes de zonas que ya no son aptas para la especie, recibiendo un influjo de alelos que ha aumentado temporalmente su diversidad alélica.

Parnassius apollo filabricus ya se encuentra actualmente casi al máximo altitudinal de Sierra de Baza-Filabres y por tanto se prevé que el calentamiento global empeore aún más su situación. Proponemos en este trabajo mejorar la conectividad entre las zonas que actualmente ocupa y otras que pudieran ser adecuadas mediante la deforestación de los pinos con los que se reforestó en los años 70.

Parnassius apollo nevadensis no parece estar en una situación tan amenazada como *P. apollo filabricus*, pero aun así es preocupante la detección de subpoblaciones con un tamaño efectivo poblacional pequeño y la constatación de limitaciones al flujo génico. De entre los posibles filtros al flujo génico que limitan las cuatro poblaciones, algunos se podrían corresponder con barreras naturales como las zonas de mayor altitud de la Sierra

o la cabecera del río Genil, y otros están más relacionados con la actividad humana como las pistas de esquí y el Puerto de la Ragua. Quizás sería recomendable de cara a su futuro y conservación tratar de mejorar el hábitat de aquellas zonas con menor cantidad de hembras adecuando la comunidad vegetal y la heterogeneidad espacial a las que nuestros modelos asocian con altas abundancias de mariposas.

Capítulo 1

INTRODUCCIÓN GENERAL

CAPÍTULO 1

Introducción General

Durante el Holoceno las condiciones del planeta tierra han sido modificadas por el ser humano de forma rápida y posiblemente irreversible por lo que algunos autores sugieren llamar a esta era el "Antropoceno" (Zalasiewicz *et al.* 2011). Aunque es cierto que en ciencia debemos intentar dejar de lado el antropocentrismo y el sesgo que consciente o inconscientemente conlleva (Brown 1995; Katz 2000), este término no fue acuñado como un acto de egolatría humana sino como un aviso y un llamamiento a la responsabilidad que tenemos en el cambio global (Crutzen 2002; Steffen *et al.* 2011). Entre las consecuencias directas del cambio global antropogénico, en el ámbito de la conservación cabe destacar una brutal pérdida de biodiversidad que afecta a todos los grupos estudiados (Dirzo *et al.* 2014). Sólo en los últimos 500 años los humanos hemos desencadenado una ola de deterioro de ecosistemas, declive de poblaciones y extinciones cuyas tasas y orden de magnitud son comparables (si no mayores) a los cinco eventos de extinciones masivas recogidos en el registro fósil (Barnosky *et al.* 2011).

Uno de los más conocidos elementos del cambio global es el cambio climático mediado por el ser humano, algunos de cuyos efectos son una menor predictibilidad del clima, mayor variación interanual, el aumento de eventos climáticos extremos y el más conocido calentamiento global (Palmer y Räisänen 2002; Schär *et al.* 2004; Jalili *et al.* 2010). La actual tendencia de aumento rápido y global de la temperatura es muy robusta y las emisiones de CO_2 continúan creciendo incluso más rápidamente que en algunos de

los modelos más pesimistas de escenarios de cambio climático (Le Quéré *et al.* 2009). Una de las principales consecuencias del aumento de la temperatura es el movimiento global de los organismos a mayor latitud y altitud, ya sea huyendo de las altas temperaturas o acompañándolas mientras se extienden (Parmesan 2006).

Aunque a menor velocidad, los cambios climáticos y los desplazamientos de las áreas de distribución de las especies han sucedido constantemente durante toda la historia del planeta (Hewitt 2004; Houghton 2004). Entre estos, los periodos glaciales son probablemente los fenómenos históricos más importantes en cuanto a la determinación de la distribución de los organismos, y fueron los ocurridos en los tres últimos millones de años los que más han afectado a la diversificación y distribución de la fauna actual (Webb y Bartlein 1992). Durante los diferentes máximos glaciares se produjo un descenso generalizado de las temperaturas, las glaciaciones expandieron los casquetes polares y los glaciares crecieron, de forma que el resultante descenso en el nivel del mar, junto con la propia extensión de la capa de hielo y del permafrost, conectaba en estos periodos zonas previamente aisladas para los organismos terrestres (Hewitt 1999). Las glaciaciones son periodos de larga duración con una periodicidad de unos 100.000 años y entre ellas se producen periodos interglaciares relativamente más cortos y de clima más cálido (Lomolino *et al.* 2006). Glaciaciones y periodos interglaciares parecen conformar un ciclo de cambio climático lento y natural.

Estas glaciaciones produjeron la extinción de muchos organismos que no estaban adaptados a temperaturas tan bajas, siendo obligatorio para su supervivencia colonizar áreas más al sur; pero también permitieron a los organismos de regiones frías extender su rango de distribución en la misma dirección, siguiendo la bajada de las temperaturas.

Al contrario, la lenta subida de las temperaturas en los periodos interglaciares permitió a muchas especies recolonizar zonas de latitudes más altas de las que habían habitado sus ancestros y provocó que de entre los organismos adaptados al frío, únicamente los de latitudes más altas sobrevivieran (Hampe y Jump 2011). Este ciclo que alterna periodos glaciares e interglaciares conlleva ciclos de aislamientos y diferenciación genética entre poblaciones que son uno de los principales factores moldeadores de la actual estructura genética de las poblaciones y especies (Avise 1998; Hewitt 2000).

Hay que entender que los cambios en la distribución no son procesos simultáneos de migración de poblaciones, sino que son procesos más o menos lentos por los cuales se establecen nuevas poblaciones en el límite de expansión de la distribución (generalmente a mayor latitud o altitud en el caso del calentamiento del clima), suponiendo en muchos casos también la extinción de las poblaciones en el límite de retroceso de la distribución (Thuiller *et al.* 2008). Los relictos glaciares boreoalpinos estaban ampliamente distribuidos por Eurasia durante los periodos más fríos del Cuaternario, pero después de la última gran glaciación su distribución experimentó una retrogresión (Hampe y Jump 2011). No es raro que durante el lento cambio en la distribución, el límite inferior en proceso de regresión deje atrás algunas poblaciones rremanentes. Estas sobreviven en enclaves aislados más benignos (refugios) que las condiciones climáticas inhóspitas que los rodean y que no permitieron la supervivencia del resto de poblaciones de su especie. Los relictos glaciares se encuentran generalmente por debajo del límite latitudinal del resto de poblaciones de su especie y su antigüedad les confiere gran importancia para la conservación de la diversidad genética (Hampe y Jump 2011).

Frente a un cambio climático generalmente se contemplan tres posibles resultados: adaptación, migración o extinción (Parmesan 2006). El cambio climático antrópico es mucho más rápido que los procesos naturales, haciendo mucho más difícil la mayoría de procesos de adaptación; aunque considerando la migración como una respuesta adaptativa sí que encontramos actualmente muchas especies que han aumentado o cambiado su rango de distribución, fenología o fecha de migración como respuesta al cambio climático (Parmesan *et al.* 1999; Hüppop 2003; Parmesan 2006, 2007). Ante el cambio climático antrópico la mayoría de estos mecanismos son prácticamente imposibles para los relictos glaciares, ya que se encuentran en "trampas climáticas" de las que les resulta imposible migrar sin caer en zonas que resultan inapropiadas para su supervivencia y los otros mecanismos de adaptación no comportamentales resultarán probablemente muy lentos (Turlure *et al.* 2009). Los relictos climáticos viven en un hábitat con unas condiciones muy específicas, raras (para la latitud a la que se encuentran) y de distribución discontinua; en este caso los cambios graduales en la distribución no son una opción, siendo la única opción (si existe) un muy limitado ascenso en altitud (Parmesan *et al.* 1999; Wilson *et al.* 2005; Turlure *et al.* 2010). La mayoría de relictos glaciales han encontrado refugio a latitudes bajas en zonas de mayor altitud y hay que tener en cuenta que el efecto del cambio climático parece ser aún mayor en los organismos de montaña, que podrían estar enfrentándose a incrementos de temperatura aún más extremos y en intervalos de espacio o tiempo menores (Nogués-Bravo *et al.* 2007; Wilson *et al.* 2007). Dada esta restricción en ocupación de nuevas zonas por los relictos glaciales y la especial sensibilidad de los ambientes montanos, la conservación de los relictos debe anticiparse a los cambios que producirá el clima en las

zonas actualmente ocupadas centrándose mucho más en los recursos ecológicos y estructura de la comunidad vegetal (Turlure *et al.* 2009).

La diversidad genética aumenta la capacidad de los organismos de enfrentarse a estos cambios en su ambiente, de forma que mantener la diversidad genética significa mantener el potencial evolutivo y es fundamental para la supervivencia a largo plazo y recuperación de las especies en peligro, y por tanto para definir su estado de conservación (Falk *et al.* 2001; Frankham 2005). Los conservadores previamente a gestionar las poblaciones deben entender su estado de conservación estudiando la diversidad genética de la especie en cuestión, la demografía, así como las características y diversidad de la comunidad de la que forma parte (Primack 2001). Es preciso comprender la distribución espacio-temporal de las poblaciones, lo que incluye distribución y procesos actuales (flujo génico, grado de aislamiento, persistencia de la población, etc.) y los procesos históricos que la originaron (historia demográfica, deriva genética, filogenia, origen biogeográfico, etc.) (Frankham *et al.* 2002; Primark 2001). En el proceso de conservar diversidad genética de una especie pueden existir factores históricos, económicos o temporales que limiten el número de poblaciones que podemos conservar o proteger de forma efectiva, pero con esta información podremos identificar los factores que afectan a su estabilidad y así también podremos identificar qué poblaciones han estado históricamente aisladas; las poblaciones históricamente aisladas generalmente forman grupos monofiléticos con varianza genética propia (unidades evolutivamente significativas) y se debe dar prioridad a su conservación ya que mantener esas poblaciones significará conservar los genotipos únicos que conforman la historia evolutiva de la especie (Moritz 1994).

Tres factores clave para entender el estatus de conservación de una especie desde el punto de vista genético son, el grado de endogamia, la perdida de diversidad genética y la acumulación de mutaciones (Frankham 2005). Además, estos tres factores dependen a su vez de otros elementos como la tasa de mutación, las respuestas comportamentales y factores demográficos como el tamaño de las poblaciones y el nivel de conectividad entre ellas (Frankham *et al.* 2002). Las tasas de mutación son generalmente bajas localmente (para cada población), por lo que en poblaciones pequeñas la tasa de variación perdida por deriva genética suele superar a la generada por mutación poligénica (Swindell y Bouzat 2005), así pues la aparición de nuevos alelos beneficiosos es poco probable y esto hace que si tenemos un pequeño tamaño poblacional la diversidad genética, y a largo plazo la viabilidad de la población, dependa críticamente de que el flujo génico distribuya genes potencialmente adaptativos entre las poblaciones (Swindell y Bouzat 2005). Para facilitar el flujo génico permitiendo la entrada de nuevos genotipos adaptados a otros ambientes y la re-colonización de hábitats adecuados disponibles, es necesario y primordial mantener las conexiones entre las diferentes poblaciones en las que se distribuye una especie (Fischer y Lindenmayer 2007; Buchalski *et al.* 2015). Cuando la fragmentación del hábitat impide el flujo génico entre poblaciones es más probable que se produzca una pérdida de diversidad genética, especialmente y más rápidamente en poblaciones pequeñas, lo que facilitará la acumulación de mutaciones deletéreas y puede ocasionar un aumento en el grado de endogamia; lo que afectará negativamente la eficacia biológica de los individuos y en última estancia perjudicará a la viabilidad de las poblaciones (Lynch *et al.* 1995; Saccheri *et al.* 1998).

Este proceso será teóricamente más rápido cuanto más pequeña sea una población, ya que en poblaciones pequeñas es mayor la probabilidad de cruzamientos entre individuos emparentados y de perdida de variación genética por deriva genética (Frankham *et al.* 2002; Segelbacher *et al.* 2010). Estos factores que afectan a la diversidad genética y a la viabilidad de las poblaciones no sólo se ven acrecentados por el pequeño tamaño poblacional, sino que además tienden a reducir el propio tamaño poblacional, reduciendo el potencial adaptativo de la especie largo plazo (Frankham 2005) y entrando en un ciclo al que se ha llamado "vórtice de extinción" (Gilpin 1986; Ugelvig *et al.* 2012). Además, las poblaciones pequeñas son más sensibles a procesos aleatorios como perturbaciones ambientales y fluctuaciones en la supervivencia o la fecundidad (Keller y Waller 2002). Por todo esto el tamaño poblacional está estrechamente relacionado con la diversidad genética y se considera una de las medidas más correlacionadas con el riesgo de extinción (O'grady *et al.* 2004; Puechmaille y Petit 2007).

Dada la estrecha relación de la diversidad genética con el potencial evolutivo y viabilidad de las poblaciones, la primera está considerada por la International Union for Conservation of Nature (IUCN), junto con la diversidad de especies y de ecosistemas, como uno de los tres principales niveles de biodiversidad en los que se recomienda hacer hincapié en cuanto a su conservación (Mcneely *et al.* 1990). De entre las principales amenazas a la biodiversidad local a las que se enfrentan los programas de conservación, dos de las más importantes son la transformación del hábitat (fragmentación, cambio en sus usos, contaminación, etc.) y la competencia con especies invasoras, ya sean especies exóticas introducidas o especies que han alterado su rango de distribución

recientemente (Wilcove *et al.* 1998). Tanto la transformación del hábitat como el avance de especies invasoras suelen estar directa o indirectamente asociados a la actividad humana. Las poblaciones pueden responder en diferentes grados y velocidad a estas amenazas según sus requerimientos y características, pero en general si no se frenan estas amenazas, el resultado es una disminución en su tamaño, pudiendo llega a la extinción de alguna de estas poblaciones y por tanto a una pérdida de diversidad global (genética y ecológica) de la especie (Hughes *et al.* 1997; Thomas *et al.* 2004).

Las mariposas, como organismos ectotermos dependen fuertemente del clima y se encuentran entre los animales más ampliamente estudiados en cuanto a distribución y demografía (Pollard y Yates 1994; Parmesan *et al.* 1999). Dada su gran sensibilidad a los cambios en el ambiente, han demostrado sobradamente ser buenos indicadores de respuesta ecosistémica a las variaciones climáticas y tener una gran vulnerabilidad al cambio climático (Parmesan *et al.* 1999; Roy y Sparks 2000; Wilson *et al.* 2005; Wilson *et al.* 2007; Forister *et al.* 2010; Wilson y Maclean 2011; Radchuk *et al.* 2013; Descombes *et al.* 2015; Oliver *et al.* 2015).

Parnassius apollo (Linnaeus, 1758) es un papiliónido (Lepidoptera, Papilionidae) con una distribución paleártica en el continente euroasiático (no incluyendo por tanto el Norte de África ni la península Arábica). Su distribución es generalmente parcheada y discontinua, encontrando múltiples poblaciones y subespecies descritas desde España hasta el sur de Fenoscandia (Carelia y Finlandia) y el este de China. En el noreste de Europa y Siberia la encontramos dispersa en prados de baja altitud; pero en el resto de su distribución como relicto de fauna glacial que es, encontramos pequeñas poblaciones restringidas a sistemas de alta montaña como Sierra Nevada, Pirineos, Alpes, Cárpatos, la

cordillera del Cáucaso o el macizo de Altái (Descimon 1995; Nakonieczny *et al.* 2007; Todisco 2008).

El origen más probable de *Parnassius apollo* (y posiblemente de todo el género) parece estar en Asia Central, desde donde esta mariposa se expandió por toda Europa (Fig 1-1). Los análisis de ADN mitocondrial indican que *P apollo* alcanzó los límites más alejados de su distribución hace al menos 60.000 años, entre el final del último periodo interglaciar (Riss-Würm) y el inicio de la última glaciación (Würm) (Todisco 2008; Todisco *et al.* 2010). Más de 200 subespecies de *P. apollo* han sido descritas, muchas fomentadas por el coleccionismo y basadas en ligeros cambios en la tonalidad de la coloración de sus alas; pero el hecho de que sus poblaciones estén tan dispersas y aisladas hace que otras tantas sí que posean diferenciación genética suficiente como para representar porciones aisladas de variabilidad genética (Todisco *et al.* 2010).

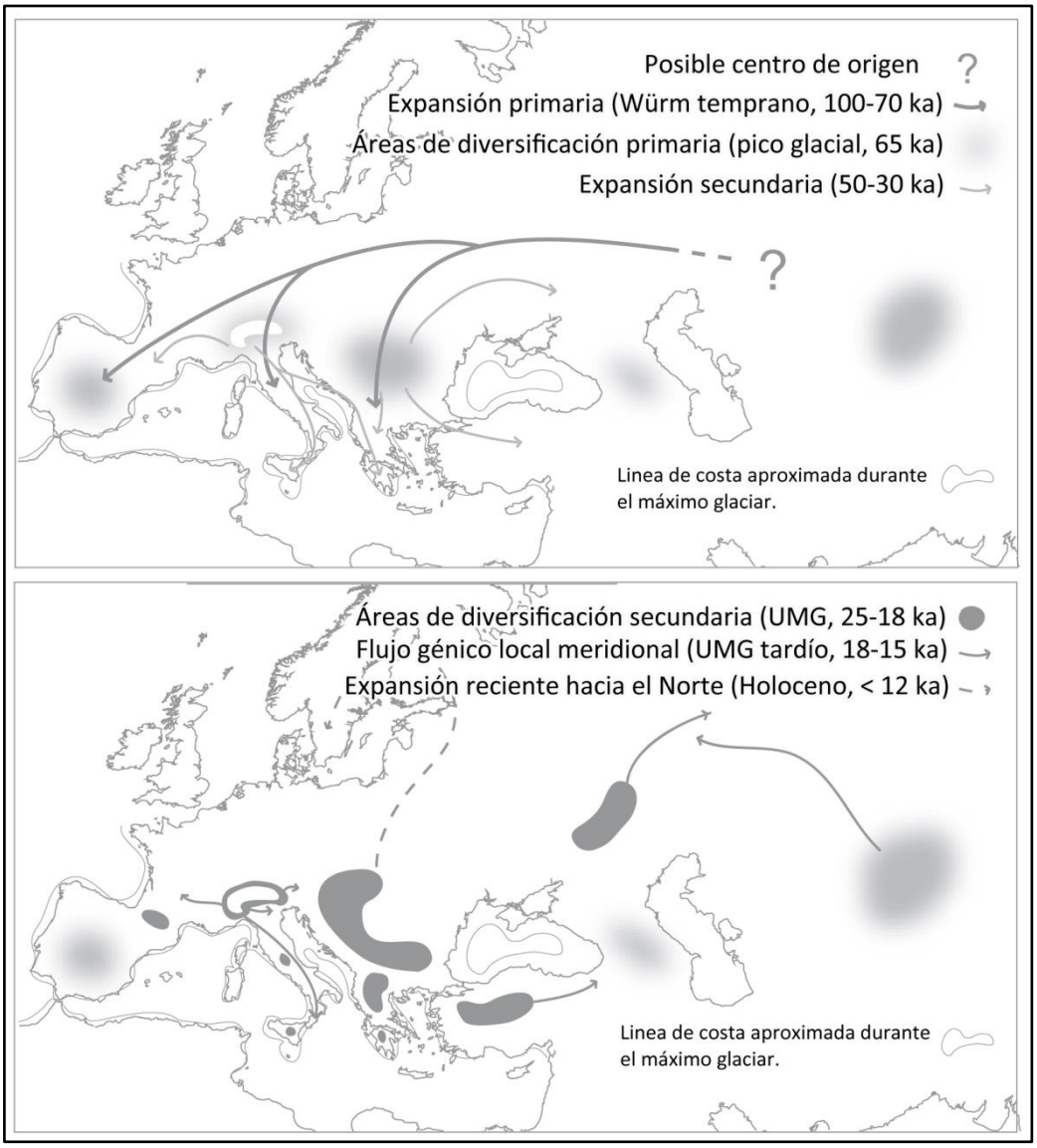

Figura 1-1: Reconstrucción hipotética de la evolución del rango de distribución de *Parnassius apollo* según los linajes exclusivos y su relación evolutiva de acuerdo con el análisis de DNA mitocondrial. UMG = último máximo glacial; 1 ka = 1000 años atrás. [Modificado de Todisco *et al*. (2010)].

Como relicto glaciar a menudo aislado en pequeñas poblaciones, *P. apollo* tiene unos requerimientos ecológicos muy particulares (Fred y Brommer 2005; Fred *et al.* 2006) y ha demostrado ser muy sensible a las alteraciones del hábitat y al cambio climático (Ashton *et al.* 2009; Todisco *et al.* 2010). El pastoreo excesivo, la contaminación, el turismo no controlado, el coleccionismo y en general la pérdida o transformación del hábitat parecen ser algunas de las causas que produjeron descensos importantes en sus tamaños poblacionales y que requirieron acciones de conservación (Gomariz-Cerezo 1998; Habel *et al.* 2009; Łozowski *et al.* 2014; Fred y Brommer 2015). Por todo esto y por el aumento de las temperaturas, desde las últimas décadas del siglo 20 hemos sido testigos del declive de numerosas poblaciones de *Parnassius apollo* en al menos 12 de los 28 países en los que se ha estudiado, y en al menos tres de ellos se denunció su extinción (Collins y Morris 1985; Van Swaay y Warren 1999; Descimon *et al.* 2006; Nakonieczny *et al.* 2007; Van Swaay *et al.* 2010). Se calculó que sus poblaciones europeas se habían reducido en un 30% durante los años 90, por lo que fue considerada una de las mariposas más amenazada de Europa por la IUCN habiendo sido catalogada como "Vulnerable" (Baillie *et al.* 1996), se encuentra recogida en el Libro Rojo de las Mariposas Europeas (Van Swaay y Warren 1999), la encontramos también listada en la directiva Habitat de la Union Europea (Habitats Directive, Annex IV, Appendix II, EEC 92/43/EWG), está también contemplada en las listas de la Convención sobre el Comercio Internacional de Especies Amenazadas de Fauna y Flora Silvestres (CITES), y considerada como sujeta a un alto riesgo de extinción (HR) por cambio climático (Settele *et al.* 2008).

Si hablamos de la sensibilidad a los cambios climáticos y los estrictos requerimientos de *Parnassius apollo* como relicto glaciar, quizás el lugar donde esto es más acentuado es en las poblaciones más meridionales, dónde sólo puede encontrar esos requerimientos para completar su ciclo vital en pequeños parches de hábitat de alta montaña. En España se han llegado a describir 21 subespecies distintas, cada una de ellas aislada en un macizo montañoso distinto (Gómez-Bustillo y Fernández-Rubio 1973). Las poblaciones meridionales de esta especie se encuentran aisladas desde el inicio del último periodo interglaciar, y en consecuencia una gran fracción de la variación genética mitocondrial de la especie está concentrada en estas poblaciones relictas meridionales (España, Sicilia y sur de Grecia) (Todisco *et al.* 2010). Las poblaciones españolas son además las más occidentales y entre las subespecies descritas se hayan al menos dos linajes mitocondriales altamente divergentes que forman dos subespecies distintas bien definidas y diferenciadas, de gran valor evolutivo: *Parnassius apollo nevadensis* (Oberthür, 1891) en Sierra Nevada (Andalucía) y *Parnassius apollo hispanicus* Oberthür, 1883 en Albarracín (Aragón) (Todisco *et al.* 2010).

Gómez-Bustillo y Fernández-Rubio (1973) agruparon a *Parnassius apollo nevadensis* (Sierra Nevada), *P. apollo filabricus* Sagarra, 1933 (Sierra de Baza-Filabres) y *P. apollo gadorensis* Rougeot y Capdeville, 1969 (Sierra de Gádor) dentro del grupo racial "Meridional". Estas subespecies españolas corresponden a las poblaciones más meridionales conocidas de *Parnassius apollo*: *P. apollo nevadensis* y *P. apollo filabricus* tienen haplotipos distintos y recientes análisis de DNA mitocondrial sugieren que podrían pertenecer a linajes diferentes (Todisco *et al.* 2010, Sánchez-Prieto *et al. en prep.*). Actualmente y debido al cambio climático, algunas subespecies españolas de

Parnassius apollo ya han sufrido regresiones en su área de distribución y un aumento de su rango altitudinal (Ronca 2005; Wilson *et al.* 2005; Ashton *et al.* 2009), llegando en algunos casos como el de *P apollo filabricus* a la desaparición de la mayoría de sus poblaciones o en el más extremo de los casos, como *P. apollo gadorensis*, a la extinción (Barea-Azcón *et al.* 2008). Las poblaciones de Sierra Nevada fueron anteriormente consideradas como amenazadas por el exceso de turismo (Gomariz-Cerezo 1998); su rango altitudinal ya era considerado de los más altos, yendo desde los 1850 hasta los 2500 metros sobre el nivel del mar (Olivares *et al.* 2011), pero en años recientes hemos podido observar un ascenso en sus límites de distribución, llegando a alcanzar en algunas zonas más de 2700 metros de altura (González-Megías *et al.* 2015).

Parnassius apollo nevadensis es por tanto el ejemplo perfecto de relicto glacial aislado en la alta montaña y un modelo ideal para estudiar los efectos de la fragmentación del hábitat y el cambio climático. Es importante además trabajar a nivel intra-específico cuando trabajamos con relictos climáticos, ya que los trabajos suelen centrarse en niveles taxonómicos mayores, pero en realidad la mayoría de estas especies solamente sufren una limitación climática en algunas poblaciones del borde de su límite de distribución, y son precisamente estas las que han acumulado distintas trayectorias evolutivas (Parmesan 2006; Hampe y Jump 2011). El estudio de estas poblaciones al borde de su límite de tolerancia es lo que proporciona un marco que permite trabajar con hipótesis y predicciones sobre las respuestas de las poblaciones naturales a los cambios en su hábitat. Por ello se insiste tanto en el caso de los relictos en la importancia de anticipar los efectos que tendrán los cambios en el clima y de centrar más la gestión en los recursos ecológicos y la estructura de la vegetación de lo que tradicionalmente se ha

hecho (Turlure *et al.* 2009). En el caso de *P. apollo nevadensis* tiene pocas o ninguna posibilidad de cambiar su distribución si el hábitat deja de ser adecuado; un entendimiento de los mecanismos ecológicos que condicionan el uso del hábitat causando los patrones de su distribución sería clave en estos casos (Turlure *et al.* 2010) y aun así hay poca o ninguna información sobre muchos de los aspectos que podrían parecer relevantes para conocer el estado de conservación y los requerimientos de esta mariposa. Por otro lado tenemos a *P. apollo filabricus*, que con el mismo grado de desconocimiento sobre su variabilidad genética o condicionantes, sí que ha sufrido un claro declive en sus poblaciones (y la extinción de la mayoría de ellas) y es un claro ejemplo de lo que podría suceder si no se consigue frenar el proceso de retroceso de sus poblaciones.

Pese a la idoneidad de *Parnassius apollo* como ejemplo de relicto glacial y a su sensibilidad al cambio climático (Ronca 2005; Wilson *et al.* 2005; Ashton *et al.* 2009) sabemos muy poco sobre su estado de conservación y su biología. Los machos de *P. apollo* como los de otras especies de lepidópteros, ponen un tapón de cópula en las hembras (*sphragis*) y muestran un comportamiento de guarda (cópula prolongada) que llega a durar horas (observación personal). En *Panassius mnemosyne* se han reportado la pérdida de *sphragis* y que su presencia no asegura que hayan sido inseminadas por un único macho (Vlasanek y Konvicka 2009). Desconocemos si esto acontece del mismo modo en *P. apollo*, pero es importante para una especie con distribución parcheada y poblaciones localmente pequeñas, ya que la poliandria puede incrementar la probabilidad de que las hembras tengan mayor eficacia biológica, ya sea por ser fertilizadas por machos más compatibles o por tener descendencia con mayor diversidad

genética (Tregenza y Wedell 2000; Zeh y Zeh 2001). La poliandria puede servir por tanto para mitigar los efectos de la endogamia y aumentar la variabilidad genética de la descendencia, aumentando así su potencial adaptativo, y podría tener un efecto sobre el tamaño efectivo poblacional a través de diferencias en la varianza en el éxito reproductivo de los machos (Franham et al 2002). Además se ha establecido una relación directa entre la poliandria y una mayor producción de descendencia en otros insectos y artrópodos (Newcomer *et al.* 1999; Arnqvist y Nilsson 2000).

Para poder determinar aspectos clave del estado de sus poblaciones, como la diversidad genética, estructura genética, flujo génico o las estrategias reproductoras y definir el estado de conservación actual de algunas las poblaciones de *P. apollo* es primordial el uso de herramientas moleculares, pero apenas hay marcadores moleculares descritos para la especie y ninguna información sobre su estructura poblacional o diversidad genética. Encontramos apenas un trabajo sobre diversidad genética en otra subespecie del Norte de Europa (Habel *et al.* 2009) en la que las poblaciones resultan monomórficas para los marcadores moleculares usados. Para estudios sobre genética poblacional actual y estudios de parentesco es necesario disponer de marcadores de regiones del genoma altamente variables, entre los cuales los más usados suelen ser los marcadores de regiones microsatélites (Selkoe y Toonen 2006). Las regiones microsatélite corresponden a *loci* codominantes, no codificantes y altamente variables en las que encontramos repeticiones en tándem de un par o más de nucleótidos (Jarne y Lagoda 1996); el hecho de que dichos *loci* sean no codificantes, es decir no estén sometidos a selección, y su alta variabilidad y rápida evolución los hace ideales en estudios de ecología (ver Capítulo 2). La escasez de marcadores de regiones

microsatélites para *Parnassius apollo* puede deberse a las dificultades asociadas a caracterizar dichas regiones en lepidópteros (Zhang 2004); en este grupo encontramos en diferentes *loci* del genoma más de una copia de algunas de las regiones microsatélites; y de las regiones que flanquean estas copias y que deberían ser usadas para amplificarlos específicamente, en ocasiones son también similares en diferentes *loci* (Meglécz *et al.* 2004; Zhang 2004).

Es preciso por tanto desarrollar las herramientas moleculares adecuadas y luego tratar de obtener toda la información que resulte relevante para definir el estado de conservación de *Parnassius apollo* en Sierra Nevada. Los marcadores de regiones microsatélites permiten estimar la diversidad genética o alélica de las poblaciones, lo que como ya hemos dicho es una estima del potencial evolutivo de la especie (Frankham 2005). Asimismo, las medidas de heterocigosidad permiten aproximar el tamaño poblacional efectivo, el nivel de endogamia o la probabilidad de que la población haya pasado por una reducción drástica en su tamaño poblacional, que haya podido ocasionar un cuello de botella genético. También comparando la información de diferentes áreas podemos saber el grado de conexión que existe entre ellas (estructura genética y flujo génico). Toda esta información es primordial para definir la viabilidad de la especie y su estado de conservación (Bohonak 1999; Frankham *et al.* 2002; Frankham 2005). Obtener esta información sobre *P. apollo nevadensis* es muy necesario con el fin de avanzar en el conocimiento de esta subespecie; conocer su estado actual y comprender mejor su contexto son posiblemente los primeros pasos para prevenir los efectos de nuestro cambio climático en una especie que ha sobrevivido ya a un considerable pero más lento

calentamiento desde que llegó por primera vez a nuestras tierras y evitar así que sufra el destino de *P. apollo gadorensis* y *P. apollo filabricus*.

Objetivos de la tesis

· 1) Identificar regiones microsatélite en el genoma de *Parnassius apollo nevadensis* y desarrollar cebadores para PCR que nos permitan amplificarlas y obtener genotipos *multilocus* para caracterizar los individuos y poblaciones (Capítulo 4).

· 2) Establecer si la presencia del tapón de cópula (*sphragis*) es realmente un indicador fiable de monogamia (Capítulo 4).

· 3) Determinar si existe estructura genética en las poblaciones de *Parnassius apollo* en Sierra Nevada y, en el caso de que exista, establecer el grado de flujo génico entre poblaciones como indicador del grado de estructuración (Capítulo 5).

· 4) Estimar el grado de diversidad genética de las poblaciones mediante parámetros como la diversidad alélica, riqueza alélica, heterozigosidad y otros indicadores, así como su tamaño poblacional efectivo y si existen diferencias entre poblaciones. Se analizará si hay indicios de recientes cuellos de botella genéticos (Capítulo 5).

· 5) Estimar el tamaño poblacional de diferentes zonas de Sierra Nevada donde habita *Parnassius apollo*, analizar las diferencias entre zonas e identificar qué variables se correlacionan con una mayor abundancia, definiendo así hábitats de mayor calidad (Capítulo 6)

· 6) Calcular la diversidad genética de la única población restante de *Parnassius apollo filabricus* conocida hasta 2016 y estimar su tamaño de censo y su tamaño efectivo de población y la posibilidad de que hayan pasado por un reciente cuello de botella y comparar dicha información con la de las poblaciones de *Parnassius apollo nevadensis* (Capítulo 7).

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Capítulo 2

METODOLOGÍA GENERAL

CAPÍTULO 2

Métodología General

Zona de estudio

El sistema Bético es un sistema montañoso de más de 600 Km de longitud formado durante la orogenia alpina (Cenozoico) que se distribuye desde Cádiz hasta las Islas Baleares. La cordillera Penibética, la más meridional del mismo, consta de numerosas sierras que encontramos en las provincias de Granada y Almería. Entre ellas se encuentran Sierra Nevada y las sierras de Baza y Filabres (Martín Martín *et al.* 2008)

Las sierras de Baza y Filabres constituyen una sola sierra continua de 60 km de largo por unos 20 de ancho y unas 150 000 hectáreas de superficie, el pico más alto se encuentra en la Sierra de Baza y alcanza los 2 271 metros sobre el nivel del mar. Pese a su continuidad, ambas sierras tienen diferente composición: La sierra de Baza (Granada) está formada principalmente por materiales pertenecientes al complejo Alpujárride, esencialmente rocas calcáreas (calizo-dolomíticas) del Triásico de 240 a 210 Ma; mientras que la sierra de Filabres (Almería) está compuesta principalmente por rocas metamórficas (esquistos y cuarcitas) pertenecientes al Complejo Nevado-Filábride con materiales de más de 250 Ma (Sanz De Galdeano y López Garrido 2016). La sierra de Baza (53 649.41 ha) está protegida como Parque Natural y dos áreas están reflejadas en la Red Natura 2000 como Lugares de Interés Comunitario (LIC, ES6140001 y ES6140010) de las cuales una (ES6140001) es considerada Zona Especial de Conservación (ZEC). Por

el contrario en la sierra de Filabres únicamente encontramos la designación de LIC y ZEC (ES6110013) en las 6 615.83 ha del área de Calares (Junta de Andalucía 2017).

Sierra Nevada constituye una unidad geográfica bien diferenciada con una disposición este-oeste de 94 kilómetros y una amplitud norte-sur de 38 kilómetros, en los puntos más alejados, llegando a abarcar más de 300 000 ha. En ella encontramos el pico del Mulhacén, que alcanza las altitudes más altas que podemos encontrar en la península ibérica (3482 m). La parte central de Sierra Nevada la forma un núcleo compuesto principalmente por los materiales metamórficos pertenecientes al Complejo Nevado-Filábride, mientras que a su alrededor a menor altitud, encontramos una zona de materiales calizos del Complejo Alpujárride (Martín Martín *et al.* 2008). En 1989 fueron declaradas Parque Natural 172 238.05 ha de Sierra Nevada y en 1999 finalmente 85 883 de esas hectáreas fueron declaradas Parque Nacional. Sierra Nevada tiene dos áreas consideradas LIC (ES6140004 y ES6140009), de las cuales una (ES6140004) es considerada ZEC y Zona de Especial Protección para las Aves (ZEPA) por la Red Natura 2000 (Junta de Andalucía 2017).

Tanto Baza-Filabres como Sierra Nevada poseen en general un clima mediterráneo de montaña y en ellas encontramos entre otros los bosques más meridionales de *Pinus sylvestris*, pero su altitud y situación meridional, junto con la heterogeneidad de su orografía posibilita múltiples mesoclimas que permiten que junto con la vegetación esclerófila propia del monte mediterráneo podamos encontrar también zonas con abundancia de robles, arces y otros caducifolios más propios de climas más húmedos (en microclimas como algunos valles) o desiertos fríos de alta montaña en las altas cumbres de Sierra Nevada (Blanca *et al.* 2001).

En concreto en Sierra Nevada, debido a su altitud, latitud y heterogeneidad espacial encontramos representados cinco de los pisos bioclimáticos de la región mediterránea: termomediterráneo, mesomediterráneo, supramediterráneo, oromediterráneo y crioromediterráneo. Existe un gran contraste entre los inviernos fríos y los veranos secos y calurosos (Blanca *et al.* 2001), pero también encontramos un gran contraste entre las temperaturas nocturnas y diurnas. Según los datos del Observatorio de Cambio Global de Sierra Nevada (Aspizua *et al.* 2012) y de WoldClim (Hijmans *et al.* 2005), a unos 2300 metros de altitud (altitud media de nuestras parcelas de muestreo) las temperaturas pueden alcanzar los 7 grados bajo cero en invierno y 27º C en verano, el rango diario anual de temperatura, es de más de 12 grados centígrados de media. Las precipitaciones también se distribuyen irregularmente, podemos observar diferencias de 325 a 98 mm entre el mes más lluvioso y el más seco.

Aunque como ya hemos dicho existen muchas variaciones en las diferentes vertientes y zonas de la sierra, en la franja de altitud en la que se distribuye *P. apollo* tanto en Sierra Nevada como en Filabres el ecosistema más abundante es el enebral-piornal, que es así mismo la formación vegetal más abundante de Sierra Nevada. Los enebrales-piornales los encontramos en zonas secas, donde el estrato arbustivo está formado principalmente por *Juniperus ssp* y *Genista versicolor* que toma formas almohadillada (pulvinulares) semiesféricas de porte bajo como respuesta a las condiciones ambientales (Blanca *et al.* 2001).

Especie de estudio

Parnassius apollo (Linnaeus, 1758) es un papiliónido (Lepidoptera, Papilionidae) de unos 35-42 mm de longitud del ala anterior. Sus alas tienen el anverso de color blanquecino-crema, con unas características zonas oscuras y en las alas posteriores encontramos sus identificativos ocelos, bordeados en negro, con el interior generalmente entre rojizo y anaranjado que aclara hacia el centro, llegando en algunas ocasiones el punto central del ocelo a ser blanco como el resto del ala (Fig. 2-1). Las apolo tienen un ciclo vital anual (univoltino) y sus larvas se alimentan de plantas del género *Sedum* y *Sempervivum*, mientras que los adultos son algo más generalistas aunque prefieren claramente libar en cardos y tomillos (Olivares *et al.* 2001).

Las hembras son mayores y más oscuras que los machos y la genitalia externa de éstos permite diferenciarlos fácilmente. Generalmente los machos patrullan a la búsqueda de hembras (Konvička y Kuras 1999; obs.pers) y después de la cópula se quedan enganchados a éstas en un comportamiento de guarda que dura unas pocas horas o toda la noche si la cópula tiene lugar al final del día (obs. pers). El macho secreta un tapón ceroso (*sphragis*) que obstruye la entrada del *ostium bursae* que en las especies que llevan *sphragis* suele encontrarse expuesto (Orr 1995), este tapón sirve como mecanismo para evitar que los competidores del macho que lo secreta puedan fecundar a la misma hembra (Eltringham 1925).

La distribución de *Parnassius apollo* (Linnaeus, 1758), suele ser parcheada, especialmente en latitudes meridionales, en donde encontramos poblaciones pequeñas y aisladas ligadas a montañas (Todisco 2008), lo que ha dado pie a la descripción de numerosas subespecies a lo largo de su distribución desde España hasta el sur de Fenoscandia y el este de China (Eisner 1976). Se llegaron a describir 23 subespecies

ibéricas de *Parnassius apollo* cada una asociada a una sierra o sistema montañoso, 21 de las cuales eran españolas (Gómez-Bustillo y Fernández Rubio 1973).

En esta tesis trabajamos con dos de dichas subespecies españolas:

Parnassius apollo nevadensis (Oberthür, 1891) es endémica de Sierra Nevada y se caracteriza por tener los ocelos de un tono amarillento, generalmente entre naranja y ocre, virando a amarillo claro hacia el centro del ocelo o cuando están las alas un poco desgastadas (Fig. 2-1). Los adultos se considera que viven entre dos y cuatro semanas (Olivares *et al.* 2011), aunque nuestros datos no registran longevidades mayores de 11 días (Capítulo 5). Se pueden observar volando desde la segunda mitad de Junio hasta el final de Julio o comienzos de Agosto, según las localidades.

Parnassius apollo filabricus Sagarra, 1933 suele tener los ocelos más anaranjados y una fenología similar a la de *P. apollo nevadensis.* Aunque anteriormente estaba distribuida por toda la Sierra de Baza-Filabres, actualmente apenas encontramos un par de poblaciones y se ha clasificado como En Peligro (EN) en el Libro Rojo de los Invertebrados de Andalucía (Barea-Azcón *et al.* 2008).

Gómez Bustillo y Fernández-Rubio (1973) categorizaron a *Parnassius apollo nevadensis*, *P. apollo filabricus* y *P. apollo gadorensis* Rougeot y Capdeville, 1969 (de la Sierra de Gádor) dentro del grupo racial "Meridional" en base al color de sus ocelos y otras características morfológicas. De estas tres subespecies andaluzas, *P. apollo gadorensis* se considera extinta (Barea-Azcón 2008) y de *P. apollo filabricus* tan solo quedan un par de parches o pequeñas poblaciones (Tinaut *et al.* 2010; Gil-T 2016). *Parnassius apollo nevadensis* parece ser la única de las subespecies meridionales en buen

estado, pero no hay muchos datos sobre su actual estado de conservación, aunque fue considerada como amenazada por el exceso de turismo y la fragmentación del hábitat (Gomariz-Cerezo 1998).

Fig. 2-1: Izquierda: *Parnassius apollo nevadensis* de Sierra Nevada (Granada y Almería). Derecha: *Parnassius apollo manleyi* (Wyatt, 1964) de Sierra Demanda (Burgos y Logroño) [Fotografías de Alberto Tinaut].

Estudio de captura - recaptura

Realizamos un estudio de captura-marcaje-recaptura (CMR) para estimar el tamaño poblacional en distintas localidades de Sierra Nevada y en una de las dos localidades conocidas en la sierra de Baza-Filabres (la única conocida en el momento de realizar el trabajo, ver Capítulo 6). Las mariposas eran capturadas con una manga entomológica y en la cara interior de la celda discoidal de ambas alas posteriores se les escribía un código numérico único con un marcador permanente antes de ser soltadas (Fig. 2-2). En el GPS se anotaba la localización exacta de la captura y el sexo del individuo. Además se anotaba el estado de desgaste de las alas como una aproximación de la edad, con cuatro categorías: fresco (cuando el individuo parecía haber pupado recientemente, sin desgaste evidente), “algo volado” (con falta de algunas escamas en las alas), “volado” (con zonas

en las alas hialinas debidas a la pérdida de escamas y alguna roturas evidentes en algunos puntos), y "muy volado" (con las alas evidentemente desgastadas y rotas en múltiples puntos). Así mismo si en sucesivas sesiones los individuos eran recapturados se apuntaba el código y el resto de información sobe su localización y estado de desgaste. Al final de la temporada si necesitábamos muestras para los análisis genéticos, los individuos recapturados en mal estado (muy volados: alas muy rotas y de vuelo dificultoso) eran llevados vivos al laboratorio en triángulos de papel y congelados hasta la extracción de ADN. En aquellas poblaciones en las que no se hizo trabajo de captura-recaptura se recolectaron unos pocos individuos para análisis genéticos eligiendo individuos volados o muy volados.

En Sierra Nevada se realizó el método de CMR en un total de seis localizaciones distintas alejadas al menos seis kilómetros entre sí y con valles o picos separándolas. A lo largo de los años que duró el estudio tratamos de ir incorporando zonas que estuviesen distribuidas a lo largo del eje este-oeste de la sierra y que se encontrasen también en las vertientes norte y sur de la misma. En 2010 trabajamos en cuatro localizaciones (Alto del Chorrillo (Chorrillo), Laguna Seca (Lagunilla), Loma de los Papeles (Papeles) y Postero Alto (Postero)), en 2011 se repitieron dos de esa localizaciones (Postero Alto y Alto del Chorrillo) y se añadieron otras dos (La Piuca y Los Campos de Otero (Otero)) y finalmente en 2012 sólo se realizó el experimento en el Chorrillo (ver Capítulo 5).). En cada zona de las seis descritas se realizó este estudio en dos parcelas de aproximadamente una hectárea, con una distancia entre sí de unos 300-900 metros. Tras observar los primeros adultos volando, las parcelas eran visitadas cada dos o tres días un total de cuatro o cinco ocasiones hasta que el número de imagos había

descendido claramente y gran número de los mismos se encontraba en mal estado (alas rotas y vuelo dificultoso); lo que tomaba generalmente unos 10-13 días. De esta forma se trató de realizar el estudio CMR en los días de máximo poblacional y evitar una clara tendencia ascendente o descendente de la abundancia durante el estudio. En el alto del Chorrillo en 2012 se siguió el mismo protocolo, pero se realizó prácticamente todos los días que el viento lo permitió mientras hubiese un buen número de mariposas frescas volando, lo que hizo un total de 10 visitas en 14 días.

En Filabres-Baza se realizó también un estudio de CMR durante los años 2011 y 2012, siguiendo el mismo protocolo que en 2010 y 2011 en Sierra Nevada, pero se muestreó toda el área (unas 30 hectáreas) de la única población conocida en aquel entonces de *P. apollo filabricus* (ver Capítulo 6).

Fig 2-2: Macho de *Parnassius apollo nevadensis,* ejemplar "volado" marcado con el número 184.

Los datos fueron analizados con el software MARK (White y Burnham 1999). Este programa es uno de los más utilizados para análisis de datos de captura y recaptura e incorpora distintos modelos usados para analizar animales marcados que anteriormente estaban disponibles en otros programas más sencillos (Lukacs y Burnham 2005). Según el modelo usado, el programa MARK permite estimar diversos parámetros poblacionales como tasa de supervivencia o tamaño poblacional, a partir del análisis de las recapturas de individuos marcados con anterioridad.

Para calcular el tamaño poblacional usamos la formulación POPAN (Schwarz y Arnason 1996) del modelo Jolly-Seber para poblaciones abiertas, este modelo postula la existencia de una superpoblación que incluye todos los individuos que nacerán en la población y usando el número de capturas y la tasa de recaptura en cada ocasión de muestreo según el tiempo transcurrido estima la probabilidad de supervivencia, probabilidad de captura, probabilidad de entrada en la población y tamaño poblacional (Fig. 2-3).

Este programa permite además la inclusión y combinación de diversas variables en los modelos usados para calcular el dichos parámetros, de esta forma podemos comprobar si los modelos están mejor ajustados a nuestros datos, con o sin dichas variables condicionando los parámetros de los modelos.

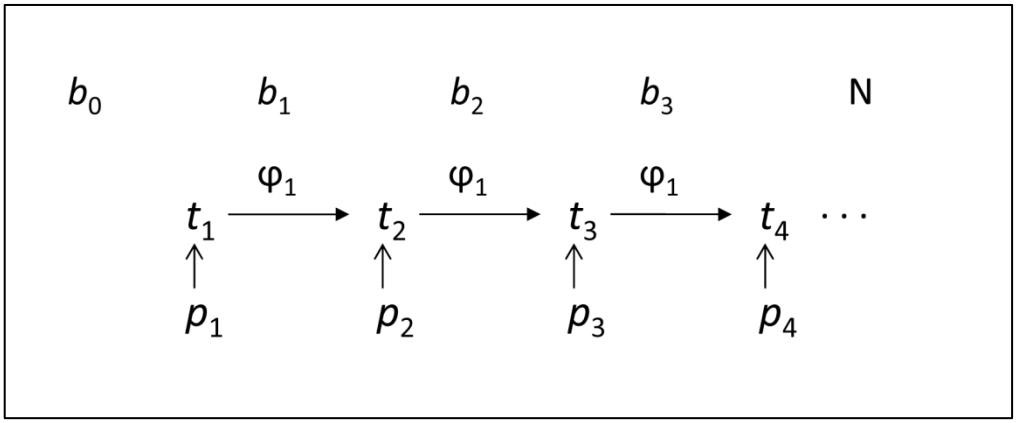

Fig. 2-3: Proceso en el que se basan los modelos de la formulación "POPAN", para cada ocasión de muestreo (t_i); p_i representa la probabilidad de captura; φ representa la probabilidad de que un animal sobreviva entre las ocasiones i e i+1; y *b* representa la probabilidad de que un animal de la super-población (N) entre en la población estudiada entre la ocasiones i e i+1 y sobreviva hasta i+1. [Redibujado basándose en Cooch y White (2016)].

Regresión de mínimos cuadrados parciales

Para averiguar qué variables afectan al tamaño poblacional utilizamos la regresión de mínimos cuadrados parciales (PLSR); este método, que está relacionado con las técnicas multivariantes, es una extensión de la regresión multiple (MR) que combina la regresión con técnicas de ordenación; y es muy útil porque relaja la colinearidad (codependencia o correlación) entre variables (Johansson y Nilsson 2002). Usamos este método para averiguar qué variables están correlacionadas con la abundancia y la supervivencia de las mariposas porque está especialmente indicado cuando sospechamos que las variables analizadas pueden estar correlacionadas (como puede acontecer con las variables ambientales) y cuando hay un problema de sobreajuste (*overfitting*) por tener más variables que observaciones (Mevik y Wehrens 2007). Este método ha probado ser más

útil identificando las variables relevantes y su grado de interacción y la magnitud de la influencia sobre la variable respuesta, que otros métodos como PCA o MR, especialmente en casos de sobreajuste (Carrascal *et al.* 2009). De forma similar a los métodos multivariantes el PLSR establece una asociación entre las variables consideradas como candidatas a explicar la variación de la variable respuesta y extrae unos factores latentes (equivalentes a los componentes principales) que maximizan la varianza explicada en la variable respuesta. Estos componentes independientes son los que se usan como variables independientes para la regresión. La aportación relativa de cada variable a la formación de los factores latentes indicará la importancia relativa de cada variable (Carrascal *et al.* 2009).

Marcadores moleculares

Las regiones microsatélite corresponden a zonas del genoma o *loci* codominantes, no codificantes y altamente variables en las que encontramos repeticiones en tándem de un par o más de nucleótidos (Jarne y Lagoda 1996).

Para poder replicar y amplificar las secuencias microsatélites es necesario diseñar marcadores específicos para las zonas adyacentes a dichas regiones, estos actuarán de cebadores o *primers*), que se unirán específicamente a ambos lados de la secuencia y servirán de punto de inicio de la replicación que iniciará la Reacción en Cadena de la ADN-Polimerasa (PCR; Saiki *et al.* 1988). Amplificando los *loci* microsatélites estos podrán ser detectados por un secuenciador para el posteriormente genotipado.

El hecho de que las regiones microsatélites sean no codificantes (es decir no sometidas a selección) y su alta variabilidad y rápida evolución, las hace ideales en estudios de ecología sobre migración, ya que son capaces de distinguir entre una situación con altas tasa de migración y una situación de panmixia. También permiten estimar el grado de parentesco entre individuos, pueden detectar cuellos de botella recientes y permiten estimar tamaño efectivo de la población (Selkoe y Toonen 2006). Los estudios comparando estas regiones con otras técnicas han demostrado que los microsatélites pueden ser más adecuados que otros marcadores moleculares también de alta variabilidad pero sin codominancia, como los AFLPs (Polimorfismo en la Longitud de los Fragmentos de restricción Amplificados) al menos en cuanto a análisis de paternidad (Gerber *et al.* 2000). Además usualmente los microsatélites tienen tasa de mutación mayor que las de los SNPs (Polimorfismo de un Solo Nucleótido) y muestran mayor diversidad alélica por *locus* que los SNPs, siendo la potencia para detectar estructura genética de los microsatélites entre 5 y 10 veces mayor que la de los SNPs (Foll y Gaggiotti 2008, Helyar *et al.* 2011).

Pese a la utilidad de éstos en estudios de ecología y conservación, sólo encontramos seis marcadores microsatélite previamente descritos para *P. apollo* (Petenian *et al.* 2005). Antes de la caracterización de marcadores microsatélite a partir de muestras de poblaciones de *P. apollo* de Sierra Nevada probamos en las mismas los seis marcadores descritos para *P. apollo* por F. Petenian y colaboradores (2005), así como cinco de *Parnassius mnemosyne* (Gratton y Sbordoni 2009) y otros cuatro descritos para *Parnassius smintheus* (Keyghobadi et al. 1999). Sólo obtuvimos productos de la PCR para uno de los *loci*, PA85 (Petenian *et al.* 2005), que resultó además ser monomórfico (un único alelo) y por tanto poco útil para estudios de estructura genética o parentesco.

Ya que los marcadores microsatélite previamente descritos en el género no resultaron útiles para nuestros objetivos, llevamos a cabo la caracterización de marcadores microsatélite a partir de muestras de *P. apollo* de Sierra Nevada. La extracción del ADN genómico fue realizada siguiendo el método del acetato amónico (Nicholls *et al.* 2000; Richardson *et al.* 2001), generalmente a partir de una sola pata en adultos, aunque en ocasiones se utilizaran varias patas o el tórax. La caracterización de microsatélites fue llevada a cabo por GENOSCREEN (Lille, Francia, www.genoscreen.fr) mediante el procedimiento descrito por Malausa y colaboradores (2011). Para crear las librerías de ADN enriquecido se usó 1 µg de ADN genómico de cinco ejemplares de *P apollo nevadensis*, incluyendo individuos de ambos sexos, y de diferentes localizaciones de Sierra Nevada. Un total de 34 963 secuencias microsatélite fueron identificadas por GENOSCREEN; 3611 de éstas eran secuencias microsatélites tenían con al menos 5 repeticiones en tándem de una secuencia dinucleotídica.

Para aumentar las probabilidades de encontrar regiones con alta variabilidad escogimos las secuencias con al menos 10 repeticiones dinucleotídicas perfectas (n=58). Diseñamos 53 pares de cebadores para PCR o *primers* usando PRIMER3 (Rozen y Skaletsky 1999), intentando que la temperatura de desnaturalización (*melting temperature*) fuera lo más próxima posible a 60º. Se realizó una amplificación preliminar con 12 muestras de diferentes localizaciones (6 machos y 6 hembras) para testar si los *primers* diseñados funcionaban y para ajustar las condiciones de la PCR para cada uno, la amplificación se realizó mediante una PCR de 2 µL totales de volumen (Kenta *et al.* 2008). Un *primer* de cada pareja lleva una molécula fluorescente en el extremo que permitirá detectar los fragmentos amplificados a partir del mismo en el secuenciador de

ADN ABI 3730; en él los fragmentos se ordenan por tamaños al migrar hacia el ánodo y pasan por un capilar donde el detector lee la fluorescencia de cada fragmento correspondiente al *locus* amplificado.

Los datos son analizados en GENEMAPPER (Applied Biosystems), donde según el tamaño, forma y separación de los picos (correspondientes a la fluorescencia de cada fragmento) podemos identificar el tamaño de los alelos presentes en la muestra y ajustar las temperaturas y tiempos de reacción, así como las concentraciones de algunos componentes si los resultados no son del todo satisfactorios. Todos aquellos *primers* que tras ser inicialmente testados con 12 individuos mostraron uno o dos picos por *locus* (correspondientes a los alelos) fueron entonces testados en 34 individuos de la misma localización (Alto del Chorrillo) para comprobar que fueran polimórficos y por tanto útiles. En los *loci* polimórficos calculamos la frecuencia de alelos nulos (alelos que al no ser detectados alteran las frecuencias alélicas esperadas) usando CERVUS (Kalinowski *et al.* 2007). Con GENEPOP *on the web* (Rousset 2008) analizamos si la población se desviaba del equilibrio de Hardy-Weinberg (HWE) para los *loci* genotipados; también comprobamos la existencia de un desequilibrio por ligamiento (*linkage disequilibrium*, LD) en la proporción de alelos, debido a que dos o más *loci* estuvieran ligados (generalmente debido a su proximidad en el genoma), y por tanto mostraran una segregación no independiente. Únicamente 20 *loci* de los 53 analizados pasaron los criterios requeridos no mostrando ningún desvío de HWE, ni alta tasa de alelos nulos ni LD en las 34 muestras del Alto del Chorrillo: Pan03, Pan16, Pan19, Pan21, Pan22, Pan26, Pan27, Pan29, Pan30, Pan32, Pan37, Pan38, Pan43, Pan44, Pan45, Pan46, Pan47, Pan49, Pan51 y Pan53.

Análisis de Paternidad

Uno de los objetivos de la tesis era usar los marcadores moleculares para comprobar la asunción de que las hembras de *P. apollo* copulan con un solo macho, lo que se asume a partir del hecho de que los machos colocan un tapón genital a las hembras durante la cópula (Eltringham 1925; Orr 1995). Para ello realizamos análisis de paternidad en los que se genotiparon las larvas que surgieron de las puestas de siete hembras grávidas de *P. apollo nevadensis*, tres de las cuales fueron capturadas copulando con un macho. Los datos de paternidad se analizaron comparando los alelos maternos (y paternos en los casos en los que había un padre candidato) con los de las larvas. Para minimizar el efecto de falsos homocigóticos (debidos a alelos nulos) y otros errores, sólo consideramos que una hembra había copulado con más de un macho cuando encontrábamos en el conjunto de larvas genotipadas para cada hembra al menos dos *loci* con tres o más alelos no maternos diferentes (dos alelos no maternos podrían pertenecer al mismo macho de ser éste heterocigótico).

Análisis de genética poblacional

Otro de los objetivos de la tesis es caracterizar el grado de variabilidad genética de las poblaciones y la existencia de estructura genética poblacional (diferenciación genética entre poblaciones y grado de conexión de estas a través de flujo génico). Para algunos de estos análisis es muy importante que todas las localidades analizadas cumplan los supuestos de equilibrio HWE y ausencia de alelos nulos (que en ocasiones son los que causan el desvío de HWE), por lo que volvimos realizar estos análisis para el conjunto de

muestras analizadas de cada localidad. No aceptamos ningún *loci* que tuviese una tasa de alelos nulos mayor del 10%; ni ninguno que mostrara un desvío significativo de HWE en alguna de las localizaciones estudiadas para cada análisis. Esto nos hizo desechar 7 *loci* para las poblaciones de Sierra Nevada (Pan03, Pan21, Pan22, Pan27, Pan37, Pan46 y Pan53), lo que nos dejó con 13 *loci* con los que se realizaron todos los análisis en estas muestras. Al usar los marcadores diseñados para *Parnassius apollo nevadensis* en *Parnassius apollo filabricus* nos encontramos que de los 20 marcadores, 4 fallaron en amplificar ningún producto (Pan26, Pan45, Pan46 y Pan51), de los restantes 16 hubo 3 con alta tasa de alelos nulos (Pan 22, Pan47 y Pan 53), los restantes 13 fueron usados para todos los análisis realizados sólo con *P. apollo filabricus*. Para estimar la diversidad genética y otros parámetros de cara a comparar con las poblaciones de Sierra Nevada y de Baza-Filabres únicamente usamos los marcadores que no mostraran desvío de HWE en ningunas de las localizaciones de ambas sierras, por lo que estos análisis se hicieron con 9 *loci* (Pan16, Pan19, Pan29, Pan30, Pan32, Pan38, Pan43, Pan44 y Pan49).

La mayoría de los análisis que usan marcadores moleculares para calcular la estructura génica y el flujo génico se basan en modelos teóricos que asumen como hipótesis nula la panmixia y un equilibrio entre pérdida de diversidad por deriva genética y entrada de alelos por inmigración (Bohonak 1999; Centeno-Cuadros 2009; Hartl y Clark 2007). Para descartarla se suele usar la comparación de las frecuencias alélicas de marcadores neutrales entre distintos grupos, usando los llamados estadísticos F de Wright (1965). Estos índices son por tanto dependientes de la asignación *a priori* de esos grupos, por lo que los hemos combinado con otros métodos de agrupación (clustering) basados en las distancias genéticas entre individuos y también con análisis multivariantes que agrupan los individuos según el aporte de cada uno a la diversidad genética global, sin requerir o suponer HWE. A estas técnicas hemos de sumar la

combinación de los métodos de agrupación con la estadística Bayesiana y las aproximaciones por Cadenas de Markov-Monte Carlo (Beaumont y Rannala 2004; Clark 2005) que nos permiten simular las diferentes posibilidades de asignación, de forma que cuando se asigne a individuos al mismo grupo de acuerdo con sus genotipos, podremos calcular la probabilidad de esa asignación en comparación con todas las otras posibles combinaciones.

Hemos calculado el índice de fijación global (F_{ST}) para detectar la existencia de estructura génica en la población, mayores valores significarán que la población está más estructurada, mientras que valores menores indicarán mayor mezcla de genotipos. Si el valor no es significativamente diferente de cero no podemos considerar que la población esté estructurada, considerándola por lo tanto como una sola población panmíctica. Complementariamente, para analizar la agrupación de individuos a priori en subpoblaciones, el Análisis Molecular de Varianza (AMOVA) estima diferenciación poblacional directamente de los datos moleculares. Se crean vectores booleanos mediante la asignación de unos y ceros a la presencia o ausencia de cada alelo y se calculan los cuadrados de las distancias euclídeas entre vectores. Las sumas de los cuadrados son analizadas en un análisis anidado equivalente al de un ANOVA que nos permite averiguar si hay mayor variación en los individuos, o dentro de cada grupo o entre grupos (Excoffier *et al.* 1992; Hartl y Clark 2007).

Otro uso que le podemos dar a los estadísticos F es el de medir la distancia genética entre parejas de grupos (localidades), para averiguar cuáles son significativamente diferentes entre sí. Estos valores además se pueden correlacionar con las distancias geográficas para ver el grado de aislamiento por distancia (IbD, Isolation by Distance). La

mayoría de estos cálculos fueron realizados en el software GENALEX (Peakall y Smouse 2012) que mezcla las muestras de manera aleatoria en las diferentes localidades y realiza los cálculos para cada conformación, de esa forma puede comparar los resultados obtenidos de la distribución real de los individuos con las de las generadas al azar y genera un p-valor de su significación (Peakall y Smouse 2006).

Para asignar los individuos de Sierra Nevada a grupos o poblaciones (no asignados a priori) se han usado dos métodos distintos: STRUCTURE (Pritchard *et al.* 2000) y GENELAND (Guillot *et al.* 2005; Guedj y Guillot 2011). Ambos métodos están basados en la estadística Bayesiana y usan simulaciones de Cadenas de Markov-Monte Carlo (MCMC) que permiten la estima de múltiples parámetros independientes en modelos complejos (Clark 2005), pero cada modelo tiene sus particulares limitaciones y supuestos. Estos métodos asignan los individuos a grupos, de forma que cada grupo formado vea minimizados el desvío del HWE y los valores de LD entre los genotipos de sus integrantes. Formando múltiples veces agrupaciones diferentes se escoge el mejor resultado, considerando que en los grupos en los que se incluya incorrectamente a individuos que pertenecen a unidades aisladas diferentes, los valores de LD serán altos y lógicamente no se cumplirían los requisitos de HWE de apareamiento al azar (Guillot *et al.* 2009).

STRUCTURE simula iteraciones MCMC para asignar a cada individuo al grupo al que pertenece con mayor probabilidad según los genotipos del resto de individuos, para cada simulación se establece un número de grupos (K) en el que se debe tratar de distribuir los individuos según sus genotipos. El modelo sin asunciones sobre la localización o agrupación *a priori* de los individuos, tiene aun así la asunción implícita de que la distribución espacial de dichos grupos no presenta una estructura espacial concreta

(Guillot *et al.* 2009). Usamos el modelo con la opción de "admixture" (mezcla) que asume que cada individuo puede tener una fracción de su genoma de cada uno de los *K* grupos, y que por tanto las frecuencias alélicas pueden ser similares debido a migración o a un ancestro común. El modelo con mezcla y correlación de frecuencias alélicas suele ser la configuración recomendada *a priori* para STRUCTURE ya que es el más probable en escenarios naturales con poblaciones abiertas (Falush *et al.* 2003).

GENELAND parte de un modelo basado en los diagramas de teselado de Voronoi, aplicados a la genética de poblaciones. Estos modelos se basan en la asunción de que el espacio ocupado por cada uno de los grupos simulados puede ser aproximado como una combinación de pequeños polígonos o células relativas a la posición y genotipo de cada individuo. Este tipo de modelos usualmente se centra en la localización de las muestras y asume que puntos adyacentes o vecinos en el mapa tienen mayor probabilidad de pertenecer al mismo grupo que dos puntos tomados al azar. Dos puntos son considerados vecinos si no hay otro punto de muestreo alrededor de uno de ellos en una línea recta que intercepte su conexión (Guillot *et al.* 2009). En el caso de GENELAND el modelo de teselación de Voronoi que usa no está asociado con individuos, sino con "territorios". Cada territorio puede por tanto agrupar numerosos individuos que se encuentren en el rango de una célula de Voronoi. La localización geográfica de cada célula, así como su número, área y forma son considerados parámetros del modelo y son estimados usando un algoritmo MCMC (François y Durand 2010). Este tipo de modelo se llama "teselación Libre de Voronoi", ya que las células de Voronoi son construidas conforme a los alelos y no restringidas a las zonas de muestreo (Guillot et al. 2009). En el caso de GENELAND el número de grupos considerado (K) y el número de iteraciones

MCMC que son eliminadas no es decidido a ciegas con anterioridad, se decide a posteriori observando la gráfica que contrapone los valores del logaritmo de la probabilidad de cada cadena de Markov-Monte Carlo simulada respecto a cada valor de K. En la gráfica observaremos una cola a la izquierda correspondiente a las primeras iteraciones MCMC con valores de probabilidad muy bajos, antes de alcanzar la meseta en la que las simulaciones se han estabilizado al coincidir con el valor de K que proporciona el mejor valor de probabilidad; las primeras MCMC correspondientes a esa cola son desechadas (Fig. 2-4).

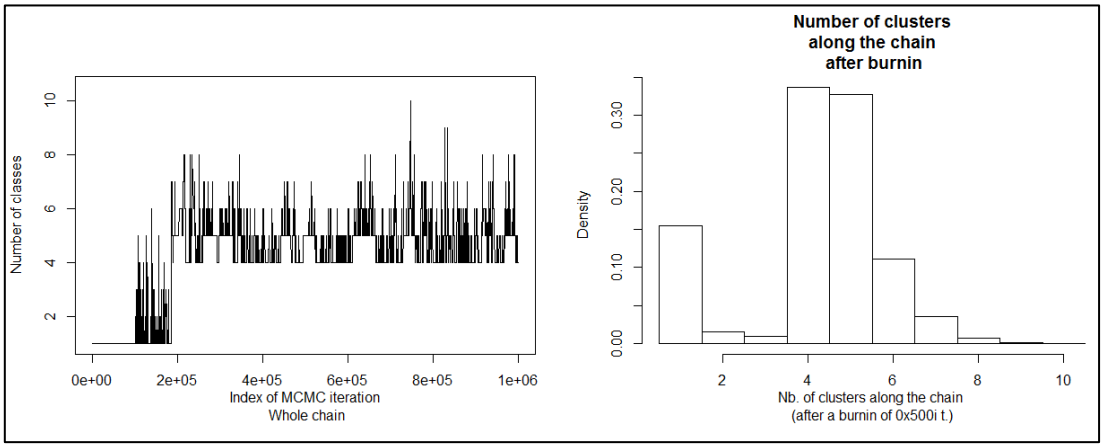

Fig. 2-4: En el gráfico de la izquierda una línea vertical muestra el número de grupos (*K; number of classes*) estimados por GENELAND en cada una de las 1,000,000 iteraciones. Se puede observar como los valores varían mucho al inicio y crecen hasta estabilizarse entre el 4 y el 5; las primeras 400.000 iteraciones (cola de la izquierda) deberán ser eliminadas antes de proseguir con el análisis. El histograma de la izquierda muestra el número de veces que aparece cada valor de *K* respecto al total de iteraciones.

Otro tipo de análisis son los realizados mediante los métodos multivariantes, que no dependen de la asignación *a priori* de las poblaciones, ni asumen HWE o la ausencia de LD y por eso se recomiendan como método complementarios a los análisis Bayesianos (Wilson *et al.* 2015). Los métodos multivariantes descomponen la varianza de los datos en un grupo de variables independientes (ortogonales), estas variables son conocidas

como componentes principales o *eigenvectors*. Estos vectores pueden a su ver descomponerse en una constante y un *eigenvalue*, la importancia de cada componente se puede expresar por la varianza de su proyección o por la proporción de la varianza total que explica (Abdi 2003).

Podemos pues analizar las frecuencias alélicas con un Análisis de Componentes Principales (PCA) como variables continuas y usar las distancias euclídeas para calcular la proximidad entre ellas. El sPCA (*spatial Principal Component Analysis*) usa además de las frecuencias alélicas, las coordenadas geográficas, para encontrar los valores de los componentes que maximizan la varianza y la autocorrelación espacial (Jombart y Ahmed 2011). Según sea la relación de la variación espacial podemos encontrar dos tipos de *eigenvalues*, los positivos y los negativos, su comparación nos permitirá ver qué tipo de estructuración predomina: los valores positivos corresponden con la predominancia de estructuras globales como parches o clinas en la distribución, en las que la varianza es mayor entre muestras alejadas; por otro lado valores negativos corresponden a estructuras locales, fuertes diferencias genéticas entre muestras cercanas de la misma zona (Jombart *et al.* 2008).

En ocasiones la representación gráfica de un PCA puede no mostrar claramente el tipo de estructura y en el caso del sPCA los *eigenvalues* pueden no indicar claramente si existen estructuras globales o locales significativas. En cualquier caso, si existe un patrón a nivel global entre las muestras, debería darse que un gran número de alelos estuvieran correlacionados con al menos uno de los susodichos *eigenvalues* positivos globales que explican su varianza. De la misma manera si existe una estructura local fuerte entre individuos (inter-poblacional), esperamos que algunos alelos estén correlacionados con

alguno de los *eigenvalues* negativos locales. El test local y global de estructura espacial implementados en ADEGENET (Jombart y Ahmed 2011), un paquete de R (R Core Team 2015), comprueban si esa correlación es significativamente diferente de una con las muestras ordenadas al azar ayudándonos a identificar la existencia de estructuras locales y globales (Jombart *et al.* 2008).

El análisis se puede ajustar según la relación entre la localización de las muestras, la conectividad geográfica y movilidad de los individuos. Para nuestros análisis seleccionamos la Red de Conexiones de Vecindad por distancia (*type 5*; *Neighbourhood by distance Connection Network*). En este tipo de red dos puntos son considerados conectados únicamente si la distancia entre ellos está por debajo de un valor asignado. Consideramos como conectadas directamente (vecinas) todas las muestras con una distancia entre sí menor de 15.38 km, ya que esta es la distancia entre las dos muestras más cercanas pertenecientes a las dos localidades adyacentes más alejadas entre sí. De este modo permitimos que todas las localizaciones adyacentes estén conectadas, y también indirectamente que todas las demás lo estén, pero únicamente a través de otras localizaciones intermedias, aproximándose a un modelo de paso intermedio (*stepping stone*).

Generalmente en los análisis multivariantes representamos las observaciones respecto al valor de aquellos componentes que explican la mayor parte de su varianza, la posición relativa de las muestras u observaciones nos puede permitir entender la relación entre ellas. En los PCAs normalmente se presentan los datos en un gráfico, en el que cada uno de los ejes representa un *eigenvalue* y los puntos se ordenan según su valor relativo (score) respecto a los dos *eigenvalues* que explican la mayor parte de la varianza. Dado que el sPCA usa las coordenadas geográficas, podemos ver los resultados en un contexto

espacial para ver si existe algún patrón geográfico que tenga sentido. Para ello ADEGENET asigna un color a cada muestra dependiendo de los *scores* de estas para los *eigenvalues* que explican mayor parte de la varianza y las distribuye de acuerdo con su posición geográfica.

Tamaño efectivo de población

El tamaño efectivo de población (N_e) indica el tamaño de una población ideal con una proporción de sexos no sesgada, tamaño poblacional constante, generaciones discretas e igual contribución de todos los individuos a la siguiente generación; pero que tiene la misma tasa de cambio en la heterocigosidad que la población real (Saarinen *et al.* 2010). La aproximación más realista sería una estima del número de parejas reproductoras (o de hembras) existentes en la población, por lo que el tamaño poblacional efectivo va a ser siempre menor que la población real. De hecho N_e suele ser más pequeño que el tamaño de censo y tanto más cuanto mayor sea el sesgo en la razón de sexos, la varianza en el éxito reproductor de los individuos (tamaño de familia) y las fluctuaciones anuales en el tamaño de censo (Frankham *et al.* 2002).

El software NeESTIMATOR (Do *et al*. 2014) es usado comúnmente con microsatélites para estimar el N_e ya que permite usar diversos métodos, cada uno con diferentes asunciones y limitaciones. Podemos encontrar dos tipos de métodos dependiendo de la información que busquemos y el tipo de muestreo. Por un lado tenemos los métodos que usan muestras de una sola generación, se considera que estos métodos dan la estima de N_e a partir de su relación con el grado de endogamia poblacional (inbreeding) y de la tasa de pérdida de variación genética que esta conlleva; generalmente miden esto basándose

en los niveles de LD causados por la deriva genética o por el exceso de heterocigosidad (Barker 2011; Saarinen *et al.* 2010). Por otro lado tenemos los métodos temporales, que estiman el N_e a partir del cambio de la varianza estandarizada del cambio en las frecuencias alélicas entre generaciones (*F*; Saarinen *et al.* 2010; Waples 1989). Los valores de ambos métodos pueden llegar a diferir mucho dependiendo de la estructura poblacional, el flujo genético o incluso el esfuerzo de muestreo (Barker 2011), por ello resulta interesante comparar varios métodos. Los requisitos para los métodos de una sola muestra son: que el muestreo sea al azar, que no haya subdivisión de la población, generaciones discretas (sin solapamiento), no inmigración, un tamaño poblacional estable, una contribución igual de los individuos a la próxima generación y no mutación ni selección (Skrbinšek *et al.* 2012). Estos prerrequisitos son difícilmente cumplidos en su totalidad, en nuestro caso, para la población de Filabres probablemente se cumplan todos menos el tamaño estable y la contribución de los individuos a la próxima generación. En cualquier caso hay muchos métodos dentro de este grupo y por ejemplo el método de LD ha demostrado ser bastante robusto a la violación de esta asunción (Waples 2006). En el caso de las poblaciones de Sierra Nevada, además tenemos posible inmigración y subdivisión de la población. Calculamos por tanto para Filabres el N_e usando 4 métodos: el método del LD corregido contra el sesgo (Waples y Do 2008), el método actualizado del exceso de heterocigosidad (Zhdanova y Pudovkin 2008), por el método del ancestro común molecular (Nomura 2008) y por último por el método temporal (Waples 1989) con las muestras de las generaciones de 2009 y 2012.

En el caso de las poblaciones de Sierra Nevada, donde se mezclan unas pocas muestras de años y localidades en grupos muy grandes, no conseguimos obtener valores válidos para todas las poblaciones por ninguno de estos métodos, así que los calculamos con el servicio online ONESAMP (Tallmon *et al.* 2008) que emplea Computación

Bayesiana Aproximada (ABC) combinando diversos estadísticos usados para calcular el N_e, lo que le confiere gran precisión y exactitud. Este método ha demostrado resultados muy estables y robustos y además resistir bien la violación de sus asunciones, aunque parece producir intervalos de confianza mayores que otros métodos (Skrbinšek *et al.* 2012).

Cuellos de botella

Un paso obligado junto con el cálculo del N_e para averiguar el estado de las poblaciones, es averiguar si han pasado recientemente por una reducción del tamaño poblacional que haya ocasionado un cuello de botella genético. La identificación de este tipo de procesos depende enormemente del tipo de mutación que produce los diferentes alelos en los *loci* utilizados, por lo que el programa BOTTLENECK (Cornuet y Luikart 1996) nos ofrece varios modelos que pueden utilizarse. El modelo de mutación paso a paso (SMM, Stepwise Mutation Model) asume que cada mutación crea un nuevo alelo añadiendo una repetición simple a la secuencia de repeticiones en tándem (de la secuencia microsatélite en nuestro caso); el modelo de alelos infinitos (IAM, Infinite Allele Model) parte de la posibilidad de que cada mutación genere un alelo diferente, independientemente de la longitud; por último el modelo de mutación en dos fases (TPM, Two-Phase Model) es un modelo mixto que combina el SMM y IAM en diferentes proporciones y varianzas. Los microsatélites y especialmente los de repeticiones en tándem perfectas como los nuestros, se considera que se ajustan bien al SMM, aunque no se descarta que puedan ajustarse a un TPM mayoritariamente mutando bajo SMM, pero con una pequeña proporción de IAM.

Usamos dos análisis diferentes para evaluar los modelos y detectar cuellos de botella, el test de signos (sign test) que es por un lado considerado más robusto ante la violación de las asunciones que el test de Wilcoxon, pero por otro lado sufre de peor poder estadístico que el segundo (Cornuet y Luikart 1996). El test de Wilcoxon para exceso o déficit de heterocigotos se considera apropiado para estudios con menos de 20 *loci* como los nuestros y para tamaños muestrales variados (Piry *et al.* 1999). Un exceso de heterocigotos se detectaría cuando ha habido una reducción reciente en el tamaño poblacional, debido a que la pérdida de alelos raros suele ser más rápida que la reducción en la heterocigosis resultante, aunque esto varía con el modelo de mutación, el tipo de marcadores y el grado de aislamiento (Maruyama y Fuerst 1984, 1985).

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Capítulo 3

VEINTE NUEVOS *LOCI MICROSATÉLITE* PARA ESTUDIOS DE PARENTESCO Y DE GENÉTICA DE POBLACIONES EN *PARNASSIUS APOLLO NEVADENSIS* (LEPIDÓPTERA, PAPILIONIDAE)

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CHAPTER 3

Twenty new microsatellite *loci* for population structure and parentage studies of Parnassius apollo nevadensis (Lepidoptera; Papilionidae).

Mira Ó, Martínez JG, Dawson DA, Tinaut A, Sánchez-Prieto C (2014) Twenty new microsatellite *loci* for population structure and parentage studies of *Parnassius apollo nevadensis* (Lepidoptera; Papilionidae). *Journal of Insect Conservation*, **18** (5): 771-779.

Introduction

The apollo butterfly, *Parnassius apollo* (Linnaeus, 1758) has a patchy distribution in the Palearctic region, from Spain to southern Fennoscandia and Eastern China (Eisner 1976), and is typically represented by small local populations, in particular in Southern Europe, where its distribution is restricted to mountain ranges (Descimon 1995; Todisco *et al.* 2010).

There are a large number of *Parnassius apollo* subspecies across the Eurasian continent, many of which have declined substantially over the last hundred years; some of them are now extinct in several mountain ranges (van Swaay *et al.* 2010). In the last decades of the twentieth century, there was a significant decline in the numbers of *Parnassius apollo* in 12 of the 28 countries that the species inhabits, and it is considered

to be extinct in three countries (Collins and Morris 1985; van Swaay and Warren 1999). Its overall European population has declined by almost 30% in the last 10 years, and consequently the species has been classified as one of the most endangered butterflies in Europe (Baillie and Groombridge 1996). This has led to the species being categorized as vulnerable (IUCN criteria A1cde), listed in the European Red Data Book (van Swaay and Warren 1999), enlisted in the annex IV of Appendix II of the Habitat Directive (EEC 92/43/EWG) of the European Union and being presently included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) lists.

In Spain there are a large number of *P. apollo* subspecies described (Gómez-Bustillo and Fernández-Rubio 1973), each of them associated with a different mountain range. *Parnassius apollo nevadensis* (Oberthür, 1891) is endemic to the mountain range of Sierra Nevada (Southern Spain) and one of the most meridional remaining population of this species.

The decline of many populations of this butterfly, together with the fact that it is very local and particular in its ecological requirements (Fred and Brommer 2005; Fred *et al.* 2006), makes it very sensitive to habitat and climate change (Ashton *et al.* 2009; Todisco *et al.* 2010), and thus a high priority species for conservation. Of particular concern is that the species may experience very low gene flow between populations. Populations (or subspecies) restricted to mountain ranges are isolated from each other, but also, at a local scale, populations within a particular mountain range might show restricted gene flow due to several causes, such as, for example, habitat fragmentation.

Studies of the population genetics of *Parnassius apollo* are of interest because this species, as other butterflies, is a suitable organism for ecological and molecular studies of the consequences of habitat fragmentation and climate change. Despite this, few articles have addressed the population genetics of species in this genus, some of them using allozymes in *Parnassius mnemosyne* (Descimon and Napolitano 1993; Meglécz *et al.* 1997; Meglécz *et al.* 1998; Napolitano and Descimon 1994), some others using Random Amplified Polymorphic DNA in *P. nomion* and *P. bremeri* in Asia (Zakharov 2001), and a few using microsatellites, as in *Parnassius smintheus* (Keyghobadi *et al.* 1999, 2005). The phylogeography of European *Parnassius apollo* has been studied using mitochondrial DNA (Todisco *et al.* 2010) but there is virtually no information on genetic diversity or population structure for most *Parnassius apollo* populations – just one single study in a German population (Habel *et al.* 2009), where all the markers used (microsatellites and allozymes) were monomorphic.

Furthermore, *Parnassius apollo* has received little attention from the point of view of its reproductive strategies, despite the implications of breeding biology on population dynamics and conservation. After the copula, males of the genus *Parnassius* employ a mating plug known as *sphragis*, a waxy structure placed in the ventral side of female's abdomen during copulation to prevent other males from mating by blocking access to the female's gonopore (Orr 1995), the male guards the female and they remain attached hours or overnight if the copula takes place late in the evening (pers. Obs.). In theory, females thus copulate only once, although there are records of *sphragis* loss in *P. mnemosyne* (Vlasanek and Konvicka 2009). In any case, even if females copulate only once, there are no data at all about variance in the reproductive success of males and females or studies of mate choice in this species, questions that could be answered by the use of molecular genetics analyses.

Microsatellites are the most widely used markers in studies of population genetic structure and gene flow, kinship and parentage (Selkoe and Toonen 2006). In spite of the utility of butterflies as model systems in ecological and evolutionary studies and the interest raised by this species, only six microsatellite markers have been developed for *P. apollo* (Petenian *et al.* 2005). This is probably because of the recorded difficulties in characterizing suitable microsatellites in Lepidoptera (Zhang 2004) as a consequence of a high frequency of interspersed repetitive sequences that compromise primer design (Meglécz *et al.* 2004; Zhang 2004).

In this paper we present twenty novel *Parnassius apollo* microsatellites isolated from and characterized in individuals belonging to the *nevadensis* subspecies (Sierra Nevada, Southern Spain) and we use these markers to perform parentage analyses of the clutches of seven females.

Materials and Methods

The subspecies *Parnassius apollo nevadensis* is endemic to the Sierra Nevada (Southern Spain). Adults have characteristic orange-yellowish wing ocelles that differ from the typically red ocelles of the nominate form (*P. apollo apollo* L.). They live in high mountain meadows, at altitudes of between 1950 and 2700 metres above sea level. Larvae feed on several species of *Sedum,* mainly *S. amplexicaule* (pers. obs.). The species is univoltine, and adults fly between the second half of June and the end of July. Females

are larger than males and the external genitalia of males, clearly visible to the naked eye, allows differentiation between the sexes.

Fifty-six individuals of *P. apollo nevadensis* were caught under permission of the National Park (Parque Nacional y Natural de Sierra Nevada) and the Consejería de Medio Ambiente (Junta de Andalucía) at the end of their flying period and then transported alive to the lab where they were frozen shortly after capture and kept at -20 ºC until DNA extraction.

In order to investigate paternity we caught a total of seven gravid females; four were captured while they were laying eggs with the mating plug on their genitalia and three of the females were caught copulating and were captured with their mate. We kept the females alive in the lab in seven individual boxes until they laid the eggs. After that, we collected the eggs and kept them in envelopes at room temperature until they hatched; the caterpillars that emerged were preserved in absolute alcohol in screw-topped rubber-sealed microfuge tubes at room temperature until DNA extraction.

Genomic DNA extraction was carried out using ammonium acetate (Nicholls *et al.* 2000, Richardson *et al.* 2001). We extracted DNA from a leg of each adult butterfly or from the whole caterpillar.

To create the enriched libraries we used a total of 1 µg of *Parnassius apollo nevadensis* genomic DNA, pooled from five individuals, 2 males and 3 females. Each one of the five individuals was from a different subpopulation across the range of distribution of the species in Sierra Nevada. The distance between the five locations of the subpopulations varied between 3.6 and 49.7 kilometers. Microsatellite sequences were isolated by GENOSCREEN, Lille, France (www.genoscreen.fr) as described at Malausa *et al.* (2011).

Briefly, total genomic DNA was sonicated or digested with *Rsa*I and enriched with Dynabeads (INVITROGEN) for AG, AC, AAC, AAG, AGG, ACG, ACAT and ATCT repeat motifs. Enriched fragments were subsequently amplified. PCR products were purified, quantified and sequenced using 454 GS-FLX Titanium pyrosequencing on a GsFLX PTP (for details see Malausa *et al.* 2011). A total of 34,963 sequences were isolated, and 3,611 microsatellite sequences possessing at least 5 repeat units were detected using QDD software (Meglécz *et al.* 2010).

We selected sequences with at least ten perfect dinucleotide repeats (n=58) and designed primers using PRIMER3 v0.4.0 (Rozen and Skaletsky 1999). The default primer design options were changed: Primer length was set to be between 16 and 30 base pairs, primer melting temperatures to between 59 and 61ºC (with a preferred temperature of 60ºC), the maximum difference in melting temperature between the forward and reverse primer was 0.5°C; the "Max poly X" was set to 3 (X indicating the maximum allowable length of a mononucleotide repeat in the primer) and with a CG clamp setting of 1 (indicating a cytosine or guanine base should be present at the 3' end of both the forward and reverse primer sequence). If no primer sets were found with these stringent settings we relaxed these parameters allowing primer sets to be designed without a CG clamp, with a Max Poly X of five, permitting a larger difference between the melting temperatures of forward and reverse primers of 1.0 or 1.5ºC and extending the allowed range of melting temperatures to between 57 and 62ºC. A total of 53 primer sets were designed.

Due to the problems previously reported regarding amplification failure caused by sequence similarity (Meglécz *et al.* 2004, Zhang 2004), we compared sequences of all

the locis using a stand-alone nBLAST (Altschul *et al.* 1997), to be sure that all *loci* were unique and the primer bind region and regions amplified by the primer set had no sequence similarity between *loci*.

Amplification was performed in a 2-µl multiplex PCR (Kenta *et al.* 2008) containing 10ng of air-dried DNA, 1µl QIAGEN Multiplex PCR Master Mix, 1µl of primer mix containing 0.2µM of the forward fluorescent primer and 0.2µM of the reverse primer and covered with a drop of mineral oil. Polymerase chain reaction was conducted in a DNA Engine Tetrad 2 thermal cycler (MJ Research) with the following cycling conditions: an initial denaturation at 95°C for 15 minutes; then 40 cycles with the three following stages: a 94°C denaturation for 30 seconds; specific primer annealing temperature (Table 3-1) for 90 s; and 60 s at 72ºC for elongation, and after those 40 cycles a final extension step at 72°C for 30min. PCRs were performed using a DNA Engine Tetrad 2 Thermal Cycler (MJ Research, BioRad UK). Products were separated on a 48-well capillary ABI3730 DNA Analyser using prism set D and a ROX size standard. Allele sizes were assigned using GENEMAPPER v 3.7 (Applied Biosystems).

To initially test *loci* for amplification and polymorphism we used 12 *Parnassius apollo nevadensis* individuals (6 males and 6 females) from different subpopulations separated more than 3000 km from each other. We started testing each *locus* with a PCR annealing temperature 2°C under the average melting temperature of the forward and reverse primers as calculated by PRIMER3, and if we found any problem with shape or strength/height of the amplified peaks observed using GENEMAPPER, or if we found mostly apparent homozygotes but with different allele sizes, we reamplified the same sample again, but increasing or decreasing the annealing temperatures by 0.5 ºC

(increasing if stutter products were observed and decreasing if weak products were observed).

All those *loci* showing amplification and displaying one or two alleles (per individual) when tested in the first 12 individuals; were tested for polymorphism with 34 individuals, including 17 males and 17 females, from the same location in Sierra Nevada (Alto del Chorrillo, 2600m above sea level, 30S 472400 4094900). The 34 individuals genotyped were confirmed to be unrelated using COLONY (Jones and Wang 2009).

Polymorphic *loci* were tested for deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) using GENEPOP on the web v4.2 (Rousset 2008). A Bonferroni correction was applied to HWE and LD p-values (Rice 1989). The number of alleles observed, observed heterozygosity, expected heterozygosity and estimated null allele frequency for each *locus* was calculated using CERVUS v3.0.3 (Kalinowski *et al.* 2007).

To determine the power of the marker set to carry out parentage analyses we used CERVUS software to calculate the combined non-exclusion probabilities for the first and the second parent. The non-exclusion probability for the first parent is the average probability (assuming HWE) that the set of *loci* will not exclude an unrelated candidate parent from parentage of an arbitrary offspring when the genotype of the other parent is unknown and therefore the non-exclusion probability for the second parent is the average probability that the set of *loci* will not exclude an unrelated candidate parent from parentage of an arbitrary offspring when the genotype of the other parent is known (second parent).

We designed multiplex reactions so that we could use more than one marker in the same reaction. We combined the selected polymorphic *loci* into suitable sets for multiplexing with MULTIPLEX MANAGER (Holleley and Geerts 2009). The software helps to compare the primer sequences between *loci* to check for incompatibilities due to sequence similarity and overlapping product sizes, we used this information to select the best multiplex sets. Primer sets were assigned into multiplex sets avoiding primer–primer sequence similarity, according to the fragment sizes, the fluorochrome label (6FAM or HEX, see Table 3-1) and the annealing temperature (calculated using PRIMER3).

Table 3-1: Details of the 20 new microsatellite markers described for *Parnassius apollo*

Locus	Primer sequence (5'-3')	Fluoro label	Repeat motif	T_a (Cº)	N	A	Exp. allele. size (bp)	Obs. allele. range (bp)	Ho	He	HWE P-Value	Null allele freq.	MultiX
Pan03	F:TTTGGGCATTCATCATTCAA	HEX	(TC)11	57	34	9	105	103-129	0.7059	0.7950	0.0301	0.0445	C
	R:GCGAAAATCCGTCAATCATTT												
Pan16	F:CGGGACTGGTTCTCTTCATAC	HEX	(TC)11	58	34	4	181	180-186	0.6765	0.6598	0.3447	-0.0350	I
	R:GATGGTACGAACAGCGAATG												
Pan19	F:CGGAATTTCAAACTTCAAGC	6FAM	(CT)13	57	34	9	111	106-126	0.8529	0.8398	0.8992	-0.0185	F
	R:AGACTGAAGACTGCACAAACAA												
Pan21	F:GATTGTAAGCGCAGCAGAAG	6FAM	(AG)11	58	34	11	160	154-189	0.7059	0.8227	0.0502	0.0763	A
	R:AACCTAGTTAGGAAGTTTGGAACG												
Pan22	F:AACGCTTCTATGGACGTTGA	HEX	(AC)12	57	34	8	180	172-226	0.7059	0.7902	0.3629	0.0491	F
	R:AAGTGCTAACTTAAGTAGGGCAAA												
Pan26	F:TAGCGTCTCCTAACGGATGG	6FAM	(TG)12	60	34	12	213	208-360	0.8529	0.8876	0.6583	0.0145	G
	R:GCTGCACTAGCCCAATCG												
Pan27	F:AAGCAAACCGAAACCGTATG	6FAM	(AG)18	59	34	9	176	164-181	0.7353	0.7682	0.6394	0.0193	H
	R:CTCGTAGCACAGATGAAAAGTAGTTC												
Pan29	F:ATGCCGTCATGTGCAAAC	6FAM	(CT)11	58	33	7	161	155-167	0.5758	0.6140	0.3928	0.0049	C
	R:CAAGTTCTTGACTCATCAAATGC												
Pan30	F:TTGTTTATGGGCAGCGATAG	HEX	(TC)11	57	34	3	228	225-229	0.1765	0.2173	0.2014	0.0865	A
	R:CGAACGAAAATAACAGTCTTGC												
Pan32	F:CGCCACAGGGTATGTATGTG	6FAM	(CA)13	59	33	6	176	169-181	0.3939	0.4951	0.2795	0.1273†	B
	R:TGACCATCAGAAAGGGAAGC												
Pan37	F:CGTTTGCCGATTCAGTGTTA	HEX	(GA)12	57	33	5	98	92-104	0.6875	0.7232	0.5618	0.0280	B
	R:GGCATTATGGAGTGGAAGTGA												

Pan38	F:CCCATCCCGTCAAATGG	6FAM	(CT)12	59	34	3	99	95-113	0.3824	0.4421	0.5793	0.0620	G
	R:TTCCGAATGTGTAGTAGTTGAACG												

Table 3-1: *Continued*

Locus	Primer sequence (5'-3')	Fluoro label	Repeat motif	T_a (Cº)	N	A	Exp. allele. size (bp)	Obs. allele. range (bp)	Ho	He	HWE P-Value	Null allele freq.	MultiX
Pan43	F:CTTGGCAGGCGGAATG	HEX	(GA)10	57	34	6	79	74-92	0.5294	0.5971	0.0363	0.0865	A
	R:ATACATGAAGGTATAACCCATCTCG												
Pan44	F:CGCTTGGGTGGATTAATGTC	6FAM	(AC)11	59	33	8	255	246-273	0.4545	0.4970	0.5069	0.0191	B
	R:GCAACTTTGTATTCCGCTTACG												
Pan45	F:CTTCCATTTGACACCAGTGC	6FAM	(TC)11	58	34	9	193	194-212	0.7576	0.7524	0.6584	-0.0058	E
	R:AGGCCAAGTCATTCTCATCC												
Pan46	F:GCTCTTCCATAACAAGTCATCTCC	HEX	(GT)11	58	34	7	125	123-180	0.3333	0.6284	0.0016*	0.3119†	E
	R:GAAGGTCGTAGTGAGAGTGATAGTAGC												
Pan47	F:GGCAGCTTCTGCAGTTGTATG	6FAM	(TC)10	58	34	5	96	80-114	0.4706	0.4425	1.0000	-0.0423	I
	R:TTACCTTAACCTATTACAGAACCCATACTC												
Pan49	F:CATGGTTTAACCTGCAATCG	HEX	(TC)10	57	34	10	186	166-210	0.7647	0.8363	0.2193	0.0414	D
	R:ACGTCAGAGATTAACTGGTAGAAGC												
Pan51	F:TCCGTGAGATGTGAATACTGC	6FAM	(GA)10	56	34	2	117	119-121	0.5000	0.5070	1.0000	-0.0004	D
	R:CGCACTTCTAGACAGAAAGGAG												
Pan53	F:CGATGGGACGGCAAAC	HEX	(AG)10	58	32	3	84	76-80	0.1563	0.2941	0.0113	0.2974†	H
	R:CAAGTTCTTGACTCATCAAATGC												

Ta: annealing temperature; N: number of unrelated individuals tested; A: number of alleles per *locus*; Ho and He: observed and expected heterozygosity; HWE P-value: probability of deviation from Hardy Weinberg Equilibrium, significant values after Bonferroni's correction are marked with *; † marks *loci* with presence of null allele equal or above 0.1; MultiX: in which multiplex reaction the marker was included. A and C at 57.5ºC; multiplex B, E, H and I at 58ºC; D at 56.5ºC; F at 57ºC and G at 59.5ºC. All values except HWE P-value were estimated using CERVUS 3.0.3; Hardy Weinberg Provability Test was estimated using GENEPOP on the web.
PCR profile used: 94 °C for 15 min; followed by 40 cycles at 94 °C for 30 s, specified Ta for 90 s, and 72 °C for 60 s; ending with a final step of 72 °C for 30 min.

To check the inheritance of the markers and assess their utility in parentage analyses we genotyped the four gravid females that were captured while they were laying, the three couples captured while copulating, and offspring from each one of the females. We compared the genotypes of all larvae in a clutch with the mother's genotype to check whether maternal alleles were present in the offspring; thus, we have estimated genotyping error rate for each *locus* (Table 3-1) as the percentage of offspring's genotypes for all the clutches that did not show maternal alleles on the *locus*.

We concluded that females copulated with more than one male when their offspring showed three or more non-maternal alleles in two or more *loci* for any given offspring. In the case of the three females caught copulating, we compared male and offspring genotypes and concluded that females copulated with more than one male if male and offspring genotypes mismatched in two or more *loci* for any given larvae

Results

Of 53 primer sets initially tested in 12 individuals, nine did not show any amplification and 22 other *loci* were abandoned, as they required additional optimization due to the presence of multiple peaks and difficulties in scoring. Twenty-two of the 53 primer sets tested amplified scorable products and displayed a maximum of two alleles per individual. According to nBLAST analysis all of the 22 *loci* with scorable products were unique.

When assessed the *loci* in 34 unrelated individuals from the same location, two out of the 22 *loci* were monomorphic (*Pan02* and *Pan07*; EMBL accession numbers HG779441 and HG779444); and the remaining 20 *loci* were polymorphic (Table 3-1). A maximum of

two alleles were amplified in each individual indicating the genome is diploid (and no *loci* displayed any evidence of duplication in the genome). We found that for all 20 polymorphic *loci*, a proportion of females and males were heterozygous indicating that none of the *loci* are located on sex chromosomes.

Focusing on the 20 polymorphic *loci*, for the 34 unrelated individuals the number of alleles observed ranged from 2 to 12 per *locus* with an average of 6.8 alleles per *locus*. Expected heterozygosity varied from 0.22 to 0.89 per *locus*, and the values of observed heterozygosity for each *locus* varied from 0.16 to 0.85 (Table 3-1). One *locus* (*Pan46*) displayed evidence of deviation from Hardy–Weinberg equilibrium ($p<0.0025$, significant after applying a sequential Bonferroni correction); this *locus* and two others (*Pan32* and *Pan53*) had an estimated null allele frequency above 10% (Table 3-1). There was no evidence of linkage disequilibrium between any groups of *loci* ($p>0.0038$ in all cases, not significant after Bonferroni correction).

The non-exclusion probability results show that this marker set provides a powerful tool for assigning paternity, as it is characterized by powerful combined non-exclusion probabilities for first and second parent (0.001084 and 0.000005 respectively).

We were able to design nine different multiplex reactions combining two or three primer sets (from the 20 polymorphic ones) per plex (Table 3-1). The annealing temperatures for the different reactions were: Multiplex sets A and C at 57.5ºC; multiplex sets B, E, H and I at 58ºC; set D at 56.5ºC; set F at 57ºC and set G at 59.5ºC. Some of the samples were amplified in reactions using just one primer set (single-plexed), as well as in the multiplex reactions, and the single-plexed and multiplexed genotypes for these individuals matched.

We amplified (using the nine multiplex sets) the three couples captured while copulating, and offspring from each one of the females. Between 19 and 46 caterpillars per female were genotyped, which represent between 44 and 100 per cent of the total eggs laid by each female (Table 3-2). Overall we can affirm that the alleles are inherited, but a few reactions failed to amplify the maternal alleles, although at a very low rate: the error rate was in all the clutches and for all the *loci* below 0.080 and the failure rate per clutche was for below 0.035 with the exception of clutch number 6 (0.052; Table 3-2). The failure rate per *loci* was below 0.050 (Table 3-1), except for *Pan29*, *Pan37* and *Pan43* (0.076, 0.052 and 0.055 respectively).

Using the criteria detailed above, it seems clear that at least some females may copulate with more than one male (Table 3-2). According to the data of the offspring genotyped, in clutch 2 the male caught copulating with the female had no paternity of any offspring, whereas in clutch 1 and 7 there is evidence that in addition to the candidate father there is another male that fathered some of the offspring. For the remaining four females (clutches 3 to 6), all but one (female 6) showed three or more non-maternal alleles in at least two *loci*, suggesting at least two different fathers.

Table 3-2: Parentage analysis on seven different clutches

Clutch	N	LG	MP	DMO	Error rate
1	22	20	13	8	0.003
2	19	19	13	19	0.022
3	47	15	7		0.033
4	36	18	5		0.013
5	36	18	2		0.013
6	23	19	3		0.052
7	22	16	4	21	0.021

Number of larvae genotyped per clutch; LG: Number of *loci* genotyped; MP: Number of *loci* showing evidences of multiple paternity (see methods); DMO: Number of offspring whose genotype mismatch that of the male (for the three cases where we captured male and female copulating); Error rate: rate of single-*locus* genotypes showing no maternal alleles for all the *loci* in that clutch.

Discussion

This article presents 20 new *Parnassius apollo* polymorphic microsatellite markers. The markers developed here present perfect repeat motifs (neither compound microsatellites nor with any interruptions), and can be successfully multiplexed. Microsatellites were isolated and characterized from individuals belonging to the *nevadensis* subspecies, and thus they are primarily of use with populations of this subspecies, but testing the primers in populations from other mountain ranges or subspecies may reveal that they are transferable between subspecies. In fact, the primers successfully amplified samples from *P. apollo filabricus*, a different subspecies from southern Spain (Chapter 6). This set of markers considerably improves the availability of microsatellites for *P. apollo*, previously limited to the six primer pairs described by Petenian *et al.* (2005). There was then a huge need for polymorphic markers for the species, not just because of the small number of species-specific markers described, but also because of the high rate of failure when using cross-species markers designed either for distant subspecies or different species of Lepidoptera, as they usually fail to amplify or are monomorphic (Habel *et al.* 2009). We have previously tested all six of the published *P. apollo* markers (Petenian *et al.* 2005), five of the *P. mnemosyne* (Gratton and Sbordoni 2009) and four of the *P. smintheus* (Keyghobadi *et al.* 1999) markers, finding that only one of the Petenian *et al.*'s markers amplified a scorable product of the size expected in *P. apollo nevadensis* (*PA85*) but it was monomorphic and was therefore omitted from this study.

The high variability of our new *Pan* microsatellite markers indicates they are appropriate for use in both population genetics and parentage analyses. Observed and

expected heterozygosities are in general high, as is allele diversity, with a mean of seven alleles per *locus*. The number of repeats affects the variability and *loci* possessing perfect repeats have been proved to be significantly more variable than imperfect repeats (Goldstein and Clark 1995). Our microsatellites were chosen with at least ten perfect dinucleotide repeat, whereas the previously published *P. apollo* markers (Petenian *et al.* 2005) had either compound or fewer repeat units.

One *Pan locus* displayed deviation from Hardy–Weinberg equilibrium (*Pan46*), that might be caused either by the presence of null alleles, sex linkage (ruled out because heterozygotes were present in both sexes) or as a result of the species' population dynamics. Two more markers displayed an estimated null allele frequency higher than 10% (*Pan32* and *Pan53*). Null alleles were also suspected to affect some of the markers isolated by Petenian *et al.* (2005). This result is in accordance with the conclusions of Zhang (2004) and Meglécz *et al.* (2004), which suggest that in some cases the high ratio of null alleles and the low efficiency of microsatellite isolation in Lepidoptera can be caused by the presence of multiples copies of some DNA regions. However, deviations from HWE might be also a consequence of the biology of the species. A metapopulation system with frequent extinctions and recolonizations, such as happens in *Euphydria aurinia*, would produce departures from HWE at some *loci* (Smee *et al.* 2013). Despite this (after screening a relatively high number of *loci* and retaining selected *loci*), our study departs from the general trend of lepidopteran microsatellite isolation articles, typically characterized by low number of *loci*, low levels of polymorphism and high deviations from HWE, and shows promising prospects for population genetics analyses of *Parnassius apollo*.

The analysis performed with the offspring from seven females and three males emphasized the value of the markers in paternity analysis, because it unexpectedly

shows that females may copulate with more than one male. In a number of genotypes, maternal alleles were not observed, which is most likely due to allelic dropout, since we were working with small quantities of template DNA (small larvae), and it is known that a low concentration of template can lead to allelic dropout (Taberlet *et al.* 1996). However, our criteria for evidence of multiple paternity was based on the presence of three or more paternal alleles (that is, non-maternal alleles but a maternal allele was also present in each offspring) at two or more *loci*, and these results can not be explained by allelic dropout. In fact, most offspring presented a large number of *loci* that either mismatched the paternal genotype or displayed three or more paternal alleles, implying that females copulated with at least two males and thus the *sphragis* may not always act as an effective blocking mechanism to avoid other males fertilising the female. *Sphragis* loss has been already described in the related species *Parnassius mnemosyne* (Vlasanek and Konvicka 2009), and we have also seen females with the *sphragis* ripped off or malformed (Mira *et al.* pers. obs.). Vlasanek and Konvicka (2009) suggested that the *sphragis* is not a good indicator of a female's mating status in *P. mnemosyne*, and our results, the first report of multiple paternity in *Parnassius apollo,* suggest that bearing a *sphragis* does not mean single paternity for a female's clutch. How common this is, and the ecological and evolutionary causes and consequences of multiple paternity, clearly deserves further research as it can affect to the genetic diversity, population size and in last instance to the fitness of the butterflies.

This unforeseen polyandry found in females of *P. apollo nevadensis* has also important implications for the population genetics of the species. Several theories suggest genetic benefits of polyandry, either because polyandry increases the probability of fertilization

by high quality males (Zeh and Zeh 2001), because females may copulate with genetically more compatible males or they can gain genetic diversity for their offspring. Seeking for genetic variation is an explanation for multiple mating when material or "good genes" benefits can be ruled out (Tregenza and Wedell 2000), and it has population and conservation implications because genetic variation influences long term sustainability of populations as it is required to evade any negative effects of inbreeding, and to allow evolutionary adaptation to a changing environment (Anthony and Blumstein 2000). Despite the mechanism involved, the direct relationship between polyandry and a direct increase in offspring production has been proved in insects (Arnqvist and Nilsson 2000) and other arthropods (Newcomer *et al.* 1999).

Polyandry may also be a form of inbreeding avoidance, because it diminishes the cost of inbreeding when females cannot avoid mating with close relatives (Brooker *et al.* 1990). This may apply to some small and/or decreasing populations of *P. apollo*.

In any case, from the population point of view, the mating system, and the occurrence and extent of inbreeding and its avoidance directly affects effective population size (N_e), genetic diversity and the viability of populations (Anthony and Blumstein 2000; Booy *et al.* 2000). So, future efforts should be directed towards a better understanding of the mating system of *Parnassius apollo nevadensis* within the context of the conservation of the species.

Molecular techniques have increasingly been used to assess population variation for purposes of conservation in a number of species. If we want to be closer to understanding the conservation status of the populations of *P. apollo nevadensis* we need to estimate their genetic diversity and effective population size, as well as the population

structure of the species within the mountain range, a task for which the markers presented here are a very useful and powerful tool.

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Capítulo 4

PARNASSIUS APOLLO NEVADENSIS: IDENTIFICACIÓN DE UNA RECIENTE ESTRUCTURA POBLACIONAL CON DINÁMICA FUENTE-SUMIDERO

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CHAPTER 4

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

Mira Ó, Sánchez-Prieto CB, Dawson DA, Burke T, Tinaut A, Martínez JG (2017) *Parnassius apollo nevadensis*: identification of recent population structure and source–sink dynamics. *Conservation Genetics* doi: 10.1007/s10592-017-0931-0.

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 with the highest populations and is allegedly threatened by excess tourism and habitat loss (Gomariz Cerezo 1993).

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 (Chapter 3) 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 4-1; Fig. 4-1), 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. 4-1).

Table 4-1 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)

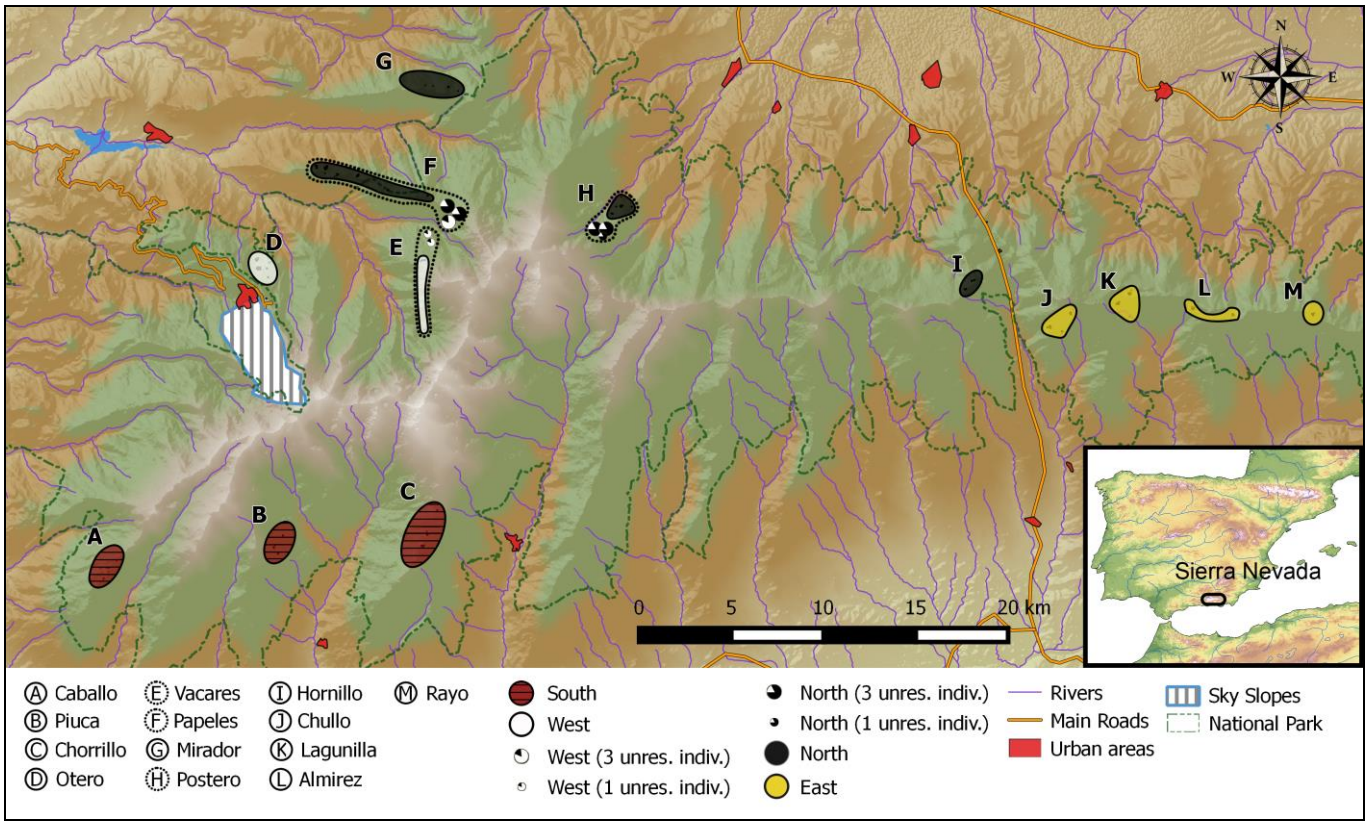

Fig. 4-1 Map including the location of all the samples clustered in 13 sampling locations. The locations are coded from A to M. A sampling location included all the sampling points from the same hillside with a continuous presence of butterflies between them. The colour of the circles surrounding the 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 4-1) 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* (Chapter 3); 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} 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.

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. 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. 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. 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 in RStudio (RStudio Team 2015) using the R (R Core Team 2015) package ADEGENET (Jombart and Ahmed 2011). sPCAs is a multivariate technique with the same characteristics that the PCA but including the geographical information.

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). 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 (Table 4-2). Global F_{ST} (0.010) shows a low but significant genetic structure ($p = 0.0001$).

Pairwise F_{ST} values are also low (Table 4-3), ranging from 0.007 to 0.029 in the significant comparisons ($p < 0.05$).

Table 4-2 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

Table 4-3 "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

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. 4-2), 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.

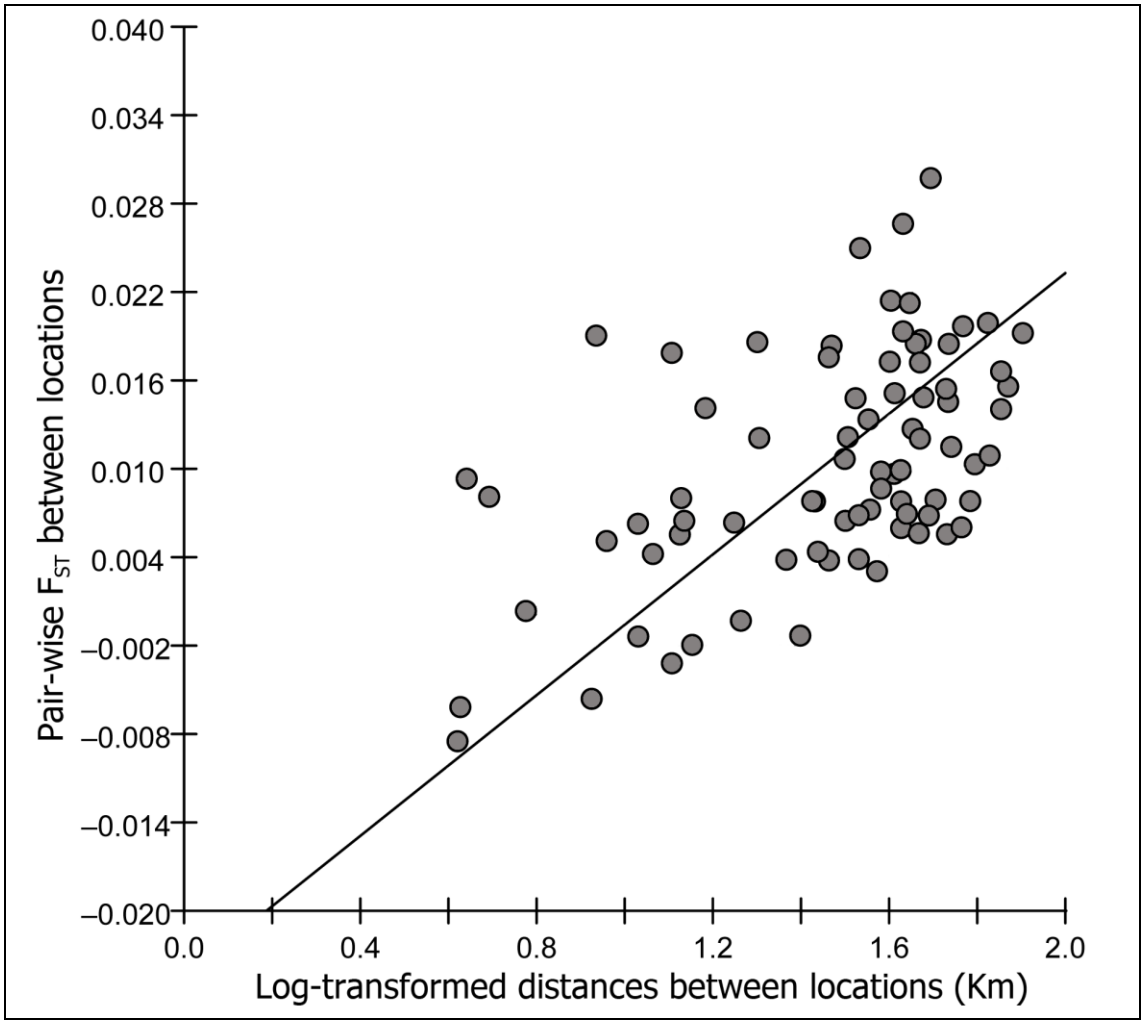

Fig. 4-2 Correlation between pairwise F_{ST} values and "travelling" geographic distances (see Methods) between locations

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.4- 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. 4-1). 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. 4-1). 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. 4-1).

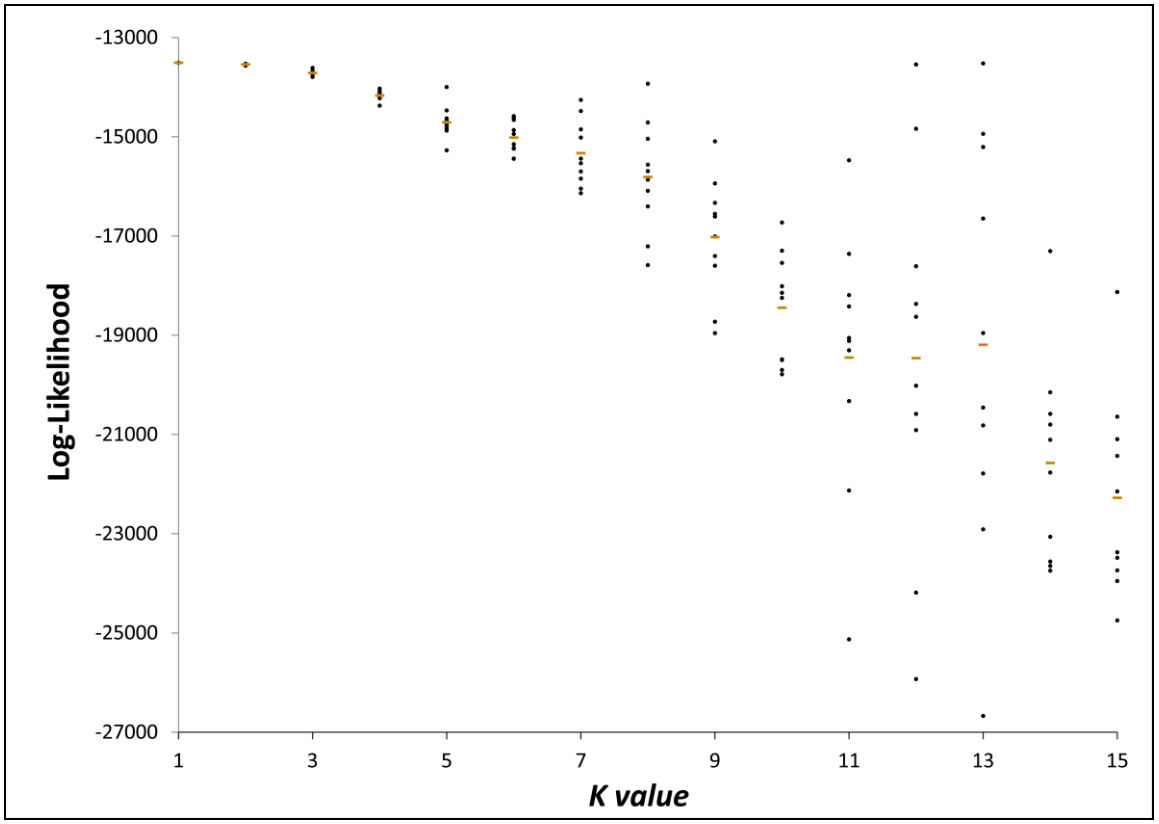

Fig. 4-3 Log-likelihood for each of the 10 independent runs for each K value; the average L(K) for each K is marked with a yellow line

Multivariate Methods

In the sPCA the components of global structure are more informative than the components of local structure (Fig 4-4). 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. 4-4). 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).

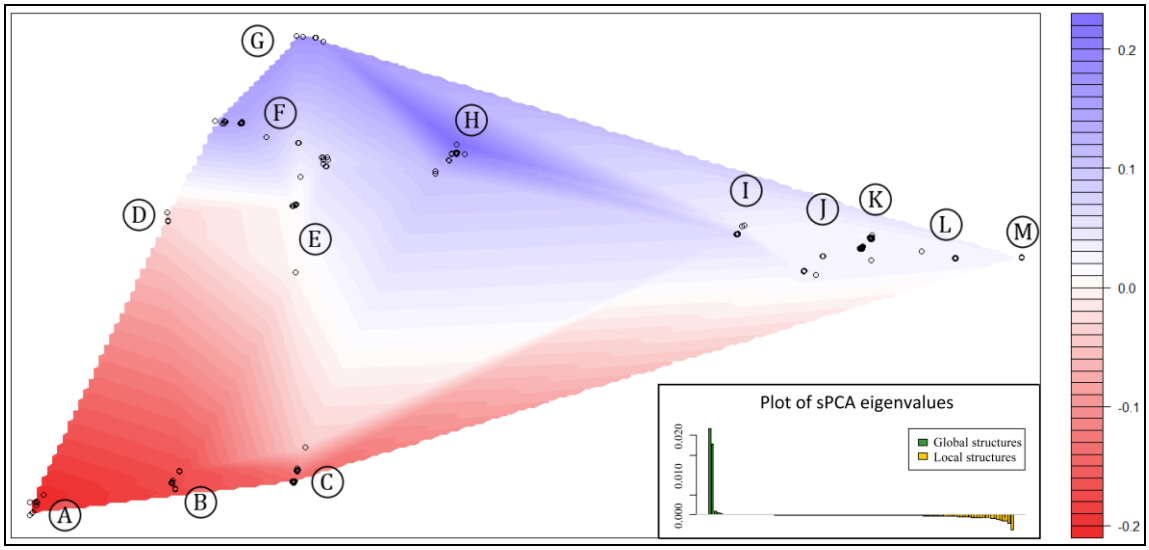

Fig. 4-4 Heat map of the sPCA results. Small black circles represent samples as they are distributed in the Sierra Nevada; the colours correspond to the first positive eigenvalue score of significant global genetic structure. Highest values are shown in red and the lowest values in blue; the magnitude 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 4-3). 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.4-4).

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 ($F_{3, 48} = 3.431$; $p = 0.024$) (Table 4-5).

Table 4-4 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

Table 4-5 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.

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 4-5).

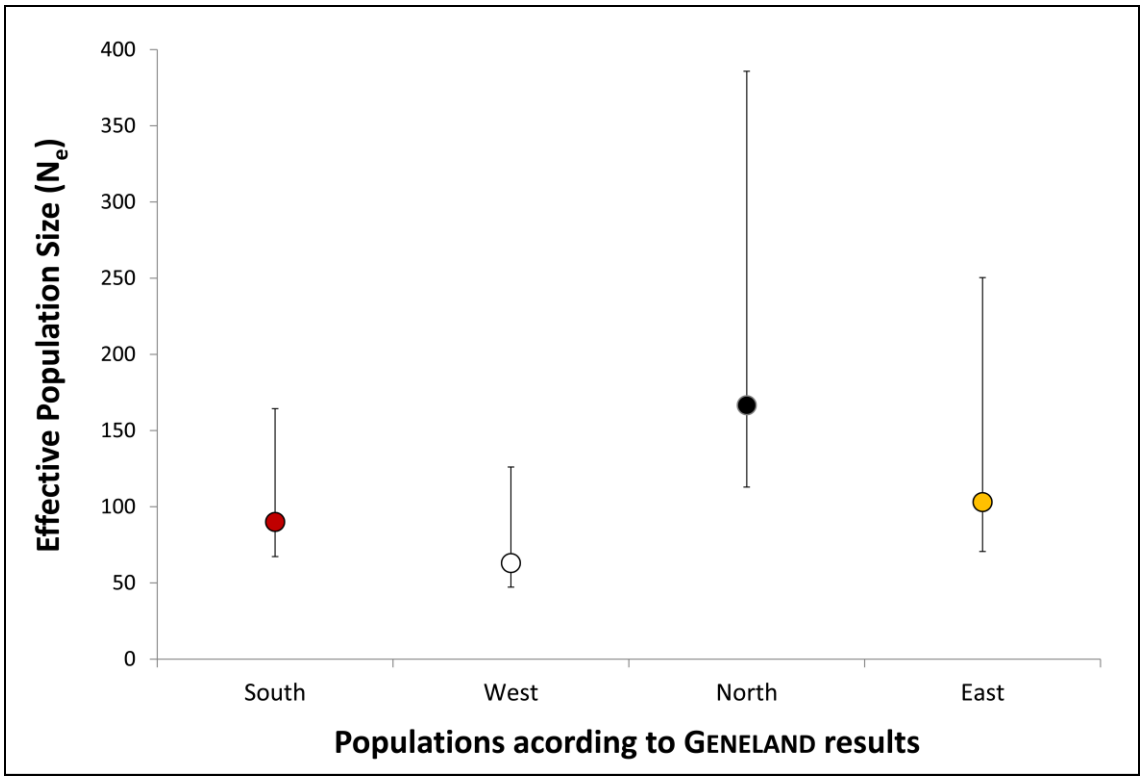

Fig. 4-5 Mean effective population size (N_e) and 95% credible intervals of the populations defined by GENELAND

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 4-6).

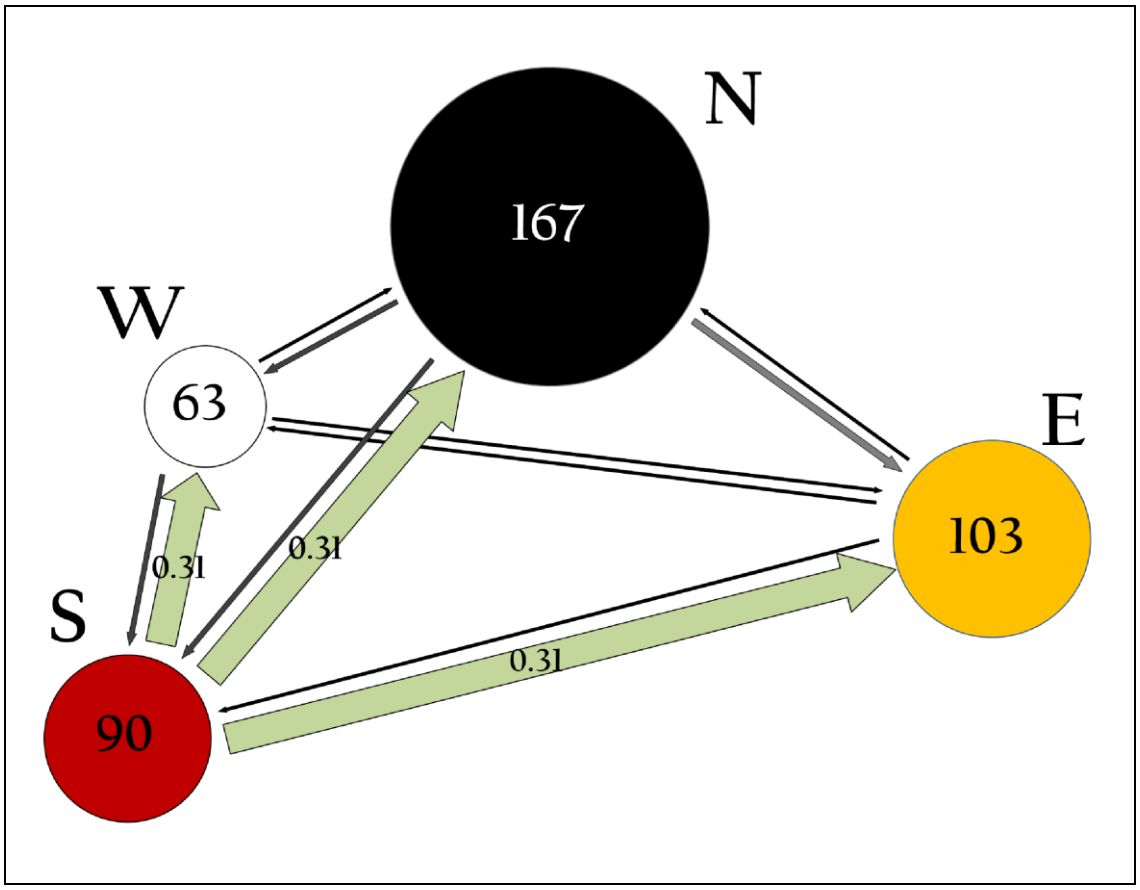

Fig. 4-6 Metapopulation flow chart displaying four asymmetrically connected populations or demes. The radius of the circles is proportional to the mean effective population size (N_e) of the populations; the arrows between the circles represent the migration rates (m) between the populations. The width 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 4-6).

Table 4-6 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. 4-3) and this confirmed that *K*=1. The bar plot from the STRUCTURE output in which are presented the different sampling locations sorted geographically from West to East reinforces this result (*K*=1) as the assignment

does not show any pattern (Fig. 4-7). 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.

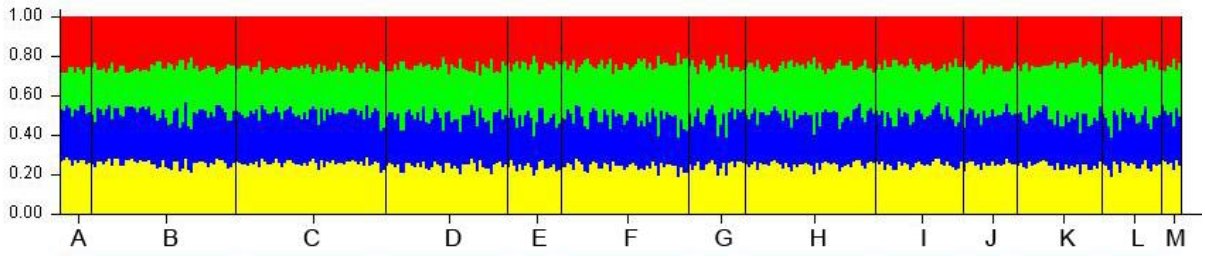

Fig. 4-7 Bar plot from STRUCTURE for K=4 showing the assignment of all the individuals sorted by sampling locations. The locations are sorted from West to East, the names or the locations (codes from A to M) are respectively A: Caballo, B: Piuca, C: Chorrillo, D: Otero, E: Vacares, F: Papeles, G: Mirador, H: Postero, I: Hornillo, J: Chullo, K: Lagunilla, L: Almirez and M: Rayo

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. The sPCA 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. 4-4). 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. 4-6).

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. 4-6). 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; Table 4-6). Significant results of the sign test (evidences of bottleneck) are usually 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 (Chapter 3) 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. 4-1), 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. 4-1). 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. 4-1) – 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. 4-1). 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. 4-1). 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; Chapter 6). 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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Capítulo 5

FACTORES DETERMINANTES DE LA ABUNDANCIA DE P. APOLLO NEVADENSIS (LEPIDOPTERA, PAPILIONIDAE): EL PAPEL DE LA CALIDAD DEL HÁBITAT

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CHAPTER 5

Factors determining the abundance of P. apollo nevadensis (Lepidoptera; Papilionidae): The role of habitat quality

Introduction

Small and isolated populations typically show high risk of extinction due to inbreeding (Keller and Waller 2002) and to their higher sensibility to environmental perturbations and random fluctuations in survival and fecundity (Lande 1988); in fact, population size (N) is one of the best correlates of extinction risk, and consequently an important factor in wildlife management and a major focus for categorization of threatened species (O'grady *et al.* 2004; Puechmaille and Petit 2007).

When the potentially endangered species have a patchy distribution it is important to know why certain populations are bigger than others, or why individuals prefer to be in some patches rather than in others. Habitat or patch quality can be defined as the group of resources and conditions present in an area that make it appropriate for individual and population persistence (Mortelliti *et al.* 2010). It has been demonstrated that habitat quality affects metapopulation dynamics, explaining significant variance in patch occupancy and vacancy (Kindvall 1996; Gyllenberg and Hanski 1997; Fleishman *et al.* 2002). Because of this, habitat or patch quality is considered the third parameter in metapopulation dynamics, often contributing more to species persistence than the area of the deme or the degree of isolation (Thomas *et al.* 2001).

Habitat quality can make a patch suitable or not for a particular species and determine how large is the size of the population that the habitat can maintain (sustainability), thus, understanding the quality of the habitat is needed for an effective conservation and management (Fleishman *et al.* 2002). The kind of variables that make an habitat suitable can vary between different species, different ecosystems or climates. As poiquiloterms, insects have been usually considered dependent from weather given that its metabolism and behaviour often depend on the temperature and insolation (Shreeve 1987), and numerous studies with insects have in fact attained good demographic predictions modelling from climatic data (Mörschel 1999; Roy *et al.* 2001; Shreeve 1987; Trân *et al.* 2007). In butterflies in particular, fluctuations in resource availability are considered important in addition to climate, and there are multiples studies where either larval host-plant or nectar sources are related with their abundance or presence in some patches (Feber *et al.* 1996; Haddad and Baum 1999; Clausen *et al.* 2001; Matter and Roland 2002; Krauss *et al.* 2004). On the other hand, butterfly population dynamics (movement and changes in abundance) seem to be frequently dependent on either weather conditions and environmental stochasticity, or on density-dependent processes such as competition or presence of natural enemies (Dempster 1983).

The butterfly *Parnassius apollo* (Linnaeus, 1758) is distributed across the Palearctic region, with the exception of North Africa and the Arabian Peninsula (Eisner 1976). *P apollo* is considered vulnerable (VU) by the International Union for Conservation of Nature (IUCN) (Baillie *et al.* 1996), and included in the European Red Data Book (Van Swaay and Warren 1999); several of its subspecies from the Eurasian continent have declined substantially over the last century, reaching in some cases the extinction

(Collins and Morris 1985; Van Swaay and Warren 1999; Descimon *et al.* 2005; Van Swaay *et al.* 2010).

As a glacial relict *P. apollo* usually dwells in cold meadows where it has a patchy distribution, and the Southern populations are even more isolated in mountain meadows were they find suitable habitats (Descimon 1995). Among the 22 subspecies described in Spain, *Parnassius apollo nevadensis* Oberthür, 1891 belongs together with *P. apollo gadorensis* Rougeot and Capdeville, 1969 and *P. apollo filabricus* Sagarra, 1993 to the Meridional racial group (Gómez-Bustillo and Fernández-Rubio 1973). These are the southernmost populations of *P. apollo* and each one of them is isolated in a different mountain range. Of these three subspecies, *P. apollo gadorensis* from Sierra de Gádor is now considered extinct and *P. apollo filabricus* has suffered a severe contraction in its distribution in Sierra de Baza-Filabres and is considered Endangered (Barea-Azcón *et al.* 2008). *P. apollo nevadensis* is endemic from Sierra Nevada (Andalucía, Spain) and was considered threatened by excess tourism and habitat modification of Sierra Nevada (Gomariz Cerezo 1993) and is categorised as Least Concern (Barea-Azcón *et al.* 2008).

It seems reasonable to think that populations of *P. apollo nevadensis* were broadly connected in the past, but now we know that there is a recent limitation to gene flow that has generated genetic structure and population differentiation (Mira *et al.* 2017). It is still unclear which factors are promoting this structuration and making some locations less suitable for the butterflies than others. Bearing in mind that it has already been reported an elevation in their altitudinal range (González-Megías *et al.* 2015), likely due to climate change, a better knowledge of the ecological variables explaining the abundance of *P. apollo nevadensis* is an important factor to understand and predict temporal and spatial population patterns in this species.

The aim of this work is to estimate the population size of *P. apollo* in different areas of Sierra Nevada and define which variables predict population size. We use data from a capture-mark-recapture experiment (CMR) performed in six different locations during three years to estimate population size, survival rate and the sex ratio of *P. apollo*. To find out which variables predict the estimated population sizes we study the correlation of the estimated abundances and survival rates with climatic, vegetation and topographic data.

Methods

Species

The altitudinal range of *Parnassius apollo nevadensis* is considered to lie between 1850 and 2500 m (Olivares *et al.* 2011), but in recent years the species has regularly been observed in the Sierra Nevada at altitudes up to 2700 metres (González-Megías *et al.* 2015*)*. The larvae feed mainly in plants from the genus *Sedum* (Olivares *et al.* 2011) and the adults feed on many flowers, but seem to prefer thistles and *Thymus sp* (Olivares *et al.* 2011; personal obs.). The adults fly between mid-June to the end of July, but usually no more than three weeks, depending on the location (Olivares *et al.* 2011).

Sampling

Sierra Nevada is a mountain range in the South of Spain, it has an East – West orientation that reaches around 94 kilometres and a North – South width of 38 km. In 2010, 2011 and 2012 we performed a capture-mark-recapture experiment (CMR) in different locations around the Sierra Nevada. We located meadows with butterflies and

selected six sampling locations (Fig. 5-1) distributed across the surface of this mountain where *P apollo nevadensis* is present, sampling zones characterized by the presence of tall bushes as *Berberis sp*. or *Prunus ramburii* as well as zones with dominance of creeping bushes (mainly *Genista versicolor*) and grass (Gramineae); we selected locations in the Western and Eastern extremes of the W-E axis of the mountain range (Otero and Lagunilla respectively), and in the North (Papeles and Postero) and South (Chorrillo and Piuca) slope facings of the mountain range. The distance between the locations was at least of six kilometres, always with a valley or mountain peaks between them. In each location we performed a CMR experiment in two areas of 100 x 100 metres each, with a distance between them of between 300 and 900 meters, depending on the availability of open meadows with no too steep or abrupt hillsides, that allowed to perform sampling. In 2010 we sampled the locations of Papeles, Lagunilla, Postero and Chorrillo (areas A to H; Table 5-1). After preliminary analyses of the data from 2010, we decided to increase the sampling effort in the following years, in 2011 we repeated sampling in Postero and Chorrillo to improve the estimates, and added Otero (areas I and J) and Piuca (K and L) to include other zones of the mountain range. In 2012 we sampled only in Chorrillo intensively for ten days (Table 5-1) so we could compare data from the same site in different years. Sampling started in each location a few days after we saw the first imagos flying, and was carried out every two days until the abundance of butterflies was clearly decreasing and most of the imagos showed signs of wear; in total, four or five sampling occasions which spanned a period of 10 to 14 days. Butterflies were caught with a butterfly net and marked on the underside of their hindwings with a unique numerical code using a soft-tipped pen; in a GPS device we introduced the precise location (coordinates), sex and a variable we called "State": as an approximation of the age we noted the state of the wings (from "0" fresh and intact, to "3" broken and worn out).

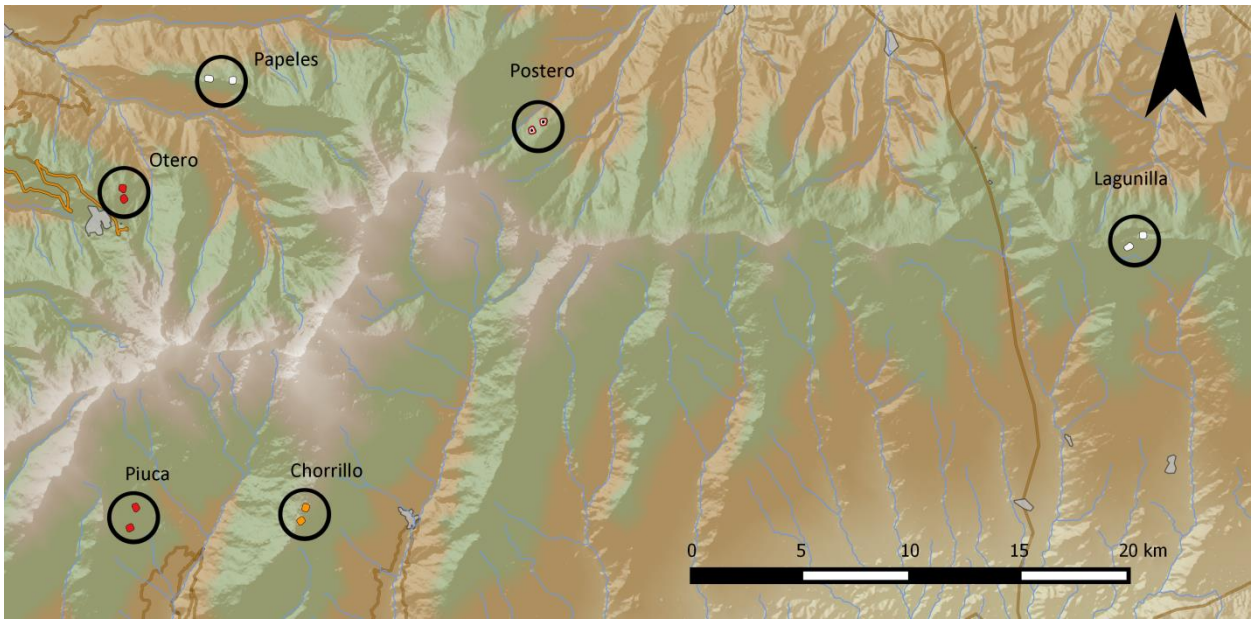

Fig. 5-1 Map of the Sierra Nevada showing the position of the 6 sampling locations and the two areas sampled in each location. In each location we sampled the two areas of 1.55 hectares. The grey polygon next to Otero marks the situation of the touristic resort of Prado Llano. Codes used for each location and area can be found in Table 5-1**.**

To characterise vegetation cover and the number of species of plants, in each one of the CMR sampling areas we performed a quadrat sampling by using six randomly distributed squares of 1.5 m^2 with 36 subdivisions. We identified all the plants or assigned them to different morpho-types when we were not able to go further in the determination.

The butterflies were handled carefully and released. All the sampling was done under permission of the National Park (Parque Nacional y Natural de Sierra Nevada) and the Consejería de Medio Ambiente (Junta de Andalucía).

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Table 5-1 Details of sampling, estimated population size, and characteristics of the best models (lowest AICc) for each location and year.

Code	Location	year	Area	Events	Span	Cap:Rec	Sex	n	ϕ (survival)	*p* (capture)	*b* (entrance)	N	AICc	Phi	N̂
ChoE10	Chorrillo	2010	E	5	13	149:15	female	21	sex+State	sex+time	Time	sex	159.9889	0.395	97.95
							male	128						0.596	697.92
ChoF10	Chorrillo	2010	F	5	13	65:13	female	7	storm	State	sex+time+wind	sex	125.1988	0.624	13.74
							male	58						0.725	127.16
LagG10*	Lagunilla	2010	G	4	11	82:10	female	18	Time+Age+State	State+wind+storm	sex	sex	104.9748	0.276	68.21
							male	64						0.276	156.26
LagH10*	Lagunilla	2010	H	4	11	72:4	female	12	State+storm	State+wind+storm	sex+wind	sex	74.738	0.088	63.75
							male	60						0.211	249.8
PapA10*	Papeles	2010	A	4	11	96:16	female	17	sex+wind+storm	time+Age+effort	State	sex	133.2758	0.115	21.83
							male	79						0.651	99.15
PapB10*	Papeles	2010	B	4	11	75:8	female	27	sex+wind+storm	time+Age	sex+State	sex	96.1494	0.002	52.74
							male	48						0.690	81.54
PosC10*	Postero	2010	C	4	11	101:12	female	17	time+State+storm	State	sex+State+wind	sex	110.9144	0.858	30.73
							male	84						0.858	138.23
PosD10	Postero	2010	D	4	11	92:12	female	18	time+wind+storm	wind*storm	sex+State+wind	sex	127.172	0.089	71.33
							male	74						0.089	221.32
ChoE11*	Chorrillo	2011	E	8	17	202:35	female	19	storm	time+wind+storm	Age+wind	sex	604.6618	0.821	100.2
							male	183						0.821	353.12
ChoF11*	Chorrillo	2011	F	5	13	70:7	female	11	State+wind	time+effort+storm	State	sex	71.0756	0.387	14.32
							male	59						0.387	79.07
OteI11*	Otero	2011	I	4	11	142:22	female	12	1	time*State	sex+wind+storm	sex	164.1208	0.544	13.14
							male	130						0.620	145.84
OteJ11*	Otero	2011	J	4	11	125:29	female	9	sex	State+storm	State	sex	190.9482	0.002	14.73
					11		male	116						0.763	170.89
PiuK11*	Piuca	2011	K	4	10	124:9	female	22	Time+Age	time+wind+storm	sex	sex	115.9817	0.454	25.36
							male	102						0.454	113.27
PiuL11*	Piuca	2011	L	4	10	150:18	female	22	sex+wind+storm	Age+State+effort	sex+time+Age	sex	157.7543	0.001	29.29
							male	128						0.703	157.23
PosC11	Postero	2011	C	4	10	92:14	female	14	State	Time+Age	sex+wind	sex	126.173	0.993	73.93
							male	78						0.999	259.24
PosD11*	Postero	2011	D	4	10	71:19	female	11	sex+State	sex+time	Time	sex	127.5358	0.106	28.77
							male	60						0.698	80.82
ChoE12	Chorrillo	2012	E	10	14	109:57	female	30	time+Age	State+wind+storm	Sex	sex	442.1609	0.793	59.32
							male	79						0.793	118.97

Events, days of CMR sampling; Span, number of days between the first and the last event (both included) of CMR sampling; Cap:Rec, total individuals captured and total recaptured in all the days of CMR study; n, number of females and males marked; ϕ, p, b, and N are the parameters of the model; AICc, corrected Akaike's Information Criterion; Phi, average estimated survival probability; N̂, estimated population size. "*" in the area codes marks those with narrower confidence intervals that were used in the PLS regression.

Demographic parameters

We used CMR data (Table 5-1) to estimate independently the population size and survival probability for each area and year using the program MARK (White and Burnham 1999). In the input file of each CMR area we included the sex as a grouping variable and the covariable with the estate of the wings ("State").

Before proceeding any further with the modelling we fitted a Cormack-Jolly-Seber (CJS) model with survival (ϕ) and recapture (p) probability depending on time (t) and sex (s) [(ϕ(t*s), p(t*s)] to test the data's Goodness of Fit (if the data meets the expectations determined by the assumptions underlying the model) with the Test 2 and 3 in the program RELEASE included in MARK. We estimated $\hat{c}$ to test for overdispersion by dividing the model's observed deviance by the mean of the simulated deviances obtained after 1000 bootstrap simulations (option "Bootstrap GOF") in MARK (White and Burnham 1999)

Once the data was verified for each CMR area, we used Rmark (Laake and Rexstad 2008), a package for R (R Core Team 2016) with an interface to MARK program, to build the models and alter the design matrix. In order to estimate population size we used the POPAN formulation of the Jolly-Seber (JS) models. POPAN is a formulation of JS for open populations, it postulates the existence of a super-population consisting of all animals that would ever be born in the population. The parameters in this formulation are ϕ (survival probability in each interval between CMR events), p (probability of capture in each CMR event), b (probability of an animal from the super-population would enter to our population in each interval between CMR events) and finally N as the number of animals that will ever be born in the super population (Schwarz and Arnason 1996). In our models each one of these parameters can be fixed to a value or allowed to

vary depending on the value of different variables and the combinations (addition or addition plus interaction) of two or more of those variables. The variables used in our models were: State (degree of wear of the butterfly on first capture), Age (a variable predefined in RMark that increases every event/time lapse unit), time (predefined variable in which the value of the parameter is allowed to change over time as a factor level), Time (predefined variable in which the change over the time is numerical and continuous), effort (a variable for the parameter *p* equivalent to the number of people participating in each CMR event), sex (sex of the individual that was used as a grouping variable), wind (coded independently for each parameter and CMR event, with a value of 1 if we recorded strong winds that affected our work during any of the days), storm (coded independently for each parameter and CMR event, with a value of 1 if we recorded rain that affected our work during any of the days), and finally "1" (indicates that the value of the parameter is constant, and therefore not depending on time or any other of the variables, equivalent to (.) in MARK).

We were interested in characterizing population sex ratio, and despite that in most of the areas the number of females captured was really low, and their recapture rate was 0 in some of those cases. In addition, after some preliminary analyses we saw that N estimates (population size) were fairly different between sexes, and because of this we decided to use sex as a grouping variable to estimate N in the same model. This allows us to compare estimates between areas and between sexes. The estimations of population size and their standard errors for males and females can be summed afterwards if we want to use the values for the whole population (Jeff Laake, personal comment).

To keep a feasible computation time instead of just running all the possible combinations for all the variables and parameters, we first selected a subset of variables for each parameter. We selected those variables that gave models with the lowest

corrected Akaike's Information Criterion (AICc) independently for each parameter (keeping the other parameters constant); and then with the selected variables we ran all the different models with all the possible combinations for each parameter and variable. To do this we set a preliminary run in a model wrapper (*mark.wrapper*) with all the possible combinations of these variables in every parameter, but with only one variable affecting one parameter each time (the other parameters were set to constant), then we sorted the models for each parameter separately to identify for each parameter which were the variables that gave models with the lowest corrected AICc. We selected the 5 variables that gave the best models for each parameter. With the 5 chosen variables of each parameter the models were re-run again using the model wrapper that calculates all the possible combinations of the chosen variables affecting the parameters. To reduce the number of possible combinations and keep a feasible computation time we set a maximum of three variables summed at the same time per parameter. We chose the best model with the lowest AICc and obtained the estimation of N and ϕ and the variance and confidence intervals for each sex. In the case that the best model has not an AICc at least two units smaller than the second best model it is recommended to average the estimates from the best models, those that have an AICc less than two units bigger than the best model (Cooch and White 2016).

For comparison purposes we built 95% confidence intervals (CIs) to compare the average estimates of population size and survival probability. The use of CIs to compare means is considered conservative as it makes harder to detect significant differences in mean (Payton *et al.* 2003), though in some degree confidence intervals are considered better than null hypothesis significance testing (NHST), because contrary to NHST, CIs show the magnitude of the differences between the means . Means with non-overlapping confidence interval are considered significantly different, however when the confidence

intervals overlap does not mean necessarily that the means are not different (Bower 2003).

Habitat characteristics and butterflies abundance

Information of the areas sampled

To perform a correlation analysis between the parameters estimated in MARK (population size and survival rate) and the explanatory habitat variables we sorted the data in four different matrices related to the CMR areas, Response matrix Y included either the values of mean estimated population size from each area or the average of the survival rates for all the CMR event in each area (Table 5-1). Explanatory matrix X contained the information about to which year belongs every observation, which was done separately of the others to make easier to leave it out of the analysis if the correlation was too strong. Explanatory matrix A was made up with 20 climatic variables downloaded from WorldClim (Hijmans *et al.* 2005). The variables included were the annual mean temperature (Tmean), maximum temperature of the hottest month (Tmaxan), minimum temperature of the coldest month (Tminan), maximum and minimum monthly temperature (for May, June and July) (Tmax1-6, Tmin1-6, Tmean1-6), temperature annual range (Trange = Tmaxan-Tminan), temperature seasonality (Tseason = SD*100), mean diurnal range (MDR = mean of monthly (maximum temperature – minimum temperature)), isothermality (Isoterm = MDR / Trange), annual precipitation (Precan), precipitation of wettest and driest months (Precmax and Precmin), and precipitation seasonality (Precseas = coefficient of variation of the precipitation). Explanatory matrix B, with the information from the quadrat sampling, including cover of the larval host-plant *Sedum sp* (Covsedum), cover of bushes (Covbush), which was mainly *Genista versicolor*, the cover of plants that were at flowering stage during July (Covflor), the total accumulative plant species richness (Stot)

and the mean plant species richness per quadrat (Smean), the Shannon-Weaver *H'* (Hsw) diversity index (Shannon *et al.* 1950) and the Simpson *D* (Dsimp) diversity index (Simpson 1949); both indexes were used as it has been shown that Simpson's D is considerably more influenced by species evenness and less by richness than is Shannon's H (Dejong 1975). Finally, explanatory matrix C included the geographical characteristics of the location, that is, degrees of slope (Slope), meters above the sea level (Elevation), distance to the closer forest track (Dcia1), and distance to the closer village or road (Dcia2). The program QGIS 2.10.1 (Qgis Development Team 2015) was used to calculate the data in the matrix C and to extrapolate the values from the WorldClim layers to each area of study.

PLS Regression

Correlation among variables can lead to unreliable estimates of regression parameters and to solve this there are two options: we can either omit variables that are strongly correlated with others (with a loss of the ability to evaluate all the variables), or we can use alternative statistical methods that allow collinearity (Geladi and Kowalski 1986; Johansson and Nilsson 2002). We used a partial least squares regression (PLSR or PLS regression) to estimate the degree of correlation between the response variable (matrix Y) and the matrices of explanatory variables characterising each CMR area (matrices A, B, C, and X). PLS regression is an extension of multiple regression analysis that combines regression and ordination, and relaxes restrictions on collinearity among variables (Johansson and Nilsson 2002). PLS is especially recommended in cases of strong collinearity and when (as in our case) there is overfitting, and the number of variables is similar to or higher than the number of observations (Mevik and Wehrens 2007). PLSR has been proved more reliable identifying relevant variables, their degree of interaction, and their magnitudes of influence, than other techniques such as Principal Component

Analysis and Multiple Regression analysis, especially in cases of small sample size (Carrascal *et al.* 2009). Similarly to the multivariate analyses, PLS regression establishes associations between the explanatory variables and extracts a few latent factors (components) that maximize the variance explained in the response variable. These components are then used as independent variables in a regression.

Because we had population size and survival probability estimates for different years of some of the locations/areas (Table 5-1), to avoid pseudoreplication and give the same weight to all sampling locations, we chose only the N estimate with the narrower confidence interval from each area for the PLS regression (Table 5-1). Prior to the PLS analysis we applied log transformations to the variables that were not normally distributed. All the variables were standardized to a mean of zero and variance of one, this procedure is recommended when there is no information on the relative importance of each variable (Johansson and Nilsson 2002).

The PLSR analyses were performed in the Add-In for Excel XLSTAT 19.1 (Addinsoft, France). For each model fitted we did first a preliminary analysis to select the number of components that gave the best quality model, and then we refitted and ran the model again with the chosen number of components fixed. Three indicators of the goodness of fit of the models were used to choose the best number of components: determination coefficient of the explanatory variables (R^2X), which shows the proportion of the variance in the matrices of explanatory variables that was used to build the models; the determination coefficient of the response variable (R^2Y), that is, the proportion of variance from the response variable that the model is able to explain (corresponds with the multiple regression coefficient, R^2); and more importantly, the cross-validated coefficient of the model (Q^2), that indicates the proportion of the variance from the response variable that the model is able to predict (Johansson and Nilsson 2002).

We repeated this process three times with different data: First, with the total N estimates from each area (calculated as the sum of N estimates for both sexes); second, only with the estimates of N for males (including the abundance of females as an explanatory covariable); and finally only with the estimates for females (including males` abundance as an explanatory covariable). For this three data arrangements we performed the PLS Regression with different combinations of the four matrices of explanatory variables (A, B, C, and X), setting X (Year) as qualitative and the rest of the data (A, B, and C) as quantitative explanatory variables. This will allow us to see which data were more important to the analysis. The same process was repeated averaging the survival probability (estimated for all the intervals between CMR events) and then using the standardized average survival probability, from the same areas that in the population size analysis, as a response matrix (Y) (Table5-1).

As a global method to evaluate the model and compare with other models, PLS is run n times, leaving out one observation each time and computing the expected value of the response variable for that observation that is missing in each run (leave-one-out cross-validation). The sum of squares of differences between the leave-one-out predictions and the real values is the root mean squared error (RMSE) (Maestre 2004). Using the RMSE we compared between the models including different data.

Relevant variables

To evaluate the variables that explain the variance in population size, we used the variable importance projection (VIP), which measures the importance of each explanatory variable in building the components included in the model. The more relevant variables should have VIP-values greater than one. The VIP value does not indicate if the correlation of the explanatory variable with the response variable is positive or negative, it only tells their relevance in building the latent factors or

components of the model (Davis *et al.* 2007). Complementary to the VIP values, the standardized coefficients (also named beta coefficients) allow comparing the relative weight of each variable in the model and the direction of its influence (sign of the coefficient). The greater the absolute value of a coefficient, the greater the weight of the variable in the model (Esposito Vinzi *et al.* 2010).

Results

Demographic parameters

CMR experiment

In one of the areas from Chorrillo (ChoF) we did not capture enough butterflies in 2012 to generate analysable data (17 captures, 0 recaptures in 9 days). In all the other CMR areas and years we obtained at least four days of data with recaptures (Table 5-1). The percentage of recaptures of individuals previously captured in a different area from the same location was in all the cases below 0.81% (zero most of times), and we never recaptured individuals from any sampling location in a different sampling location. The variable "State" shows a bigger proportion of fresh males than females, but the rate of probably older butterflies with broken wings is higher in females (Fig 5-2).

MARK models

All Test 2 of the program RELEASE for all the CMR areas were non-significant (all p-values > 0.4507). According to program MARK manual (Cooch and White 2016) to perform the Test 3, RELEASE needs to compare recapture stories of individuals recaptured more than once and in different events to check if their probability of capture or survival depends on when they were marked or on if they were recaptured. For this reason we were only

able to apply Test 3 to the area E (2010, 2011 and 2012), and it was non-significant in the three cases (all p-values > 0.5036). For all the areas, several models showed similar support, with difference between them and the best model (lower AICc) smaller than 2 units. Therefore, we averaged the estimates of the parameters for all the highly supported models (Table 5-1).

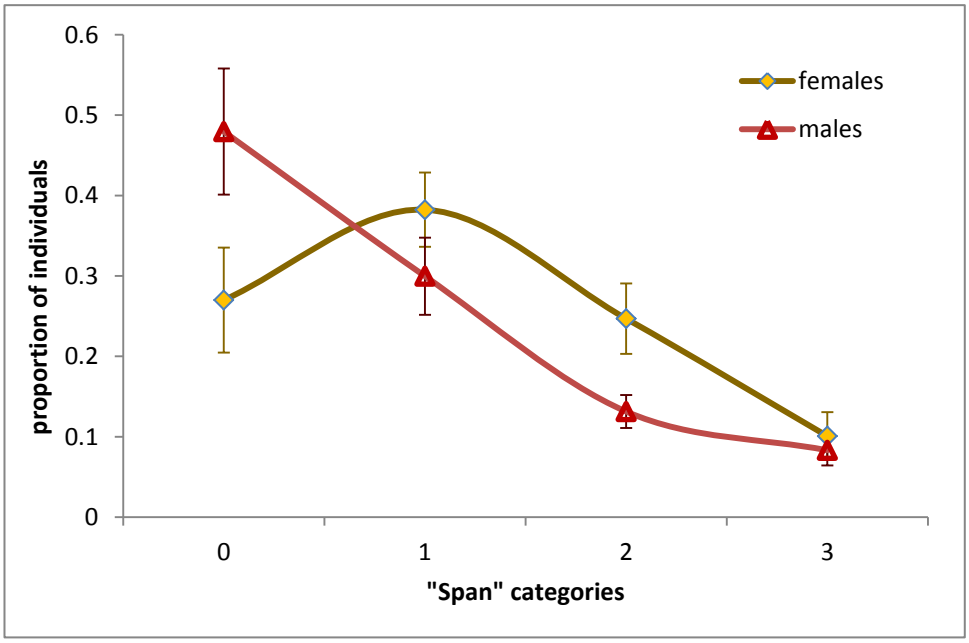

Fig. 5-2 Proportion of individuals from each sex found belonging to each of the Span categories, from 0 (fresh and clean), to 3 (worn out and with broken wings).

Survival rate

The maximum longevity recorded for a butterfly was of 11 days. The average survival probabilities range from 0.001 to 0.999 (Table 5-1); in the cases where the survival parameter depends from sex, male survival seems to be higher than female survival (Fig. 5-3).

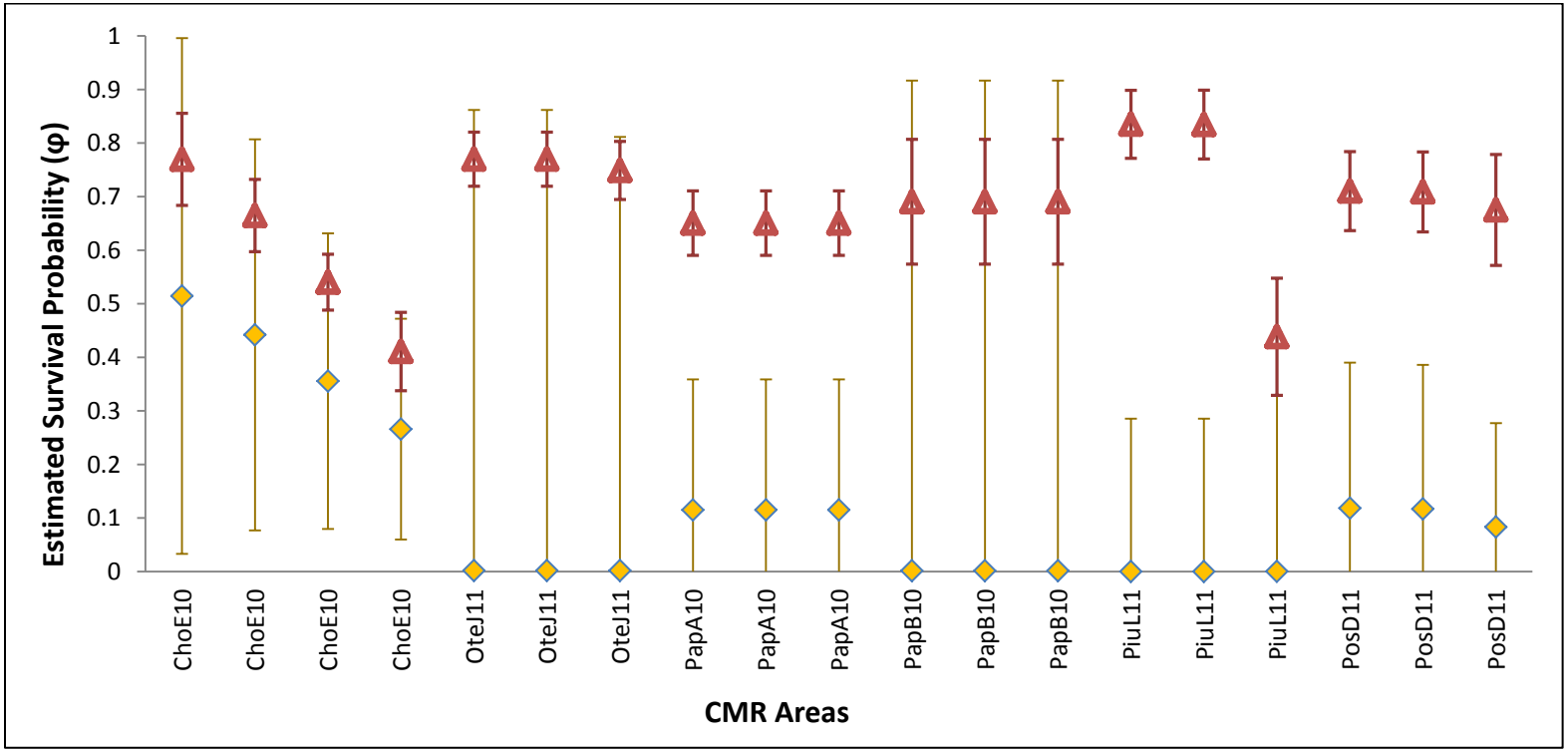

Fig. 5-3 Average estimated Survival Probability (ϕ) and 95% confidence intervals for males (△) and females (◊) from the areas in which this parameter is sex-dependent, every value from the same area and year correspond to the estimate from a different CMR event for that area and year

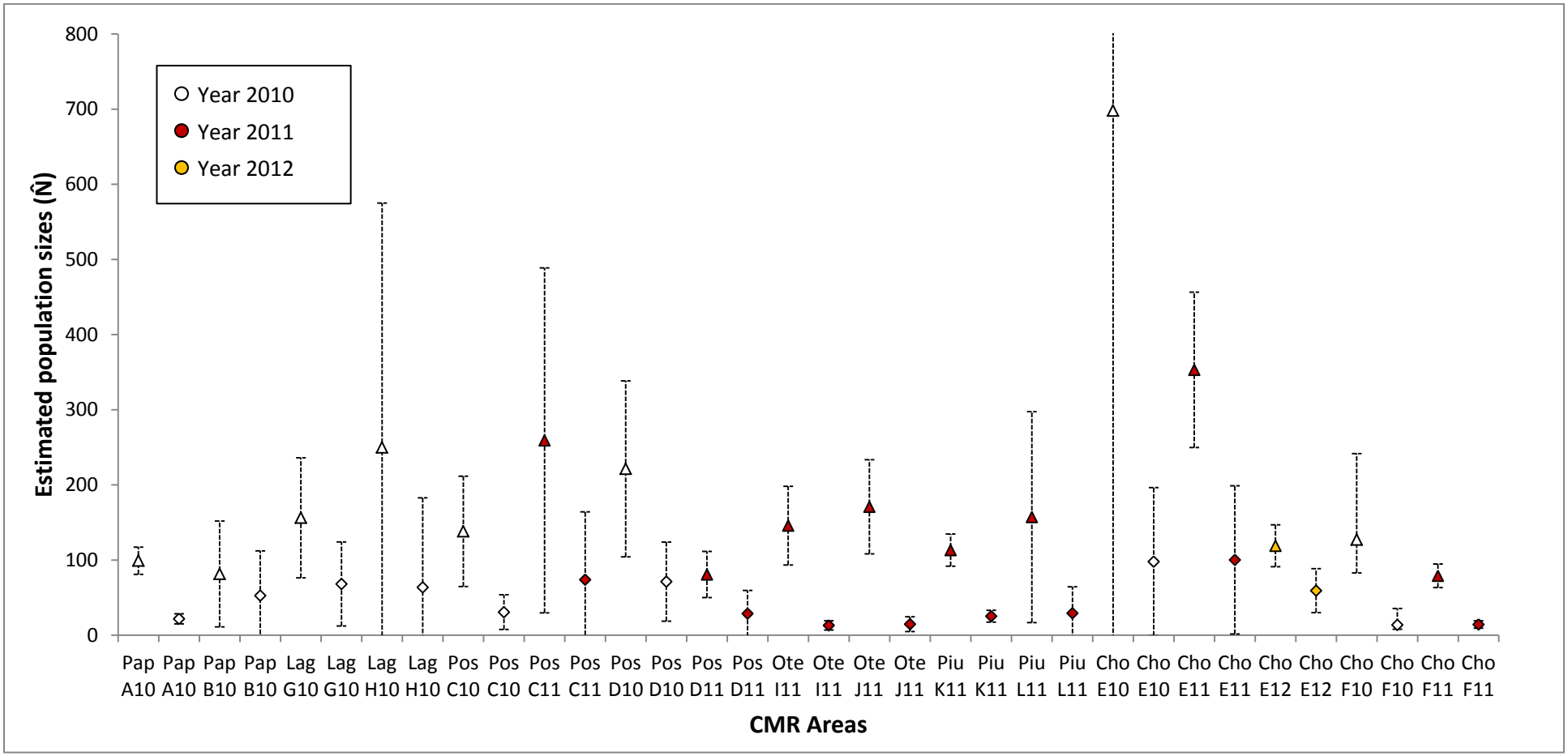

Fig. 5-4 Average population size and 95% confidence intervals estimated with Mark separately for males (△) and females (◊), for every CMR area and year sampled. Codes for the locations and areas are shown in Table 5-1

Population size

The estimates of total population size of the areas (N̂ of males and females summed together) range from 109 (Area D in Postero in 2011) to 795 (area E in Chorrillo in 2010, Fig. 5-4). Overall area ChoEll from Chorrillo has significantly larger population size than most of the areas from other locations either considering total N or male population size (see Fig. 5-4 and 5-5). In general we can observe that the population sizes of females are always smaller than population sizes of males of the same area, and the areas with more males are usually also the areas with more females and vice versa.

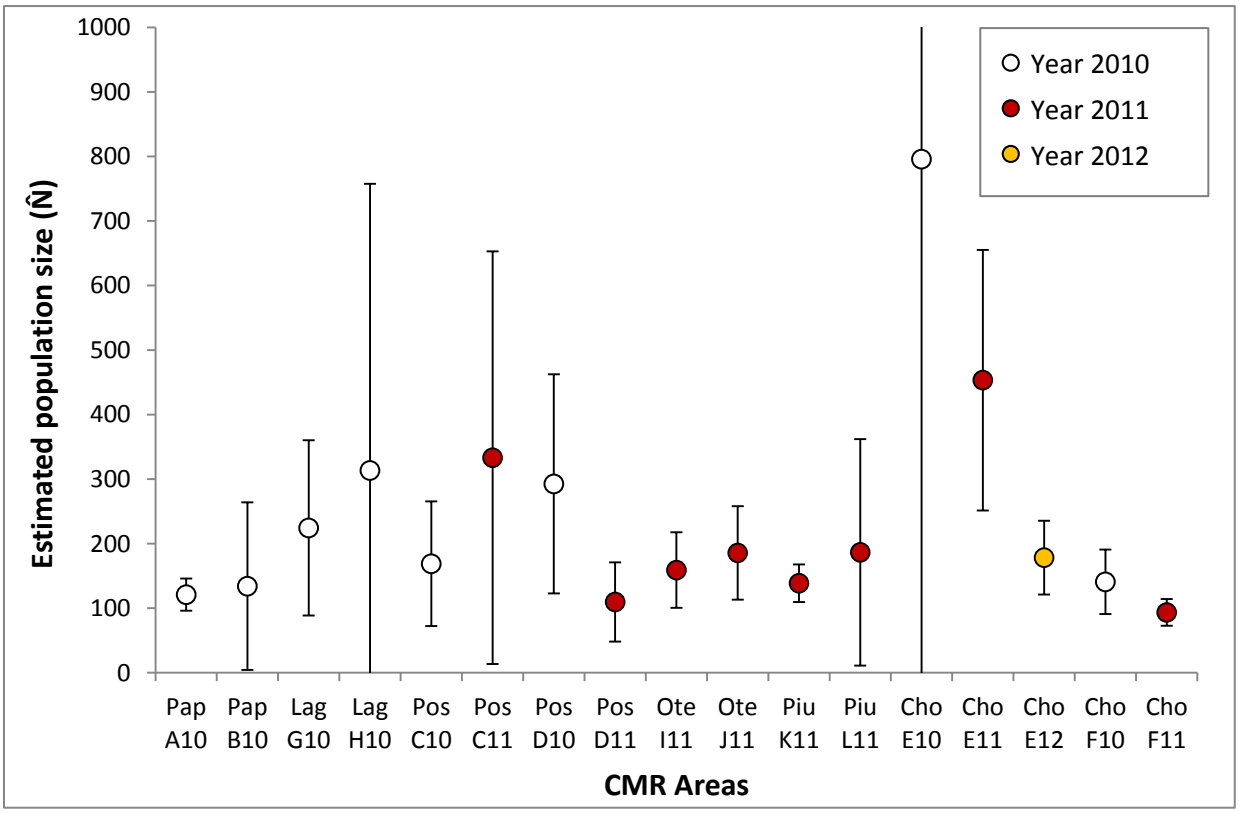

Fig. 5-5 Population sizes and 95% confidence intervals estimated with MARK for each CMR area and year. The N estimates and SE have been summed for both sexes. Codes for the areas are from Table 5-1.

Table 5-2 Explanatory variables matrices B and C, and sex ratio for all the areas and years sampled

	B							C				Sex ratio	
Code	H'sw	*D*simp	*S*tot	*S*mean	Covsedum	Covbush	Covflor	Elevation	Slope	Dcia1	Dcia2	captured	estimated
ChoE10	1.450	3.222	10	4.17	0.199	0.792	0.144	2591.07	6.29	0.91	4052.98	6 : 1	7 : 1
ChoF10	2.345	8.132	17	9.50	0.060	0.250	0.259	2626.10	5.71	123.70	4238.96	8 : 1	9 : 1
LagG10	2.155	6.152	17	6.83	0.093	0.255	0.380	2269.80	3.09	216.77	6267.95	4 : 1	2 : 1
LagH10	1.407	3.146	9	5.00	0.005	0.690	0.093	2309.07	16.45	55.08	5560.55	5 : 1	4 : 1
PapA10	2.380	8.548	20	11.17	0.398	0.352	0.514	2097.87	11.87	486.71	5510.93	5 : 1	5 : 1
PapB10	2.700	9.902	35	13.50	0.431	0.194	0.583	2049.53	10.64	95.58	4694.82	2 : 1	2 : 1
PosC10	2.785	13.833	24	10.83	0.208	0.310	0.713	2041.80	21.55	1684.89	8729.17	5 : 1	4 : 1
PosD10	1.809	12.325	27	12.33	0.315	0.458	0.676	1984.63	19.44	1035.18	8093.08	4 : 1	3 : 1
ChoE11	1.927	4.823	17	6.00	0.208	0.745	0.403	2591.07	6.29	0.91	4052.98	10 : 1	4 : 1
ChoF11	2.016	5.627	18	6.50	0.042	0.394	0.519	2626.10	5.71	123.70	4238.96	5 : 1	6 : 1
OteI11	2.453	9.253	22	10.14	0.369	0.500	0.468	2199.67	19.66	21.64	1245.29	11 : 1	11 : 1
OteJ11	2.318	7.014	25	8.71	0.238	0.611	0.421	2290.50	19.95	502.54	895.12	13 : 1	11 : 1
PiuK11	2.101	5.767	21	7.71	0.119	0.504	0.345	2283.77	10.19	1492.77	3552.46	5 : 1	5 : 1
PiuL11	1.933	4.865	14	6.50	0.079	0.444	0.303	2416.10	10.59	859.99	3465.64	6 : 1	5 : 1
PosC11	3.025	16.019	33	13.33	0.361	0.319	0.648	2041.80	21.55	1684.89	8729.17	6 : 1	4 : 1
PosD11	2.695	9.921	34	11.50	0.329	0.407	0.690	1984.63	19.44	1035.18	8093.08	5 : 1	3 : 1
ChoE12	1.995	4.712	27	6.37	0.131	0.701	0.391	2591.07	6.29	0.91	4052.98	3 : 1	2 : 1

Matrix B: H'sw; Shannon-Weaver H' diversity index; Dsimp, Simpson D diversity index; Stot, total plant species richness from all the quadrats sampled in the area; Smean, average plant species richness; Covsedum, percentage of Cover of Sedum sp; Covbush, cover of bushes; Covflor, cover of flower plants that bloom in July. Matrix C: Elevation (meters above the sea level); Slope (degrees); Dcia1, distance (m.) to the next forest track; Dcia2, distance (m.) to the next road or village. Sex ratio expressed as ration of females : males, estimated directly from the number of captures of each sex, and from the N estimates.

Habitat characteristics and butterflies abundance

Information of the areas sampled

We found high variability in plant diversity (D_{simp}), plant species richness (S_{tot}) and floral cover (3.5 to 16.02; 9 to 35; and 0.09 to 0.71, respectively). We found that some of the areas have big differences in most of their variables (Table 5-2), for example Chorrillo is the highest location (2591 m) and together with Lagunilla includes the areas with the lowest plant diversity (Dsimp and H'sw), floral cover, and *Sedum* cover; on the other hand Postero is the lowest location (1984m) and the farthest from any village or road, and jointly with Papeles includes the areas with the highest plant diversity and floral cover. Papeles and Otero have the highest *Sedum* cover, and Otero in addition is the closest location to a road. Finally we can see how in the locality of Chorrillo there is great heterogeneity in the cover of bushes, sharing the highest Covbush with LagH (Lagunilla) and the lowest with Papeles.

PLS Regression and variables relevant for abundance

In the PLSR, the best model for males was ABCm, the model not including the information about the year (RMSE = 0.226) and the best models for females was XBCf, the model without the environmental data matrix (RMSE = 0.262). The best models for males and females separately have better prediction power (lower RMSE) and are better fitted (Q^2 = 0.226 and 0.535, respectively) than the best model for the data from both sexes summed, which has higher RMSE (0.274) and a Q^2 of 0.052 (Table 5-3); this is below 0.097, the minimum level of significance usually accepted for Q^2 (Fridén *et al.* 1994; Johansson and Nilsson 2002) and therefore we only consider the best models that include male and female data separately for the subsequent analyses.

There are six relevant variables (VIP > 1) correlated with the population size of females (Table 5-4). The variable most strongly positively correlated with the abundance of females is the abundance of males; the distance to the nearest road or village and the total plant species richness of the area are also positively correlated with female abundance. We can see also an effect of the year in female abundance (Table 5-4). In the modelling of the males we found only five variables correlated with population size (Table 5-4), being the abundance of females the most strongly positively correlated variable. The cover of bushes and flowers and the minimum temperature in June (Tmin6) are negatively correlated with male abundance, while the degrees of slope in the area are positively correlated with male population size (Table 5-4).

PLS Regression and relevant variables for survival rate

The PLS regression models with the survival probability are generally worse fitted and have lower predictability, than those with the population size, indicating that the variables included are not as good predicting survival probability as they are predicting population size. Models with the female's average survival probability have in all the cases a cross validated coefficient (Q^2) below zero indicating a lack of fit; and therefore should be rejected. The models with the average survival probability of the males show a better fit than the models with female survival probability, the best model is the model is ABCm with a RMSE of 0.556 (Table 5-3).

Table 5-3 Summary of PLS models fitted to explain population size and survival probability. The table shows the matrices of predictor variables (A,B, C, and X) included in each model, and the best number of components (ncom). Root mean square error (RMSE), determination coefficient of the explanatory variables (R^2X), the response variable (R^2Y) and the model (Q^2) are shown as a measure of the goodness of fit.

Data	Model	ncom	R^2X	R^2Y	Q^2	RMSE
total population size	ALL+	1	0.642	0.232	-0.414	0.289
	ABC+	1	0.677	0.228	-0.374	0.290
	ABX+	1	0.673	0.239	-0.386	0.288
	AXC+	1	0.728	0.159	-0.860	0.303
	XBC+	1	0.494	0.310	0.052	0.274
males population size	ALLm	2	0.747	0.756	0.266	0.246
	ABCm	2	0.760	0.793	0.321	0.226
	ABXm	2	0.788	0.738	0.262	0.255
	AXCm	1	0.709	0.360	-0.016	0.398
	XBCm	1	0.464	0.557	0.294	0.331
females population size	ALLf	2	0.746	0.732	0.156	0.444
	ABCf	2	0.746	0.777	0.061	0.405
	ABXf	2	0.776	0.730	0.079	0.446
	AXCf	2	0.819	0.731	0.278	0.445
	XBCf	3	0.744	0.906	0.535	0.262
	XBCf	2	0.646	0.733	0.223	0.443
average males survival probability	ALLm	2	0.777	0.642	0.167	0.573
	ABCm	2	0.793	0.662	0.231	0.556
	ABXm	2	0.821	0.614	0.135	0.595
	AXCm	2	0.816	0.551	0.057	0.642
	XBCm	1	0.443	0.480	0.186	0.690
average females survival probability	ALLf	1	0.629	0.154	-0.330	0.881
	ABCf	1	0.655	0.157	-0.307	0.879
	ABXf	1	0.664	0.141	-0.206	0.888
	AXCf	1	0.714	0.123	-0.146	0.897
	XBCf	1	0.115	0.519	-1.281	0.664

The values showing the best goodness of fit in each category are underlined. Models used in the rest of the analysis are shown in bold. The first three letters of the model's name indicate whether it was fitted with all the explanatory variables matrices (ALL), or otherwise which combination of the four matrices of explanatory variables it included (A, B, C, and X); the last letter (lowercase) indicates whether it was done with the estimates of males (m), females (f), or both estimations summed (+).

The number of relevant variables correlated with the survival probability of the males is higher than the number of variables related to population size, but the strength of the correlation is weaker. The two variables most strongly positively correlated with male survival probability are the *Sedum* and floral cover, and only two variables are negatively correlated with male survival, the minimum temperature in May and in June (Table 5-4).

Table 5-4 Influence (VIP) and coefficient of the explanatory variables used in the PLS models. Only the most influencing variables (VIP > 1) are shown

Model	Variable	VIP	coefficient
Males abundance (ABCm)	Nfem	2.260	0.291
	Covbush	2.102	-0.271
	Covflor	1.107	-0.140
	Tmin6	1.125	-0.128
	Slope	1.120	0.112
Females abundance (XBCf)	Nmal	1.705	0.716
	Dcia2	1.018	0.336
	Stot	1.091	0.218
	y2010	1.169	0.128
	y2011	1.169	-0.128
Males survival (ABCm)	Covsedum	1.699	0.175
	Covflor	1.404	0.144
	Precseason	1.383	0.140
	Hsw	1.243	0.121
	Dsimp	1.132	0.107
	MDR	1.290	0.103
	Tmin6	1.372	-0.100
	Trange	1.175	0.082
	Tmin5	1.124	-0.065

Discussion

We very rarely captured a butterfly marked in an area in the other area of the same location, which agrees with a limited movement by apollos; *Parnassius ssp* have a low daily movement rate and travel short distances; below 300 metres in average (Bromer and Fred 1999; Välimäki and Itamies 2003; Auckland *et al.* 2004) but according to the gene flow between areas (Chapter 4) this movement is enough to occasionally connect some areas.

The lower densities according to our data are of 52.6 males/Ha (ChoFl1) and 8.6 females/Ha (OteIl1) which summed is at least six times bigger than the other estimation that has been described for Sierra Nevada, of 1.1-10 indiv/Ha (Gomariz-Cerezo 1993). The estimates are not however comparable to ours, since data were taken with a different method (transects); however in 2012 and 2013 we performed a CMR experiment in the Sierra de Baza-Filabres (Spain) with the same method described here and the population densities for Sierra Nevada are around one order of magnitude above our results for *Parnassius apollo filabricus* in the Sierra de Baza-Filabres: 2.12 males/Ha and 1.05 females/Ha in 2012; and 2.90 males/Ha and 4.66 females Ha in 2013 (Chapter 6). This indicates that the populations of Sierra Nevada are far bigger than those from Baza-Filabres and probably of less concern.

On the other hand female population size is at least 35.3% smaller than male's, and in several of the study areas the confidence intervals of population size of males and females do not overlap and are reasonably small (see Figure 4), suggesting that in all of the areas sex ratio is male-biased. The average sex ratio is 5:1 (2:1 to 11:1; males:females) with very similar values between the sex ratio of the raw data of animals captured and the sex ratio of the estimated population sizes for both sexes (Table 5-2). There are

several explanations for a male-biased sex ratio in butterflies. In *Parnassius ssp.* the sex ratio has been reported in some cases to be up to 2:1 biased towards males (Konvička and Kuras 1999; Matter and Roland 2002), and in *Panassius apollo* it has been proposed that the bias is at least partially an artefact caused by differences of behaviour between sexes (Adamski 2004). The adults are conspicuous while flying, but when resting on the ground with the wings open, the black and white pattern confers them a good camouflage. Females spend most of the time on the ground laying eggs, basking or resting, being less conspicuous if compared with the patrolling males (Konvička and Kuras 1999), which suggests a smaller catchability for females than for males. Our data however shows that in only two of the models (ChoE10 and PosDI1, Table 5-1) the probability of capture (p) depends on the sex, indicating that sex might not determine capture probability in general and therefore we can conclude that at least in some populations sex ratio is really biased towards males.

Biased sex ratio may betemporary, as in many butterfly species males emerge before females and patrol around waiting for the females to emerge (Gorbach and Kabanen 2010), but our population size estimates correspond to the total number of individuals using the area throughout sampling, and we designed sampling to encompass most of the flying period of the species, meaning that our estimates of sex ratio include all individuals using the sampling area along the flying period. In fact “time” and “Time” are the two variables more rarely affecting survival rate (ϕ) and probability of entrance (b) in both sexes indicating that the changes on those parameters do not show a trend over the period of the study that affected their estimate neither for males or females, and therefore it is not likely that a difference in the emergence times of the adults could explain a male-biased sex ratio. This biased sex ratio means that even if populations sizes seem to be large in some cases (from 109 to 795), the population effective size (N_e),

which is limited by the number of females, is quite smaller ranging from 13 to 100 in the areas studied. A low N_e is a problem for the conservation of the species as it is linked to loss of evolutionary potential, reduced variability, reduced effectiveness of selection (Frankham 2005; Charlesworth 2009) and higher inbreeding and extinction risk of the population (Saccheri et al. 1998). In fact, effective population sizes calculated from genetic markers (Chapter 4) show values between 63 and 167, which is closer to the estimated population size of the females than to those of the males.

In other species of butterflies, male-biased sex ratio is usually produced by a combination of higher pre-adult mortality in females and either lower female adult survival rate or higher female emigration, or a combination of both (Ehrlich *et al.* 1984). Male harassment to mated females is usually the cause of higher migration rates and lower survival rates of females that can cause male-biased sex ratio (Shapiro 1970; Ehrlich *et al.* 1984; Baguette *et al.* 1998).

In our case according to MARK models the survival rate does not seem to depend on the sex in most of the cases (only 32% of the models) and seems to be more dependent of the weather (Storm, Table 5-1); similarly, in the PLSR survival is more dependent on climate (Temperature) and vegetation (Table 5-4). However in those models were survival depends on sex, in general survival probability is larger for males than for females (Fig. 5). In the case of probability of entrance in the population (*b*), the most frequent variable determining it is "sex" (57% of the models), implying that there are differences between males and females in their probability of entrance (either by birth/emergence rate or by migration), in fact females have an higher average probability of entrance than males (0.250 and 0.153 respectively). According to our data, the differences in N estimates could be partially due to the higher mortality of adult females, however this alone will not explain the bias in sex-rate found in all the areas studied,

which suggest that the lowest proportion of females could be due to other reasons that our experience could not detect as for example higher pre-adult mortality of females (Ehrlich et al. 1984).

The fact that we have a large variation between areas of the same locations (such as Chorrillo, with largest and smallest total population size values in its two areas, Fig.4) may mean that the scale is very important determining the variation in apollo abundance. In an extensive work with multiple mountain butterfly species, Gutierrez *et al.* (2010) found that topoclimatic factors drive the species distribution at fine-resolution, probably because in mountains climatic conditions can vary greatly during the day or even in short distances . Accordingly, numerous studies on *P. apollo* and other mountain butterflies (specifically on other glacial relicts) reveal the importance of considering habitat heterogeneity, micro-scale and spatial context in understanding habitat use (Ashton *et al.* 2009; Turlure and Van Dyck 2009; Turlure *et al.* 2009). The Chorrillo location may consist in a few small patches with high quality for butterflies (area E) surrounded by low-density sub-optimal patches (area F). The individuals that we see in F may be there by pure effect of proximity to E, even if those are in fact suboptimal patches.

There are 5 variables relevant in predicting the abundance of males and 6 for the abundance of females (Table 5-4), Unexpectedly there are not relevant variables in common in males and females related with the different abundances found in the areas sampled and there is not a general model capable of predicting the abundance of butterflies for both sexes. Even if their abundances are correlated (Table 5-4), this underlines the difference of behaviour and requirements in both sexes. This differences in the use of resources between males and females has been already reported with the

nectar sources in the Sierra de Guadarrama (Baz 2002), where males feed on the same nectar sources as females (such as *Armeria arenaria*, or *Carduus carpetanus*), but females also incorporate in their diet other species with short stems, near the ground surface (*Jurinea humilis*, *Thymus bracteat*a and *Anthyllis lotoides*, Baz 2002). The wider range of nectar sources used by females may be the reason why the abundance of females is correlated positively with the total richness of plant species (Stot).

The strong negative correlation of male abundance with bush cover (Table 5-4) seems to disagree with other studies of *Parnassius apollo* in which the percentage of shrubs was one of the main factors explaining the selection of sites to lay eggs by the females (Ronca 2005). In Sierra Nevada, *Genista versicolor* flowers are rarely used as nectar sources by apollos (Baz 2002, pers. observ.) and so this very abundant shrub and other shrubs are probably mainly used as shelter against the bad weather (the most important variable affecting survival in MARK models). However, in some zones *Genista* can be the most dominant plant (reaching a 43% of cover in PapB10), probably competing with other plants that may be also needed by the butterflies. In fact, canopy cover and bush densities are an important factor for butterflies abundance and distribution, but in most cases a percentage too high or too low can become a limiting factor (Bergman 2001; Gutiérrez *et al.* 2013).

The negative correlation of bush and floral cover with male abundance could be related with thermoregulation behaviour. In a local scale the vegetation often buffers the small-scale temperature variation (Ashcroft *et al.* 2009; Hampe and Jump 2011). Adults of *Parnassius sp.* in cold environments have been seen in basking posture in open zones seeking the sun and directly in contact with the substrate (Guppy 1986). It is possible that males are found in bigger numbers in open zones with less bush and floral cover (where usually *Sedum* is found) to keep their body temperature high as they are usually

more active than the females (Konvička and Kuras 1999; Adamski 2004). This would explain why the survival probability of males is positively correlated with floral cover and sedum cover but their abundance is negatively correlated with floral and bush cover, and would also explain why the climatic variables (matrix A) predict better male abundance than female abundance (Table 5-3).

It has been suggested that presence-absence data can be better than abundance estimates from transects to characterise the habitat requirements for *Parnassius apollo*. This methodology is able to identify a smaller number of variables with higher correlation than the abundance estimates (Gutiérrez *et al.* 2013), and it is important given that distribution studies usually require less effort than mark-recapture studies and provide valuable information that could explain some questions about the use of the space from males and females. Our results with data obtained from mark-recapture methods differ greatly of those of Gutiérrez *et al.* (2013) in which they worked with adults and larvae distribution. In Sierra de Guadarrama there is a relationship of both, abundance and distribution of *P. apollo* with altitude and bush cover. But as discussed above, a too high degree of bush cover will have a detrimental effect in the presence and abundance, as the larvae and the adults bask in bare ground (Guppy 1986; Ashton *et al.* 2009).

It is well documented that the demography of butterflies and other insects depends strongly on the temperature and the stochastic changes of the weather (Dempster 1983; Shreeve 1987; Roy *et al.* 2001), and some stochastic events have been the cause of extinction of other *Parnassius apollo* populations (Descimon *et al.* 2005). Accordingly, in our models the variable more often determining "survival probability" is "Storm" (42%).

In addition it has been recorded for *P. apollo* that they occupy cooler microhabitats in lower elevations (Gutierrez *et al.* 2010), because at lower elevation there are higher minimum temperatures. This preference for cooler temperatures is the explanation to why apollo butterflies are responding to the actual global warming with an elevation in their distribution (Wilson *et al.* 2005; Ashton *et al.* 2009; González-Megías *et al.* 2015). In our case, higher minimum temperatures in June are related with lower male abundance and survival, and higher minimum temperatures in May seem to affect negatively to the survival of the adults (Table 5-4). In May and (early) June *Parnassius apollo* is in larval phase or pupa depending of the altitude of the area. If the temperature has negative effects in larvae or in the pupation as it has in other relict butterflies (Turtule 2010), the survival rate as adults of those individuals affected by high temperatures in larval or pupae phase could be affected, and in general adult abundance in the zones with higher temperatures would be lower than in cooler zones if the high temperatures increase the mortality of the larvae.

The cover of *Sedum*, the larval host plant (Covsedum) does not appear as an important variable predicting the abundance of butterflies. Probably the importance of *Sedum sp.* will have a strongest association with larvae abundance, since *Parnassius apollo* does not lay the eggs directly on the host-plants (Ashton *et al.* 2009; Fred and Broomer 2010), and during the mating season this may not be the most important factor for predicting Parnassius butterflies abundance (Auckland et al. 2004). In any case it has been seen that laying eggs in a host-plant is more determined by the quality of the plant (size, number of flowers and number of leaves or aggregation of plants) than by the density or cover of them (Czekes et al. 2014). In some populations of P. apollo the larvae seemed unable to track their host-plant (Fred and Broomer 2010), and those cases a high aggregation of plants could probably be as important as a high density, as it would imply

less random movement for the larvae between patches randomly looking for Sedum. Another possible explanation is that the minimum cover of *Sedum* found by us (0.5%) implies an abundance of the plant enough to sustain the populations of *P.* apollo and the variation or increment in their abundance will no suppose any change as there are other limiting factors.

Regarding the effect of roads, even if in most cases roads are not inside the patches occupied by apollos, in a metapopulation the quality of the matrix surrounding a suitable area in fragmented landscapes can be as important as the quality of the patch itself, because it influences dispersal ability, edge effects, permeability, and isolation (Ricketts 2001). There are numerous studies showing the effects of roads in animal communities (Forman and Alexander 1998; Fahrig and Rytwinski 2009), and their importance for butterflies (Davis *et al.* 2007). In our case the distance to the nearest town or road (Dcia2) has one of the strongest positive correlations with the abundance of females (table 5-4). It is interesting that the location Otero is the closest to a road and has one of the areas with the highest *Sedum* cover but is the population with the smallest estimates for female population size. Accordingly, previous genetic analyses identified Otero as the deme with the lowest effective population size (Chapter 4) probably as a consecuence of the low number of females (Table 5-1).

Slope is positively correlated with the abundance of males. Slope as a part of habitat heterogeneity has an important role in the variation of temperature (Suggitt *et al.* 2011), but it can also be related with predation risk (Huey and Hertz 1984; Bland and Temple 1990; Dunbar and Roberts 1992). For small flying insect as butterflies the slope probably does not suppose an impediment to the movement, but it may dissuade or difficult the movement of bigger animals, and thus may be related with easier predator-evasion for a

light or flying animal and can be an appropriate indicator of habitat quality for the potential preys, given that predators are habitually bigger than their preys and they usually prefer to feed on level terrains, while the preys are less vigilant when they feed on slope terrains and it seems easier for them to scape uphill (Huey and Hertz 1984; Bland and Temple 1990; Dunbar and Roberts 1992). This would apply mainly to males, if the main predators of males are flying animals, whereas may not apply to females if their predators chase or find them on the ground. Although apollos predators are not well known, we have anecdotically observed birds (kestrels) chasing males in flight and females being consumed by crickets and spiders.

Conclusions

Our analysis shows a clear contrast between male and female' s population sizes, as well as a different set of variables explaining abundance of both sexes. However we have been able to identify a small set of explanatory variables that are actually relevant in predicting their population sizes, such as the distance to roads and plant richness for females and the slope degree or negative correlation with bush and flower plant cover (interpreted as a preference for open habitats) for males. *Parnassius apollo nevadensis* survival and abundance are negatively affected by higher minimum temperatures in June and seems to be affected by also by stochasticity of the climate, this implies a serious problem in the future with the predicted rise of temperatures and stochasticity that has already affected other populations and species (Parmesan *et al.* 1999; Roy *et al.* 2001; Wilson *et al.* 2005; Wilson *et al.* 2007; Ashton *et al.* 2009; Forister *et al.* 2010; Todisco *et al.* 2010; Matter *et al.* 2011).

The conservation efforts should focus on preserving or augmenting the quality of the habitat and the patches identified as high-quality patches in terms of abundance which

according to our results should be far away from roads and heterogeneous, thus combining patches with high plant diversity, high floral and host plant cover (correlated with female abundance and male survival), and others patches with more bare ground and open habitat which seems relevant for male abundance. The patches of smaller N are the ones of greater concern, especially those with smaller female population size as Otero (OteI and OteJ) and ChoF (Chorrillo) or PiuK (Piuca). But the conservation of the patches will be pointless if we do not help the immigration or colonization by improving and maintaining an adequate connectivity (Matter *et al.* 2009). We should keep in mind that *Parnassius apollo nevadensis* is a glacial relict species; that are discontinuously distributed, and because it is "trapped" in a high-mountain habitat, gradual distribution range shifts are not an option for its populations and it cannot easily escape unsuitable conditions. In these cases conservation efforts need to anticipate the changes that threaten the current habitat; an ecological understanding of these environmental tolerances and of the use of resources is essential for effective conservation in changing and irregular environments, specifically for glacial relict species (Turlure *et al.* 2009).

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Capítulo 6

TAMAÑO POBLACIONAL Y VARIABILIDAD GENÉTICA DE UNA POBLACIÓN RELICTA DE UNA ESPECIE EN PELIGRO: *PARNASSIUS APOLLO FILABRICUS*

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CHAPTER 6

Population size and genetic variability of a relict population of an endangered butterfly, *Parnassius apollo filabricus*: recommendations for management

Introduction

Small, declining and/or isolated populations typically show a high risk of extinction due to demographic stochasticity and genetic effects (Lande 1988; Keller and Waller 2002; O'Grady *et al.* 2004). In particular, theory predicts causal links between neutral genetic diversity, effective population size (N_e) and inbreeding coefficient in closed random mating populations (Spielman *et al.* 2004; Frankham 2005), and a link between N_e and evolutionary potential of populations (Frankham 2005; Charlesworth 2009). In fact there is empirical support for the relationship between N_e and evolutionary potential (Frankham *et al.* 1999) as well as population inbreeding and extinction risk (Saccheri *et al.* 1998; Spielman *et al.* 2004). Thus, a reliable estimation of N_e and the degree of inbreeding of small, isolated populations is crucial in conservation biology. The rate of decay in genetic diversity and the increase in inbreeding depend upon N_e rather than the actual or census size, and N_e is typically much smaller than the number of potentially breeding adults in populations, averaging an order of magnitude lower than census population sizes (Frankham 2005). Early detection of population declines is important as it allows for rapid management actions to avoid irreversible loss of genetic variation

and increased risk of extinction due to genetic and demographic factors (Antao *et al.* 2010).

Population fragmentation and isolation may result from the demographic history of species, such as, for example, the Southern European refuge of many taxa during the glacial periods and the recolonization of Central Europe afterwards (Hewitt 1996), as well as the adaptation to particular environmental conditions during these movements. The apollo butterfly, *Parnassius apollo* (Linnaeus, 1758) is a good example of a glacial invader in Southern and Western Europe of which part of its nowadays distribution range is characterized by isolated populations restricted to mountains, in particular in Southern Europe (Descimon 1995; Todisco *et al.* 2010). The apollo presents a patchy distribution along the Palearctic region, excluding with the exception of North Africa and the Arabian Peninsula; it is found from Spain to southern Fennoscandia and the Balkan Peninsula including northwestern Peloponnesus (Todisco *et al.* 2010). There are a large number of *Parnassius apollo* subspecies across the Eurasian continent, many of which have declined substantially over the last hundred years, and some of them now extinct (van Swaay *et al.* 2010). Its overall European population has declined by almost 30% in the last years, and consequently the species has been classified as one of the most endangered butterflies in Europe (IUCN 1996), being listed in the European Red Data Book (van Swaay and Warren 1999) and the annex IV of Appendix II of the Habitat Directive (EEC 92/43/EWG) of the European Union.

In Spain there are a large number of *P. apollo* subspecies described (Gómez-Bustillo and Fernández-Rubio 1973), each of them associated to a mountain range. In the South of Spain we found the meridional group with the three southernmost subspecies, *P. apollo nevadensis, P. apollo gadorensis* and *P. apollo filabricus*. *P. apollo gadorensis* is

considered now extinct from the Sierra de Gádor (Barea-Azcón 2008), and we have seen that *P. apollo nevadensis* in the Sierra Nevada has experienced a recent elevation in altitude and a subdivision of its populations (Chapter 4). *Parnassius apollo filabricus* (Sagarra, 1933) is endemic of the Sierra de Baza-Filabres in Southern Spain (Fig. 6-1). This subspecies was distributed over much of the Sierra de Baza-Filabres range during the twentieth century, but it has gradually disappeared from most of its known locations, becoming rare and considered endangered in the Red Data Book of Andalusia Invertebrates" (Endangered B2ab(i,v); C2a(ii)); Barea-Azcón *et al.* 2008); nowadays it is only present in two localities, one of them found in 2009 and another very recently discovered, in 2016 (Fig. 6-1, Tinaut *et al.* 2010**;** Gil-T, 2016)**.** *P. apollo filabricus* represents an independent evolutionary lineage within the species, as suggested by its unique mitochondrial DNA composition (Todisco *et al.* 2010; Sánchez-Prieto *et al.* in prep.), and thus it harbors a portion of the genetic diversity of the species that would disappear if the subspecies went extinct. Because of the clear recent decline of the species in its distribution range, it is of great importance to characterize these populations in terms of abundance and genetic diversity in order to gain insights into the demographic history of the populations as well as a discussion of possible management actions. In this paper we aim to: (i) characterize census and effective population size of the only known population until 2016, (ii) estimate genetic diversity, and (iii) discuss the recent demographic history of the species as well as suggest possible management actions to reduce extinction risk.

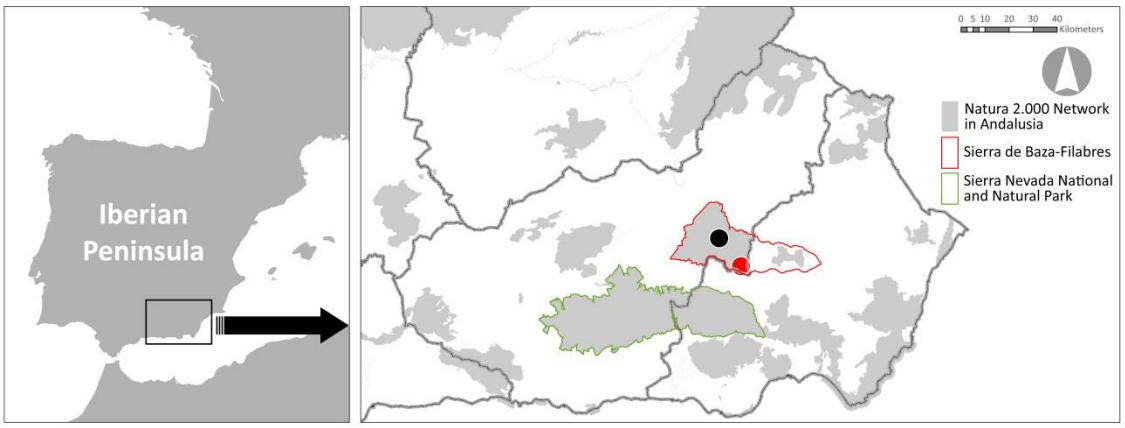

Fig. 6-1: Location of Sierra Nevada and Sierra de Baza-Filabres in Southeastern Spain. Dots show the approximate location of the two known populations of *P. apollo filabricus*. In red, the population presented in this study. In black the recently found population described in Gil-T (2016).

Methods

Species

Parnassius apollo filabricus as the rest of the Iberian apollo subspecies, lives in high mountain meadows (Gómez-Bustillo and Fernández-Rubio 1973), at altitudes of between 1800 and 2700 meters above sea level depending on the mountain range (Olivares *et al.* 2011). Larvae feed on several species of *Sedum,* mainly *S. amplexicaule* (pers. obs.). The species is univoltine, and adults fly between the second half of June and July. Females are larger and darker than males, and the external genitalia of males is visible to the naked eye allowing differentiation between the sexes.

Study area

Despite its two different names, Sierra de Baza and Sierra de Filabres, the Sierra de Baza-Filabres is a mountain of around 60 km long and 20 km wide, with a total surface of approximately 150,000 ha, occupying the provinces of Almeria and Granada in southeastern Spain (Fig. 6-1). Sierra de Baza is completely protected (53,649.41 ha) as Natural Park and as a Special Area of Conservation (SAC code: ES6140001), while only a part of Sierra de Filabres, Calares de la Sierra de los Filabres (6615.83 ha), is protected as Special Area of Conservation (SAC code: ES6110013). The highest peaks in the mountain reach 2271 in the west (Sierra de Baza) and 2168 meters in the east (Sierra de Filabres), and quite a large area of the range is over 1800 meters. Apollos dwell between 1800 and 2500 meters in the near Sierra Nevada mountain (Olivares *et al.* 2011), while in Baza-Filabres most of their known historical locations are around 2000 meters. Apollos live in open habitats, meadows and pasture land where the larvae's host plant (*Sedum sp.*) and nectar food plants (i.e. Thymus spp. and Cardus spp.) are abundant. However a large proportion of the habitat within 1800 and 2100 meters in Baza-Filabres is now occupied by forest, mainly pine (*Pinus sp.*) plantations, a habitat that is not used by apollos, that may fly in the proximities of trees, but not inside closed forests (pers. observ).

Butterfly sampling.

Sierra de Baza-Filabres was surveyed in search of *P. apollo* during the months of June and July of 2008 and 2009. In 2009 the study population was discovered and the area used by butterflies roughly delimited (Cerro de Quintana, Sierra de Baza, 37.27N -2.70W, an area of roughly 30 Ha, Fig. 6-1 and Fig. 6-2 area A). All butterflies were captured or observed between 1800 and 1950 m.a.s.l. In 2010 we confirmed that butterflies occurred only in this area by a detailed prospection of the known historical localities for the

species in Baza-Filabres and the proximities of the newly detected population. We only detected two adults in the near proximities (300 and 600 meters away) of the 30 Ha area where capture-mark-recapture (CMR) work was done (Fig. 6-2, areas B and C). In 2012 and 2013 we carried out CMR work. Butterflies were captured and marked in 5 sampling occasions during June both years trying to encompass most of the adult flight period, starting a few days (3-5) after the first adult was seen. Sampling period lasted 14 days, until all butterflies captured showed signals of being worn out. They were caught with a butterfly net and marked in their wings with a numerical code using a soft-tipped pen. We also recorded capture position with a GPS (3 meters precision). In order to perform genetic analyses we captured worn out individuals, 11 in 2009 and 18 in 2012. All the individuals were transported alive to the lab where we kept them at -20ºC until DNA extraction. All individuals were caught under permission of Consejería de Medio Ambiente (Junta de Andalucía). *P. apollo filabricus* is included in the Junta de Andalucía´s Red List of Invertebrates as endangered (Barea-Azcón *et al.* 2008), and also is protected under the Andalusian List of Wild Species under Special Protection Regime. The survey was designed to minimize effects to the population, by capturing a small number of individuals that were already worn out and thus near death; the effect of sampling on the productivity of the population should be negligible.

Population size

Demographic parameters were estimated for each year (2012 and 2013) separately using the Jolly-Seber method for open populations with the POPAN formulation in program MARK 6.2 (White and Burnham 1999). Analyses were performed in two steps: first with the software RELEASE in MARK we tested data for goodness of fit of the most general model that assumes survival (φ) and capture probability (p) dependence from sex (s) and

time (t) dependence of under the Cormack-Jolly-Seber (CJS) model ([φ(s*t)p(s*t)] using the standard MARK notation). The model fitted the data both years (χ^2= 5.01, p= 0.89 and χ^2 = 6.79, p= 0.45 respectively) and we did not correct for overdispersion because the c-hat value (χ^2/d.f.) was smaller than 1.0 (Lebreton *et al.* 1992).

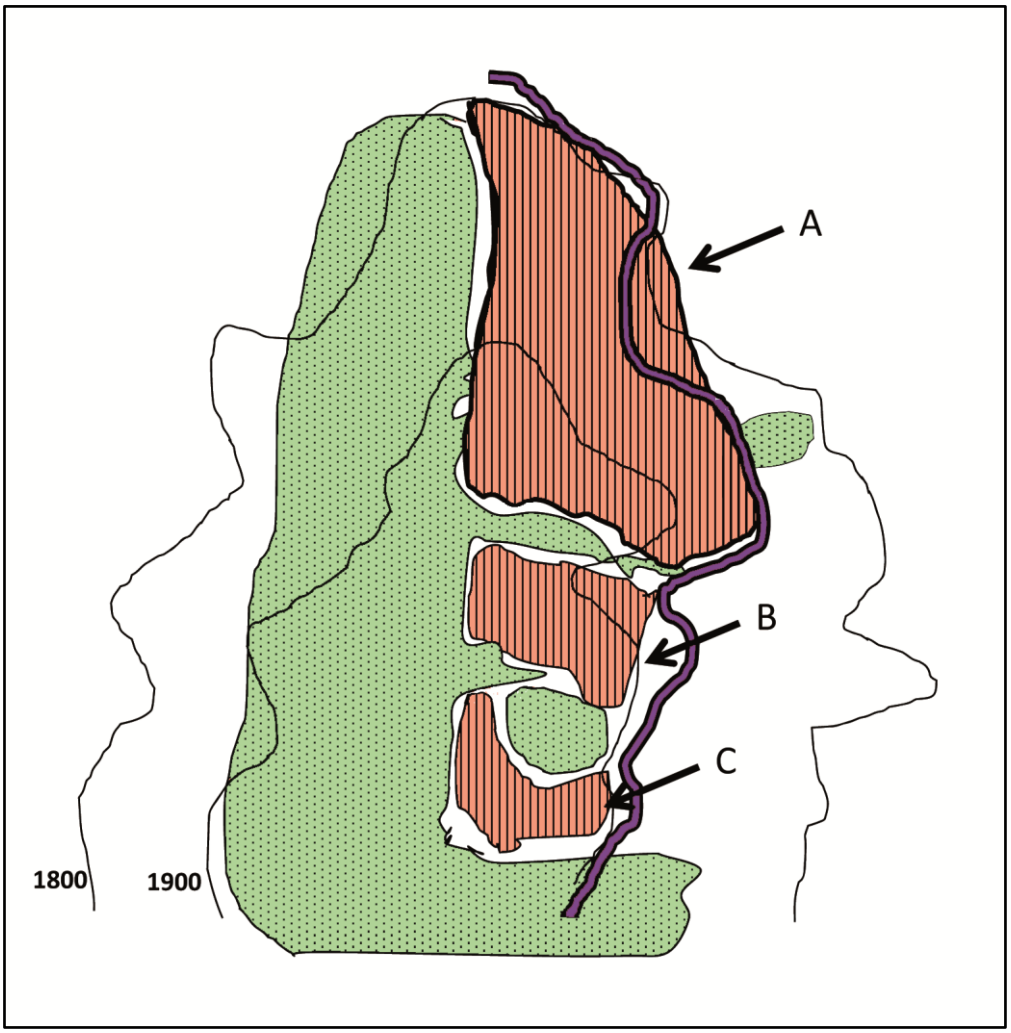

Fig. 6-2: Schematic presentation of the patches of habitat suitable for apollos (A, B and C, stripped red polygons) and the areas occupied by pines (dotted green areas). The 1800 and 1900 m.a.s.l. contour lines and the situation of a track that crosses the study area (double line) are also depicted.

Since we were interested in testing the possible effect of sex in probability of capture and survival, we run different models, starting with the general model and relaxing the sex and time dependence of parameters in simpler models. Model selection was carried out by comparing AICc between candidate models, being the one with the smaller AICc the one with more support. In a second step, we fitted a Jolly-Seber model using POPAN with a setup for φ and p as in the most parsimonious CJS model. POPAN estimates the parameters, survival (φ), probability of capture (p), probability of entrance in the population (b), which is equivalent to recruitment, and population size (N). Model selection was performed again to find the most supported model, using as general models those suggested by CJS analyses and relaxing the sex and time dependence of b in simpler models. The sex dependence in the models was also relaxed for N, so we could estimate separately population size for the whole population for each sex. Because in both years several models showed similar support to the data, that is, differences between AIC were smaller than 2, we estimated the values of parameters using model averaging (Cooch and White 2016).

Molecular methods.

DNA extraction was carried out using an ammonium acetate salt extraction method. We extracted DNA from a leg of each adult. Butterflies were genotyped for a set of 20 microsatellite *loci* previously characterized for *P. apollo nevadensis*: Pan03, Pan16, Pan19, Pan21, Pan22, Pan26, Pan27, Pan29, Pan30, Pan32, Pan37, Pan38, Pan43, Pan44 - Pan47, Pan49, Pan51 and Pan53 (Chapter 3). Amplification was performed in a 2 µl multiplex PCR containing 10ng of air-dried DNA, 1µl Qiagen Multiplex PCR Master Mix (Qiagen Inc), 0.2µM reverse primer and 0.2µM forward fluorescent primer and covered with a drop of mineral oil with the following cycling conditions described in Chapter 1.

Genetic variation

Loci were checked for evidences of null alleles using Micro-Checker version 2.2.3 (van Oosterhout *et al.* 2004) and departures from Hardy–Weinberg equilibrium (HWE, related with a heterozygote deficit) were assessed with GENEPOP 4.0 (Raymond and Rousset 1995). With the *loci* with no evidence of null alleles and in HWE (see results) observed (H_o) and expected (H_e) heterozygosity were calculated in GENEPOP 4.0. FSTAT v. 2.9.3 (Goudet 1995) was used to calculate allelic richness (AR, the number of alleles corrected for sample size) per population and *locus*. The degree of population inbreeding was characterized by F_{IS}, estimated using FSTAT.

For estimating genetic diversity we have used the data from the 18 individuals of *P. apollo filabricus* captured in the population in 2012; as this was the las year sampled and with bigger sample size. We have compared their genetic diversity with that of 13 populations of *P. apollo nevadensis* (Sierra Nevada). Since some of the markers showed evidences of null alleles and/or where significantly out of HWE in the populations either of Baza (see results) or Sierra Nevada (Chapter 4), we have made the comparison of genetic variation between Baza and Sierra Nevada excluding these markers, and have used only the 9 *loci* that did not show evidence of null alleles and were in HWE (Pan16, Pan19, Pan29, Pan30, Pan32, Pan38, Pan43, Pan44 and Pan49).

To compare genetic diversity of Baza with that of the other populations, we used a single sample t-test as implemented in STATISTICA 6.0 (Statsoft 2001), in which we looked for differences between allelic richness, H_o and H_e averaged across *loci* between Baza (a single value) and the mean value for the Sierra Nevada populations.

Estimating N_e.

N_e was estimated using the software NeEstimator V2 (Do *et al.* 2014). We used both single-sample and temporal methods to calculate N_e, as implemented in NeEstimator, that includes (1) a bias-corrected version of the linkage disequilibrium method (LD, Waples and Do 2008), (2) an updated version of the heterozygote-excess method (Zhdanova and Pudovkin 2008), (3) a new implementation of the molecular coancestry method (Nomura 2008), and (4) the standard temporal method (Waples 1989). Different estimators of N_e are characterized by different properties and assumptions, and in fact each of them estimates a different type of population size. It is assumed that single-point methods give an estimate of the inbreeding effective size of the parental population, whereas temporal methods estimate variance effective size (Saarinen *et al.* 2010, Barker 2011) and they may perform differently depending on factor such as population structure, gene flow or sampling effort (Luikart *et al.* 2010, Barker 2011); thus it is interesting to use different estimates altogether to evaluate the conservation status of a population (Waples 2005); the online approximate Bayesian computation online service OneSamp (Tallmon et al. 2008) used in Chapter 4 was no longer available. The population studied meets well most assumptions of the single-sample approaches to the estimation of N_e (Skrbinsek *et al.* 2012): random sampling, no subdivision, discrete generations, no immigration, no mutation and no selection. Stable population size and equal contribution of individuals might be violated however, although it is difficult to establish to what degree. Sex ratios are likely to be near parity (see results), but nothing is known about the variance in contribution of males and females to the next generation. In any case, the LD method is largely robust to the violation of this assumption (Waples 2006).

For estimating N_e using the temporal method we have used the genotypes of the samples taken in 2009 (n= 11) and the samples taken in 2012 (n= 18), whereas in the rest of the estimates we have only used genotypes from 2012; in all cases the estimates are based upon 13 microsatellites markers, excluding three *loci* with evidences of presenting null alleles in this set of samples (see results). We screened out rare alleles with frequencies below 0.02, and used parametric chi-square tests for computing confidence intervals; we used the F_k option (Pollak 1983) for computing the standardized variance in allele frequency in the temporal method (Waples1989).

Bottleneck test

We have tested for recent bottlenecks in the population in samples from 2012 using the Wilcoxon test implemented in BOTTLENECK (Cornuet and Luikart 1996). Of the different tests available in BOTTLENECK, the Wilcoxon test provides relatively high power when the number of individuals and *loci* used is small, as in our case.

Results

Population size

The most supported CJS models differed between years, showing that φ and p are dependent on sex on 2012, and dependent on sex and time in 2013. So, the general models for POPAN analyses were *φ(s)p(s)b(s*t)* in 2012 and (*φ(s*t)p(s*t) b(s*t)* in 2013. The analyses show (Table 6-1) that survival was dependent on sex in 2012 and sex and time in 2013, probability of capture was only dependent on sex both years and recruitment depended on time. Population sizes estimated were similar between years,

although the standard error for the population size of females is high both year, and in 2013 was too high, rendering the 95% confidence interval for this estimate inconclusive. In any case, when sex was not included in the analyses, total population sizes estimated for both years were similar 62.5 (SE 6.2) and 84.2 (SE 14.3) for 2012 and 2013 respectively).

Table 6-1: Comparison of the models used to estimate population size in both years of study

Year	Model	AICc	ΔAICc	AICcw	N_{males} (SE; 95% CI)	$N_{females}$ (SE; 95% CI)
2012	*φ(.)p(s)b(t)*	209.26	0.00	0.39	63.6 (6.8; 50.2 - 77.1)	31.4 (13.2; 5.5 - 57.4)
	φ(s)p(s)b(t)	209.46	0.20	0.35		
	φ(.)p(.)b(t)	210.86	1.60	0.17		
2013	*φ(s)p(s)b(t)*	175.68	0.00	0.41	86.9 (14.9; 57.7 - 116.2)	139.7 (139.5; -133.8 - 413.3)
	φ(t)p(s)b(t)	176.12	0.43	0.32		

ΔAICc: difference with the most supported model; AICcw: relative weight of the model; N, weighted average estimated number of males or females, with its standard error (SE) and the 95% confidence interval for the weighted average estimate.

Genetic variation

Loci Pan26, Pan45, Pan46 and Pan51 failed to amplify in most of the samples. *Loci* Pan22, Pan47 and Pan53 showed evidence of null alleles in the *filabricus* population, and thus only the remaining 13 *loci* were used in the calculations of N_e and Bottleneck. Pan03, Pan21, Pan27 and Pan37 showed evidence of significant deviation from HWE in some populations of the Sierra Nevada (Chapter 4). The rest of the *loci* (9) met Hardy–Weinberg expectations and showed no evidences of null alleles neither in Baza or any of the Sierra Nevada populations and were therefore used to estimate heterozygosity, allelic richness and in the comparisons.

Average heterozygosities were higher in Baza than in Sierra Nevada, both H_e (0.59 (0.21) versus 0.56 (0.02), Table 6-2; single sample t test = 2.81, p= 0.01, df = 13) and H_o

(0.58 (0.22) versus 0.55 (0.03), Table 6-2; t = 3.40, p = 0.004, df = 13, Fig. 6-3). Allelic richness was also slightly but significantly higher in Baza (3.91 (1.82)) than the average for populations in Sierra Nevada (3.76 (0.12), Table 6-2; t = 3.97, p = 0.01, df = 13, Fig.6-3). F_{IS} values were 0.017 and 0.027 (0.05) respectively (Table 6-2), and were not significantly different (t= 0.75, p = 0.46, df = 13); the F_{IS} value for Baza was not significantly different from zero (test of heterozygote deficit in Genepop, p= 0.14).

Table 6-2: Measures of genetic diversity of Baza and the Sierra Nevada populations.

Population	AR (SD)	H_o (SD)	H_e (SD)	F_{is}
Baza	3.91 (1.82)	0.58 (0.22)	0.59 (0.21)	0.017
Sierra Nevada	3.76 (0.12)	0.55 (0.03)	0.56 (0.02)	0.028
Almirez	3.87 (1.73)	0.59 (0.25)	0.58 (0.23)	-0.026
Caballo	3.46 (1.32)	0.51 (0.23)	0.52 (0.22)	0.025
Rayo	3.88 (1.65)	0.58 (0.28)	0.58 (0.23)	0.011
Chorrillo	3.72 (1.39)	0.55 (0.20)	0.57 (0.19)	0.039
Chullo	3.73 (1.62)	0.47 (0.18)	0.55 (0.22)	0.143
Otero	3.77 (1.44)	0.56 (0.23)	0.59 (0.19)	0.045
Hornillo	3.90 (1.47)	0.60 (0.20)	0.59 (0.19)	-0.007
Lagunilla	3.69 (1.50)	0.52 (0.20)	0.55 (0.22)	0.055
Mirador	3.78 (1.86)	0.55 (0.32)	0.53 (0.28)	-0.045
Papeles	3.82 (1.65)	0.52 (0.23)	0.57 (0.22)	0.090
Piuca	3.60 (1.45)	0.57 (0.24)	0.55 (0.22)	-0.042
Postero	3.77 (1.66)	0.56 (0.24)	0.57 (0.21)	0.019
Vacares	3.84 (1.88)	0.53 (0.26)	0.56 (0.23)	0.059

AR, Allelic richness corrected for sample size averaged across *loci*, as estimated by FSTAT; H_o, mean observed heterozygosity across *loci*, H_e, mean expected heterozygosity across *loci*, as estimated by Genepop; and F_{is} value per population as estimated by FSTAT. Values given (except for F_{is}) are means and standard deviations (SD). "Sierra Nevada" includes the average values for all the sampling sites below

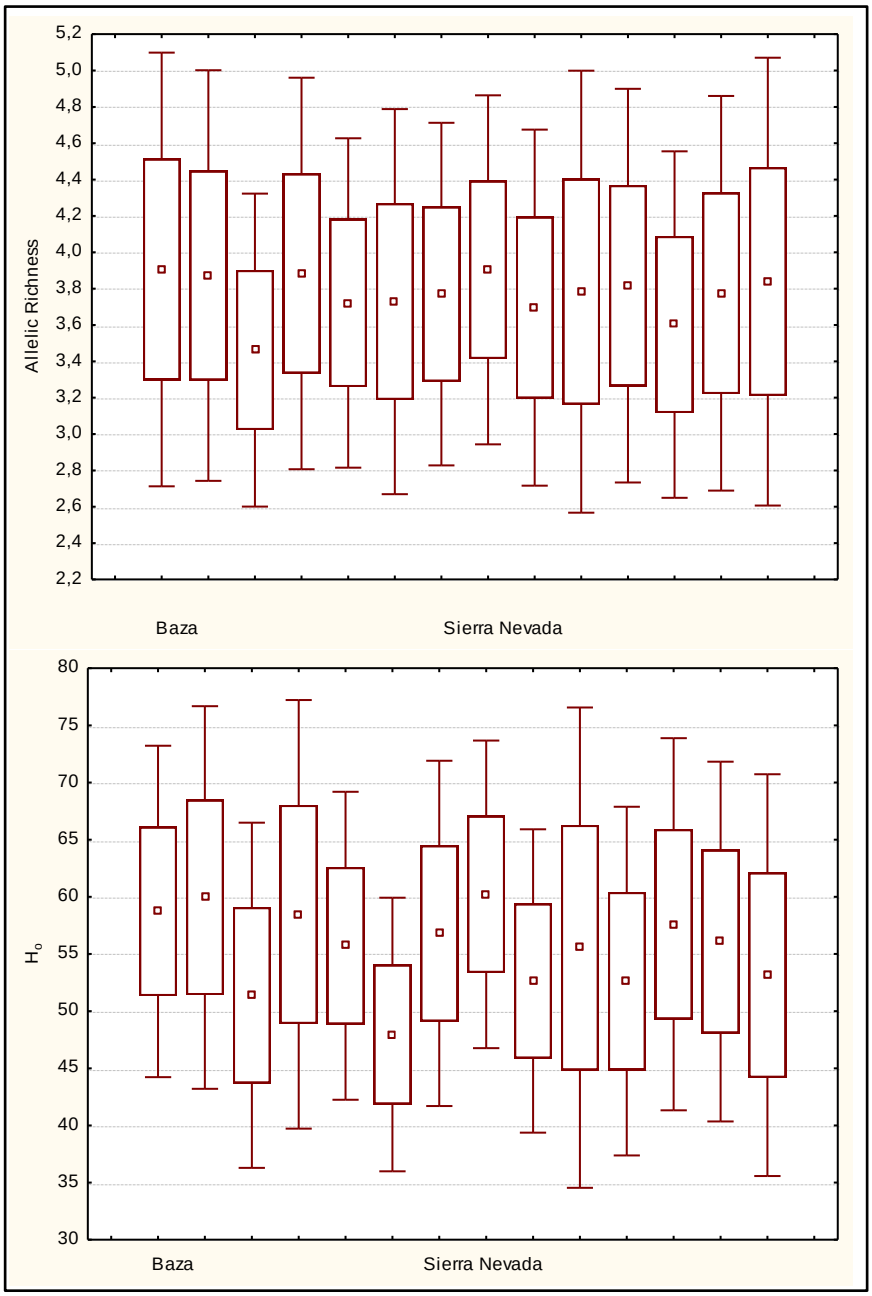

Fig. 6-3: Allelic Richness and observed Heterozygosity in the populations studied. The first column corresponds to the filabricus population in Baza, the rest to the different populations studied in Sierra Nevada. Mean (squares), SD (boxes) and SE (whiskers) of the estimated allelic richness and observed Heterozygosity per population.

Estimates of N_e and signals of bottlenecks

Estimates of N_e differ depending on the method used, but suggest an effective population size of a few tens individuals. LD and Heterozygote excess methods give too large confidence intervals, but the coancestry and the temporal method show estimates comparable and with reasonable confidence intervals (Table 6-3). The ratio N_e /N was 0.20 and 0.46 when using the coancestry and the temporal methods respectively and the census size obtained for 2012 in the POPAN analyses not considering sex. If we consider sex in the analyses, total population size is 95, and N_e /N lowers to 0.14 and 0.32.

The Wilcoxon test estimated by BOTTLENECK was not significant for any of the mutation models considered (p= 0.27, 0.73 and 0.63 for the infinite allele model, the two-phased allele model and the stepwise mutation model respectively).

Table 6-3: Estimated values of Ne and its 95% interval confidence using four different methods as implemented in NeEstimator. See methods for details.

Method	N_e	95% CI
LD	51.1	17.1 - Infinite
H excess	239	8.0 - Infinite
Coancestry	13.2	5.9 - 23.4
Temporal method	30.2	13.8 - 184.3

Discussion

The population sampled is very small, with an abundance below 150 individuals suggested by the CJS analyses, which altogether with an occupied area of roughly 30 Ha supposes a low density population, of around 2 to 3 individuals per hectare. Apollo populations seem to be quite variable in their densities, although they normally seem to appear in larger areas and census sizes; however, there are few published studies reporting census size and/or densities and the estimations are based on different methods. For example, Gomariz (1993) reported densities of between 11 and 10 ind/Ha in different study plots in Sierra Nevada, based on 300 to 1000 meters transects. Fred *et al.* (2006) found densities of around 1 female/Ha in a coastal habitat in Finland using CMR in a large area (300 Ha), although these figures are not the estimation of population size but are calculated from the total number of females captured in the sampled area during the whole study period. Doing so with our own data would give an estimate of around 0.5 females/Ha (17 females in 2012 and 19 in 2013). Our own data based on CMR in different locations from Sierra Nevada (Chapter 4) suggests much larger densities (of at least the double of individuals per hectare, and in some locations hundreds). In any case, even if the density is within normal numbers for the species, census size seems to be compromising long-term viability of the population. Despite the controversy around the 50/500 rule (see for example Jamieson and Allendorf 2012), a population of around 100 individuals should be considered facing a high risk of extinction either due to stochastic or genetic factors, and more likely both of them (Reed *et al.* 2003, Spielman *et al.* 2004).

Small populations are typically characterized by low genetic diversity, as a result of the combined action of processes such as genetic drift and inbreeding (Frankham 2005). We cannot, however, say that the *apollo* population in Baza harbours little genetic

variation. Its comparison with other populations in Sierra Nevada renders the conclusion that the *filabricus* population is not genetically impoverished. Surprisingly both allelic richness and observed heterozygosity are higher in Baza than the mean values of the populations sampled in Sierra Nevada. The values for observed heterozygosity are very similar to those calculated for other non endangered butterflies, ranging from 0.39 to 0.48 (Fauvelot *et al.* 2006, Saarinen *et al.* 2014, Sarhan 2006). In particular, in other *Parnassius sp.* populations studied with microsatellite *loci* (Keyghobadi *et al.* 1999, 2002, Meglécz *et al.* 1998; Petenian *et al.* 2005) the observed heterozygosity ranges between 0.32 and 0.67. Apollo populations characterized as extremely isolated and subjected to recurrent bottlenecks in the past show a severe depletion of genetic variation (Habel *et al.* 2009).

Our single-sample N_e estimates (LD, H excess an coancestry) do not offer similar results. Two of them render infinite values in the confidence intervals (LD and H excess). Infinite values in the confidence interval may be interpreted as no evidence for variation in the genetic characteristic caused by a finite number of parents (Do *et al.* 2014), but may also be due to a large sampling error; if the actual amount of sampling error is larger than expected, it is possible to obtain a negative estimate of N_e that is interpreted as infinity (Waples and Do 2010, Do *et al.* 2014). The coancestry and temporal methods are quite precise and offer similar estimates of N_e, suggesting that effective population size has been small in the last few generations in this population. Different simulations using methods for estimating Ne show that some of these methods (in particular LD) can detect declines in population size only a few generations after they occur (Antao *et al.* 2010, Tallmon *et al.* 2012).The effective size is typically much smaller than the number of potentially breeding adults in populations, averaging an order of magnitude lower than census population sizes (Frankham 2005). Meta-analyses have found that N_e /N ratios

average only 0.11, ranging from 0.04 to 0.60, depending on the life history of the organism and method used to generate Ne (Frankham 1995, Frankham *et al.* 2002). Ratios N_e / N for our population, whatever estimate we use (see results), are larger than the average, which suggests that effective size is not particularly smaller than expected from what is known in other animal populations.

Despite that there has been some criticism regarding the use of genetic methods based on a single temporal sample to infer bottlenecks, because they require assumptions about microsatellite evolution and mutations models (Peery *et al.* 2012), the results of the Wilcoxon test, considered appropriate when the number of individuals and *loci* used is small as in our case (Cornuet and Luikart 1996), suggest no bottleneck in the population. Theory and empirical data show that populations that have suffered size reductions have reduced genetic diversity and high levels of inbreeding (Frankham *et al.* 1999, Groombridge *et al.* 2000, Frankham *et al.* 2002), and the Baza population does not seem to show reduced allelic richness, heterozygosity or inbreeding (F_{IS}; see above). In fact, a very recent bottleneck would show a very small reduction in heterozygosity, difficult to detect, but a larger effect on allelic diversity (Frankham *et al.* 2002), but our data do not suggest any of the effects. We acknowledge that the right comparison should be between present and past genetic diversity in the Baza-Filabres range, but we believe that diversity values of populations in Sierra Nevada should be a good proxy of the expected values of diversity at equilibrium. In the case of *P. apollo filabricus* we indeed know that there has been a severe reduction in the distribution range (and thus population size), and therefore, we should consider that this remnant population may have a higher allelic diversity than expected because of the input from all the other extinct subpopulations, if there was in fact some gene flow between them and the

population was acting as a sink. This is known as the Refugee Model (Porter 1999) and it seems suitable for butterflies and other mobile species in fragmented habitats.

Taken altogether our results suggest a very small population, both in terms of census and effective population size, which however harbours a genetic diversity similar to other larger populations, and that does not show any sign of recent bottleneck, although a very recent and not to severe reduction of population size cannot be disregarded. However, small N_e and N are among the best predictors of population extinction risk, disregarding ecological stochasticity, that may drive small populations to extinction (O'Grady 2004); theory predicts links between neutral genetic diversity, N_e, evolutionary potential and extinction risk (Frankham 2005) that have received some empirical support (Frankham *et al.* 1999, Spielman *et al.* 2004, Frankham *et al.* 2013). We must that consider that the long-term evolutionary potential of the population is seriously jeopardized. Considering that there are only two known locations with presence of the species in Sierra de Baza-Filabres, the one considered in this study and the one very recently reported by Gil-T. (2016), of which we have no data on population size, density or genetic parameters, the future of this lineage or subspecies, *P. apollo filabricus*, seems very compromised and demands a management conservation plan.

Conservation measures

Considering our results, urgent conservation actions are needed. We should consider that the majority of the pine forests that are currently common in this mountain at the potential altitudinal range for the apollo butterflies are product of recent reforestations. The plan to reforest with pines Sierra de Filabres dates from 1961, as a response to an alleged loss of forest surface during the XIX and beginning of the XX century as a consequence of an increase in human activities such as mining and ploughing (Gómez

Mendoza and Mata Olmo 2002). Most reforestations took place in the seventies, although they continued until the eighties, that is around 35 years ago. The landscape before these reforestations is supposed to have been historically, at least since the XVI century, devoted to extensive agriculture and the breeding of ovine cattle, and characterized by an expansion of areas of pasture and meadows and a reduction of forested areas (Sanz Herraiz *et al.* 2002). These pastures were likely to be appropriate for the apollo, and its extension diminished severely after the reforestations that were carried out at the potential altitudinal range for the species (Sanz Herraiz *et al.* 2002). Reforestation also brought other likely negative effects for the species, mainly the use of insecticides during the eighties and the beginning of nineties to control the pine processionary moth (*Thaumetopoea pityocampa*). There is coherence between the beginning of these pest control actions through chemical methods and the decline in *Parnassius apollo filabricus* population at many sites of its range.

So, during the last few centuries the species may have suffered a process of expansion, following the increase in suitable areas, and later severe contraction and fragmentation due to the heavy reforestation work and then a diminution in the population size as a consequence of the pest control. The species might have been functioning as a set of discrete populations connected through gene flow in different degrees depending on their situation and the characteristics of the surrounding habitat. We found a contemporary reduction in gene flow leading to a weak but significant genetic structure of populations in the apollo populations of Sierra Nevada (Chapter 4), that seems in part related to changes in land use and landscape characteristics. We may be watching nowadays in Baza-Filabres the end of a process of extinction of local populations (either due to genetic or ecological causes), with little or no connectivity among them.

Changes in climate may have acted synergistically with land use changes in the decline of *Parnassius apollo filabricus* populations. Theses populations live at the edge its climate optimum and we consider that climate warming has likely been implicated in the decline of this subspecies in many areas of the Baza-Filabres mountain. In the nearest Sierra Nevada and regarding to the annual average maximum temperature, 82,51 % of the mountain range showed a positive trend during the last 50 years (Pérez-Luque *et al.*, 2016a). These changes seem to be especially important during the 70s decade and more intense above 2000 m.a.s.l. This warming is coherent with data from other mountain ranges at the southern edge of the Iberian Peninsula (Galan *et al.*, 2001). Moreover, in Sierra Nevada climate simulations forecast a rising in annual maximum and minimum temperatures from now to the end of the present century (Pérez-Luque *et al.*, 2016b). Under theses simulations minimum temperatures increase may be between 1 to 4 °C. Higher minimum temperatures affect negatively to population size in *P. apollo* (Chapter 5) and together with the current habitat degradation situation should greatly reduce the habitat suitability for this subspecies, so this aspect must be taken in to account in order to define a conservation strategy and to select areas where habitat managing and ex situ actions may be priority. Currently, the optimum sites for apollos in Sierra de Baza-Filabres are located above 1800 masl, and there are not many extensions above this elevation in the mountain range. This imply that a slight increase in the temperature and thus in the lower limit of the suitable habitat for apollo butterfly should imply a drastic reduction in the availability of climatic appropriate areas to hold its populations.

Management actions should aim to improve and maintain population size and genetic variation in the extant populations, ease genetic connectivity through individual dispersal between occupied patches and the founding of new populations in patches of suitable habitat.

A potential measure that would enhance population connectivity through dispersal and the founding of new populations is the clearing of pine forests surrounding the known subpopulations. At least in the case of the patch occupied by the population studied in this article, the suitable areas for the species are nearly completely surrounded by pine forests (Fig. 6-2, areas A, B and C). Pine forest must be considered a non suitable habitat for the species for two reasons: apollos avoid to fly within close forests (pers. observ.), and the soil below pines is not adequate for the growing of *Sedum* sp. and other Crassulaceae plants that are the only food to *apollo* caterpillars neither for the nectar sources preferred by adults. This is a possible reason why areas B and C (Fig. 6-2) do not seem to hold a stable population of apollos despite the close proximity of the main study area (area A). Despite the proximity and similarity of the three areas, there are dense pine plantations surrounding the area occupied by butterflies to the North, South and West, hindering movement between nearby patches.

The appearance of new populations may happen through effective dispersal of females between patches of suitable habitat, which seems difficult in the current circumstances or by translocation of eggs, caterpillars or adults from the known populations to nearby appropriate areas, in our study population areas B and C in Fig. 6-2. Translocation is a measure of common use in conservation actions (Seddon *et al.* 2007). Fred and Brommer (2015) report on a translocation plan performed with *Parnassius apollo* in Finland, where larvae were translocated to a number of "empty" suitable patches, but the reintroduction succeded only in a few areas (3 out of 25). The authors concluded that empty suitable habitat consists of only a few sites where population establishment is possible, and so the key to the success was starting the translocation in many sites but then "zooming in" on a smaller set of sites showing evidence of successful establishment. However, in order to do this, a large number of

larvae would be needed (which in the Finland study was more than 3000 larvae), and so a captive breeding program would be needed to avoid taken individuals straight from the wild, as it was the case in Fred and Brommer's (2015) study.

Translocation of few individuals, or the offspring (larvae) of few individuals, can result in substantial loss of genetic variation owing to founder effects which may then be exacerbated by high variance in reproductive success among founders post-translocation (see Ramstand *et al.* 2013 and references herein). In addition, small population size can lead to elevated inbreeding within new established further reducing genetic diversity. To reduce this risk and enhance long-term viability of the species, it would necessary starting out breeding programs with individuals from the two known populations in Baza, and to accompany this measure with the above proposed actions of clearing pine forests in order to ease patch connectivity trough adult dispersal.

The two known *filabricus* populations are inside a protected area (Sierra de Baza Natural Park), thus a high degree of protection seems to be *a priori* ensured. An effective protection of these sites is essential for the survival of this subspecies. In order to ensure this we recommend avoiding any change in the land use in this area and in its surroundings, to control the possibility of illegal captures, to include these areas in the network of reserve areas of the Natural Park (sites that holds the higher protection status inside the Park) and to maintain sufficient measures to avoid the impact of forest fires potentially affecting these areas. Moreover, one of the most important recommendations is to limit the control of the pine processionary moth pest through chemical methods in these sites. Insecticide spraying has been demonstrated as an inappropriate management practice to control pine processionary moth in Mediterranean environments from the cost-efficiency point of view (Cayuela *et al.* 2011), while the effects on biodiversity are

dramatic, not only for *Parnassius apollo filabricus* but also for another invertebrates and the vertebrate communities that depend on them.

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Capítulo 7

DISCUSIÓN GENERAL

CAPÍTULO 7

Discusión General

En la presente tesis se han presentado, evaluado y utilizado con éxito marcadores moleculares microsatélite para *Parnassius apollo nevadensis*. Estas herramientas nos han permitido realizar estudios de paternidad y estudiar la genética de poblaciones de estas mariposas. Aunque estos marcadores han sido diseñados para esta subespecie han demostrado también su utilidad en *P. apollo filabricus* lo que pone de manifiesto su posible utilidad para otras subespecies.

En general se considera muy difícil el desarrollo de marcadores para regiones microsatélites de lepidópteros (Zhang 2004), debido a que las zonas adyacentes a dichas regiones se pueden encontrar duplicadas en algunas zonas de su genoma (Meglécz *et al.* 2004; Zhang 2004). Dos de nuestros loci presentaron una frecuencia de alelos nulos mayor del 10% (Pan32 y Pan53) y en muchos de los 33 loci desechados (de los 53 escogidos inicialmente) se obtuvieron múltiples bandas o picos indicando poca especificidad en la unión de los cebadores. La poca especificidad y alta frecuencia de alelos nulos podría deberse a dichas repeticiones de los loci en varias zonas del genoma (Meglécz *et al.* 2004; Zhang 2004). Otros tres loci muestran un desvío significativo del equilibrio de Hardy-Weinberg, en algunas de las poblaciones de Sierra Nevada (Pan03, Pan22 y Pan27), estos resultados, dado que no están causados por una elevada frecuencia de alelos nulos (como la de Pan32 y Pan 53), podrían deberse a la propia dinámica poblacional de la especie que puede ser la causa de un desvío significativo del equilibrio de Hardy-Weinberg (Smee *et al.* 2013)

Pese a esto, en el capítulo 3 se describen y utilizan con éxito 20 marcadores para loci únicos y polimórficos, de 53 loci aislados, lo que supone un grado de éxito del 38%. Esto es un número total y un porcentaje de éxito mucho mayor que los 6 marcadores de 45 loci analizados conseguidos anteriormente para la misma especie (Petenian *et al.* 2005), los 4 marcadores de 23 loci obtenidos para *P. smintheu*s (Keyghobadi *et al.* 1999) y los 3 de 14 y 5 de 22 descritos en otros dos trabajos con *P. mnemosyne* (Meglécz y Solignac 1998; Gratton y Sbordoni 2009). En nuestras muestras los cinco marcadores descritos por Gratton y Sbordoni (2009) y los cuatro descritos por Keyghobadi *et al.* (1999) no amplificaron ningún producto válido, en el caso de los loci descritos para *P. mnemosyne* puede deberse a diferencias en las regiones adyacentes al locus donde debe unirse el marcador, lo que es comprensible, ya que a pesar de que el género *Parnassius* es monofilético, *P. mnemosyne* y *P. apollo* se encuentran en grupos filogenéticamente muy alejados dentro del género (Omoto *et al.* 2004); *P. smintheus* por el contrario se encuentra en el mismo grupo que *P. apollo,* siendo especies muy próximas (Omoto *et al.* 2004), pero nuestros resultados mostraron que los marcadores de *P. smintheus* no se unieron efectivamente o específicamente al ADN de *P. apollo nevadensis*. Esto no es sorprendente dado que tampoco los marcadores descritos por Petenian *et al.* (2005) para *P. apollo* funcionaron con los individuos de Sierra Nevada (con excepción de un loci que resultó ser monomórfico). Estas incompatibilidades son posiblemente debidas al alto grado de aislamiento de las poblaciones de este género y de *P. apollo* en concreto (Descimon y Napolitano 1993; Descimon 1995), y concuerda con la filogenia molecular del género que muestra que las poblaciones francesas y españolas representan haplotipos distintos muy alejados, con al menos 11 mutaciones de diferencia en COI y dos haplotipos intermedios (Todisco *et al.* 2010).

Nuestros marcadores tiene como mínimo 10 repeticiones dinucleotídicas perfectas, mientras que los otros marcadores descritos para esta especie (Petenian et al. 2005) tienen o bien menos repeticiones o bien las unidades repetidas son compuestas. Probablemente debido a que el número de repeticiones está directamente ligado a la variabilidad del locus (número de alelos diferentes), y a que los loci con repeticiones perfectas son significativamente más variables que los que tienen repeticiones imperfectas (Goldstein y Clark 1995), nuestros marcadores son más polimórficos que los descritos por Petenian et al (2005).

Estos marcadores nos han permitido estudiar la diversidad genética de las poblaciones, lo que es un indicador de su potencial evolutivo y por tanto de su estado de conservación (Falk *et al.* 2001; Frankham 2005). Además nos han permitido estimar factores clave como son el tamaño efectivo de las poblaciones y el nivel de conectividad entre ellas (Frankham *et al.* 2002; Frankham 2005). El flujo génico es uno de los factores que determinan la supervivencia o extinción de una población a largo plazo (Frankham 2005), La conexión entre zonas permite la recolonización de hábitats adecuados (que podrían haber sido desocupados por eventos estocásticos) y mantiene la diversidad genética al contrarrestar los efectos de la deriva genética distribuyendo alelos entre poblaciones (Frankham *et al.* 2002; Segelbacher *et al.* 2010).

En el Capítulo 4 describimos un proceso de incipiente estructuración genética en las poblaciones estudiadas de Sierra Nevada donde los datos sugieren que se ha pasado de una población panmíctica a un sistema metapoblacional en el que las 13 localidades analizadas se agrupan en 4 demes o subpoblaciones interconectadas entre sí por un flujo génico limitado. En dicha dinámica metapoblacional el flujo de individuos posiblemente sigue una dinámica de paso intermedio (*stepping stone*) en la que los individuos se

mueven a las áreas adecuadas circundantes sin recorrer largas distancias. Esto concordaría con una baja tasa de movimiento, menor de 300 metros diarios en *Parnassius sp.* (Brommer y Fred 1999; Välimäki y Itämies 2003; Auckland *et al.* 2004). Esta baja tasa de movimiento produciría una tasa de dispersión anual baja en estas especies que dificulta la conexión entre poblaciones (Roland *et al.* 2000; Matter y Roland 2002; Matter *et al.* 2004), limita el flujo génico y podría explicar en parte la bajísima tasa de recaptura entre parcelas de la misma localidad (Capítulo 5).

Nuestras estimas de aislamiento por distancia (Capítulo 4) son más significativas cuando usamos unas medidas de distancia calculadas recorriendo los hábitats intermedios a un intervalo de altitud adecuado para la especie, en vez de las tradicionales distancias en línea recta que teóricamente *Parnassius apollo* podría sobrevolar. Esto encajaría también con una dinámica de paso intermedio asociada a la baja movilidad diaria. Además se ha observado que cuando una población se ajusta a un modelo de paso intermedio las distancias (tradicionales en línea recta) se ajustan peor y muestran un grado de correlación menor con la estructura genética (Kimura y Weiss 1964), cosa que aparentemente se corrige (al menos en parte) con nuestro método de calcular las distancias, ya que produce un mayor grado de correlación. La baja tasa de movimiento estaría acentuando el aislamiento por distancia, al provocar que las poblaciones más cercanas estuvieran conectadas, pero limitan el flujo genético con poblaciones más alejadas.

Nuestros resultados muestran que el flujo génico entre las subpoblaciones estudiadas (o demes de la metapoblación) es muy asimétrico lo que limita sus efectos y provoca que algunas poblaciones apenas reciban individuos de otras áreas, mientras que otras subpoblaciones parecen ser sumideros de variabilidad genética del que raramente

escapan individuos a otras áreas. De las subpoblaciones que actúan como sumidero, la subpoblación más oriental (East) se encuentra separada del resto de Sierra Nevada por el puerto de montaña de la Ragua, a menor altitud que las zonas circundantes y con una carretera y algunas instalaciones turísticas, y parece actuar como filtro limitando el flujo genético; North es la subpoblación más grande y aparentemente conectada con las demás, pero pese a ello hay evidencias de que ha pasado recientemente un cuello de botella genético y West parece tener una baja abundancia de hembras (al menos la población de la que tenemos datos de tamaño poblacional, Otero, ver Capítulo 5) y pequeño tamaño efectivo poblacional (Capítulo 4). La única subpoblación que actúa como fuente (South), contiene la parcela que tiene un N significativamente mayor que el resto de parcelas (ChoE, Capítulo 5), y tiene un menor número de alelos privados (y por tanto menor variación genética única), seguramente debido al movimiento ocasional de algunos individuos (y alelos raros) a todas las otras subpoblaciones.

Cuanto más limitado esté el flujo génico, más determinante es el tamaño poblacional para la supervivencia o extinción de una población (Frankham *et al.* 2002; Swindell y Bouzat 2005; Segelbacher *et al.* 2010). De hecho, las estimas de tamaño poblacional y tamaño efectivo poblacional se consideran junto con la estocasticidad ambiental los mejores predictores de riesgo de extinción de una población (O'grady *et al.* 2004).

Nuestras estimas de tamaño efectivo poblacional para las subpoblaciones de Sierra Nevada muestran valores de entre 63 y 167, lo que en algunos casos estaría por debajo de los 100 individuos mínimos, y en cualquier caso muy por debajo de los miles recomendados que se consideran un tamaño mínimo para una población viable (Reed *et al.* 2003). Poblaciones como las estudiadas, por debajo de estos tamaños, en las que el flujo genético se viera más limitado podrían sufrir efectos de deriva genética y

endogamia, lo que les conferiría una baja diversidad genética (Frankham 2005). Por esto se asocia una baja N_e a un bajo potencial evolutivo y a un alto riesgo de extinción, como han demostrado empíricamente algunos trabajos (Frankham et al. 1999, Spielman et al. 2004, Frankham et al. 2013).

Las estimas de tamaño poblacional por otra parte confieren una aproximación más reciente al estado de la población que la de las estimas de N_e que se remontan unas cuantas generaciones atrás dependiendo del método (ver discusión en el Capítulo 6). Las estimas de tamaño poblacional en Sierra Nevada muestran una gran variación entre áreas de la misma zona (93 a 705) y en general parecen gozar de un tamaño poblacional mayor del indicado por otros trabajos (Gomáriz-Cerezo 1998), aunque dichas estimas han sido calculadas con diferentes métodos y con diferentes limitaciones. En sierra de Baza-Filabres se hizo el estudio de marcaje-recaptura en toda la población conocida (unas 30 hectáreas), mientras que en Sierra Nevada se trata de múltiples subpoblaciones abiertas, por lo que eso no es posible y por tanto las estimas no son completamente comparables; aun así la abundancia de *P. apollo nevadensis* en las zonas estudiadas parece ser mayor que la de *P. apollo filabricus* (237.01 y 160.80 de media respectivamente), y la densidad en Sierra Nevada es de un mínimo de 8.6 hembras/hectárea (Otero en 2011), lo que es prácticamente el doble de la densidad de la población estudiada en Baza-Filabres el último año muestreado. A esto hay que añadir que alrededor de la población estudiada en Filabres no existen zonas próximas con presencia de mariposas según los trabajos de muestreo realizados en 2009 y 2010. Aunque muy recientemente se ha descubierto otro núcleo poblacional de *filabricus,* de características demográficas desconocidas (Gil-T 2016), es muy probable que no exista flujo génico entre la población estudiada y la recientemente descubierta dado que se encuentra a una distancia considerable (en torno a 10 km). Por ello, las bajas estimas de

tamaño poblacional que hemos obtenido nos llevan a concluir que *P. apollo filabricus* se enfrenta a un alto riesgo de extinción (Reed et al. 2003, Spielman et al. 2004).

El relativo mayor tamaño poblacional de *P. apollo nevadensis* debe interpretarse con cautela, ya que existe un gran sesgo en la proporción de sexos a favor de machos, lo que quiere decir que el N_e, que está limitado por el número de hembras reproductoras, en Sierra Nevada sería bastante menor que el tamaño de censo tal y como sugieren los métodos moleculares.

La proporción de sexos calculada en nuestras poblaciones (5 : 1 de media) es mucho mayor que el de 2 : 1 de 2012 (o 1 : 2 de 2013) de la subespecie de Sierra de Baza-Filabres (Capítulo 6) y del 2 : 1 de otras especies del mismo género (Konvička y Kuras 1999; Matter y Roland 2002). Este sesgo a favor de los machos frente a hembras no se explica en las poblaciones estudiadas por las diferencias en su comportamiento o en su probabilidad de captura. En otros lepidópteros se ha visto que una sesgo a favor de los machos frente a las hembras están relacionados con una mayor mortalidad pre-adulta de las hembras y una combinación de mortalidad (adulta) y salida de hembras hacia otras zonas instigada por el acoso de los machos a hembras que ya se han apareado (Shapiro 1970; Ehrlich *et al.* 1984; Baguette *et al.* 1998), aunque desconocemos si estos sistemas de apareamiento son comparables a los de *P. apollo nevadensis y* si sería esta la causa de la sex ratio encontrada por nosotros.

Conocer mejor las estrategias reproductivas de la especie (sistemas de apareamiento, poliandria, elección de pareja, competencia intra e inter sexual) puede ser muy relevante, ya que tiene repercusión en el tamaño poblacional efectivo, la diversidad genética y la viabilidad de las poblaciones (Anthony y Blumstein 2000; Booy *et al.* 2000). Respecto a esto, el Capítulo 3 muestra que la poliandria podría ser común en *P.* apollo y que el

sphragis no parece asegurar la paternidad única al macho, y no debe por tanto, ser tomado como un indicador fiable de la monogamia de las hembras. Desconocemos qué mecanismos han desarrollado las hembras (u otros machos) para evitar la monogamia impuesta por la implantación del s*phragis*, aunque es cierto que los machos parecen secretar peores *sphragis* en copulas sucesivas (O'grady *et al.* 2004) y que hay hembras en las que el *sphragis* se pierde o es arrancado (Vlasanek y Konvicka 2009; obs. per). En los parnasiinae generalmente encontramos que las hembras muestran un *ostium bursae* más expuesto que en otras especies, lo que hace más difícil el que sea taponado efectivamente, pero también facilita que sean fecundadas contra su voluntad (Kitching 1999); esto podría estar relacionado con la pérdida de efectividad del *sphragis* y la tasa de paternidad múltiple encontrada. De todos modos sean cuales sean los mecanismos del sistema de apareamiento implicados, la poliandria puede aumentar la diversidad alélica de las puestas, permitir a las hembras escoger "buenos genes", y disminuir el efecto de la endogamia si se diera (Zeh y Zeh 1996, 1997; Tregenza y Wedell 2000; Zeh y Zeh 2001).

La endogamia es uno de los problemas genéticos que amenazan las poblaciones de pequeño tamaño y que pueden ser incluso más graves que los eventos estocásticos (Lande 1988; Keller y Waller 2002; O'grady *et al.* 2004; Spielman *et al.* 2004). Afortunadamente las subpoblaciones de Sierra Nevada no están totalmente aisladas y parecen mantener un tamaño poblacional suficiente para mantener unos niveles de diversidad alélica medios-altos (H_o = 0.580 y A_R = 7.12) que encajan con los descritos para otros parnasinos (H_o [0.33–0.68]) en trabajos similares (Meglécz et al. 1998; Keyghobadi et al. 1999, 2002; Petenian et al. 2005), y no parece haber evidencias de *inbreeding* poblacional (valores de F_{IS} no significativos). Esto *a priori* indicaría un buen estado general de las poblaciones nevadenses; sin embargo, estos datos han de interpretarse con cautela dado que la población estudiada en la sierra de Baza-Filabres

(Capítulo 6) parece tener unos valores similares de diversidad genética (mayor heterocigosidad observada y riqueza alélica que las nevadenses), pero es bien conocido que aunque esta población no se haya extinguido, la subespecie ha pasado por una reducción reciente de su área de distribución en las últimas décadas (Barea-Azcón *et al.* 2008) y nuestros datos confirman un tamaño de censo muy pequeño (Capitulo 6). Aunque los altos valores podrían significar que esta población de *P. filabricus* no se ha visto afectada por los mismos problemas que el resto de las ya extintas, una mayor diversidad alélica también sería posible en las subpoblaciones remanentes de una población que ha sufrido una retrogresión de su área de distribución, si existe flujo genético entre las subpoblaciones (Porter 1999). El proceso por el cual el hábitat se hace menos adecuado ya sea debido a la temperatura o a la transformación del hábitat (Wilson *et al.* 2005; Wilson *et al.* 2007; Forister *et al.* 2010; Oliver *et al.* 2015) puede afectar de forma diferencial a la supervivencia o abundancia de machos y hembras debido a sus diferentes requerimientos (Baz 2002; Capítulo 5), así como a la de larvas y adultos (Auckland *et al.* 2004; Radchuk *et al.* 2013). Debido a esto, el proceso de degeneración del hábitat puede no afectar drásticamente a todas las fases de su ciclo y ser suficientemente lento como para que, si existe cierta conexión entre subpoblaciones, cada temporada unos pocos individuos logren desplazarse desde zonas que se estén volviendo inadecuadas a las subpoblaciones adecuadas remanentes. De esta forma, las últimas poblaciones que queden de un sistema contendrán alelos que han llevado los "refugiados" de todas las otras áreas y por tanto, pese a la población haber pasado por una reducción en su tamaño poblacional, temporalmente la diversidad alélica de las poblaciones remanentes se verá incrementada (Porter 1999).

Tras una reducción del tamaño poblacional se espera que la frecuencia de alelos raros (alelos con frecuencias muy bajas) descienda más rápidamente que la heterozigosidad,

que también descendería, pero más lentamente (Maruyama y Fuerst 1984, 1985). El moderado flujo genético descrito entre subpoblaciones, junto con el hecho de que nuestros loci tienen repeticiones perfectas de dinucleotidos, que se espera muten principalmente siguiendo el modelo SMM (Cornuet y Luikart 1996; Capítulo 2 y 4), explicaría también porqué algunas subpoblaciones de Sierra Nevada (East y North) presentan indicios de haber pasado un cuello de botella reciente (Sign test, Capítulo 4), pero presentan una heterocigosidad menor que la esperada en comparación con la frecuencia de alelos raros (Wilcoxon test, Capítulo 4). Esto se debería a que en algunos casos, en loci evolucionando estrictamente bajo el SMM la heterozigosis puede descender más rápidamente que la frecuencia de alelos raros, que además, podría mantenerse alta por la dispersión de unos pocos individuos provenientes de poblaciones diferenciadas genéticamente (Maruyama y Fuerst 1984, 1985; Cornuet y Luikart 1996). Así pues pese a que aún exista un moderado flujo génico, consideramos nuestros datos de Sierra Nevada indican cierto grado de reducción en la distribución (reciente estructura genética), y además en el tamaño poblacional (Bottleneck) de las poblaciones almerienses y las de la ladera Norte (East y North respectivamente).

En poblaciones pequeñas y aisladas los eventos estocásticos que afectan a la supervivencia pueden ser devastadores y ocasionar la extinción de la misma (O'grady *et al.* 2004; Spielman *et al.* 2004). Actualmente ya se han reportado extinciones de poblaciones francesas de *P. apollo* (Descimon *et al.* 2005) debido a eventos estocásticos ("falsa primavera" en invierno). Nuestros datos muestran que "storm" (fuertes vientos y lluvia) es la principal variable determinando la tasa de supervivencia (en un 42% de los modelos). Precisamente uno de los efectos previstos del cambio climático es el aumento en la frecuencia de este tipo de eventos (Palmer y Räisänen 2002; Schär *et al.* 2004; Jalili *et al.* 2010), que ya se ha visto pueden afectar dramáticamente a las mariposas (Dempster

1983; Roy y Sparks 2000; Roy *et al.* 2001; Descimon *et al.* 2005; Wilson *et al.* 2005; Parmesan 2006, 2007; Wilson y Maclean 2011; Descombes *et al.* 2015; Oliver *et al.* 2015).

Parnassius apollo ha demostrado ser muy sensible a pequeños cambios de temperatura durante su estadio larval (Ashton *et al.* 2009; Turlure *et al.* 2010) y en concreto las poblaciones de *P. apollo nevadensis* estudiadas parecen verse negativamente afectadas por altas temperaturas mínimas durante la fase de larva y pupa (Capitulo 5). Ante una subida general de las temperaturas (otro de los efectos de cambio climático) *P. apollo nevadensis* tiene una mayor capacidad de respuesta, ya que aún hay cierto grado de flujo genético entre sus poblaciones y Sierra Nevada alcanza mayores altitudes, a las que las mariposas pueden desplazarse para combatir el incremento de las temperaturas. De hecho *Parnassius apollo nevadensis* ya ha desaparecido de algunas zonas de menor altitud como el Puerto de La Ragua, a 2000 m (González-Megías *et al.* 2015), y ha ascendido el límite superior de su distribución como ha sucedido con otras subespecies de *P. apollo* (Wilson *et al.* 2005; Ashton *et al.* 2009). Esto posiblemente habrá restringido aun más el flujo génico y la disponibilidad de hábitats adecuados para *P. apollo nevadensis*, pero el destino de *P. apollo filabricus* es aún más complicado, dado que está prácticamente ya en las cotas máximas de altitud de la sierra de Baza-Filabres en la que habita.

Para mejorar el estado de las poblaciones o prevenir futuros problemas se debería de actuar en dos frentes, facilitando el crecimiento de las poblaciones (N y N_e) mejorando la calidad el hábitat y facilitando su interconectividad (flujo génico). Para ello en nuestro trabajo hemos descrito las variables que definirían un hábitat de calidad y que pueden darnos indicaciones de dónde y cómo sería más eficiente actuar. Las mariposas parecen preferir parcelas alejadas de carreteras y deben de ser heterogéneas ya que por un lado

deben de tener una buena riqueza de especies vegetales y cobertura de *Sedum* (planta hospedadora de la larva) y de fuentes de néctar para los adultos, pero por el otro lado también deben de tener heterogeneidad espacial, incluyendo zonas más despejadas que parecen ser preferidas por los machos y que facilitarían también la termorregulación de las larvas según otros trabajos (Ashton *et al.* 2009). Esto incluiría por tanto considerar si la densidad de planta hospedadora (*Sedum*) o fuentes de néctar es baja y se debe facilitar su crecimiento y además estudiar adecuar el "ambiente térmico" para que haya un nivel adecuado de elementos como rocas y arbustos, ya que esto es un elemento clave en los planes de conservación de mariposas (y específicamente de relictos glaciales) que tienen unas condiciones térmicas muy precisas (Turlure *et al.* 2009; Turlure *et al.* 2010).

En el caso concreto de Sierra Nevada, aunque las poblaciones parezcan estar en un relativo mejor estado habría que poner especial atención en el mantenimiento de las zonas con mayor abundancia como el Chorrillo, ya que además pertenece a la subpoblación que actúa como fuente (Capítulo 4) pero tiene un N_e menor de lo esperado para su tamaño poblacional. También se podría considerar restaurar las zonas con baja abundancia de hembras como Otero o Piuca que puedan precisar de actuación. Respecto al flujo génico entre zonas, no pudiendo actuar sobre las barreras o filtros naturales como valles o picos, quizás si se podría estudiar algunas zonas intermedias entre localizaciones con mariposas y tratar de mejorar las condiciones descritas más arriba, sembrando o facilitando *Sedum* y fuentes de néctar en caso de que sea necesario y adecuando la heterogeneidad espacial.

En el caso de Baza-Filabres se proponen medidas más drásticas como la deforestación de zonas previamente reforestadas con pino, para crear corredores que comuniquen parches que podrían ser adecuados para la especie. La formación de corredores entre

poblaciones ha demostrado ser muy eficiente con otras especies de mariposas (Haddad 1999). Otra medida que se podría probar sería un proceso de cría y reintroducción, pero esto necesita la suelta de un gran número de larvas para evitar la endogamia y por su bajo índice de éxito en *Parnassius apollo* (Adamski y Witkowski 2007; Fred y Brommer 2015), es una medida que parece arriesgada.

En cualquier caso sería muy interesante y quizás necesario complementar este trabajo con un estudio de presencia/ausencia de la especie en Sierra Nevada, ya que estos métodos han mostrado ser muy útiles para explicar la distribución de las apolo de sierra de Guadarrama (Gutiérrez *et al.* 2013), el estudio de las condiciones de zonas en las que no hay mariposas podría indicarnos qué carencias tienen esas zonas y que medidas hacen falta tomar. También han demostrado ser muy útiles los estudios a menor escala (Ashcroft *et al.* 2009; Ashton *et al.* 2009; Turlure y Van Dyck 2009; Hampe y Jump 2011) que quizás permitirían explicar las causas de que encontremos el mayor y menor N en parcelas de la misma zona (Chorrillo, capítulo 5) y que muestren el uso que hacen del hábitat machos y hembras y que hace que sus abundancias sean tan diferentes y se vean correlacionadas con variables distintas.

Una de las conclusiones de esta tesis debería ser precisamente esa diferencia en abundancias que produce una *sex-ratio* mucho más sesgada hacia machos que en cualquier otra población descrita y la diferencia en las variables que predicen sendas abundancias. Además hemos visto que esos tamaños poblacionales son de media mayores que los de *P. apollo filabrius*, y que *P. apollo nevadensis* no muestra indicios de endogamia o de baja diversidad genética. No obstante ha habido una subida altitudinal de su distribución y retrogresión de la misma que podría ser la causa de la estructura genética de reciente formación, en la que existe un flujo génico limitado entre algunas de

las subpoblaciones y en algunas de estas ha habido una reciente reducción de su tamaño poblacional. Por todo esto se considera que la viabilidad de las poblaciones no se debería encontrar amenazada críticamente, pero que se debería trabajar en facilitar la conexión entre parches con hábitats adecuados y restaurar si es posible y necesario aquellos parches con menor cantidad de mariposas que tengan unas condiciones alejadas de las descritas como hábitat de calidad en este trabajo.

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8

CONCLUSIONES

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8 Conclusions

1. The new 20 *Parnassius apollo* polymorphic microsatellite markers present perfect repeat motifs (neither compound microsatellites nor with any interruptions), and can be successfully multiplexed.

2. We successfully used 13 of the markers for population genetics studies in *P. apollo nevadensis* and other 13 (9 in common) in *P. apollo filabricus*. Due to the high variability of our Pan microsatellite markers we have been able to use them in population genetics and parentage analyses

3. Six of seven analysed females of *P. apollo nevadensis* copulated with at least two males and thus the *sphragis* may not always act as an effective blocking mechanism to avoid other males fertilising the female.

4. *P. apollo* populations in Sierra Nevada are undergoing a strong process of isolation by distance and a recent genetic structure.

5. The 13 locations studied in Sierra Nevada are grouped in four demes or subpopulations with asymmetric gene flow between them. The subpopulations West, East, and North act as a sink while South is the only source. Sink subpopulations East and North show some signs of recent bottleneck, on the other hand has South a significantly smaller private allelic richness, which reinforces its role as a source deme.

6. East is separated from the rest of subpopulations by the mountain pass of Puerto de la Ragua, in which there was butterflies years ago but recently *P.* apollo do not fly there, probably due to the lower altitude of this zone and the climate change.
7. The studied population of *P. apollo filabricus* is isolated and seems to have lower population density and effective population size than *P. apollo nevadensis*, indicating its worrying situation.
8. We do not found signs of recent bottleneck in populations from sierra de Baza-Filabres, and heterozygosity seems to be medium-high for both *P. apollo nevadensis* and *P. apollo filabricus*; however given the recent process of retrogression found in nevadensis and the extinction of most of the subpopulations in filabricus, this is probably a result of the refugee effect.
9. We have a low recapture rate, and the recapture rate between areas from the same locality is almost zero. This indicates that the butterflies probably wander out the areas, but normally don not go over the distance that separate the areas. This agrees with the typically low movement rate of the Genus.
10. The studied localities of *P. apollo nevadensis* show a great range in population size (109-795) and the greater variation is found between two areas from the same location (Chorrillo).
11. We found a sex ratio highly biased toward males (5 : 1 of mean), this is more biased than the reported in other populations of the species.
12. There are not variables in common in males and females predicting abundance, highlighting the differences in requirements of both sexes.

13. According to our data an habitat of quality for *P. apollo nevadensis* should be far from roads, with high plant species richness (variables correlated with female abundance); in addition should contain high host plant (*Sedum ssp.*) cover and nectar sources cover (variables correlated with survival rate). However they should also be heterogenic, with no overdominance of bushes (*Genista sp.*) and zones with bare-ground (correlated with male abundance).

14. Higher temperatures (in particular the minimum temperature of June) affect negatively to both abundance and survival of *P. apollo nevadensis*, this is probably due to an effect in the larval stage.

15. *P. apollo nevadensis* does not seem to require urgent management measures but it will be recommended to manage the heterogeneity and plant cover of zones with lower abundance of females to try to improve its situation.

8 CONCLUSIONES

1. Los nuevos 20 marcadores microsatélites para *Parnassius apollo*; presentan repeticiones perfectas de dinucleótidos (sin secuencias compuestas ni interrupciones), son polimórficos y pueden ser usados conjuntamente en reacciones "multiplex".

2. De los 20 marcadores descritos, 13 han sido usados con éxito en todas las subpoblaciones estudiadas de *P. apollo nevadensis* y otros 13 (9 en común) en *P. apollo filabricus*. Debido a su alta variabilidad, estos marcadores (*Pan*) han demostrado ser útiles para análisis de parentesco y de genética de poblaciones.

3. Seis de las siete hembras de *P. apollo nevadensis* analizadas copularon con al menos dos machos y por tanto podemos afirmar que el *sphragis* no siempre resulta efectivo como mecanismo para evitar que otros machos fertilicen a una hembra.

4. Las poblaciones de *P. apollo* de Sierra Nevada se encuentran ante un fuerte proceso de aislamiento por distancia y existe una estructura genética reciente.

5. Las trece localidades estudiadas en Sierra Nevada han sido agrupadas en cuatro subpoblaciones o demes con flujo génico asimétrico entre ellos. Las subpoblaciones West, East y North actúan como sumideros, mientras que South es la única subpoblación fuente. De entre las que actúan de sumidero East y North muestran signos de haber pasado por un reciente cuello de botella; por otro lado South tiene una riqueza de alelos únicos significativamente menor que el resto, lo que refuerza su rol de deme fuente.

6. La subpoblación East se encuentra separada del resto por el puerto de la Ragua, en donde años atrás se podían observar apollos, pero recientemente parece haber una total ausencia de estas, probablemente debido a una menor altitud de la zona y el efecto del cambio climático.

7. La población estudiada de *P. apollo filabricus* se encuentra aislada y parece tener menor densidad poblacional y tamaño poblacional efectivo que *P. apollo nevadensis*, lo que indicaría una preocupante crítica de la primera.

8. *apollo nevadensis P. apollo nevadensis* y *P. apollo filabricus* parece ser moderadamente alta, pese a esto encontramos un proceso reciente de retrogresión en la distribución de esta especie en Sierra Nevada y la mayoría de poblaciones de poblaciones previamente existentes en sierra de Baza-Filabres se han extinguido; por lo que probablemente el grado alto de diversidad genética es un efecto derivado del “efecto refugiado”.

9. La tasa de recaptura es en general baja, pero concretamente la tasa de recaptura entre áreas de la misma localidad es prácticamente cero. Esto sugiere que las mariposas entran y salen de las áreas, pero no suelen desplazarse a distancias como las que separan nuestras áreas de muestreo, lo que concuerda con la típica baja tasa de movimiento del género.

10. Encontramos un rango amplio en los tamaños poblacionales de las áreas estudiadas (109-795) y la mayor variación se encuentra entre dos áreas de la misma localidad (Chorrillo).

11. Hemos hallado una proporción de sexos altamente sesgada a favor de los machos (5 : 1 de media), el sesgo es mucho mayor que el encontrado en otras poblaciones de la especie.

12. No hay variables en común que predigan la abundancia de machos y hembras, lo que pone de manifiesto las diferencias en los requerimientos de ambos sexos.

13. Según nuestros datos un hábitat de calidad para *P. apollo nevadensis* debería de encontrarse alejado de carreteras y con una gran riqueza de especies vegetales (variables correlacionadas con la abundancia de las hembras); además debería de tener una gran cobertura de planta hospedadora (*Sedum ssp.*) y de fuentes de nectar (variables correlacionadas con la supervivencia). Sin embargo el hábitat debería también ofrecer una condiciones heterogeneas, sin sobredominancia de arbustos (*Genista sp.*) y con algunas zonas despejadas de suelo desnudo (variables correlacionadas con la abundancia de machos).

14. Altas temperaturas (en particular altas mínimas mensuales en junio) afectan negativamente a la abundancia y supervivencia de *P. apollo nevadensis*, probablemente a un efecto sobre su fase larvaria.

15. La situación de *P. apollo nevadensis* no parece requerir acciones inmediatas pero quizás sería recomendable actuar en cuanto a la cobertura de las plantas relevantes descritas en las zonas con baja abundancia de mariposas, para tratar de mejorar su situación.

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