UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Departamento de Biología

i-UNAT
INSTITUTO UNIVERSITARIO DE ESTUDIOS AMBIENTALES Y RECURSOS NATURALES

Conservación genética de tres endemismos vegetales canarios amenazados: *Bethencourtia* spp., *Sambucus palmensis* y *Viola cheiranthifolia*
Priscila Rodríguez Rodríguez
2017

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Departamento de Biología

TESIS DOCTORAL

Conservación genética de tres endemismos vegetales canarios amenazados: *Bethencourtia* spp., *Sambucus palmensis* y *Viola cheiranthifolia*

Priscila Rodríguez Rodríguez

Las Palmas de Gran Canaria

2017

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Facultad de Ciencias del Mar

ANEXO I

Dª MARÍA ISABEL PADILLA LEÓN, SECRETARIA DE LA FACULTAD DE CIENCIAS DEL MAR, ÓRGANO RESPONSABLE DEL PROGRAMA DE DOCTORADO EN GESTIÓN SOSTENIBLE DE RECURSOS PESQUEROS DE LAS PALMAS DE GRAN CANARIA.

CERTIFICA

Que el Consejo de Doctores del Programa de Doctorado en Gestión Sostenible de Recursos Pesqueros, en su sesión de fecha 5 de junio de 2017, tomó el acuerdo de dar el consentimiento para su tramitación a la tesis doctoral titulada:
"Conservación genética de tres endemismos vegetales canarios amenazados: *Bethencourtia spp., Sambucus palmensis y Viola cheiranthifolia* ",
presentada por la doctoranda: **Dª Priscila Rodríguez Rodríguez** y dirigida por el Doctor D. Pedro Sosa Henríquez.

Y para que así conste, a efectos de lo previsto en el Artº 6 del Reglamento para la elaboración, tribunal defensa y evaluación de tesis doctorales de la Universidad de Las Palmas de Gran Canaria, firmo el presente en Las Palmas de Gran Canaria, a cinco de junio de dos mil diecisiete

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Anexo II

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UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

Departamento de Biología

Instituto Universitario de Estudios Ambientales y Recursos Naturales

PROGRAMA DE DOCTORADO:

GESTIÓN SOSTENIBLE DE RECURSOS PESQUEROS

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Conservación genética de tres endemismos vegetales canarios amenazados: *Bethencourtia* spp., *Sambucus palmensis* y *Viola cheiranthifolia*

Presentada por la Lcda. Priscila Rodríguez Rodríguez

Dirigida por el Dr. Pedro A. Sosa Henríquez

El Director,
Pedro A. Sosa

La Doctoranda,
Priscila Rodríguez

Las Palmas de Gran Canaria, a 31 de mayo de 2017

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Departamento de Biología

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INSTITUTO UNIVERSITARIO
DE ESTUDIOS AMBIENTALES
Y RECURSOS NATURALES

El Dr. Pedro A. Sosa Henríquez, Catedrático de Botánica de La Universidad de Las Palmas de Gran Canaria,

CERTIFICA:

Que el trabajo de investigación "Conservación genética de tres endemismos vegetales canarios amenazados: *Bethencourtia* spp., *Sambucus palmensis* y *Viola cheiranthifolia*" realizado bajo su dirección por la Lcda. Priscila Rodríguez Rodríguez, se considera finalizado y puede ser presentado para su exposición y defensa como Tesis Doctoral en el Departamento de Biología de la Universidad de Las Palmas de Gran Canaria.

Y para que así conste y surta los efectos oportunos, firmo el presente certificado en Las Palmas de Gran Canaria, a 31 de mayo de 2017.

Fdo: Pedro A. Sosa Henríquez

"*Una vez que una especie se extingue*
ninguna ley puede hacerla regresar:
se ha marchado para siempre."

Allen M. Solomon

A mis padres,
mi hermana,
y Dani.

Esta tesis ha sido cofinanciada por la Agencia Canaria de Investigación, Innovación y Sociedad de la Información (ACIISI) y por el Fondo Social Europeo (FSE), eje 3, prioridad de inversión 10.2, objetivo específico 10.2.1, con una tasa de cofinanciación del 85% del Programa Operativo FSE de Canarias 2014-2020. La ACIISI también financió dos ayudas para Estancias Breves en el extranjero durante los años 2015 y 2016.

Además, este trabajo ha formado parte del proyecto de investigación titulado 'Biología de la conservación de endemismos vegetales de los Parques Nacionales canarios: caracterización genética y demográfica' financiado por el Organismo Autónomo de Parques Nacionales del Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente (referencia 255/2011).

Gobierno de Canarias
Consejería de Economía, Industria, Comercio y Conocimiento
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MINISTERIO DE AGRICULTURA Y PESCA, ALIMENTACIÓN Y MEDIO AMBIENTE
ORGANISMO AUTÓNOMO PARQUES NACIONALES

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Mi inmersión en el mundo de la Biología comenzó en la Universidad de La Laguna, en el máster de Biodiversidad Terrestre y Conservación en Islas. No podría cuantificar todo lo que aprendí en La Laguna en esas salidas de campo y en los viajes a La Palma y La Gomera. Me mostraron la biodiversidad de mi tierra, y por ello, un enorme gracias a todos los profesores del máster. A José Ramón Arévalo, por confiar en mí para el trabajo de fin de máster y brindarme la oportunidad de continuar investigando, y a José María Fernández Palacios por mostrarme el apasionante mundo de la biogeografía insular. Esa época no hubiera sido lo mismo sin mis compañeras de fatiga Alexandra y Raquel. A todos los integrantes del grupo de ecología, por todos los buenos momentos, en especial a Silvia, Lea, Javi y Cristina.

De vuelta a la ULPGC, mil gracias a todas las personas del Departamento de Biología que de alguna manera u otra iluminan los días oscuros de nuestro edificio. En especial a mis compañeras de laboratorio: Leticia Curbelo por encargarse siempre de organizar mis desastres, a Isabel Saro por darme ideas para análisis y escucharme siempre que hacía falta, y a Carolina Medina, por esa calma que transmite en todo momento. A Montse, por el apoyo mutuo que nos hemos brindado en este frenético final, y a Javi y Marina por nuestras inspiradoras conversaciones en el B5.

El trabajo de la violeta del Teide no hubiera sido posible sin la colaboración de muchas personas. Por un lado, los muestreos se llevaron conjuntamente con el personal del proyecto VULTEIDE: Jaume Seguí, Marta López, Benito Pérez, Manuel Nogales, Francisco Valladares y Anna Traveset. Alejandro de Topcon nos brindó una ayuda valiosa en la georreferenciación del Refugio. Olivier Lepais me cedió amablemente sus scripts de R para poliploides y Lindsay Clark siempre respondió rápida y eficazmente a mis preguntas sobre el Polysat. A Jaume Seguí, porque nuestros encuentros aquí y allí siempre traen divertidas sorpresas y por sus aportaciones al artículo de la violeta. En la elaboración de este artículo también ha colaborado Alejandro González de Castro, que además de realizar los modelos de nicho, ha mejorado con creces la calidad del manuscrito.

A todo el personal de los Parques Nacionales de Garajonay y El Teide por poner todos los medios para la recolección de muestras. En Garajonay, la inestimable ayuda de Ángel Fernández, Sito Chinea y Ángel García fue esencial en los trabajos de *Bethencourtia* y *Sambucus palmensis*. En el Teide, Manolo Marrero y José Luis Martín Esquivel también ayudaron a la recolección de muestras, junto a los otros integrantes que se unieron en esa larga excursión a Pasajirón.

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y la hizo parecer menos horrible. Mil gracias a Anna Ansebo por toda la ayuda en el laboratorio y a Romina Batista, por la amistad que forjamos en esos fines de semana de laboratorio, y para la que el océano Atlántico no supone una barrera. Un tremendo gracias a todos los integrantes de Botaniska, y a los miembros del Antonelli Lab, por integrarme en sus reuniones de una calidad científica increíble.

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Resumen

El estudio de la genética de poblaciones constituye un pilar fundamental en la gestión de especies amenazadas. Así, el conocimiento de la distribución y niveles de la variabilidad genética entre y dentro de las poblaciones puede permitir, entre otros, resolver incertidumbres taxonómicas, detectar cuellos de botella en las poblaciones, identificar individuos genéticamente, o estimar la adaptación de las especies a las perturbaciones externas. En este trabajo de investigación se ha procedido a la caracterización genética de tres taxones vegetales amenazados y endémicos del archipiélago canario con diferentes problemáticas y necesidades de actuación desde el punto de vista de la biología de la conservación.

Bethencourtia hermosae (Pit.) Kunkel y *B. rupicola* (B. Nord.) son dos endemismos de la Isla de La Gomera que requerían su caracterización y diferenciación genética mediante marcadores moleculares. Se procedió al análisis de todas las poblaciones conocidas para *B. hermosae* y *B. rupicola* y dos poblaciones de la tercera especie del género descrita en Canarias (*B. palmensis*), analizándose 9 poblaciones y 276 individuos. Con el análisis mediante 10 marcadores microsatélites (SSR) de nuevo desarrollo se pudieron resolver las incertidumbres taxonómicas en este género endémico. Los tres taxones se encuentran diferenciados genéticamente y albergan unos escasos niveles de diferenciación genética entre poblaciones dentro de especie. Debido a la baja diversidad genética encontrada en *B. hermosae* y *B. rupicola* junto a su reducida distribución y autoincompatibilidad reproductiva, se sugieren medidas de conservación, así como reevaluar su categoría de amenaza.

En el caso del saúco canario (*Sambucus palmensis* Link.), se estudiaron los efectos de las traslocaciones sobre la estructura poblacional y diversidad genética en el Parque Nacional de Garajonay, después de 30 años de reforzamiento. Para ello se analizaron 402 ejemplares representativos de todas las las localidades restauradas, además de todos los individuos naturales (divididos en 15 áreas geográficas). Mediante el genotipado de estas muestras con 7 SSR se pudo estimar el número de genotipos clónicos presentes, además de aquellos individuos con alelos raros. Estos resultados han permitido presentar un método de gestión y actuación para optimizar el refuerzo y la

monitorización de dichos ejemplares para conservar y aumentar la diversidad genética. Por otro lado, se detectó que las translocaciones no han disminuido la diversidad genética de la especie en la isla, y se ha homogeneizado la estructura genética debido al trasvase de genotipos.

Finalmente, se estudió, a través de 14 SSR, la estructura, variabilidad genética e historia demográfica de las poblaciones de la violeta del Teide (*Viola cheiranthifolia* Humb. et Bonpl.) en el Parque Nacional del Teide. Se recolectaron 266 muestras de 9 localidades distribuidas en el complejo Teide-Pico y las zonas más altas del borde de Las Cañadas. A pesar de presentar altos niveles de autofertilización, la violeta del Teide muestra moderados niveles de diversidad genética, posiblemente debido a su condición poliploide. La estructura genética de la especie se ve condicionada por su sistema reproductivo, siendo Las Cañadas, la principal barrera para el flujo genético. La población de Guajara, en lo alto de Las Cañadas, en un sustrato más antiguo, puede ser la población actual más ancestral, dando lugar a la recolonización del Teide después de eventos eruptivos. Además, mediante la modelización del nicho climático, se proyectaron posibles variaciones en la distribución altitudinal de las poblaciones de *V. cheiranthifolia* debido al cambio climático. Estos resultados, junto a la simulación de la pérdida de heterocigosidad esperada, han permitido confirmar la vulnerabilidad de *V. cheiranthifolia* ante el cambio global. Aunque la violeta del Teide es una especie resiliente y adaptada a los cambios bruscos geológicos y climáticos de la zona alpina de Tenerife, se sugieren medidas para su conservación, como la supervisión de la distribución de las poblaciones a largo plazo, reforzamiento en el caso de una disminución poblacional severa y el control de los herbívoros introducidos.

Abstract

The study of population genetics constitutes a fundamental pillar in the management of endangered species. Thus, knowledge of the distribution and levels of genetic variability within and between populations can allow, among others, to resolve taxonomic uncertainties, to detect bottlenecks in the natural populations, to identify individuals genetically, or to estimate species adaptations to external disturbances. This research work has been focused on the genetic characterization of three threatened and endemic plant taxa of the Canary Islands with diverse problems and management needs from the biological conservation point of view.

Bethencourtia hermosae (Pit). Kunkel and *B. rupicola* (B. Nord.) are two endemic species of La Gomera, requiring their characterization and genetic differentiation with the use of molecular markers. We proceeded to the analysis of all known populations of *B. hermosae* and *B. rupicola* and two populations of the third species of the genus described in the Canary Islands (*B. palmensis*), analyzing 9 populations and 276 individuals. With 10 newly developed microsatellite markers (SSR), we could resolve the taxonomic uncertainties of this endemic genus. The three species are genetically differentiated and contain low levels genetic differentiation between population within species. Due to the low genetic diversity found in *B. hermosae* and *B. rupicola* along with their reduced distribution and reproductive self-incompatibility, conservation measures are suggested, as well as the re-evaluation of their conservation status.

In the case of the Canary elderberry (*Sambucus palmensis* Link.), we studied the effects of the translocations on the population structure and genetic diversity in the Garajonay National Park, after 30 years of population reinforcement and reintroductions. In total, 402 specimens were analyzed, including a representation of the restored areas, as well as all natural individuals (divided into 15 geographic areas). The genotyping of these samples was performed with 7 SSR, estimating the number of clonal genotypes present, in addition to those individuals with rare alleles. These results have allowed the presentation of a management method to optimize the monitoring at the individual level to conserve and increase genetic diversity. On the other hand, we have detected that the translocations did have not diminished the genetic diversity of the species on the island, and it has homogenized the genetic structure due to the shift of genotypes.

Finally, we studied, through 14 SSR, the genetic structure, genetic variability and demographic history of the Teide violet populations (*Viola cheiranthifolia* Humb. et Bonpl.) in the Teide National Park. 266 samples of 9 locations distributed in the highest zones of Las Cañadas Wall and the Teide-Pico complex were collected. Despite high levels of self-fertilization, *V. cheiranthifolia* shows moderate levels of genetic diversity, possibly due to its polyploid condition. The genetic structure of the species is conditioned by its mating system, being Las Cañadas, the main barrier to gene flow. The population of Guajara, on Las Cañadas Wall, in a most ancient substrate, may be the most ancestral current population, and could have recolonized Teide after the construction of the stratovolcano. In addition, we projected possible variations in the altitudinal distribution of *V. cheiranthifolia* populations due to climate change through niche modeling methods. These results, together with the simulation of heterozygosity loss, allowed to confirm the *V. cheiranthifolia* vulnerability to global change. Although the Teide violet is a resilient species, adapted to the dramatic and abrupt geological and climatic changes of the Tenerife alpine zone, we suggest conservation measures, such as the surveillance of niche shifts in a long term basis, the population reinforcement in the case of population reduction and the control of introduced herbivores.

Estructura

La presente Tesis Doctoral está organizada en una introducción general, un resumen global y 3 capítulos. La introducción y el resumen global están escritos en castellano, mientras que los capítulos están escritos en inglés y forman parte de artículos que están siendo revisados, o se encuentran en su fase final de elaboración. Cada uno de estos capítulos está dedicado a uno de los taxones objeto de esta tesis.

1. Introducción general. Se trata de una introducción en castellano sobre la temática general de la tesis. Primero, se hace una introducción global sobre la importancia de la biología de la conservación en especies amenazadas, y posteriormente, se hace una revisión de los patrones de variabilidad genética en endemismos insulares, con énfasis en el archipiélago canario como punto caliente de biodiversidad.

2. Resumen global. Incluye los objetivos de la tesis, un resumen de la metodología empleada, así como los principales resultados obtenidos y las conclusiones finales (en castellano y en inglés).

3. Species delimitation and conservation genetics of the Canarian endemic *Bethencourtia* (Asteraceae). En este capítulo se estudió la caracterización genética del género endémico *Bethencourtia* con el fin de esclarecer incertidumbres taxonómicas entre las especies del género a nivel molecular. Además, se evaluó la variabilidad genética dentro y entre poblaciones con fines de conservación.

4. The restoration of the endangered *Sambucus palmensis*: Genetic assessment and monitoring after 30 years of conservation actions in the Garajonay National Park. Este trabajo consistió en el asesoramiento genético sobre las poblaciones reforzadas y reintroducidas del endemismo canario *Sambucus palmensis* en La Gomera.

5. Alpine species in dynamic insular ecosystems through time: Conservation genetics and niche shift estimates of the Teide Violet (*Viola cheiranthifolia*) in Tenerife Island. En este último capítulo se estudió la variabilidad genética y estructura poblacional en el presente y el futuro de *Viola cheiranthifolia*, así como la historia demográfica de las poblaciones. Con el fin de estimar las posibles fluctuaciones en la distribución poblacional como consecuencia del cambio climático se incorporaron modelos de nicho climático con alta resolución.

Parte de los resultados ya han dado lugar a dos publicaciones científicas, y a otros dos manuscritos que se encuentran en estos momentos en elaboración y serán sometidos a revistas incluidas en el SCI.

(I) Rodríguez-Rodríguez P, González-Pérez MÁ, Culley TM, Carqué E, Sosa PA (2015) Isolation and characterization of 16 microsatellite loci in the endemic *Viola cheiranthifolia* Humb. & Bonpl. (Violaceae) and their transferability to *Viola palmensis* Web & Berthel. *Conservation Genetic Resources,* 7, 455–458.

(II) Rodríguez-Rodríguez P, Pérez de Paz PL, Sosa PA (2017) Species delimitation and conservation genetics of the Canarian endemic *Bethencourtia* (Asteraceae). *Genetica. En revisión*

Índice general

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4. THE RESTORATION OF *Sambucus palmensis*

5. THE ALPINE *Viola cheiranthifolia*

Foto: Pedro Sosa

INTRODUCCIÓN GENERAL

1. INTRODUCCIÓN GENERAL

Biodiversidad se puede definir como "la diversidad de la vida a todos los niveles de organización biológica" (Gaston & Spicer 2013), incluyendo la variación entre genes, especies y rasgos funcionales. Desgraciadamente, los niveles de biodiversidad están disminuyendo dramáticamente antes de que seamos capaces de cuantificar la vida sobre La Tierra (Cardinale *et al.* 2012). En concreto, se ha estimado que del 22 al 47 % de la Flora mundial se encuentra amenazada (Pitman & Jørgensen 2002). Como respuesta a esta pérdida de biodiversidad, la Biología de la Conservación es una joven disciplina que surgió en los años 80, con el último fin de proteger y preservar en el tiempo la biodiversidad sobre el planeta Tierra. Actualmente, es conocida como una meta-disciplina, ya que se nutre de diversas áreas del conocimiento, desde las ciencias biológicas básicas hasta las ciencias sociales y humanitarias. Ha sido denominada una "disciplina de crisis", comparándola con la biología del cáncer, ya que debe anticiparse a los acontecimientos, proponiendo acciones y actuaciones incluso antes de que se confirmen las hipótesis y teorías planteadas (Soulé 2008).

Dentro de esta meta-disciplina, la conservación genética de especies amenazadas se está convirtiendo en un pilar fundamental dentro de la Biología de la Conservación. De hecho, y siguiendo a DeSalle y Amato (2004), la conservación genética contribuye a la asistencia en el diseño de programas de conservación, genética de poblaciones y sistemática, así como al estudio de ADN fósil o de organismos preservados. En estas áreas de estudio, el rol principal de la conservación genética se centra en entender los procesos genéticos y evolutivos que se ciernen sobre las poblaciones, además de definir los patrones que son relevantes para la conservación de poblaciones amenazadas (Figura 1.1). Entre los procesos descritos por DeSalle y Amato (2004), podemos destacar el conocimiento de la variación genética y flujo genético[1] en las poblaciones naturales, así como la identificación de los procesos de endogamia[2] y pérdida de variación genética. Para evitar y resolver los problemas derivados de la pérdida de diversidad genética, es necesario determinar las relaciones entre las distintas poblaciones y su estructura genética. Además, la resolución de incertidumbres taxonómicas también es fundamental para la detección de unidades de conservación (González-Pérez *et al.* 2009c; Crawford & Stuessy 2016). Las acciones más comunes para la recuperación de especies amenazadas incluyen la reintroducción[3] de

poblaciones o el reforzamiento de las ya existentes. Para ello, el conocimiento de la variación genética de los individuos a reintroducir aumentará las posibilidades de éxito de la restauración ecológica. Se puede considerar que un programa de reintroducción o reforzamiento se ha completado con éxito cuando las poblaciones resultantes son capaces de sobrevivir, reproducirse y adaptarse a los cambios ambientales (Godefroid *et al.* 2011). Por lo tanto, el establecimiento y el éxito reproductivo de las poblaciones restauradas debe monitorizarse a largo plazo, tomando la acciones necesarias para aumentar la capacidad reproductiva y de dispersión en cada caso. Dada la importancia de los estudios genéticos en los programas de conservación de especies amenazadas, el estudio de la genética de poblaciones con marcadores moleculares[4] con capacidad para identificar un proceso biológico y cuantificar la variabilidad genética es fundamental en dichos programas (Sosa *et al.* 2011; Allendorf 2017).

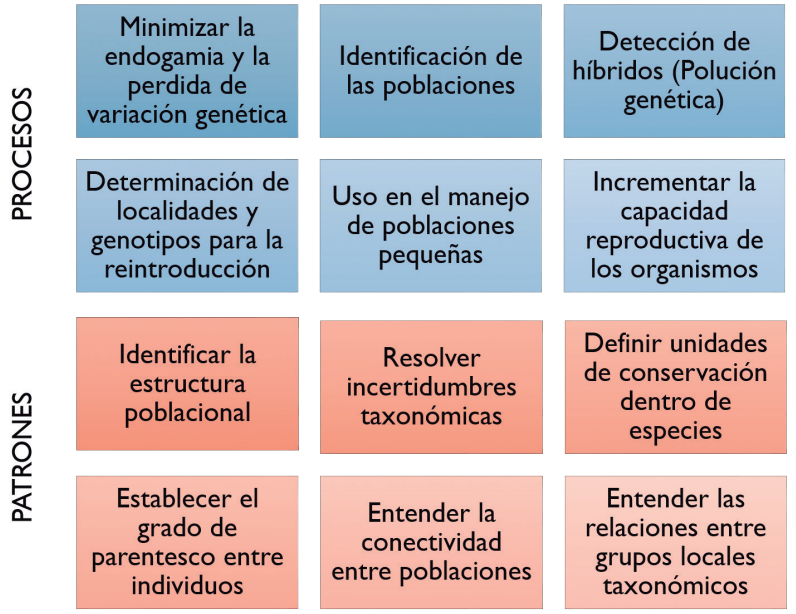


Figura 1.1 Roles de la conservación genética en plantas. Adaptado de DeSalle y Amato (2004) y Sosa *et al.* (2011)

1.1. Genética de poblaciones: variabilidad y estructura genética.

La genética de poblaciones estudia el origen, cantidad y distribución de la variación genética presentes en las poblaciones de los organismos y el destino de esta variación a través del tiempo y el espacio. Estos cambios en la variación genética constituyen la base de la microevolución (evolución dentro de especies) y a su vez tendrán un impacto en el origen de nuevas especies,

y por tanto, en la macroevolución (Templeton 2006). Una población puede ser definida como un grupo de individuos que se reproducen y coexisten en el tiempo y el espacio (Hedrick 2011). Aunque a veces se asume que una población está bien definida geográficamente, no ocurre en la mayoría de los casos, ya que hay múltiples factores que influyen en su estructura geográfica.

Los patrones de variación genética en las poblaciones naturales se pueden abordar desde tres puntos de vista. En primer lugar, determinando la cantidad de variabilidad genética (diversidad genética), en segundo lugar, estableciendo la distribución de ésta variabilidad a lo largo de las poblaciones de una especie (estructuración genética), y finalmente conociendo el grado de similitud entre individuos y/o poblaciones (distancia o diferenciación genética) (Lowe *et al.* 2004).

Los métodos para cuantificar la diversidad genética en las poblaciones se basan en las variaciones alélicas entre individuos para unos genes determinados. Para el caso de los marcadores moleculares neutrales, estas variaciones alélicas se verán influenciadas por la acción conjunta de la mutación, la migración y la deriva genética, que a su vez definirán la estructura geográfica de las poblaciones (Avise 1994; Templeton 2006). Mientras que las tasas de mutación determinan la velocidad a la que se añaden nuevas variantes a los genes, eventos ambientales estocásticos que afectan a las poblaciones finitas, pueden provocan la eliminación de gametos al azar (deriva genética[5]), y por lo tanto reducir la diversidad genética. La deriva genética se puede ver compensada con la migración de individuos de otras poblaciones, que pueden aportar alelos nuevos o aumentar las frecuencias alélicas existentes. Estos conceptos serán importantes a la hora de establecer el manejo de poblaciones amenazadas, aumentando el flujo génico natural o restringiendo el flujo artificial, y sobrellevar así eventos estocásticos que pueden dar lugar a deriva genética y endogamia (Frankham *et al.* 2002).

Una de las medidas más utilizadas para cuantificar la diversidad genética es la heterocigosidad esperada[6], también llamada "diversidad génica" (Nei 1987), que tiene significancia biológica para organismos tanto haploides como diploides con diferentes sistemas reproductivos (Hedrick 2011). En el caso de especies poliploides se pueden estimar valores de heterocigosidad si se combinan las frecuencias alélicas de los subgenomas existentes, asumiendo que éstos presentan herencia polisómica (Meirmans & Van Tienderen 2013). Otros estadísticos de utilidad, como la heterocigosidad observada[7], el número de alelos por población, el número medio de alelos por locus, la proporción de

alelos privados[8] o el porcentaje de loci polimórficos también son ampliamente utilizados. Cada uno de ellos ofrece una visión diferente y complementaria de los niveles de diversidad genética. Algunos de estos estadísticos se ven altamente influenciados por diferencias en el tamaño muestral, por lo que la rarefacción de acuerdo al tamaño muestral para el cálculo de la riqueza alélica[9] y la riqueza de alelos privados permite una comparación más equitativa entre poblaciones (Petit *et al.* 1998; Hedrick 2011).

De acuerdo con Templeton (2006), la estructura poblacional está determinada por el sistema de cruzamiento de la especie, el tamaño de la población, el grado de intercambio genético con otras poblaciones y el patrón de edades de los individuos dentro de la población. En los organismos vegetales, el sistema de cruzamiento cobra especial importancia, debido a las múltiples formas de reproducción que éstos presentan (alogamia, autogamia o reproducción asexual). Normalmente, al menos dos de ellas están presentes en las poblaciones dando lugar a sistemas mixtos (Richards 1996). Al contrario del cruzamiento aleatorio, en la que todos los individuos tienen las mismas posibilidades de entrecruzarse (panmixia), en el apareamiento selectivo positivo, los individuos tendrán más posibilidades de entrecruzarse con aquellos con los que comparten fenotipo. El caso más extremo de apareamiento selectivo positivo sería la autofertilización o autocompatibilidad completa, que da lugar a una disminución de la heterocigosidad respecto a la panmixia (Lowe *et al.* 2004). Aunque un estudio exhaustivo de la biología reproductiva de las especies no es objetivo de este trabajo, su conocimiento previo es fundamental para interpretar los factores que influyen en la estructura geográfica y la variabilidad genética. De hecho, se ha determinado que los sistemas de cruzamiento y los sistemas de dispersión de polen y/o semillas influyen significativamente sobre la diversidad genética y en la estructuración genética a fina escala (Hamrick & Godt 1996; Nybom 2004; Vekemans & Hardy 2004). En este sentido, la ley de Baker (1955) postula que las especies de plantas con autocompatibilidad reproductiva tienen más éxito a la hora de establecerse en el nuevo territorio, debido a la facilidad para reproducirse en la ausencia de compañeros (Crawford *et al.* 2009). Una forma de medir estos procesos es a través de los estadísticos *F*.

Los estadísticos *F* fueron desarrollados por Wright (1951), y posteriormente ampliados por Weir y Cockerham (1984), y aunque clásicos, siguen siendo ampliamente utilizados en el estudio de la genética de poblaciones para el tratamiento jerárquico de la variabilidad genética (entre individuos dentro una

población, entre poblaciones y entre regiones o metapoblaciones). El estadístico F_{IS} mide la correlación entre los alelos de un individuo respecto a la subpoblación en la que se encuentra. Además, el estadístico F_{IS} es equivalente a la desviación de frecuencias genotípicas respecto al equilibrio de Hardy-Weinberg dentro de la población, indicando un defecto de heterocigotos cuando es positivo, y un exceso de heterocigotos cuando es negativo (Holsinger & Weir 2009; Hedrick 2011). El estadístico F_{ST} (entre 0 y 1) mide la variación en frecuencias alélicas entre pares de poblaciones, y por consecuencia, al grado de semejanza entre individuos dentro de poblaciones (Templeton 2006; Holsinger & Weir 2009). En consecuencia, el estadístico F_{ST} es un coeficiente de diferenciación genética entre poblaciones, y por tanto, una medida de distancia genética. En los modelos de aislamiento por distancia, en los que se estima que el grado de diferenciación poblacional aumenta a medida que aumenta la distancia, las estimaciones a pares F_{ST} son más convenientes (Templeton 2006). Sin embargo, los índices de distancia genética basados en la identidad alélica, como el F_{ST}, asumen bajos niveles de mutación genética. De esta manera, la diferenciación poblacional dirigida por altos niveles de mutación no puede ser detectada con estos estadísticos (Hardy *et al.* 2003; Templeton 2006). En los estudios mediante microsatélites, si los niveles de mutación explican la variabilidad genética en mayor medida que la deriva y el flujo génico, el uso de estadísticos que tengan en cuenta los procesos de mutación pueden ser más adecuados. El estadístico R_{ST} (Slatkin 1995) es uno de los índices más utilizados en el análisis con microsatélites, si se asumen modelos de mutación paso a paso (SMM; "stepwise mutation model"). Finalmente, el análisis molecular de la varianza (AMOVA) desarrollado por Excoffier *et al.* (1992), es un método comúnmente utilizado para la estimación de los estadísticos *F* en todos los niveles de jerarquía.

Desde la isoenzimas hasta los más recientes SNPs ("single nucleotide polymoprhisms"), los marcadores moleculares han sido de gran utilidad en el estudio de la genética de poblaciones, y en consecuencia en la conservación de especies (Allendorf 2017). En concreto, los microsatélites (SSR o STR; "simple sequence repeats" o "short tandem repeats") han sido uno de los marcadores más populares para estimar la diversidad genética de los organismos (Hodel *et al.* 2016). Son secuencias cortas de 1 a 6 pares de bases que se repiten hasta un máximo de 60 veces, y están distribuidos a lo largo del genoma de células eucariotas en regiones codificantes y no codificantes (Goldstein & Pollock 1997; Zane *et al.* 2002). La alta tasa de mutación de los microsatélites los convierte en marcadores hipervariables, lo cual supone una ventaja,

debido a que no existen límites para detectar la variabilidad genética de ningún organismo, aunque esta variación sea intrínsecamente baja (Allendorf 2017). Han sido ampliamente utilizados para detectar cuellos de botella[10], la identificación de individuos, la estimación de la estructura poblacional a escala espacial o la historia demográfica de las poblaciones (Sosa *et al.* 2010; Saro 2015). Sin embargo, su mayor desventaja es que necesitan ser desarrollados de novo para cada especie, a no ser que exista transferibilidad de microsatélites[11] caracterizados para especies cercanas (Kalia *et al.* 2011). Aunque los SNPs y la genómica de poblaciones amplían el rango de opciones, como la capacidad de detectar genes bajo selección o el grado de hibridación, los microsatélites siguen siendo una opción viable cuando la inclusión de un gran número de individuos es una prioridad (Hodel *et al.* 2016).

La poliploidía es una característica típica de los genomas vegetales, y ha propiciado el éxito evolutivo de las angiospermas, con múltiples eventos de duplicación de genes o genomas completos a lo largo de su historia evolutiva (Jiao *et al.* 2011). A pesar de la importancia de la poliploidía en la evolución, nuestro estado del conocimiento sobre los organismos poliploides es aún menor que el de los diploides. De hecho, el estudio genético a nivel poblacional de especies poliploides es un reto, ya que se dificulta la estimación de las frecuencias alélicas y hay una dependencia sobre el conocimiento de los distintos patrones de herencia (Dufresne *et al.* 2014). La poliploidía resulta de la fusión de dos gametos de la misma especie (autopoliploide; duplicación del mismo genoma) o de dos especies diferentes (alopoliploide; hibridación interespecífica). Aunque existe un continuo entre las estrictas autopoliploidía y alopoliploidía, normalmente los autopoliploides presentan herencia polisómica[12] y los alopoliploides, herencia disómica[13] (Dufresne *et al.* 2014). Por otro lado, muchas especies presentan alopoliploidía parcial, y una mínima recombinación entre genomas basta para igualar las frecuencias alélicas, y analizar la base de datos de una especie aparentemente alopoliploide como autopoliploide (Meirmans & Van Tienderen 2013). El hecho de distinguir entre los dos tipos de herencia es crucial para determinar la dosificación alélica, es decir, el número de veces que repite cada alelo por locus en un mismo individuo. Incluso siendo capaces de detectar el tipo de herencia, la dosificación alélica muchas veces es imposible de descifrar, especialmente en genes con un gran número de alelos, dificultando un genotipado[14] exacto y el correspondiente cálculo de las frecuencias alélicas (Trapnell *et al.* 2011). A pesar de estas dificultades, la investigación sobre el análisis de datos de

especies poliploides va en un aumento y en la actualidad existen numerosos estadísticos y programas infórmáticos específicos para analizar su variabilidad genética (Hardy & Vekemans 2002; Bruvo *et al.* 2004; Markwith *et al.* 2006; Clark & Jasieniuk 2011).

En un reciente estudio de conteo cromosómico en especies canarias, se detectó que un 33.7 % de las plantas vasculares son poliploides (Suda *et al.* 2003). En general, las especies poliploides son más vigorosas y presentan mayores niveles de diversidad genética que sus relativos diploides debido a la redundancia de genes (Abbott *et al.* 2007; García-Verdugo *et al.* 2013). Esta redundancia de genes provoca que los poliploides puedan sufrir en menor medida las consecuencias de la depresión endogámica (Frankham *et al.* 2002). Sin embargo, la auto-fertilización y la reproducción asexual son comunes entre los poliploides (Comai 2005), lo cual a su vez puede reducir la diversidad genotípica (García-Verdugo *et al.* 2013), pero también puede ser una ventaja en ausencia de polinizadores, especialmente en ecosistemas insulares (Busch & Delph 2012).

1.2. Patrones de variabilidad genética de la flora vascular en islas oceánicas

Los ecosistemas insulares son una importante fuente de biodiversidad en La Tierra. De hecho, las plantas endémicas insulares representan el 25% de las especies de plantas vasculares descritas (Kreft *et al.* 2008). Pero las características únicas de los organismos insulares, debido al aislamiento y tamaños poblacionales pequeños, las hacen vulnerables a las perturbaciones de origen antrópico (Whittaker & Fernández-Palacios 2007). Se estima que del 5 a 10% de estas especies endémicas pueden estar altamente amenazadas y que de un 3 a un 4% podrían estar en peligro crítico de extinción (Caujapé-Castells *et al.* 2010).

Por lo tanto, no es de extrañar que las islas hayan sido y sigan siendo "laboratorios naturales" para el estudio de la biogeografía, ecología, sistemática y evolución. Desde el desarrollo de la Teoría de la Biogeografía Insular de MacArthur y Wilson (1967) y la Teoría de la Evolución por Selección Natural de Darwin y Wallace no se han cesado de hacer aportaciones a los estudios sobre la evolución en islas y la extrapolación de este conocimiento a los hábitats continentales fragmentados (Simberloff & Abele 1982; Laurance 2008). Aunque

otros archipiélagos, como el de las Islas Galápagos o Hawái, han despertado la atención de los biólogos a lo largo de la historia, el archipiélago canario ha sido foco de expediciones históricas y numerosas investigaciones durante las últimas décadas. De hecho, en la actualidad, éste es uno de los mejores estudiados del mundo (Whittaker & Fernández-Palacios 2007). Además, el conocimiento sobre la caracterización de la flora en todas las islas es excepcional (Emerson & Kolm 2005). Gracias a los avances en biología molecular, los estudios sobre la diversidad genética y filogeografía de las plantas insulares, y en concreto del archipiélago canario, se acumulan de manera exponencial (Rumeu *et al.* 2014; Saro *et al.* 2015; Sosa *et al.* 2014; Sun *et al.* 2016).

El archipiélago canario posee una historia geológica y biogeográfica compleja que forma un sistema de islas heterogéneo y con multitud de hábitats. La cercanía al continente y la peculiar ontogenia de las islas han propiciado la colonización y diversificación de sus organismos y de su biodiversidad, resultando en una extensa y variada especiación. Por ello, el archipiélago canario está considerado como uno de los componentes clave de la cuenca mediterránea, un "hot-spot" de biodiversidad a nivel global (Medail & Quezel 1997). En total, en Canarias se han descrito más de 12.000 especies nativas, de las cuáles el 30 % de éstas, son especies endémicas del archipiélago (Fernández-Palacios & Whittaker 2008).

En cuanto a la flora vascular terrestre en Canarias, existen alrededor de 1.300 especies de plantas, 44 % de las cuales son endémicas, y 22 géneros endémicos (Whittaker & Fernández-Palacios 2007; Reyes-Betancort *et al.* 2008). Además, el archipiélago canario, siendo tan sólo el 1.5 % del territorio nacional, alberga más del 50% de las especies vegetales endémicas de España. Muchas de estas especies están restringidas a una sola isla, encontrándose en áreas reducidas y aisladas incluso dentro de la misma isla. Además, aproximadamente el 26 % de las plantas vasculares canarias se encuentra con algún grado de amenaza, siendo la región con la mayor densidad de especies amenazadas por área de todo el territorio español (Bañares *et al.* 2003; Moreno-Saiz *et al.* 2015). Se estima que las principales causas de amenaza sobre la flora canaria son: el sobrepastoreo por herbívoros introducidos; la hibridación interespecífica; la competición con especies invasoras y la reducción del hábitat debido al desarrollo urbano y agrícola (Caujapé-Castells *et al.* 2010).

En los estudios de conservación genética, se ha constatado que las especies raras generalm.0ente presentan menor variabilidad genética que las especies

de amplia distribución (Gitzendanner & Soltis 2000; Cole 2003). Esta idea también se ha aplicado a las especies insulares respecto de sus congéneres en el continente, haciéndolas más vulnerables a la extinción (Frankham 1997). Estas asunciones se deben principalmente a los mayores tamaños de población efectiva de las especies de amplia distribución, lo que posibilita el mantenimiento de la variabilidad genética (Charlesworth 2009). Los efectos de la reducción de los tamaños poblaciones sobre la diversidad genética han sido ampliamente discutidos, dado que conllevan a la deriva genética y endogamia de las poblaciones (Barrett & Kohn 1991; Ellstrand & Elam 1993). Muchas de las plantas insulares endémicas presentan una distribución restringida debido a la propia ontogenia de las islas, que mediante la adaptación local y el aislamiento geográfico ha dado lugar a la radiación adaptativa[15] de muchos grupos filogenéticos (Kim *et al.* 1999; Jorgensen & Olesen 2001; Vitales *et al.* 2014b). Pero también existen especies de amplia distribución, que aunque aportan menor riqueza específica a las islas, albergan una importante variabilidad genética en sus poblaciones (Saro *et al.* 2015; Mairal *et al.* 2015).

Por otro lado, en un estudio comparativo de especies analizadas con marcadores microsatélites, se pudo apreciar que muchas especies canarias presentaban menores valores de diversidad genética que sus congéneres continentales, mientras otras mantenían valores moderadamente altos (Sosa *et al.* 2011). De hecho, a medida que aumentan los estudios con marcadores moleculares, se revela que hay considerables excepciones a la regla, y que hay muchas especies insulares, restringidas o de amplia distribución, con altos niveles de variabilidad genética (García-Verdugo *et al.* 2015; Meloni *et al.* 2015; Silva *et al.* 2016). Además, en estudios comparativos entre islas oceánicas, se ha detectado que la flora canaria presenta niveles sorprendentemente altos de variabilidad genética en comparación con otros archipiélagos (Francisco-Ortega *et al.* 2000). La mayor cercanía al continente (< 100 km) es una de las explicaciones más aludidas, ya que puede haber permitido múltiples eventos de colonización, con propágulos provenientes de diferentes áreas geográficas. La hibridación entre estos múltiples colonizadores puede ser el origen de esta alta diversidad (Francisco-Ortega *et al.* 2000; Caujapé-Castells *et al.* 2017).

Además, en la revisión sobre los niveles de diversidad genética de plantas vasculares en Canarias (Pérez de Paz & Caujapé-Castells 2013), se detectó que el tamaño poblacional no estaba positivamente correlacionado con la diversidad genética. Sin embargo, sí que se determinó que los factores bióticos

como la poliploidía, la autoincompatibilidad reproductiva, o la dispersión a larga distancia influyen positivamente sobre la variabilidad genética. En concreto, se detectó que los taxones auto-compatibles presentan menor variación genética y mayores niveles de aislamiento que aquellos total o parcialmente auto-incompatibles (Pérez de Paz & Caujapé-Castells 2013). Pero al contrario que en otros archipiélagos oceánicos más aislados del continente, el archipiélago canario presenta una gran proporción de especies de plantas con auto-incompatibilidad reproductiva, posiblemente favorecido por la ventaja de los múltiples eventos fundadores ya descritos (Crawford *et al.* 2009).

Cuadro 1 Glosario

[1]**Flujo genético**: Transferencia de alelos de genes de una población a otra. En las plantas vasculares, esta transferencia se produce generalmente mediante la dispersión del polen, las semillas u otros propágulos.

[2]**Endogamia**: producción de descendencia entre individuos genéticamente emparetados. La endogamia puede reducir la capacidad reproductiva y de supervivencia de la descendencia, y en general, reducir la eficacia biológica de la especie.

[3]**Reintroducción**: incorporación de individuos o poblaciones en áreas naturales o en proceso de restauración ecológica. En un sentido estricto, implica que la especie en cuestión habitase las áreas a reintroducir en el pasado, habiéndose extinguido localmente.

[4]**Marcadores moleculares**: segmentos de ADN con una ubicación física identificable dentro del genoma, y cuya herencia se puede rastrear, para la identificación de procesos biológicos.

[5]**Deriva genética**: es uno de los mecanismos básicos de la evolución, junto a la selección natural, la mutación y la migración. Implica la pérdida aleatoria de alelos entre generaciones, dando lugar a cambios en las frecuencias alélicas.

[6]**Heterocigosidad esperada**: fracción de individuos heterocigóticos para cualquier locus tomado al azar. Constituye una medida del nivel de diversidad genética, la cual también se denomina diversidad génica. Es menos sensible al tamaño muestral que la heterocigosidad observada y es el tipo de heterocigosidad que se tiene en cuenta en el equilibrio de Hardy-Weinberg.

[7]**Heterocigosidad observada**: número de individuos heterocigóticos para cada locus dividido por el total de individuos muestreados.

[8]**Alelos privados**: alelos (distintas formas de un gen), que sólo están presentes de manera exclusiva en un grupo, población, región geográfica o especie.

[9]**Riqueza alélica**: número medio de alelos por locus. Debido a que se ve influenciada por el tamaño muestral, normalmente se calcula con rarefacción.

[10]**Cuello de botella**: reducción drástica y temporal del tamaño poblacional, con la consecuente pérdida de diversidad genética.

[11]**Transferibilidad de microsatélites**: amplificación de marcadores microsatélites con los mismos cebadores en especies cercanas a aquella para la que se desarrollaron los marcadores en primer lugar.

[12]**Herencia polisómica**: modo de herencia típico de especie autopoliploides, en el que las variantes del mismo cromosoma se pueden recombinar durante la meiosis.

[13]**Herencia disómica**: modo de herencia típico de especies alopoliploides, en el que la recombinación durante la meiosis ocurre preferentemente entre los cromosomas procedentes de cada ancestro. Por lo tanto, no se espera que haya recombinación entre los distintos subgenomas.

[14]**Genotipado**: conjunto de procesos encaminados al análisis de las variaciones genéticas individuales de un organismo, de forma que pueda ser identificado y diferenciado.

[15]**Radiación adaptativa**: proceso que describe la rápida evolución de una o varias especies para ocupar diferentes nichos ecológicos.

Foto: Claudio Moreno

RESUMEN GLOBAL

2. RESUMEN GLOBAL

2.1. OBJETIVOS

El objetivo principal ha sido determinar, mediante el uso de marcadores moleculares, el estado de conservación genética de las poblaciones (naturales y restauradas) de los siguientes endemismos vegetales canarios amenazados: tres especies del género *Bethencourtia* (*B. hermosae, B. rupicola* y *B. palmensis*); *Sambucus palmensis* y *Viola cheiranthifolia*.

2.1.2. Objetivos específicos

- Desarrollar y caracterizar marcadores hipervariables y polimórficos (microsatélites) en las tres especies del género endémico *Bethencourtia*, *Sambucus palmensis* y *Viola cheiranthifolia* con el fin de determinar la diversidad genética de sus poblaciones.

- Analizar la variación genética dentro y entre las poblaciones (naturales y restauradas), así como la estructura geográfica, para todas las especies objeto de estudio con el fin de evaluar su estado de conservación genética.

- Estimar y analizar mediante técnicas moleculares el grado de autogamia de *Bethencourtia* y *V. cheiranthifolia* y los efectos del sistema de cruzamiento sobre su diversidad genética.

- Resolver las incertidumbres taxonómicas dentro del género *Bethencourtia*, así como analizar las relaciones genéticas entre las tres especies descritas en Canarias: *B. palmensis*, *B. hermosae* y *B. rupicola*.

- Identificar el número de individuos clónicos mediante el genotipado individual en las localidades naturales y restauradas de *S. palmensis* en el Parque Nacional de Garajonay.

- Estimar los efectos de la restauración de *S. palmensis* sobre la diversidad y estructura genética de las poblaciones, como base para el asesoramiento sobre futuros programas de conservación genética.

- Inferir la historia demográfica reciente de las poblaciones de *V. cheiranthifolia*.

- Predecir los cambios futuros en la distribución y diversidad genética en las especies de alta montaña en islas oceánicas debido al cambio climático, mediante la modelización de nichos climáticos y usando como modelo a *V. cheiranthifolia* en el Parque Nacional del Teide.

- Proponer acciones de conservación a los gestores de los Parques Nacionales de Garajonay y El Teide a corto y largo plazo, con el fin de reducir las amenazas que se ciernen sobre las especies de estudio y mantener o mejorar su diversidad genética.

2.2. ESPECIES DE ESTUDIO

El género *Bethencourtia* Choisy ex Link (Asteraceae) es endémico de las Islas Canarias y comprende tres especies: *B. rupicola*, *B. hermosae* (La Gomera) y *B. palmensis* (La Palma y Tenerife). *Bethencourtia rupicola* y *B. hermosae* son raros endemismos, ambos conocidos como turgaite gomero, y anteriormente considerados como *Senecio hermosae*, disponen de una distribución restringida en varios de los pitones fonolíticos del centro y norte de La Gomera. Sus ejemplares están sometidos a cierta presión antrópica (proximidad de cultivos, senderos y vías de escalada), desconociéndose aspectos clave de su dinámica poblacional, reproductiva o genética (Cuadro 2.1).

No fue hasta la descripción morfológica realizada por Nordenstam (2006a), que se diferenció a *B. rupicola* como una especie independiente de *B. hermosae*. Así tendríamos *B. hermosae* para las poblaciones de Vallehermoso y *B. rupicola* para la población de Los Roques (Nordenstam, 2006a), dentro del Parque Nacional de Garajonay y del Monumento Natural de Los Roques (Fernández-López & Velázquez Barrera, 2011). Después de Nordenstam (2006a; b), la clasificación taxonómica sería la siguiente:

- *Bethencourtia hermosae* (Pit.) Kunkel (= *Senecio hermosae* Pit. = *Canariothamnus hermosae* (Pit.) B. Nord.)
- *Bethencourtia rupicola* (B. Nord.) B. Nord. = *Canariothamnus rupicola* B. Nord.

- *Bethencourtia palmensis* (Nees) Choisy (= *Senecio palmensis* Buch = *Cineraria palmensis* (Buch) Nees = *Canariothamnus palmensis* (Buch) B. Nord.)

Sin embargo, esta clasificación no se tiene en cuenta en la listas o catálogos de especies amenazadas, lo que dificulta la protección y seguimiento de las poblaciones.

Cuadro 2.1 Características principales del género *Bethencourtia*


Bethencourtia hermosae


Bethencourtia palmensis

Nombres comunes: turgaite gomero (*B. rupicola* y *B. hermosae*); turgaite (*B. palmensis*)

Distribución: endemismos canarios

- *B. rupicola* y *B. hermosae*: La Gomera
- *B. palmensis*: La Palma y Tenerife

Características destacables:

- Especies diploides
- Hermafroditas con posible autoincompatibilidad reproductiva
- Polinizadores desconocidos
- Dispersión anemócora

Estado de conservación:

- ***B. hermosae***:
  - UICN: vulnerable
  - Lista Roja nacional: vulnerable
  - Catálogo nacional: en régimen de protección especial
  - Catálogo regional: especie de "intéres para los ecosistemas canarios"
- ***B. rupicola***: catalogada junto a *B. hermosae*
- ***B. palmensis***: sin catalogar

Sambucus palmensis Link. (*Sambucus nigra* ssp. *palmensis* (Link) R. Bolli); Sambucaceae. El saúco es un endemismo canario citado inicialmente para los Sauces, en la isla de La Palma, y que con posterioridad se añadieron otras localidades en la misma isla, además de Tenerife, Gran Canaria y La Gomera (Marrero *et al.* 2015). A pesar de su distribución en varias islas, el saúco es un rarísimo endemismo cuyos efectivos naturales censados no alcanzan más de 70 de individuos (Sosa *et al.* 2010; Marrero *et al.* 2015). Constituye un elemento muy singular de las manifestaciones mejor conservadas del monteverde de fondos de barranco y ocasionalmente como rupícola de paredones rezumantes en el ámbito forestal. Su representación esporádica y escasez de efectivos poblacionales le confiere una extraordinaria fragilidad; posibles fenómenos de autoincompatibilidad reproductiva y endogamia pudieran constituir las causas de su bajísima capacidad de renovación y esporádica mortalidad de su descendencia (Cuadro 2.2).

Cuadro 2.2 Características principales de *Sambucus palmensis*

Nombre común: sabuco, sayugo, saugo, saúco, sauco

Distribución: endemismo canario (Gran Canaria, Tenerife, La Palma y La Gomera)

Características destacables:

- Diploide
- Hermafrodita con posible autoincompatibilidad reproductiva
- Polinización entomófila generalista
- Dispersión ornitócora

Estado de conservación:

- UICN: en peligro
- Catálogo nacional: en peligro de extinción
- Lista Roja nacional: en peligro crítico
- Catálogo regional: vulnerable

Viola cheiranthifolia Humb. et Bonpl., Violaceae. La violeta del Teide es un endemismo exclusivo de Tenerife que se limita a los dominios del Parque Nacional del Teide, ya que sólo crece de forma silvestre por encima de los 2.100 metros de altitud (es la planta que florece a mayor altura en España) y bordes meridionales del mismo: Teide, Pico Viejo, y Montaña Blanca, cumbres del Circo de Las Cañadas, etc. A pesar de que ya, en 1724, el astrónomo y naturalista Louis Feuillée hace referencia a esta planta, fue en 1808 cuando Alexander von Humboldt y Aimé Bonpland hacen su primera descripción botánica en su *Plantae aequinoctiales*.

Ecológicamente participa en comunidades de la alianza *Spartocytison nubigenii* y forma comunidad propia en sustratos de pómez junto con *Silene nocteolens*. Más rara en comunidades rupícolas. Especie bien adaptada a las pedreras, a veces móviles, de pómez, con ramificación abundante y hojas pequeñas, de bordes solo ligeramente lobulados. La presión turística del Parque Nacional del Teide, junto a los efectos de la herbivoría por conejos y muflones y el cambio climático se encuentran entre las principales amenazas para esta especie alpina (Cuadro 2.3).

Cuadro 2.3 Características principales de *Viola cheiranthifolia*

Nombre común: violeta o pensamiento del Teide

Distribución: endemismo de Tenerife

Características destacables:

- Poliploide
- Hermafrodita con autocompatibilidad reproductiva
- Polinización entomófila, principalmente por himenópteros
- Dipersión balística con posible mirmecocoria

Estado de conservación:

- Lista Roja nacional: vulnerable

2.3. MATERIAL Y MÉTODOS

Cada uno de los trabajos que componen esta Tesis Doctoral consta de una metodología concreta. La recogida de muestras, el desarrollo y caracterización de los microsatélites ha sido similar para todos los trabajos, excepto ligeras diferencias, mientras que algunos de los análisis estadísticos variaron para cada especie dependiendo de las necesidades de cada una, los objetivos planteados, y de las circunstancias específicas de la especie (ej. poliploidía). Por lo tanto, el resumen de la metodología se puede dividir en: (i) recogida de muestras, (ii) desarrollo y caracterización de microsatélites (iii) análisis de datos por grupo o especie.

2.3.1. Recogida de muestras

La recogida de muestras para todas las especies se realizó entre los años 2012 y 2014. Se intentó recolectar hojas jóvenes de cada individuo, con su posterior conservación en bolsas plásticas con gel de sílice. La georreferenciación de todos los individuos fue realizada mientras las condiciones lo permitiesen.

Para *Viola cheiranthifolia*, el muestreo tuvo lugar en las inmediaciones del Parque Nacional del Teide entre las estaciones de primavera y verano en 2013 y 2014. Se recolectaron 266 muestras de 9 localidades para el posterior genotipado (Figura 5.1, pág. 91).

Por otro lado, en el Parque Nacional de Garajonay se procedió a las recolecciones de muestras foliares de *Bethencourtia hermosa*e, *B. rupicola* y *Sambucus palmensis*. Debido a las particularidades del hábitat escarpado donde habita *Bethencourtia*, surgió la necesidad de contratar un escalador profesional para la recolección de las muestras. Con el fin de completar el muestreo de las especies del género *Bethencourtia*, se añadieron 3 poblaciones de *B. palmensis* (2 en La Palma y 1 en Tenerife). En total, se recolectaron 276 muestras de 11 localidades de *Bethencourtia* para su genotipado (Tabla 3.1. pág. 41). En este caso, por la verticalidad del terreno, la georreferenciación fue imposible, por lo que se tomaron coordenadas de referencia, y mediante un croquis se extrapolaron las coordenadas gráficas de todos los individuos. En cuanto a *S. palmensis*, se procedió a la recolección de los 402 individuos. Todos los individuos de origen natural conocidos y una importante representación de aquellos translocados fueron incluidos en el muestreo. La distribución de los individuos en 15 áreas de estudio se decidió conjuntamente con el personal de

Parque Nacional de Garajonay, de acuerdo a la localización de los individuos y facilidades de manejo (Figura 4.1, pág. 66).

2.3.2. Desarrollo de microsatélites y genotipado

Los marcadores microsatélites empleados en los trabajos de esta tesis fueron generalmente desarrollados de novo y específicamente para cada grupo o especie. Solo en el caso de *S. palmensis*, se emplearon algunos marcadores desarrollados para la especie cercana *S. nigra*, y que ya habían sido testados en un trabajo anterior. En cada capítulo se detalla la metodología empleada, por lo que a continuación se describe un resumen general del desarrollo y caracterización de los microsatélites, así como el genotipado de los individuos.

El desarrollo de las regiones hipervariables con motivos microsatélites se encargó a dos empresas especializadas en el sector. En los casos de *Bethencourtia* y *V. cheiranthifolia*, con la colaboración del laboratorio Savannah River Ecology (Universidad de Georgia, E.E.U.U.), se procedió a la detección de estos marcadores moleculares y al diseño de los cebadores específicos para cada marcador. Por otro lado, los nuevos marcadores específicos para *S. palmensis* fueron desarrollados por la empresa AllGenetics (Universidade de A Coruña).

Después de recibir todas las secuencias de microsatélites para cada especie en concreto, se eligió inicialmente un número determinado de marcadores para su caracterización. La selección de los microsatélites se basó básicamente en la idoneidad de los cebadores diseñados para la amplificación de los fragmentos. También se consideró que el tamaño de los productos de amplificación (en pares de bases; (pb)) fuera significativamente distinto, con el fin de poder analizar conjuntamente varios microsatélites en el secuenciador. Los microsatélites preseleccionados se ensayaron en las condiciones de amplificación mediante la reacción en cadena de la polimerasa (PCR) que se detallan en cada capítulo específico. La observación de los fragmentos resultantes de la PCR se observó en geles de agarosa. Aquellos microsatélites que no amplificaron correctamente fueron sometidos a condiciones de PCR menos restrictivas, que implicaban la disminución de la temperatura de hibridación o el aumento de la concentración de $MgCl_2$. De los cebadores que ofrecieron una mejor amplificación, se volvieron a sintetizar los cebadores "reverse", pero esta vez con la adición de un fluorocromo, (6-FAM, VIC, PET, NED, ROX, TAMRA) con el fin de que el producto de amplificación fuera detectable en un secuenciador capilar

(ABI 3770). La interpretación de los resultados obtenidos del secuenciador capilar se realizó con el programa GENEMAPPER 4.0 (Applied Biosystems), el cual permitió determinar el tamaño en pares de bases (pb) de los fragmentos obtenidos en la amplificación de los microsatélites. Después esta segunda selección, se descartaron aquellos marcadores que no ofrecían un suficiente grado de polimorfimo para los análisis posteriores.

Una vez realizado el filtrado final de los marcadores microsatélites a amplificar para cada especie, se procedió a la amplificación de todas las muestras individuales. Los protocolos de extracción y posterior PCR se detallan para cada caso particular en los capítulos correspondientes. Los fragmentos resultantes fueron interpretados nuevamente con el programa GENEMAPPER 4.0, a través del cual se obtuvieron bases de datos con los tamaños (pb) para cada locus y muestra.

En el caso del género *Bethencourtia* se desarrollaron los microsatélites con muestras de *B. hermosae* y se testó la transferibilidad de los cebadores para *B. rupicola* y *B. palmensis*. De los 38 pares de cebadores inicialmente escogidos, 10 de ellos amplificaron correctamente en las tres especies y con suficiente polimorfismo para el total de muestras (Tabla 3.2, pág. 43).

Para *S. palmensis* se utilizaron 3 de los marcadores implementados para *S. nigra* (Clarke & Tobutt 2006) y 4 de nuevo desarrollo, específicos para la especie. Para la caracterización de los nuevos marcadores se sintetizaron inicialmente 20 parejas de cebadores, de las cuales, 4 amplificaron consistentemente con suficiente grado de polimorfismo. En total se emplearon 7 marcadores microsatélites para el estudio de *S. palmensis* en La Gomera (Tabla 4.1, pág. 67).

Por último, para *V. cheiranthifolia* se sintetizaron inicialmente 67 pares de cebadores, de los cuales 16 amplificaron correctamente y con un marcado polimorfismo. Estos 16 marcadores se describen en la publicación sobre la caracterización de los microsatélites para *V. cheiranthifolia* y su transferibilidad en la especie cercana *V. palmensis* (Rodríguez-Rodríguez *et al.* 2015). Sin embargo, después de esta publicación, y realizar el genotipado del total de muestras se descartaron dos marcadores debido a las dificultades de interpretación de los resultados en algunas localidades. Por lo tanto, en el estudio genético de *V. cheiranthifolia* se han incluido 14 marcadores microsatélites (Tabla 5.1, pág. 92).

2.3.3. Análisis de datos

A continuación, se resumen los principales análisis estadísticos empleados en el estudio para cada especie en concreto. En la Tabla 2.1 se indican los principales programas informáticos empleados en el estudio de la genética de poblaciones en esta tesis, y los análisis que se han implementado en cada uno de ellos. Algunos de estos programas informáticos se han usado repetidamente a lo largo de los capítulos, mientras que otros cubrieron necesidades más específicas, como, por ejemplo, el análisis de datos poliploides.

El género *Bethencourtia*

Para la estimación de la variabilidad entre especies y dentro de especies, se calcularon los siguientes índices de diversidad genética: número de alelos, número de alelos privados, riqueza alélica, riqueza de alelos privados, heterocigosidad observada y esperada, así como los coeficientes de endogamia y la significancia de la desviación sobre el equilibrio de Hardy-Weinberg para todas las poblaciones. Además, para valorar la eficacia de los cebadores y marcadores microsatélites empleados, se detectó la presencia de alelos nulos por locus y por población y se estimó el desequilibrio de ligamiento entre pares de poblaciones y loci. Con el fin de identificar eventos de cuellos de botella en las poblaciones, se emplearon test específicos para ello.

Por otro lado, para inferir el sistema de cruzamiento en *Bethencourtia*, se calculó el nivel de autogamia para cada especie. Por último, para estimar las diferencias genéticas entre las especies y la estructura genética a nivel poblacional se desarrollaron los siguientes análisis estadísticos: AMOVA con la agrupación entre especies y entre poblaciones dentro de especie, una matriz de F_{ST} entre todos los pares de poblaciones, PCoA con la distancia genética entre individuos, UPGMA con la distancia genética entre poblaciones, y análisis bayesianos de la estructura genética.

Sambucus palmensis

La identificación genotípica individual de todos los individuos muestreados de *S. palmensis* requirió la realización de un genotipado lo más exacto posible, sin alelos perdidos o no detectados ("missing data"). Una vez revisada y completada la base de datos, se procedió a un sondeo exhaustivo

de los genotipos multilocus presentes en cada localidad estudiada (Tabla S.4.1, pág. 78). Ello permitió el conteo de los genotipos compartidos entre y dentro de las localidades, así como la cantidad de genotipos únicos en cada una de ellas.

En cuanto al cálculo de la diversidad genética, se calcularon los siguientes índices de diversidad: números de alelos, número de alelos privados, números de alelos poco frecuentes (raros), riqueza alélica, riqueza de alelos privados y los índices de heterocigosidad observada y esperada. A su vez, para estimar los efectos de las acciones de restauración sobre la diversidad genética, estos índices se calcularon por separado tanto para los individuos naturales como para los individuos resultantes de la restauración. También se estimó la presencia de alelos nulos por locus en las localidades de estudio, así como la detección de cuellos de botella en los individuos naturales.

Con un enfoque especialmente dirigido a la gestión de las localidades, se elaboró una lista de aquellos individuos más apropiados para la elaboración de un vivero destinado a la obtención de esquejes y/o semillas. La presencia de genotipos únicos o poco frecuentes, alelos raros y una alta heterocigosidad individual fueron los criterios a tener en cuenta para elaborar dicha lista.

Para el estudio de la estructura genética actual, se realizó un AMOVA con agrupación acorde a la localidad de estudio o al origen (natural o restaurado), un PCoA con la distancia genética entre individuos (naturales o restaurados), y análisis bayesianos de la estructura genética.

Viola cheiranthifolia

Los análisis de *V. cheiranthifolia*, debido a su condición poliploide, hubo que realizarlos con software que incorporasen algoritmos específicos. Los índices de diversidad genética se pudieron calcular mediante el software SPAGeDi 1.5, que tiene en cuenta organismos con ploidía variable, asumiendo heredabilidad polisómica. Se calcularon los índices (número medio de alelos por locus, número medio de alelos privados, riqueza alélica, heterocigosidad observada y heterocigosidad esperada) para todas las localidades de estudio, además de para las poblaciones detectadas mediante los análisis bayesianos. Además, con el fin de corroborar la autocompatibilidad de la especie, se midieron los niveles de autogamia con un método para poliploides implementado en SPAGeDi (Hardy 2015). Para

detectar patrones filogeográficos en las poblaciones y determinar el índice de distancia genética a emplear, se implementó un test de permutación de tamaño de los alelos (pR_{ST}) (Hardy *et al.* 2003), que ayuda a detectar si los niveles de mutación infieren en la estructura poblacional más que la deriva y el flujo génico.

La estructura genética fue estudiada mediante análisis bayesianos, un AMOVA a nivel jerárquico (entre localidades y poblaciones detectadas en el análisis bayesiano), PCoA con la distancia genética de Bruvo (Bruvo *et al.* 2004) entre individuos y la distancia genética de Rho (Ronfort *et al.* 1998) entre pares de poblaciones. Los análisis de la estructura espacial a fina escala (SGS) fueron realizados para las dos principales poblaciones (Teide y circo de Las Cañadas; detectadas en STRUCTURE). El método de computación bayesiana (ABC) se utilizó para explorar la historia demográfica que pudo haber dado lugar a la estructura genética actual y los tamaños de población efectiva para cada grupo o población. Con el fin de simplificar los análisis y respetar el requisito de aislamiento poblacional que impone la metodología, los individuos fueron agrupados en tres poblaciones (Teide, Guajara y Pasajirón), de acuerdo con los resultados del STRUCTURE y la distancia genética de Rho.

La segunda parte del trabajo sobre *V. cheiranthifolia* tiene dos principales componentes. Por un lado, se incorporaron datos de alta resolución topográfica, gradientes de temperatura y mapas de cobertura de nieve para elaborar modelos de nicho a partir del rango actual de distribución de la especie. Los modelos se proyectaron sobre distintos escenarios de cambio climático para determinar el alcance de los cambios en idoneidad ambiental y testar los cambios en la distribución en un gradiente altitudinal. Por otro lado, para valorar si los cambios en la idoneidad climática de las poblaciones se corresponderían con cambios en la diversidad genética, se realizaron simulaciones sobre la pérdida de heterocigosidad esperada a medida que se perdían individuos y cambiaban las frecuencias alélicas. Las tasas de migración entre poblaciones obtenidas mediante los resultados del flujo genético fueron incluidas para la construcción de las frecuencias alélicas en cada simulación. La matriz de datos fue dividida en las principales poblaciones de estudio resultantes del STRUCTURE (Teide y circo de Las Cañadas), tal y como se hiciera para los análisis anteriores. Los análisis de esta segunda parte se llevaron a cabo es distintos paquetes específicos de R, detallados en el apartado de material y métodos para *V. cheiranthifolia* (5.4).

Software	Referencia	Análisis implementados
GENEPOP 4.2	(Rousset 2008)	Desequilibrio de ligamiento
		Desviaciones del equilibrio de Hardy-Weinberg
MICROCHECKER 2.2.3	(Van Oosterhout *et al*. 2006)	Estimación de alelos nulos por locus y/o población
GENALEX 6.5	(Peakall & Smouse 2012)	Índices de diversidad genética
		Análisis de coordenadas principales (PCoA)
		Identificación de individuos por su genotipo multilocus
HP-RARE 1.0	(Kalinowski 2005)	Rarefacción de la riqueza alélica y alelos privados
SPAGeDi 1.5	(Hardy & Vekemans 2002)	Índices de diversidad genética (con cualquier nivel de ploidía)
		Niveles de autogamia (para diploides y poliploides)
		Permutaciones pR_{ST} para patrones filogeográficos
		Estructura genética espacial a fina escala (SGS)
		Cálculo del estadístico Rho (Ronfort et al. 1998) entre pares de poblaciones para poliploides
BOTTLENECK 1.2.02	(Cornuet & Luikart 1996)	Detección de cuellos de botella
ARLEQUIN 3.5.2.1	(Excoffier & Lischer 2010)	Análisis Molecular de la Varianza (AMOVA)
		Cálculo de F_{ST} entre pares de poblaciones.
POPULATIONS 1.2. 28	(Langella, 2002)	Cladograma UPGMA
STRUCTURE 2.3.4	(Pritchard *et al*. 2000)	Análisis bayesiano de la estructura genética
STRUCTURE HARVESTER	(Earl & vonHoldt 2012)	Interpretación de los resultados de STRUCTURE
		Método de Evanno *et al*. (2005) para la elección de K
CLUMPP 1.1.2	(Jakobsson & Rosenberg 2007)	Iteración entre las repeticiones para cada K del STRUCTURE.
DIYABC 2.1	(Cornuet *et al*. 2014)	Análisis de la historia demográfica de las poblaciones con ABC (Beaumont 2010)
Polysat 1.4-1	(Clark & Jasieniuk 2011)	Tratamiento de datos de especies poliploides. Exportación de formato para otros software
		Cálculo de distancia genética específica para poliploides (Bruvo et al. 2004)
Adegenet 2.0.1	(Jombart 2008)	AMOVA anidado, con las frecuencias alélicas obtenidas en Polysat
Poppr 2.3.0	(Kamvar *et al*. 2014)	

Tabla 2.1. Lista de los principales programas informáticos empleados en este trabajo para el estudio y análisis de la genética de poblaciones de *Bethencourtia*, *Sambucus palmensis* y *Viola cheiranthifolia*.

2.4. RESULTADOS Y DISCUSIÓN

El género *Bethencourtia*

Todos los resultados de estructura genética revelaron que existe un alto grado de diferenciación entre *Bethencourtia hermosae*, *B. rupicola* y *B. palmensis*, acorde con la clasificación morfólogica y taxonómica realizada por Nordenstam (2006a; b) (Figuras 3.3, 3.4, 3.5, págs. 49-50). Comparando el grado de diferenciación genética con otros géneros de Canarias, se encontraron valores similares para especies de *Ilex* y *Cheirolophus* (Sosa *et al.* 2013; Vitales *et al.* 2014a). Por lo tanto, para optimizar los esfuerzos de conservación, *B. rupicola* debería estar incluida como especie en las listas de especies silvestres y catálogos de especies amenazadas.

Por otro lado y, dentro de cada especie, se encontraron bajos niveles de diferenciación genética, con abundante flujo genético entre las poblaciones estudiadas, posiblemente debido a la dispersión anemócora y a la corta distancia geográfica entre localidades (Tabla 3.4, pág. 47). Sin embargo, la diferenciación entre las poblaciones de *B. palmensis* (La Palma y Tenerife) es más pronunciada, al encontrarse en islas diferentes, siendo un patrón ampliamente detectado en las especies de islas oceánicas que no presentan dispersión a larga distancia (García-Verdugo *et al.* 2014).

Las tres especies presentaron altos niveles de alogamia detectados con el método de David *et al.* (2007), lo cual concuerda con experimentos de germinación anteriormente realizados para *B. hermosae* (Ortega & González 1986). Las especies autoincompatibles son más propensas a la pérdida de diversidad genética debido a la fragmentación del hábitat y los cuellos de botella que las especies autógamas (Aguilar *et al.* 2008). Además, se detectaron signos de cuello de botella reciente en algunas poblaciones de *B. hermosae* y *B. rupicola*, probablemente debido a las perturbaciones que están afectando a estas especies. En consecuencia, los reducidos tamaños poblacionales debido a la especificidad por el hábitat, junto a eventos de cuello de botella y las dificultades para reproducirse pueden ser los principales factores que reducen la diversidad genética en *Bethencourtia*, especialmente *B. hermosae* y *B. rupicola*.

Sambucus palmensis

Mediante el análisis y genotipado con marcadores microsatélites, se ha caracterizado a nivel individual una representación importante de los individuos reintroducidos y todos los individuos naturales de *Sambucus palmensis* de la

isla de La Gomera. Para un total de 402 muestras se detectaron 147 genotipos diferentes. Algunos de estos genotipos contaban con una amplia representación, debido al método de propagación utilizado para la restauración poblacional, ya que se han extendido numerosos clones de algunos individuos, al igual que por la propia naturaleza reproductiva de la especie (Tabla 4.2, pág. 70).

Los índices de diversidad genética indican que *S. palmensis* presenta unos niveles moderados para su restringida distribución, lo cual puede ser signo de una mayor distribución en el pasado (Beltrán *et al.* 1999; Sosa *et al.* 2010). Por otro lado, la heterocigosidad observada de las localidades restauradas ha aumentado respecto a la de las naturales, fruto del intercambio genético promovido con las translocaciones, establecidas en las últimas décadas en la isla (Tabla 4.3, pág. 71).

En cuanto a la estructura genética, se ha detectado una alta homogenización entre las localidades, a excepción de la población de Liria que presentó un mayor número de alelos privados y una mayor diferenciación en los resultados del STRUCTURE (Figura 4.3, pág. 73). A pesar de que se han utilizado ejemplares de Liria para la obtención de esquejes, la población sufrió una expansión demográfica a raíz de un incendio ocurrido en Garajonay en 2008 (Fernández-López *et al.* 2014), por lo que actualmente existen individuos con genotipos que no han sido translocados. Probablemente, debido a esta expansión en Liria, no se hayan detectado cuellos de botella en los individuos naturales, ya que la mayoría de los especímenes naturales pertenecen a esta población.

Finalmente, se proponen acciones concretas para la conservación genética de este endemismo en La Gomera. Por un lado, si se siguen empleados esquejes para la propagación de los individuos, sólo se deberían tomar de aquéllos con genotipo único o en baja frecuencia. Por otro lado, se proponen una serie de individuos idóneos para su propagación en vivero. Para ello se tuvo en cuenta que presentasen genotipos únicos o presentes en menos de 3 individuos, y que además contuviesen alelos raros (presentes en 4 localidades o menos) y una alta heterocigosidad individual (Tabla S.4.3, pág. 82).

Viola cheiranthifolia

Los valores de diversidad genética en las poblaciones naturales de *Viola cheiranthifolia* fueron moderadamente altos a pesar de que la especie presenta una distribución restringida y altos niveles de autogamia. Sin embargo, se

detectó por primera vez que la especie es poliploide, encontrando un máximo de cuatro alelos para todos los loci estudiados. Por lo tanto, los efectos de la autogamia y la rareza de la especie sobre la diversidad genética se pueden haber visto compensados por su condición poliploide. Guajara y Pasajirón fueron las poblaciones que presentaron mayor y menor diversidad genética respectivamente (Tabla 5.2, pág. 100).

Los análisis empleados para estimar la estructura genética mostraron que existe una clara diferenciación entre las localidades del complejo Teide-Pico Viejo y las del circo de Las Cañadas (Guajara y Pasajirón) (Figuras 5.2 y 5.3, pág. 100-101). Por lo tanto, debido a la corta capacidad de dispersión de la especie y que los principales polinizadores (himenópteros) (Seguí *et al.* 2017) normalmente no viajan largas distancias (Zurbuchen *et al.* 2010), Las Cañadas actúa como barrera geográfica para el flujo génico. Los resultados de los estadísticos para calcular la estructura genética espacial a fina escala fueron significativos, principalmente debido a los niveles de autogamia encontrados, junto al corto sistema de dispersión de las semillas (Figura 5.5, pág. 103).

De acuerdo con los resultados del análisis bayesiano para inferir la historia demográfica de las poblaciones, se sugiere que hubo un evento de recolonización desde las poblaciones del alto de Las Cañadas después de los eventos eruptivos que dieron lugar al estratovolcán del Teide (Tabla 5.6, pág 105). Aunque han ocurrido erupciones volcánicas después de la edad de divergencia estimada entre las poblaciones del Teide y Guajara (hace 21.425 años), éstas han sido menores en los últimos 30.000 años (Carracedo *et al.* 2007).

Al contrario de la suposición generalizada de que las especies alpinas se extinguirán con el cambio climático (IPCC Working Group I 2013), nuestros resultados sugieren que el nicho climático de *V. cheiranthifolia* se mantiene en cualquiera de los escenarios. Sin embargo, se detectó una reducción de la idoneidad climática en todas las predicciones, en el que las poblaciones a menor altitud se ven perjudicadas, pero con grandes variaciones entre los distintos escenarios proyectados (Figuras 5.6 y 5.7, págs. 107-108). Además, las simulaciones sobre la pérdida de la heterocigosidad esperada a medida que se reducía la idoneidad climática, mostraron un importante decrecimiento después del año 2060, especialmente para las localidades del circo de Las Cañadas (Figura 5.8, pág. 109).

Por lo tanto, para asegurar la conservación a largo plazo, se deben cumplir una serie de condiciones. En primer lugar, el control de herbívoros

introducidos debe ser una prioridad, ya que se ha demostrado que la herbivoría por parte de los conejos disminuye la densidad y tamaño vegetativo de las violetas, y aumenta los niveles de autofertilización (Seguí *et al.* 2017). En segundo lugar, asegurar que no existen impedimentos para la dispersión de las semillas, realizando una monitorización sobre los cambios en la distribución de las poblaciones. En tercer lugar, evitar que la presión antrópica afecte seriamente a las áreas de idoneidad climática de la especie, situación poco probable debido al alto grado de protección que posee el Teide. Por último, y quizás a largo plazo, podría ser necesario el reforzamiento en aquellas poblaciones que se verían reducidas si se cumplen las predicciones, por ejemplo, en Guajara y Pasajirón, donde se estima que la pérdida de diversidad genética a largo plazo sea más acusada.

2.5. CONCLUSIONES

1. La caracterización genética del género endémico *Bethencourtia* reveló que las tres especies del mismo se encuentran bien diferenciadas, a la vez que relacionadas, en concordancia con sus diferencias morfológicas y su estatus taxonómico actual. Las dos especies de La Gomera (*B. hermosae* y *B. rupicola*) están más próximas entre sí, respecto a *B. palmensis* (La Palma y Tenerife).

2. Los bajos niveles de diferenciación genética dentro de cada especie pueden deberse a las características reproductivas de las mismas (autoincompatibilidad), junto a la proximidad geográfica de sus poblaciones.

3. Los reducidos niveles de diversidad genética detectados en *B. rupicola* y especialmente en *B. hermosae*, podrían ser la consecuencia de cuellos de botella recientes, así como de una reducida distribución geográfica.

4. Es necesario establecer acciones de conservación para *B. hermosae* y *B. rupicola*, como la catalogación de *B. rupicola* como especie amenazada y la realización de estudios demográficos de ambas especies a largo plazo.

5. En las localidades de *Sambucus palmensis* en La Gomera, se detectaron numerosos individuos clónicos, debido tanto al método de propagación establecida por los técnicos del Parque Nacional (por esquejes), como a la reproducción vegetativa que la especie presenta. Pero también se identificaron genotipos únicos, posiblemente propios de algunos individuos naturales o fruto de la regeneración sexual de los individuos reintroducidos.

6. Los efectos de las acciones de conservación llevados a cabo por el Parque Nacional de Garajonay en *S. palmensis* han sido positivos. Desde el punto de vista genético, se han mantenido los niveles de variabilidad genética respecto a los individuos naturales, detectándose además un ligero aumento de la heterocigosidad observada.

7. La población de Liria, cuenta con una mayor presencia de genotipos únicos y alelos privados, lo que la diferencia del resto de las poblaciones estudiadas. Las translocaciones de genotipos de unas áreas a otras del Parque Nacional de Garajonay probablemente han propiciado el intercambio genético de *S. palmensis* en la isla.

8. Como medidas de conservación genética a tener en cuenta para *S. palmensis* se ha propuesto una lista de individuos que cumplen con los siguientes requisitos: poseer un genotipo único o poco frecuente, disponer de alelos raros y de una alta heterocigosidad individual. Con la propagación de estos ejemplares se podría conservar un alto porcentaje de la variabilidad genética de la especie en Garajonay.

9. *Viola cheiranthifolia* presentó un patrón poliploide, al igual que otros congéneres de la sección *Melanium*. Probablemente por su condición poliploide, mantiene unos moderados niveles de diversidad genética, a pesar de su distribución restringida y los altos niveles de autogamia detectados.

10. Las localidades de *V. cheiranthifolia* se encuentran genéticamente diferenciadas en dos grupos: el complejo Teide-Pico Viejo y el circo de Las Cañadas. La caldera de Las Cañadas actúa como barrera para el flujo génico debido a la ausencia de idoneidad climática en esta zona y a la corta capacidad dispersiva de la especie.

11. La historia demográfica de las poblaciones de *V. cheiranthifolia* se ha visto marcada por el vulcanismo y construcción del estratovolcán del Teide. Se estima que después de la finalización del complejo Teide-Pico Viejo, la zona se pudo haber recolonizado con violetas procedentes del circo de Las Cañadas.

12. Los modelos de nicho climático indican que *V. cheiranthifolia* mantiene áreas de idoneidad climática en todos los escenarios propuestos, a pesar de la dramática reducción de las poblaciones periféricas en los modelos más pesimistas. Además, las estimaciones de la pérdida de diversidad genética están correlacionadas con la pérdida de idoneidad climática, siendo más acusada para las localidades del circo de Las Cañadas.

13. Los programas de conservación de *V. cheiranthifolia* deben enfocarse en el control exhaustivo de la herbivoría, la monitorización de la distribución de las poblaciones a largo plazo, y en el caso de presentarse una drástica reducción de los tamaños poblacionales, promover acciones para el reforzamiento genético.

2.5. CONCLUSIONS

1. The genetic characterization of the endemic genus *Bethencourtia* revealed that the three species described for the genus are well differentiated, while related, in accordance with their morphological differences and their current taxonomic status. The two species of La Gomera (*B. hermosae* and *B. rupicola*) are more related between them than to *B. palmensis* (La Palma and Tenerife).

2. The low levels of genetic differentiation detected within each species may be due to their reproductive characteristics (self-incompatibility), along with the geographic proximity of the populations.

3. The reduced levels of genetic diversity detected in *B. rupicola* and especially in *B. hermosae*, could be the result of recent bottlenecks, as well as a reduced geographical distribution.

4. It is necessary to establish conservation actions for *B. hermosae* and *B. rupicola*, as including *B. rupicola* as an endangered species and carrying out demographic studies of both species in a long-term perspective.

5. Numerous clone individuals were detected in the *Sambucus palmensis* localities in La Gomera, due both to the propagation method established by the technicians of the National Park (by cuttings), and to the vegetative reproduction that the species presents. But we also identified unique genotypes, possibly typical of some natural individuals or because of the sexual regeneration of the reintroduced individuals.

6. The effects of conservation actions carried out by the Garajonay National Park in *S. palmensis* are positive from the genetic point of view. The levels of genetic variability were maintained in comparison with the natural individuals, and a light increase in the observed heterozygosity was detected.

7. The Liria population has a greater presence of unique genotypes and private alleles, which makes it different from the rest of the populations studied. Translocations of genotypes from some areas to others in the Garajonay National Park have probably led to genetic exchange of *S. palmensis* in the island.

8. As a conservation measure for *S. palmensis*, we have proposed a list of individuals who meet the following requirements: to present a unique or rare genotype, rare alleles and a high individual heterozygosity. The propagation of these specimens could serve to preserve a high percentage of the genetic variability of the species in Garajonay.

9. *Viola cheiranthifolia* presented a polyploid pattern, like other congeners of section *Melanium*. Probably due to its polyploid condition, it maintains moderate levels of genetic diversity, despite its restricted distribution and the high levels of autogamy detected.

10. The *V. cheiranthifolia* localities are genetically differentiated in two groups: the Teide-Pico Viejo complex and Las Cañadas Wall. Las

Cañadas caldera acts as a barrier to gene flow due to the absence of climatic suitability in this area and the short dispersal capacity of the species.

11. The demographic history of *V. cheiranthifolia* populations has been marked by volcanism and construction of the Teide stratovolcano. It is estimated that after the completion of the Teide - Pico Viejo complex, the area could have been re-colonized with violets from Las Cañadas Wall.

12. The climatic niche models indicate that *V. cheiranthifolia* will keep climate suitability areas in all the scenarios proposed, despite the dramatic reduction of peripheral populations in the most pessimistic models. In addition, the estimates of the genetic diversity loss are correlated with climate suitability loss, being more pronounced for the Las Cañadas Wall localities.

13. Conservation programs for *V. cheiranthifolia* should be focused on the thorough control of herbivores, the monitoring of the populations distribution in the long term, and in case of a drastic reduction of the population sizes, promoting actions for genetic reinforcement.

Foto: Claudio Moreno

THE ENDEMIC GENUS *Bethencourtia*

3. Species delimitation and conservation genetics of the Canarian endemic *Bethencourtia* (Asteraceae)

ABSTRACT

Bethencourtia Choisy ex Link is an endemic genus of the Canary Islands and comprises three species. *Bethencourtia hermosae* and *B. rupicola* are restricted to La Gomera, while *B. palmensis* is present in Tenerife and La Palma. Despite the morphological differences previously found between the species, there are still taxonomic incongruities in the group, with evident consequences for its monitoring and conservation.

The objectives of this study were to define the species differentiation, perform population genetic analysis and propose conservation strategies for *Bethencourtia*. To achieve these objectives, we characterized 10 polymorphic SSR markers. Eleven natural populations (276 individuals) were analyzed (three for *B. hermosae*, five for *B. rupicola* and three for *B. palmensis*). All grouping analyses such as AMOVA, PCoA and Bayesian analysis confirmed the evidence of the well-structured groups corresponding to the three species. At the intra-specific level, *B. hermosae* and *B. rupicola* did not show a clear population structure, while *B. palmensis* was aggregated according to island of origin. This is consistent with self-incompatibility in the group and high gene flow within species. Overall, the genetic diversity of the three species was low, with expected heterozygosity values of 0.302 (*B. hermosae*), 0.382 (*B. rupicola*) and 0.454 (*B. palmensis*). Recent bottleneck events and a low number of individuals per population are probably the causes of the low genetic diversity. We consider that they are naturally rare species associated with specific habitats. The results given in this article will provide useful information to assist in conservation genetics programs for this endemic genus.

3.1. INTRODUCTION

The Canarian archipelago has a significant representation of the tribes of Asteraceae compared to other oceanic islands (Crawford *et al.* 2009), including the Senecioneae tribe. Within the Senecioneae tribe, *Pericallis* contains many single-island endemics in the Macaronesia, and *Bethencourtia* is the only endemic genus of the Canary Islands (Nordenstam *et al.* 2009).

Bethencourtia Choisy ex Link (Asteraceae) includes three species: *Bethencourtia hermosae* (Pit.) G. Kunkel, *Bethencourtia rupicola* (B. Nord.) B. Nord. and *Bethencourtia palmensis* Choisy. *Bethencourtia hermosae* and *B. rupicola* are island-exclusive to La Gomera. *Bethencourtia hermosae* can be found in Vallehermoso with three main localities and *B. rupicola* is located in the Monumento Natural de Los Roques within the Garajonay National Park, on the steep slopes of the phonolitic outcrops of Agando, Ojila, Carmona and Las Lajas (Figure 3.1). *Bethencourtia palmensis* is present in Tenerife in the Barranco de El Río and is more widespread in La Palma, including at the summit of the Caldera de Taburiente National Park.

B. palmensis and *B. hermosae* were considered to be under the genus *Senecio*: *Senecio palmensis* (C. Sm. in Buch) Link and *S. hermosae* Pit. Nevertheless, enough morphological differences were found to include them in a separate genus (*Canariothamnus* B. Nord.) (Nordenstam 2006a; b). This separation of *Bethencourtia* was confirmed with a phylogeny of the Senecioneae tribe, and the genus appeared closely related to *Jacobaea* sp. from the Iberian Peninsula (Pelser *et al.* 2007).

Moreover, Nordenstam found enough morphological differences between the populations in the Monumento Natural de Los Roques and Vallehermoso in the island of La Gomera to describe the new species *B. rupicola* as a different species from *B. hermosae*. He also emphasized the low number of individuals per locality of *B. rupicola* that he found. The morphological differences between these three species are clear, the especially obvious dissimilarity being the length of the leaf lobes. Leaf lobes are 1-5 mm long in *B. palmensis*, 5-10 mm in *B. rupicola* and 10-25 mm in *B. hermosae* (Nordenstam 2006a) (Figure 3.2). In addition, the breeding system of these species is not completely understood (Crawford *et al.* 2009). The dispersal syndrome is anemochorous and the pollinators are still unknown (Fernández-López & Velázquez-Barrera, 2011).

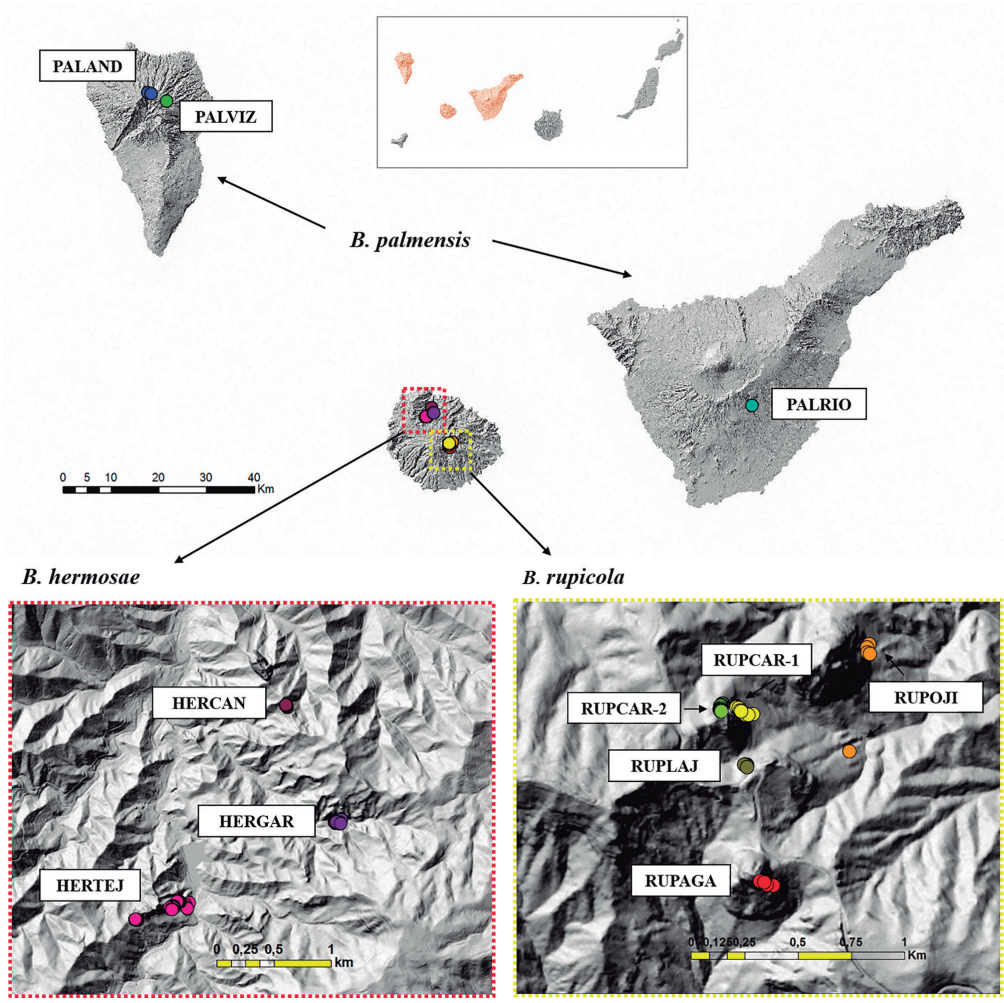


Figure 3.1 Map of the distribution of *Bethencourtia* localities sampled for this study. The maps at the bottom correspond to *B. hermosae* (left) and *B. rupicola* (right). Population codes are detailed in Table 3.1.

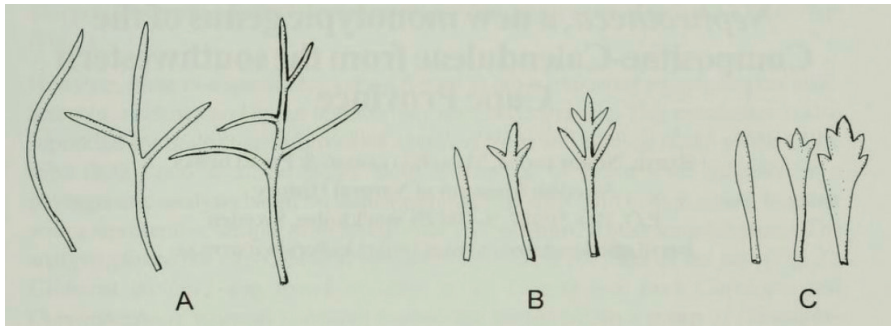


Figure 3.2 Drawings of the leaf morphology of the three *Bethencourtia* species. Extracted from Nordenstam (2006a). (A) *B. hermosae*, (B) *B. rupicola*, (C) *B. palmensis*.

Bethencourtia palmensis and *B. hermosae* are considered within the genus *Senecio* in the official lists of species (Arechavaleta *et al.* 2010; BOC 2010), but *B. rupicola* has not been included as a taxonomic unit separate from *B. hermosae* (=*S. hermosae*). Thus, the reports on the demography and distribution of *B. hermosae* and *B. rupicola* consider all localities as a single species, which might result in an underestimated evaluation of the threat status for each of them separately. As management policies state that a species is the minimum unit for legal protection, this may become an important issue from a conservation standpoint (IUCN 2012). In the IUCN Red List, *B. hermosae* and *B. rupicola* (= *C. hermosae*) are listed as Vulnerable due to their extremely narrow distribution, being affected by competition with exotic species and grazing (Martín Osorio *et al.* 2011). *Canariothamnus hermosae* is also cited as Vulnerable in the Spanish Red List of Vascular Flora (Moreno-Saiz 2008), and it is catalogued within the category "de interés para los ecosistemas Canarios" ("of interest for the Canarian ecosystem") in the regional list (BOC 2010). *Bethencourtia rupicola*, despite the low number of individuals, and possibly due to its recent description, is not considered in any list as a single taxon, but its populations are usually included together with *B. hermosae*. Unfortunately, after our sampling season in 2012, a fire occurred in the Garajonay National Park, which affected the populations of *B. rupicola* in the Monumento Natural de los Roques. Nevertheless, a significant recovery of the individuals has been detected (Fernández-López *et al.* 2014). *Bethencourtia palmensis* is not currently threatened, as it presents a wider distribution with higher population sizes. Moreover, there are no detailed studies, either molecular or morphological, of the species delimitation in the genus *Bethencourtia* with a thorough sampling of its distribution.

The aims of this research were (1) to assess with molecular evidence the differentiation between *B. hermosae*, *B. rupicola* and *B. palmensis*; (2) to estimate the intra- and interpopulation genetic diversity and structure; (3) to understand traits of their reproductive biology such as the selfing rate and (4) to propose conservation actions. To accomplish these objectives, we have developed 10 microsatellite markers for the three species, which are described in this article for the first time. These results will be relevant for taxonomical issues, as well as for the management and conservation of *Bethencourtia*.

3.2. MATERIAL AND METHODS

3.2.1. Sample collection

Leaf samples were collected in 2012 and 2013 from La Gomera in all the previously known locations for *Bethencourtia hermosae* and *B. rupicola*, and three localities of *B. palmensis* in La Palma and Tenerife. Number of individuals per population, geographic coordinates and population codes are listed in Table 3.1. For the collection of the individuals in La Gomera the assistance of a professional climber was necessary, especially for the *B. rupicola* populations, due to the height and gradient of the phonolitic outcrops and cracks which they inhabit. All specimens were georeferenced in ArcGIS 10.4 (ESRI) (Figure 3.1). In order to homogenize sample sizes across populations, Roque Carmona was subdivided in two populations (RUPCAR1-RUPCAR2), according to the distances between individuals on the outcrop. Leaf samples were stored in plastic bags with silica gel, and herbarium voucher specimens were brought to the TFC Herbarium at the University of La Laguna. Herbarium specimens were only collected from those populations with individuals in bloom during the sampling period.

Species	Island	Location	Population code	Voucher	N
B. hermosae	La Gomera	Lomo Las Tejas	HERTEJ	-	41
		Presa del Garabato	HERGAR	TFC-50.736	49
		Roque Chico-Roque Cano	HERCAN	-	31
Subtotal					121
B. rupicola	La Gomera	Roque Carmona	RUPCAR1	TFC-50.735	18
			RUPCAR2	-	41
		Roque Las Lajas	RUPLAJ	-	11
		Roque Ojila	RUPOJI	-	5
		Roque Agando	RUPAGA	TFC-50.731	4
Subtotal					79
B. palmensis	La Palma	Los Andenes	PALAND	TFC-50.713	20
		Fuente Vizcaína	PALVIZ	TFC-50.712	22
	Tenerife	Barranco de El Río	PALRIO	TFC-50.714	34
Subtotal					76
Total					276

Table 3.1 *Bethencourtia hermosae*, *B. rupicola* and *B. palmensis* localities sampled and included in this study. Voucher = Reference from TFC Herbarium, La Laguna University. N = sample size.

3.2.2. Microsatellite development

We describe the characterization of 10 SSR markers in the endemic genus of the Canary Islands *Bethencourtia* Choisy ex Link, indicating their effectiveness in identifying patterns of genetic diversity.

Genomic DNA for the development of markers was extracted from leaf tissue using the (Dellaporta 1983) protocol. For subsequent analysis, the whole set of samples was extracted with Invisorb DNA Plant HTS 96 KIT INVISORB.

Microsatellite loci were selected from an Illumina paired-end shotgun library developed by Savannah River Ecology Laboratory (University of Georgia) using 3 probe mixes of *B. hermosae*. We initially chose 38 primer pairs of this library of which 19 yielded some product and were labelled. Finally, 10 primer pairs amplified consistently in the three species and were used for further analysis (Table 3.2). For the initial testing, PCR (polymerase chain reaction) was conducted individually with each primer pair in a 25 uL total reaction volume, which contained approximately: 20 ng of DNA 10 pmol of each primer, as well as PCR Master Mix until 25 uL were completed (Reddy-Mix, ABgene, Surrey, UK). Reverse primers were color-labelled at the 5'-end with 6-FAM, PET, NED or VIC. Amplifications were performed using the following conditions: 3 min denaturation at 95°C, 35 cycles of 30 s denaturation at 95°C, 30s annealing at 60°C, and 72°C for 1.5 min; followed by 5 min elongation at 72°C.

Once primers pairs known to generate products were labelled, we conducted the subsequent multiplex amplifications using the QIAGEN Multiplex Kit (QIAGEN). PCR were performed in 15 µL reaction volumes: 7.5 µL of Multiplex PCR Master Mix, 1.5 µL primer mix (containing 0.2 µM of each primer in TE), 1.5 µl of Q-solution, 20-40 ng of DNA and dH_20. Multiplexing was carried out in two primer groups as indicated in Table 3.2. Following the instructions of the manufacturer, PCR consisted of a Touchdown protocol with the thermal conditions: 15 min at 95°C, 10 cycles of 30 s at 94°C, annealing for 90 s at 65°C with a decrease of 0.5°C per cycle and 60 s at 72°C, following by 20 cycles of 30 s at 94°C, annealing for 90 s at 55°C and 60 s at 72°C, with a final extension of 30 min at 60°C.

All the products from both simple PCR and multiplex PCR were detected on an ABI 3730 Genetic Analyzer and fragments were sized against the

LIZ (500-250) size standard (Applied Biosystems, Inc.) and visualized using Genemapper 4.0 (Applied Biosystems, Inc.). We identified allele peak profiles at each locus and assigned a genotype to each individual.

Locus	EMBL ID	Motif	PCR primer sequence (5' -> 3')	Range (bp)	Dye
BetTet-2	LN794613	$(AAAT)_{32}$	F: TGAAATTCGAAGTTGACGTGG R: AGGTCAATGACTAGCCTCGG	217-247	6-FAM *
BetTet-3	LN794614	$(AAAT)_{32}$	F: GAGGTAACTCTAGAGATGATAACTGTAAGG R: TTGTGTTATACTGCAGAGATTGAAGG	131-143	NED *
BetTet-5	LN794618	$(ATCT)_{32}$	F: AAAGTGATGGACAAATAAACGG R: TGGTGGTAGGTGTAGAGGAGG	268-292	NED *
BetPen-1	LN794611	$(AAATC)_{30}$	F: GACCACCATAGTCAGAAGATCTAACC R: CCGATTCTTCTCAACTGATAATTGC	271-286	VIC ‡
BetPen-2	LN794612	$(TCTCC)_{30}$	F: AAGAAGAGAGTTATTTATTTGTAAGTGGG R: TTTACTTCCACCTTCCATTTCC	196-211	NED ‡
BetPen-5	LN794616	$(AAAAC)_{30}$	F: CGTTGGCTCAAACAAACACC R: CAAGATCCCTTACAACGGGC	187-212	PET *
BetPen-6	LN794617	$(AATGC)_{30}$	F: GAATCCTACTCTACTAAACTATCCTCCG R: TTGAAAGTTTGAACCAAATGCC	156-180	6-FAM ‡
BetHex-3	LN794609	$(TTGCTG)_{42}$	F: CTTCTGAGAATCAGGATGTAAGTCAGC R: CCGGTACTACCTTCTTTCAGTCACC	168-204	VIC ‡
BetHex-4	LN794610	$(ATAGAG)_{36}$	F: TCTCTCTACATCCTTGTCTCTGTAGC R: CCAAACCTAAACTCTCCGGC	301-319	PET ‡
BetHex-6	LN794615	$(TGTGGG)_{36}$	F: AAATCAAGCTTTACCAACTCTCCC R: GAGTGTAAGGAAGGTGGTGGG	128-158	PET ‡

Table 3.2 Ten microsatellite primer pairs developed and characterized for *Bethencourtia hermosa*e and transferred to *B. rupicola* and *B. palmensis*. EMBL ID: Accession number per locus. Dye: Name of each fluorescent dye per locus. Multiplex; ‡Load A, *Load B.

3.2.3. Statistical analysis

Linkage disequilibrium and deviation from the Hardy-Weinberg equilibrium (HWE) were calculated using GENEPOP 4.2 (Rousset 2008). For all tests, a sequential Bonferroni correction for multiple comparisons was applied (Rice 1989). Estimation of null alleles for each population was carried out with MICROCHECKER 2.2.3 (Van Oosterhout *et al.* 2006). Basic genetic diversity

indices such as number of alleles (*NA*), number of private alleles (*PA*), observed (H_O), and unbiased expected (H_E) heterozygosities for each locus were estimated with GENALEX 6.5 (Peakall & Smouse 2012). Measures of allelic (A_R) and private allelic richness (PA_R) with a hierarchical method between and within species were calculated using HP-RARE 1.0 (Kalinowski 2005), which uses rarefaction to correct for sampling error.

Estimates of selfing (David *et al.* 2007) were calculated for each species and implemented in SPAGeDi 1.5 (Hardy & Vekemans 2002). BOTTLENECK 1.2 software was used to identify any recent genetic drift events in the natural populations. (Cornuet & Luikart 1996). The two-phase mutation model (TPM), which is a modification of the stepwise mutation model (SMM), was implemented and is shown to be a better fit for most microsatellite data sets (Piry *et al.* 1999). In the TPM model, to optimize the most sensitive values for microsatellites, a proportion of SMM in the TPM = 0.000 and a variance of the geometric distribution for TPM = 0.36 were chosen.

Allele frequency information was analyzed using a nested analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) with ARLEQUIN 3.5. The analyses were conducted with two different data sets: (i) all populations grouped by species (3 species, 9 populations); and (ii) the data sets for each species individually. Matrices of pairwise F_{ST} values (Weir & Cockerham 1984) were also obtained from ARLEQUIN. Significance values were estimated over 100 permutations.

In addition, a principal coordinate analysis (PCoA), using the covariance standardized method of pairwise codominant genotypic distances among individuals, was implemented with GENALEX. At the population level, a genetic distance matrix (Nei *et al.* 1983) between localities, and the resulting UPGMA tree, were estimated using POPULATIONS 1.2 software (Langella 2002), with 100 bootstraps on each locus. The tree was visualized and edited on FigTree (Rambaut 2009).

To check if the taxonomic status of this endemic genus is in concordance with the genetic structure, all the genotypes were screened using a Bayesian admixture procedure with the software STRUCTURE 2.3.4 (Pritchard *et al.* 2000). The model was assumed to be of population admixture and correlated allele frequencies. 10 independent runs were conducted for each value of K (from 1 to 15) and analysis consisted of a 10^5 burn-in period replicated

and a run length of 10^6 replicates. The optimal number of clusters was found by the ΔK method (Evanno *et al.* 2005) visualized with STRUCTURE HARVESTER (Earl & vonHoldt 2012). Results of 10 replicates of the best fit K were processed using CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007) to determine the optimal clustering. The STRUCTURE HARVESTER results for the election of the optimal K are presented in the Figure S.3.1 for all analysis. These analyses were carried out at the interspecific level, including the whole set of samples, and at the intraspecific level to test the genetic structure within species with 1-10 values of K.

3.3. RESULTS

The ten tested primer pairs amplified consistently and were polymorphic for the whole set of samples. Specifically, all the loci showed polymorphism in *Bethencourtia palmensis*, but BetPen-6 was monomorphic for both *B. hermosae* and *B. rupicola* with different allele sizes in each species. BetPen-5 was monomorphic only for *B. rupicola*. RUPCAR2 in *B. rupicola* was the only population that deviated from HW after a Bonferroni correction and there was no significant linkage disequilibrium for all the pairwise tests analyzed. We detected the presence of null alleles in *B. rupicola* in the populations RUPCAR1 (Hex4, Tet2) and RUPCAR2 (Hex4, Tet3, Tet5) and *B. palmensis* in the populations PALAND (Pen2, Tet3), PALVIZ (Tet3) and PALRIO (Pen2).

The highest values for all the genetic diversity parameters calculated were found in *B. palmensis*, with the highest value of H_E for PALAND (0.420). On the other hand, *B. hermosae* presented the lowest genetic diversity in HERGAR (H_E = 0.212). The average of rarefied allelic richness over all loci ranged from 1.57 in *B. hermosae* (HERCAN, HERGAR) to 2.34 in *B. palmensis* (PALAND). The highest presence of private alleles was found in *B. palmensis* equaling 0.240 (PALRIO) and *B. hermosae* with the value of 0.180 (HERTEJ) (Table 3.3).

The estimates of the selfing rate (from 0 to 1) based on the distribution of multilocus heterozygosity, showed low and similar values for *B. hermosae* (0.093±0.248), *B. rupicola* (0.000±0.109) and *B. palmensis* (0.000±0.027). These results, in accordance with the Hardy Weinberg equilibrium for all the populations within species, corroborate the lack of selfing reproduction in this group.

Species	Population	N	NA	A_R	PA	PA_R	H_O	H_E	F_{IS}	Bottleneck test L	Hd/He	P
B. hermosae	HERCAN	30.8	16	1.57	0	0.01	0.237	0.252	0.059 ^{ns}	5	0/5	0.016**
	HERGAR	48.9	19	1.57	0	0.01	0.208	0.212	0.017 ^{ns}	7	3/4	0.289 ^{ns}
	HERTEJ	40.9	27	2.00	3	0.18	0.335	0.365	0.083 ^{ns}	9	2/7	0.018**
Total		120.6	27	2.14	4	0.35	0.258	0.302				
B. rupicola	RUPAGA	4.0	19	1.90	0	0.02	0.400	0.321	-0.297 ^{ns}	6	2/4	0.281 ^{ns}
	RUPLAJ	10.9	22	1.86	1	0.11	0.336	0.309	-0.093 ^{ns}	7	3/4	0.344 ^{ns}
	RUPOJI	4.9	23	2.15	0	0.11	0.240	0.356	0.351 ^{ns}	7	5/2	0.812 ^{ns}
	RUPCAR1	18.0	23	1.94	0	0.02	0.311	0.356	0.129 ^{ns}	7	2/5	0.019**
	RUPCAR2	41.0	29	2.07	2	0.08	0.278	0.358	0.226***	8	5/3	0.578 ^{ns}
Total		78.8	33	2.62	6	0.50	0.297	0.382				
B. palmensis	PALAND	19.9	31	2.34	0	0.11	0.391	0.420	0.072 ^{ns}	10	3/7	0.312 ^{ns}
	PALVIZ	22.0	25	2.03	0	0.07	0.414	0.379	-0.093 ^{ns}	10	4/6	0.161 ^{ns}
	PALRIO	33.9	28	2.00	2	0.24	0.359	0.358	-0.003 ^{ns}	10	5/5	0.161 ^{ns}
Total		75.8	38	2.99	10	1.18	0.383	0.454				

Table 3.3 Basic genetic diversity indices for *Bethencourtia hermosae, B. palmensis* and *B. rupicola* populations. Population codes are detailed in Table 3.1. N = mean sample size over loci; NA = number of alleles; A_R = rarefied allelic richness; PA = number of private alleles; PA_R = rarefied private allelic richness; H_o = observed heterozygosity; H_e = unbiased expected heterozygosity; F_{IS} = inbreeding coefficient; L = number of polymorphic loci used in the bottleneck tests; Hd/He = number of loci with heterozygote deficiency and heterozygote excess (respectively) according to the TPM model; P = probability of the Wilcoxon test for heterozygote excess. Not significant (ns); ***P < 0.001; **P < 0.05

The results from BOTTLENECK (Table 3.3) showed significant values for heterozygosity excess only in two *B. hermosae* populations and one in *B. rupicola*, showing evidence of recent bottleneck events. The populations HERCAN, HERTEJ and RUPCAR1 revealed significant values of heterozygosity excess under TPM ($P < 0.05$).

The analysis of molecular variance (AMOVA) at the species level indicated that 45.3% of the variation was found between species, while 9.1% was explained by the variation between populations within species and 45.7 % within populations. The AMOVA analysis at the intraspecific level showed a similar pattern for the three species, with most of variation found within populations (86.3%, 87.4% and 77.3%) for *B. hermosae*, *B. rupicola* and *B. palmensis* respectively (Table 3.4).

Source of variation	Degrees of Freedom	Sum of squares	Variance components	Percentage of variation	F-statistics
B. hermosae* vs *B. rupicola* vs *B. palmensis					
Among species	2	619.3	1.605	45.3%	F_{CT} = 0.453***
Among populations within species	8	128.6	0.321	9.1%	F_{SC} = 0.165***
Within populations	541	875.8	1.619	45.7%	
Total	551	1.623.7	3.545		F_{ST} = 0.543***
B. hermosae					
Among populations	2	37.1	0.217	13.7%	
Within populations	239	325.6	1.362	86.3%	
Total	241	362.7	1.579		F_{ST} = 0.137***
B. rupicola					
Among populations	4	32.9	0.251	12.6%	
Within populations	153	267.1	1.746	87.4%	
Total	157	300.0	1.997		F_{ST} = 0.126***
B. palmensis					
Among populations	2	58.5	0.557	22.7%	
Within populations	149	283.1	1.900	77.3%	
Total	151	341.6	2.457		F_{ST} = 0.226***

Table 3.4 AMOVA analysis for *Bethencourtia hermosae*, *B. palmensis* and *B. rupicola* at the interspecific and intraspecific hierarchical levels. ***$P < 0.001$

Pairwise F_{ST} values (Table S.3.1) ranged from 0.011 (between RUPCAR1 and RUPCAR2) to 0.669 (between HERGAR and PALRIO). The average values between species ranged from 0.490 (between *B. hermosae* and *B. rupicola*) to 0.601 (between *B. palmensis* and *B. hermosae*), with the intermediate value of 0.513 (between *B. palmensis* and *B. rupicola*). It is interesting to compare these high F_{ST} values found between species with the averages among populations within species, these being 0.136 (*B. hermosae*), 0.156 (*B. rupicola*) and 0.199 (*B. palmensis*). The highest F_{ST} values among populations of the same species were detected in *B. palmensis*, between the two populations of La Palma and the one in Tenerife, these being of 0.276 (PALAND – PALRIO) and 0.266 (PALVIZ – PALRIO). In contrast, the lowest value among populations of the same species were found in *B. rupicola*, between RUPCAR1 and RUPCAR2 (0.011), this also being the only non-significant value.

Consistent with the AMOVA results, the first two axes of the PCoA accounted for a high proportion of the total variance (53.26 %) with 34.80 % explained by the first axis and 18.45% by the second (see Figure 3.3). This revealed three clearly differentiated groups of individuals of each species.

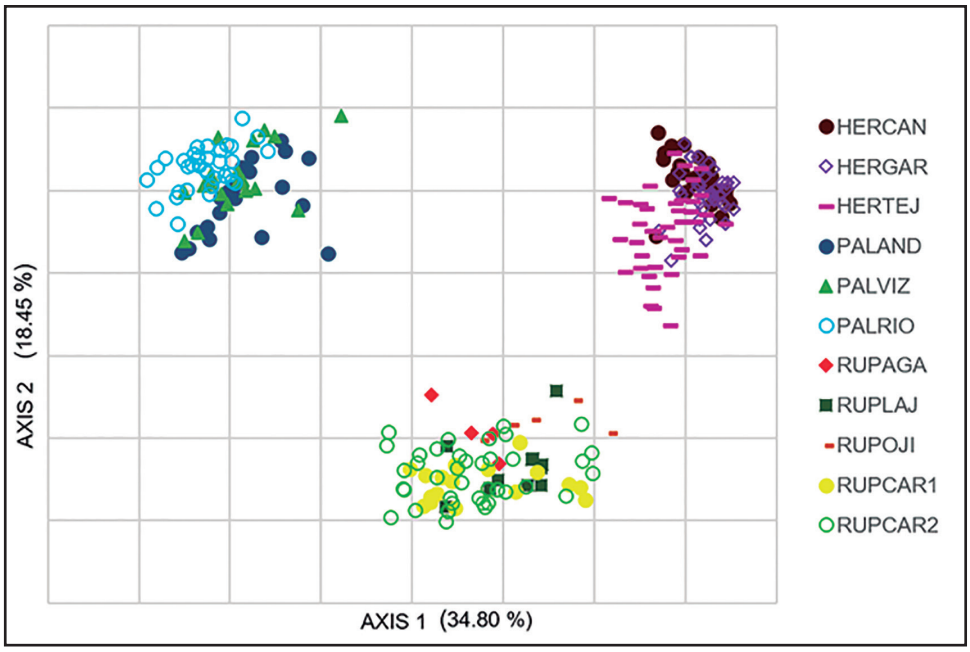


Figure 3.3 Principal coordinates analysis (PCoA) based on the genetic distance among individuals. The first two axes explained 53.26 % of the total variation. Percentage of variation for each axis are indicated within brackets. Population codes are detailed in Table 3.1.

In addition, the UPGMA tree confirmed the differentiation between the three species, supported by high values of bootstraps, in agreement with the topology found by Pelser *et al.* (2007) (Figure 3.4).

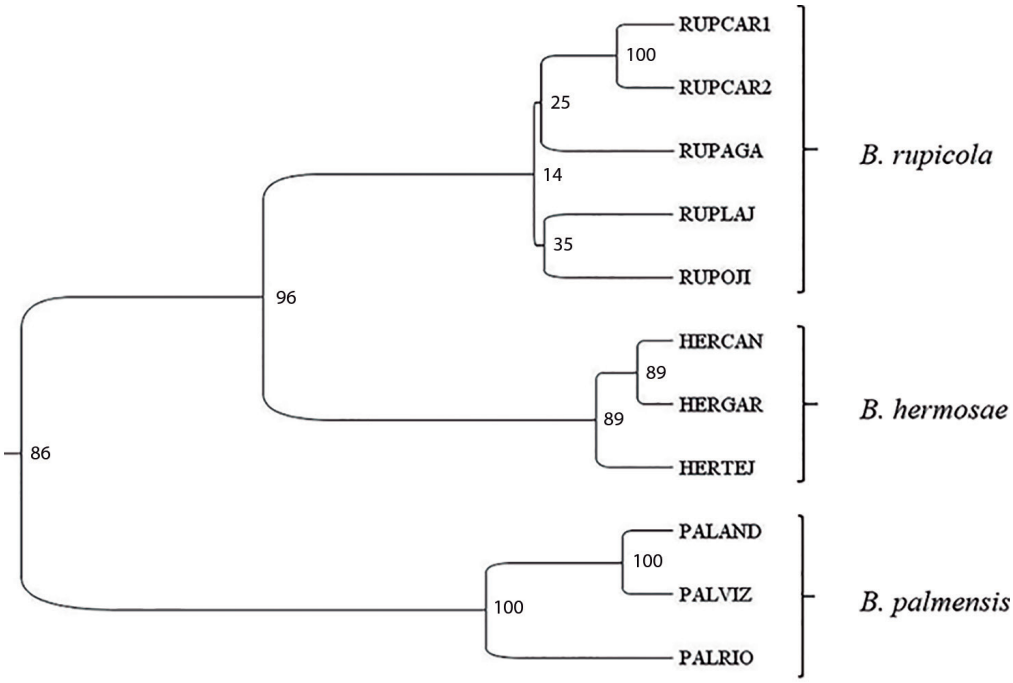


Figure 3.4 UPGMA based on (Nei, 1983) genetic distance among the eleven localities of *Bethencourtia hermosae*, *B. rupicola* and *B. palmensis*. Bootstraps values are indicated on the right of the nodes. Population codes are detailed in Table 3.1.

Including the whole set of samples, the Bayesian structure analysis identified three genetic clusters based on the highest ΔK (K = 3, fig. S.3.1). This is congruent with the other results in the species differentiation, since all individuals were aggregated according to their taxonomic origin with more than 80% of assignation. However, in the analysis for each species individually, we detected that each of the three species was divided into two genetic clusters (K = 2), assuming the admixture model with correlated frequencies (Figure 3.5). In accordance with the AMOVA and F_{ST}, the strongest genetic structure was found in *B. palmensis*, separating the Tenerife population from the ones in La Palma. On the other hand, we did not find a clear structure in *B. rupicola*, while *B. hermosae* presented more assignation in one of the clusters in the HERTEJ population.

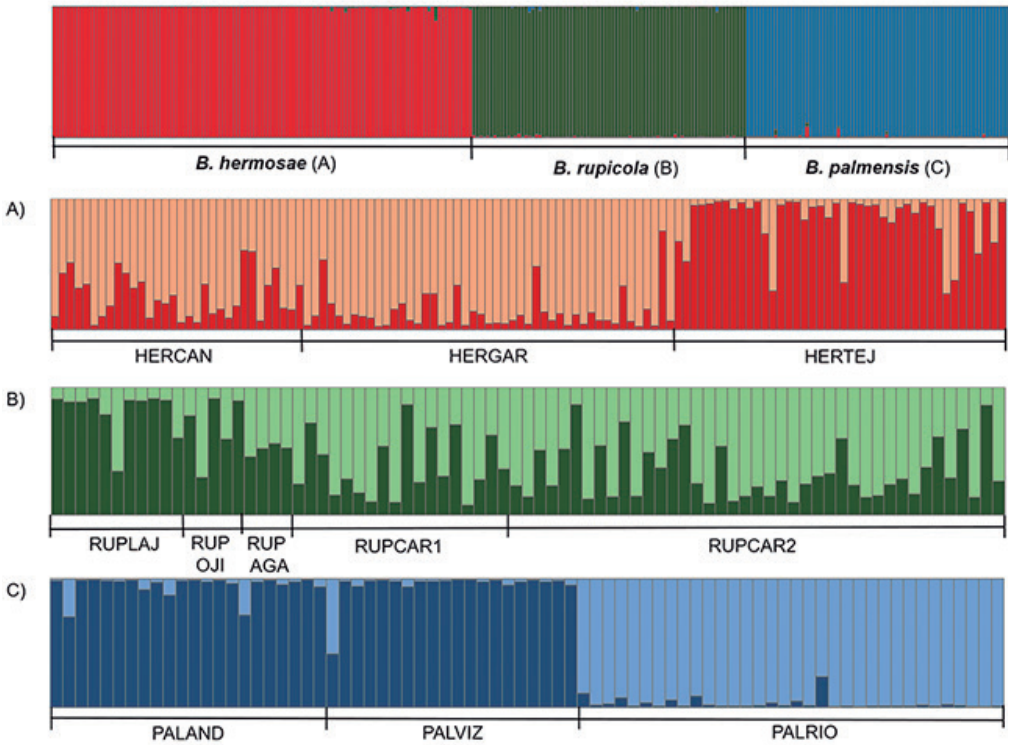


Figure 3.5 Bar plots for the proportion of coancestry inferred from Bayesian cluster analysis implemented on STRUCTURE and CLUMPP. The first plot includes the whole set of *Bethencourtia* samples (K= 3), (A) *B. hermosae*, (B) *B. rupicola* and (C) *B. palmensis* with (K=2) each. Population codes are detailed in Table 3.1.

3.4. DISCUSSION

One of the main objectives of this article was to determine the level of differentiation among populations within *Bethencourtia*, in order to resolve taxonomic uncertainties for conservation purposes, an important issue in conservation Biology (Frankham *et al.* 2002). The results of the analysis for genetic differentiation and structure in the endemic genus *Bethencourtia*, such as the PCoA and UPGMA, strongly reveal that the three species conform to different entities to a high degree, in concordance with their morphological differences (Nordenstam 2006a). The Bayesian analysis implemented in STRUCTURE for the whole set of samples also indicates a clear grouping of the three taxa, along with the results at the species-hierarchical level in AMOVA (45.3% of variation between species). Consistently, pairwise F_{ST} values between species were clearly higher than those between populations within species. Although *B. hermosae* and *B. rupicola* share the same island, the differentiation

levels between these two species are similar to the differentiation between each of them and *B. palmensis*. The high F_{ST} value found between species (F_{ST} = 0.543) reveals the lack of interspecific gene flow.

Similar percentages of differentiation were detected between the different lineages of *Ilex* in Macaronesia, with 36.38% between species (Sosa *et al.* 2013). Within the *Cheirolophus* genus (Asteraceae), a percentage of explanation among taxonomical groups of 38.63% was also found by Vitales *et al.* (2014a). In addition, the results obtained for the differentiation between *B. hermosae* and *B. rupicola* in La Gomera are higher than other related species inhabiting the same island, such as *Crambe tamadabensis* and *C. pritzelii* (8.84 % of the total variation between species) (Soto *et al.* 2016).

The genetic structure at the intraspecific level showed different degrees of homogenization. On the one hand, *B. rupicola* and *B. hermosae* presented a high degree of gene flow among the populations studied. In *B. rupicola*, this is supported by the lack of high assignation of any of its individuals to the two clusters in the STRUCTURE analysis. All results indicate that RUPCAR1 and RUPCAR2 represent the same panmictic unit. This was as expected because they are geographically close in the same phonolitic outcrop. In *B. hermosae*, HERTEJ presented a higher assignation to one cluster and more private alleles. As occurred in *B. rupicola*, the pairwise F_{ST} values and intraspecific variation were low. The anemochorous dispersal syndrome of this group and the reduced geographical distance between localities could be promoting gene flow within species.

On the other hand, in *B. palmensis*, the genetic structure is different: two defined clusters (K = 2) to which the Tenerife (PALRIO) and La Palma populations (PALAND and PALVIZ) were assigned. The AMOVA results at the intraspecific level were higher than in *B. hermosae* and *B. rupicola*, with 22.8 % of the variation found between populations. This was much as expected due to the differences between the islands of Tenerife and La Palma with the ocean acting as a natural barrier. The relatively high F_{ST} values detected between the two islands and the high presence of private alleles in PALRIO also indicate lower levels of gene flow between islands. Although there may be speciation processes taking place in this species due to isolation, we did not find enough evidence to treat the two island populations as different units. Although we only used the PALRIO locality for our research, to test this differentiation,

further studies at the morphological and/or molecular level could include more localities from Tenerife. Indeed, due to their habitat complexity (Mairal *et al.* 2015) high population differentiation is common in the Canary Islands, as well as within the same species between islands (González-Pérez *et al.* 2009a), leading to adaptive radiation in plant genera (Pérez de Paz & Caujapé-Castells 2013). Moreover, dry fruits like those of *Bethencourtia* usually lead to species-rich lineages with divergence between islands (García-Verdugo *et al.* 2014).

According to the selfing rate results from this study, the species in *Bethencourtia* would be partially or totally self-incompatible. This conclusion agrees with Ortega and González (1986), who mentioned low seed viability in *in vitro* cultivated individuals, possibly caused by self-incompatibility. It has also been pointed out that total or partial self-incompatibility is a common trait in island colonizers in the Asteraceae family. When multiple colonization events have been possible, as in the Canaries due to their proximity to the continent (Francisco-Ortega *et al.* 2000; Pérez de Paz & Caujapé-Castells 2013), outcrossing or pseudo-self-compatible ancestors could have provided a higher genetic diversity than selfing ones (Crawford *et al.* 2009). The high homogenization in *B. rupicola* and *B. hermosae* can also be supported by this reproductive system. Furthermore, the HW equilibrium found in almost all populations shows a lack of inbreeding that usually occurs in selfing species (Jarne & Charlesworth 1993).

However, the overall genetic diversity values obtained are low for an outcrossing group. Indeed, the genetic diversity values detected in *Bethencourtia*, specially *B. hermosae* and *B. rupicola* are much lower than in other oceanic endemics in Asteraceae, such as the self-incompatible *Tolpis azorica* (H_E = 0.716) (Silva *et al.* 2016), and *Leontodon filii* (H_E = 0.530) (Dias *et al.* 2014). The values found in *Bethencourtia* were also lower than in other Canarian endemics analyzed with microsatellite markers, such as the insular endemics *Ruta oreojasme* (H_E = 0.687) (Meloni *et al.* 2015), *Parolinia ornata* (H_E = 0.515) (González-Pérez & Caujapé-Castells 2014) and *Silene nocteolens* (H_E = 0.780) (Sosa *et al.* 2011), the critically endangered *Sambucus palmensis* (H_E = 0.500) (Sosa *et al.* 2010) and the related species in the Canary Islands *Senecio chrysanthemifolius* (H_E = 0.700) (Brennan *et al.* 2012).

In addition, significant analysis in BOTTLENECK for HERCAN, HERTEJ and RUPCAR1 indicate that the populations from La Gomera could have

suffered a recent reduction of their effective size. The possible causes of this reduction are difficult to infer, although land use and competition with invasive species may have diminished all populations in La Gomera. In fact, low numbers of seedlings and juveniles have been detected in both species (Gobierno de Canarias 2009).

Outcrossing species with most of their genetic variability within populations are bound to suffer a greater diversity loss due to habitat fragmentation than selfing species (Aguilar *et al.* 2008). Naturally rare species that are inherently associated with specific habitats and endemics that form small populations usually have reduced genetic diversity due to bottlenecks, genetic drift and inbreeding. Moreover, populations with low effective sizes are less capable of confronting external disturbances (Barrett & Kohn 1991; Ellstrand & Elam 1993; Frankham 1998). These rare endemic species are commonly expected to have lower variance than widespread ones (Cole 2003) and insular endemics with less variation than continental species (Frankham 1997; Sosa *et al.* 2011). *Bethencourtia* is a typical case of rare insular endemics with habitat specificity, so low genetic diversity values are to be expected, in consonance with those hypotheses.

Accordingly, further studies of the conservation status of this genus should be considered, with demographic and reproductive studies that would help understand the causes of the bottleneck events and the low genetic diversity found. In this vein, the conservationists of the Garajonay National Park have started to monitor *B. hermosae* and *B. rupicola*, with annual population censuses (Fernández-López & Velázquez-Barrera, 2011). In extreme cases, if the germination in-situ continues to be unsuccessful, in-vitro propagation could be provided for the maintenance of the populations (Ortega & González 1986). Indeed, to maintain and enhance genetic diversity within each species on La Gomera, propagules from all localities should be taken to construct a reservoir in case of stochastic events or decline of the populations in the near future.

In conclusion, reduced population sizes due to habitat specificity, coupled with bottleneck events and the difficulties in finding available mates may be the most important factors affecting genetic diversity in *Bethencourtia*. Consequently, these aspects should be considered when conservation programs are designed.

3.4.1. Implications for conservation

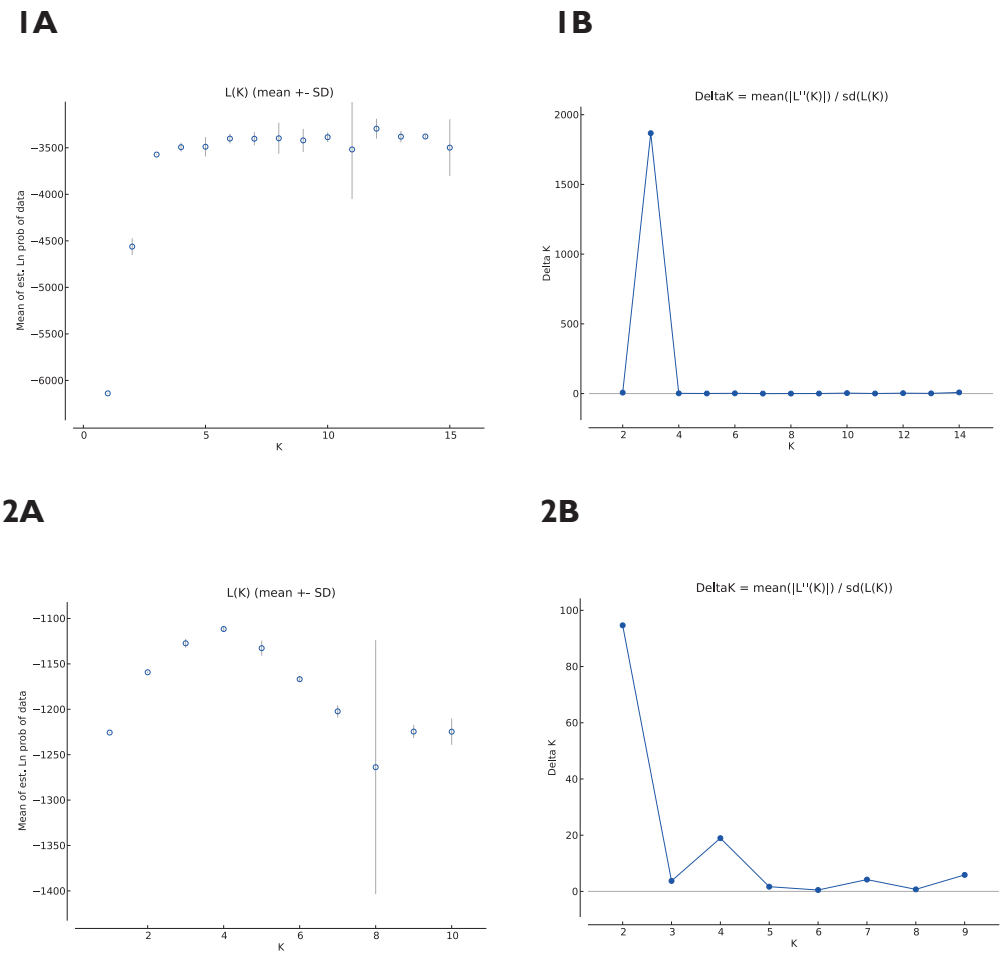
We genetically characterized the endemic genus *Bethencourtia* in the Canary Islands. The delimitation of the three species has been successfully clarified through microsatellite markers, especially needed between *B. hermosae* and *B. rupicola*. Consequently, the inclusion of *B. rupicola* in the official checklists should be of primary importance to its conservation. Certainly, the facts of its reduced geographical distribution and its presence in only five localities with a low number of individuals in Agando and Ojila, are sufficient reasons to catalog this taxon as Endangered or Vulnerable by the IUCN.

Considering that *Bethencourtia* species are possibly self-incompatible, we do not expect 'inbreeding depression' (Ellstrand & Elam 1993), therefore, efforts should be focused on reinforcing the natural populations with individuals belonging to the same area, and preserving the genetic structure which has been found. The population genetic structure within species was low, apart from the divergence in *B. palmensis* between islands. Therefore, conservation strategies ought to be focused on avoiding anthropological translocations among the distinct populations found in this research. First, the transfer of propagules from *B. rupicola* to *B. hermosae* localities should be avoided. In Vallehermoso, the HERTEJ population was found to be distinct with higher genetic diversity, which should be considered for management purposes. Secondly, further attention must also be paid to *B. palmensis* populations, trying to avoid the introduction of individuals from Tenerife to La Palma or vice versa. Homogenization of already differentiated localities could lead to the loss of genetic diversity and interrupt speciation processes.

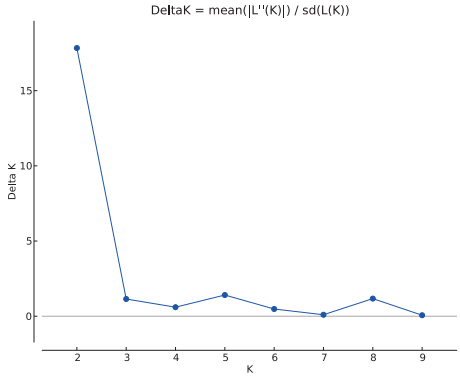
Table S.3.1 Table of pairwise F_{ST} values among *Bethencourtia* populations. Values in bold and italics: $P < 0.05$; only bold: $P < 0.001$; (ns): not significant. Population codes are indicated in Table 3.1 in the manuscript.

	HERCAN	HERGAR	HERTEJ	RUPAGA	RUPLAJ	RUPOJI	RUPCAR1	RUPCAR2	PALAND	PALVIZ
HERGAR	**0.151**									
HERTEJ	**0.114**	**0.144**								
RUPAGA	**0.561**	**0.581**	**0.413**							
RUPLAJ	**0.548**	**0.594**	**0.415**	**0.206**						
RUPOJI	**0.474**	**0.506**	**0.293**	***0.187***	***0.155***					
RUPCAR1	**0.540**	**0.558**	**0.404**	**0.177**	**0.187**	**0.128**				
RUPCAR2	**0.515**	**0.527**	**0.404**	**0.154**	**0.214**	**0.141**	0.011^{ns}			
PALAND	**0.586**	**0.642**	**0.513**	**0.435**	**0.515**	**0.476**	**0.466**	**0.466**		
PALVIZ	**0.608**	**0.648**	**0.534**	**0.482**	**0.553**	**0.511**	**0.500**	**0.500**	**0.056**	
PALRIO	**0.635**	**0.669**	**0.573**	**0.538**	**0.588**	**0.544**	**0.548**	**0.533**	**0.276**	**0.266**

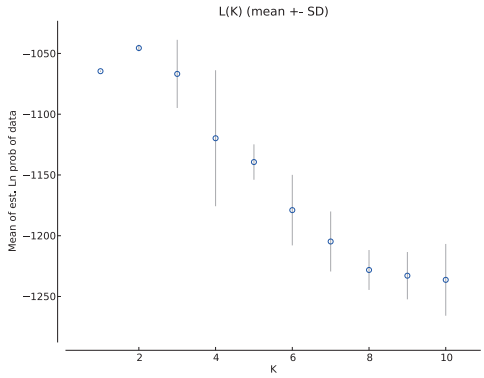
Figure S.3.1. Output results from STRUCTURE HARVESTER. Two graphs are shown for each STRUCTURE analysis: (A) The mean of log-likelihood values for each value of K; (B) Ad hoc statistic based on the rate of change in the log probability of data between successive K values (ΔK, following Evanno *et al.* (2005)). 1A: Log-likelihood for each value of K (1-15) for the whole *Bethencourtia* dataset. 1B: ΔK values for each K for the whole dataset. 2A: Log-likelihood for each value of K (1-10) for *B. hermosae*. 2B: ΔK values for each K for *B. hermosae*. 3A: Log-likelihood for each value of K (1-10) for *B. rupicola*. 3B: ΔK values for each K for *B. rupicola*. 4A: Log-likelihood for each value of K (1-10) for *B. palmensis*. 4B: ΔK values for each K for *B. palmensis*.



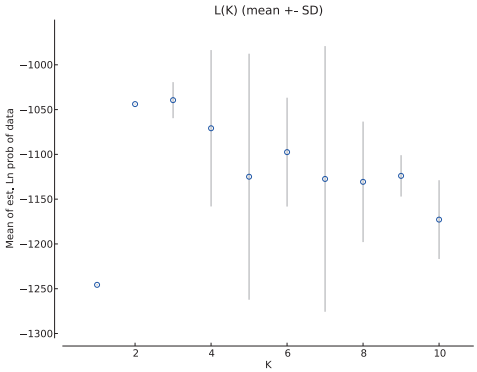
3A


DeltaK = mean(|L''(K)|) / sd(L(K))
Delta K
K

3B


L(K) (mean +- SD)
Mean of est. Ln prob of data
K

4A


L(K) (mean +- SD)
Mean of est. Ln prob of data
K

4B

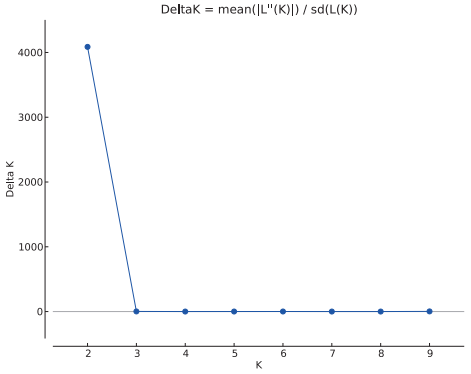

DeltaK = mean(|L''(K)|) / sd(L(K))
Delta K
K

Foto: Claudio Moreno

THE RESTORATION OF *Sambucus palmensis*

4. The restoration of the endangered *Sambucus palmensis:* Genetic assessment and monitoring after 30 years of conservation actions in the Garajonay National Park.

ABSTRACT

The translocation of individuals or the reinforcement of populations are common measures in the genetic rescue of endangered species. *Sambucus palmensis* Link. is a critically endangered endemic present in four of the Canary Islands. During the past 30 years, the Garajonay National Park (La Gomera) has carried out an intensive program of translocations for the recovery of this species in the island. The main propagation technique used was taking cuttings, due to the low germination rates of seeds. We collected 402 samples from all the restored sites and from all known natural individuals. Seven microsatellite markers were used for genotyping, of which four were newly developed. Fifteen localities were identified according to the geographical location of individuals and the needs of the National Park managers.

In addition to identifying the number of genotypes per locality, we also proceeded to estimate changes in diversity and genetic structure after the restoration programs. The results show that there is a high proportion of clone specimens, because of the propagation method, and the natural clonal growth of the species. The observed heterozygosity has increased with the restorations, but there still are private alleles in the natural populations that have not been considered in the reforestation. The population of Liria constitutes a very important genetic reservoir for the species. Except for Liria, which is genetically differentiated, the genetic structure reveals that there is low variation and a high homogenization across localities due to the translocation of genotypes. To optimize future reintroductions from the genetic point of view, we have proposed a list of specimens that are suitable for the extraction of seeds or cuttings in a greenhouse.

4.1. INTRODUCTION

The preservation of endangered plant species usually involves population restorations, either by reinforcement of the extant populations or the reintroduction of new populations. Before starting restoration programs, biological information on the species must be first gathered in order to determine the most important factors limiting the growth of the founding population (Heywood & Iriondo 2003). The biological purpose of the restorations is to increase the species' survival prospects by recovering its evolutionary potential and autonomous ecological behavior (Godefroid *et al.* 2011). These measures often involve translocating genotypes across geographic ranges. This is a very controversial practice in which the need to maximize genetic diversity and avoid inbreeding depression is balanced against the maintenance of coadapted gene complexes (outbreeding depression; Ellstrand & Elam 1993; Storfer 1999; González-Pérez *et al.* 2009b). In this respect, it has been extensively argued that the increasing of gene flow largely improves fitness and evolutionary potential of inbred populations, without high risks of outbreeding depression (Frankham 2015). Nevertheless, many restoration programs have taken place without prior knowledge of the genetic background of the populations. The genetic variability within and between natural populations should be considered before starting reintroductions and translocations of genotypes.

Islands ecosystems are generally threatened either by stochastic causes (inherent to islands) or deterministic causes (related to human activities). The deterministic causes are mainly habitat loss or degradation, introduction of alien species or direct predation (Whittaker & Fernández-Palacios 2007). In Macaronesia, the laurel forest is one of the best representations of this insular vulnerability and it has experienced a major reduction since human colonization. Despite its slow recovery due to the abandonment of agricultural land, the extant laurel forest represents 20 % of its original area (Fernández-Palacios & Whittaker 2008). However, the forests in the Garajonay National Park in La Gomera, are considered to be the best relicts of laurel forest, which have suffered less due to human colonization than in the other islands (Nogué *et al.* 2013).

Sambucus palmensis Link, (*Sambucus nigra* ssp. *palmensis* (Link) R. Bolli) (Sambucaceae), Sauco or Canary elderberry is a rare endemic of the Canarian archipelago and is present in four of the seven main islands: Tenerife, La Gomera, La Palma and Gran Canaria. It grows in the laurel forest vegetation

zone between 600-1000 m a.s.l., with a preference for shady and humid places. It can also be found on the margins of agricultural fields being grown by locals due to its historical use for medicinal purposes (Beltrán *et al.* 1999; Abdala *et al.* 2014). This tree can reach up to 4-6 m, is a hermaphroditic taxon and the fruits can be dispersed by birds (Marrero *et al.* 2011). Also, the species extensively propagates by vegetative reproduction, which has facilitated its reintroduction by cuttings. However, the low regeneration capacity of the species and the high mortality rates of new individuals leads one to think that *S. palmensis* presents reproductive self-incompatibility and inbreeding (Marrero *et al.* 1998).

Sambucus palmensis is recognized as Critically Endangered in the Spanish Red List of Vascular Flora (Moreno-Saiz 2008), while it falls within the category of In danger of Extinction in the Spanish Catalogue of Threatened Species and as Endangered C2a in the IUCN Red List (Marrero *et al.* 2011). In fact, it is one the tree species at highest risk of extinction in the Canary Islands. These considerations are due to the small number of naturally occurring individuals (<100) in the four islands. Other factors that could affect this threatened species are herbivory by goats and rats, habitat loss as well as forest fires and change in land use (Fernández-López & Velázquez-Barrera 2011; Marrero *et al.* 2011). And in fact, signs of a recent bottleneck were found in two populations in La Palma and Tenerife (Sosa *et al.* 2010).

The highest number of individuals are currently found in La Gomera, (1090), distributed in more than 12 localities in the surroundings of the Garajonay National Park. Despite the high number of specimens, only 25 have been considered to be of natural origin, while the rest are the result of restoration programs developed over more than 30 years (Marrero *et al.* 2015). By the 1980s, the populations of La Gomera were reduced to a few individuals, which led to the urgent need of restoration activities (Marrero *et al.* 1998; 2015). Later on, due to a fire that occurred in 2008 in La Gomera which cleared the laurel forest canopy, the locality of Liria went through a population expansion, from four known individuals to the 40 individuals that we analyzed in this article (Fernández-López *et al.* 2014). In the early stages of the conservation program, since germination rates were very low, the reintroduced specimens were obtained through vegetative propagation by cuttings (Marrero *et al.* 1998). Surprisingly, after these restorations, sexual propagation has been detected in good years, increasing the size of the restored sites (Marrero *et al.* 2015). Consequently, individuals obtained by cuttings or descendants of old

reintroductions have been translocated to other localities, possibly leading to a homogenization of the genetic structure. Although there has been a significant increase in the number of individuals, the genetic background has not been considered in any of the restorations. In the previous study of *S. palmensis* covering its entire distribution (Sosa *et al.* 2010), low genetic diversity, a high number of identical genotypes and overall exclusive alleles were found in La Gomera. A strong connection with Tenerife was also detected, possibly due to the historical transfer of individuals between islands. However, in the study by Sosa *et al.* (2010), only three markers were polymorphic for La Gomera, which could have led to misinterpretation of the number of genotypes.

The aims of this study were (1) to estimate changes in genetic diversity after the restoration programs in La Gomera, (2) to identify the current genetic structure and the number of identical genotypes in the populations and (3) to provide a genetic background for future conservation programs in the Garajonay National Park (La Gomera), performing a more extensive sampling and the inclusion of more polymorphic markers.

4.2. MATERIAL AND METHODS

4.2.1. Sample collection

Leaf samples from all the naturally occurring individuals (47), present in the localities of Acebiños, Ancón de Candelaria, Liria, Meriga and Presa de Las Rosas, and a significant representation of the reintroduced individuals from the whole distribution in La Gomera (355) were collected during 2012 and 2013. The individuals from Liria and Presa de Las Rosas are all natural, with no reintroduced individuals. In total, 402 samples were collected and genotyped for the study. They are distributed in 15 sampling sites according to their geographical distribution or management area for the National Park. The distribution of the individuals and the sampling sites are represented in Figure 4.1. Young leaves for all specimens were collected and stored in plastic bags with silica gel for their conservation.

4.2.2. Microsatellite development and genotyping

In this study, seven SSR markers were used for the genotyping of *Sambucus palmensis* individuals in La Gomera. Five markers that were developed for *S.*

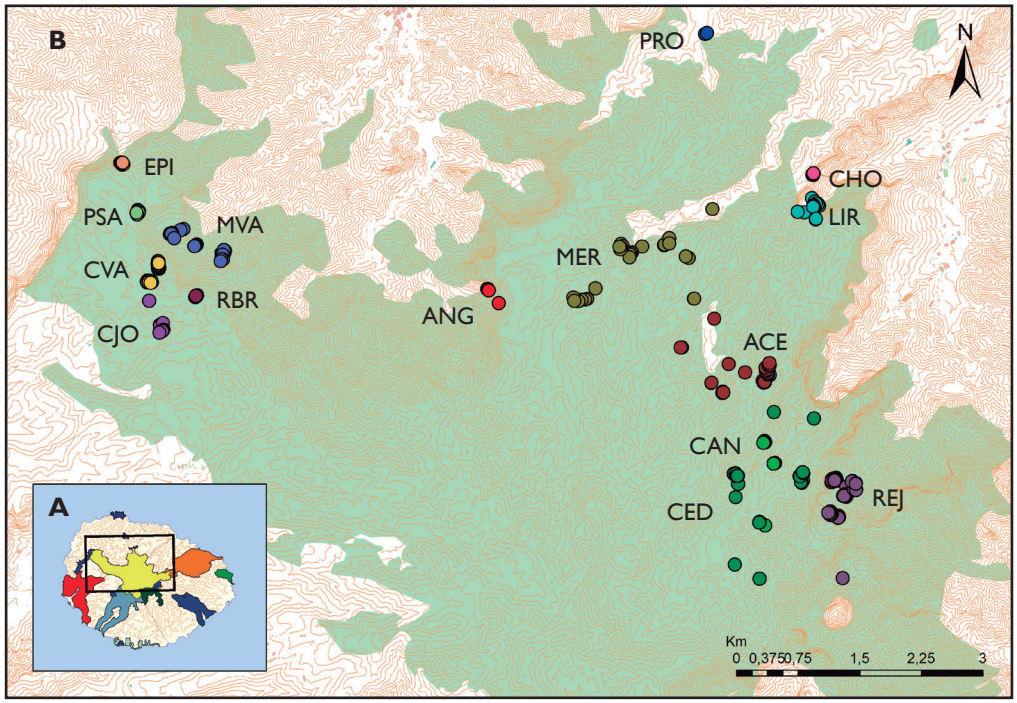


Figure 4.1 (A) La Gomera Island with the representation of natural protected areas (Garajonay National Park, in yellow) (B) Map of the distribution of the *Sambucus palmensis* individuals sampled. The 15 areas described for population management are indicated, see the locality codes in Table 4.2.

nigra (Clarke & Tobutt 2006) and characterized for *S. palmensis* (Sosa *et al.* 2010) were tried for our set of samples. However, only three of these markers (EMSn017, EMSn025, EMSn003) yielded enough polymorphism in the La Gomera populations to be considered. Therefore, we developed four new microsatellite markers for *S. palmensis* to improve the accuracy of the genotypes, coded as Sam_Tet2, Sam_Hex2, Sam_Hex1 and Sam_Tri8 (Table 4.1)

DNA was extracted from silica-gel-dried young leaves using a modified 2 x CTAB protocol (Doyle & Doyle 1987). DNA was subsequently purified with the commercial kit Gene Elute PCR Clean-up (Sigma). After the characterization of the microsatellites, the whole set of samples was extracted with Invisorb DNA Plant HTS 96 KIT INVISORB.

Microsatellite loci were selected from an Illumina paired-end shotgun library developed by the company AllGenetics (University of A Coruña) using three probe mixes of *S. palmensis*. We initially chose 20 primer pairs from this library of which 16 yielded some product and were labelled. Finally, four primer pairs amplified consistently with more than two alleles and were

Locus	EMBL ID	Motif	PCR primer sequence (5' -> 3')	Range (bp)	Dye
EMSn017	AM086423	$(AG)_{21} – (AG)_{24}$	F: GGTATTGCTTGAACAATCATCG R: GCCTTTTGCCCAAACTATCC	202-206	6-FAM ‡
EMSn025	AM086426	$(AC)_{13} – (AC)_{8}$	F: AATGCATCGCAAGAAAAAGG R: GGTAAGATAAATGATACAATGTTTTGG	191-197	6-FAM *
EMSn003	AM086420	$(CT)_{11}$	F: TCGTCTTTTCCGACTCTAAAGC R: CTGGACATTTGCGATCTGG	202-224	NED *
Sam_Tet2	LT600693	$(AGAT)_{12}$	F: AAATGCACTGAACAGTGGTTGA R: CCCTAGTCCTCCAACCCATC	118-146	VIC ‡
Sam_Hex2	LT600694	$(AATAAC)_{6}$	F: TGATGATGGTTGTTGGTAGATCA R: GGCAGAATTCTAGGGCCAGT	218-224	PET ‡
Sam_Hex1	LT600695	$(AGAGGT)_{5}$	F: GCAGTGGTGGAAGAGATTGC R: AAATTTGCATAGGGCAGCAC	199-211	NED ‡
Sam_Tri8	LT600696	$(AAC)_{21}$	F: AATCCCGACACAACCTCAAA R: CGGTGGTAGAGCAAGTGAGG	100-127	PET *

Table 4.1 Characteristics of the 7 microsatellite markers implemented for *Sambucus palmensis* in La Gomera. EMBL ID: Accession number per locus. Dye: Name of each fluorescent dye per locus. Multiplex; ‡Load A, *Load B. F: Forward. R: Reverse.

selected to complete the genotyping for all samples. For the initial testing, PCR (polymerase chain reaction) was conducted individually with each primer pair in a 25 uL total reaction volume, which contained approximately: 20 ng of DNA 10 pmol of each primer, as well as PCR Master Mix until 25 uL were completed (Reddy-Mix, ABgene, Surrey, UK). Amplifications were performed using the following conditions: 3 min denaturation at 95°C, 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 50-62°C, and 72°C for 1.5 min; followed by 5 min elongation at 72°C. Reverse primers were color-labelled at the 5'-end with 6-FAM, PET, NED or VIC.

Once the new markers where characterized, we conducted the subsequent multiplex amplifications for the seven primer pairs using the QIAGEN Multiplex Kit (QIAGEN). PCR were performed in 15 µL reaction volumes: 7.5 µL of Multiplex PCR Master Mix, 1.5 µL primer mix (containing 0.2 µM of each primer in TE), 1.5 µl of Q-solution, 20-40 ng of DNA and dH_20. Multiplexing was carried out in two primer groups as indicated in Table 4.1. Following the manufacturer's instructions, PCRs consisted of a Touchdown protocol with the thermal conditions: 15 min at 95°C, 10 cycles of 30 s at 94°C, annealing for 90 s at 65°C with a decrease of 0.5°C per cycle and 60 s at 72°C, followed by 20 cycles of 30 s at 94°C, annealing for

90 s at 55°C and 60 s at 72°C, with a final extension of 30 min at 60°C. All the products from both simple and multiplex PCR were detected on an ABI 3730 Genetic Analyzer and fragments were sized against the LIZ (500-250) size standard (Applied Biosystems, Inc.) and visualized using GENEMAPPER 4.0 (Applied Biosystems, Inc.). We identified allele peak profiles at each locus and assigned a genotype to each individual.

4.2.3. Statistical analysis

To estimate the incidence of clonality and identify the different genotypes for *S. palmensis* in La Gomera, multilocus matches were identified with GENALEX 6.5 (Peakall & Smouse 2012). With the same software, basic genetic diversity indices: average of alleles per locus (*A*); number of private alleles (*PA*), rare alleles (present in four localities or less; *RA*), observed (H_O), and unbiased expected (H_E) heterozygosities for each locus and locality were estimated. Measures of allelic (A_R) and private allelic richness (PA_R) were calculated using HP-RARE 1.0 (Kalinowski 2005), which uses rarefaction to correct for sampling error. To detect differences in genetic diversity before and after the restoration programs, the indices calculated for each locality were also estimated for the restored and natural individuals separately. Individual heterozygosity was also calculated to provide a list of the most suitable individuals for restoration programs, in order to increase the fitness of the founder individuals and their offspring (Engelhardt *et al.* 2014). On this list, individuals with unique genotypes, a high individual heterozygosity and presence of rare alleles were identified.

Estimation of null alleles for each locality was carried out with MICROCHECKER 2.2.3 (Van Oosterhout *et al.* 2006). BOTTLENECK 1.2.02 software was used to identify any recent genetic drift events in the original set of individuals. (Cornuet & Luikart 1996). The two-phase mutation model (TPM), which is a modification of the stepwise mutation model (SMM), was implemented and shows to be a better fit for most microsatellite data sets (Piry *et al.* 1999). In the TPM model, to optimize the most sensitive values for microsatellites, a proportion of SMM in the TPM = 0.00 and a variance of the geometric distribution for TPM = 0.36 were chosen.

Allele frequency information was analyzed using a nested analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) with ARLEQUIN 3.5. The analyses

were conducted with two different approaches, with the individuals being grouped either by locality or their origin (natural versus restored). In addition, a principal coordinate analysis (PCoA), using the covariance standardized method of pairwise codominant genotypic distances among individuals, was implemented with GENALEX 6.5.

To estimate the current genetic structure of the populations, all the genotypes were screened using a Bayesian admixture procedure with the software STRUCTURE 2.3.4 (Pritchard *et al.* 2000). The model was assumed to be of population admixture and correlated allele frequencies. Ten independent runs were conducted for each value of K (from 1 to 15) and analysis consisted of a 10^5 burn-in period replicated and a run length of 10^6 replicates. The optimal number of clusters was found by the ΔK method (Evanno *et al.* 2005) visualized with STRUCTURE HARVESTER (Earl & vonHoldt 2012). Results of 10 replicates of the best fit K were processed using CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007) to determine the optimal clustering. The STRUCTURE HARVESTER results for the election of the optimal K are presented in the Figure S.4.1.

4.3. RESULTS

All microsatellite markers used for this study yielded enough polymorphism to identify the possible number of genotypes in the sampling sites analyzed and to detect possible identical genotypes. Out of the 402 individuals sampled, 147 different genotypes were found. 84 of these were unique genotypes (from only one individual), and 63 were shared genotypes (from more than one individual) (Table 4.2). We found some genotypes corresponding to a high number of individuals, for example the genotype JJ that matched 81 samples, the genotype V that matched 30 genotypes and the genotype UU matched with 15 individuals. The remaining genotypes matched between 2 and 10 individuals each. A detailed list of genotypes per locality is shown in Table S.4.1. The sampling site with the highest number of unique genotypes was the locality of Liria (29), where all individuals are natural, followed by the restored sites of El Rejo (18) and Meriga (11). The percentages of unique genotypes per locality were also higher in Presa de las Rosas (100 %) and Liria (82.86 %) than in the restored sites, which ranged from 0.00 % to 66.67 %.

Locality	Acronym	N	Natural individuals	Total number of genotypes	Shared genotypes	Unique genotypes	% of unique genotypes
Acebiños	ACE	31	1	25	21	4	16.00
Ancón de Candelaria	CAN	17	1	6	5	1	16.67
Angola	ANG	5	-	5	4	1	20.00
Cañada Jorge	CJO	13	-	1	1	0	0.00
Cordillera Vallehermoso	CVA	25	-	22	16	6	27.27
El Cedro	CED	35	-	21	17	4	19.05
El Chorrillo	CHO	9	-	2	2	0	0.00
El Rejo	REJ	129	-	58	40	18	31.03
Epina	EPI	13	-	13	12	1	7.69
Liria	LIR	40	40	35	6	29	7.00
Meriga	MER	36	3	26	15	11	42.31
Meseta Vallehermoso	MVA	38	-	18	15	3	16.67
Palo que salta	PSA	7	-	7	5	2	28.57
Presa Las Rosas	PRO	2	2	2	0	2	100.00
Raso de La Bruma	RBR	3	-	3	1	2	66.67
Average per locality		26.9	9.4	16.2	10.6	5.6	31.71
Total		402	47	147	63	84	57.14

Table 4.2 *Sambucus palmensis* localities sampled in La Gomera. N = sample size. The number of natural individuals, shared and unique genotypes per locality are indicated. Localities were grouped according to their geographical distribution or management area required by the National Park.

Basic genetic diversity indices, such as allelic richness and expected heterozygosity values were similar across localities (Table 4.3). The expected heterozygosity ranged from 0.357 (Presa de las Rosas) to 0.495 (Raso de la Bruma). The observed heterozygosity values did show higher differences across localities, ranging from 0.366 (El cedro) to 0.714 (Cañada Jorge). Liria and Meriga were the only sites with private alleles, and Liria presented the highest rarefied private allelic richness at 0.26. Moreover, eight localities

Locality	*N*	*A*	*PA*	*RA*	A_R	PA_R	H_O	H_E
ACE	31	2.71	-	1.00	1.73	0.02	0.406	0.373
CAN	17	2.86	-	2.00	1.95	0.04	0.605	0.491
ANG	5	2.14	-	-	1.87	0.00	0.600	0.467
CJO	13	1.71	-	-	1.65	0.00	0.714	0.371
CVA	25	2.57	-	-	1.88	0.02	0.606	0.466
CED	35	2.57	-	-	1.71	0.01	0.366	0.368
CHO	9	2.14	-	-	1.74	0.00	0.698	0.415
REJ	129	2.86	-	2.00	1.93	0.05	0.549	0.479
EPI	13	2.14	-	-	1.89	0.00	0.692	0.486
LIR	40	2.29	3.00	5.00	1.73	0.26	0.421	0.376
MER	36	2.71	1.00	1.00	1.7	0.03	0.425	0.359
MVA	38	2.86	-	2.00	1.94	0.03	0.564	0.484
PSA	7	2.29	-	1.00	1.71	0.03	0.469	0.364
PRO	2	1.57	-	2.00	1.57	0.23	0.500	0.357
RBR	3	2.14	-	-	1.93	0.00	0.667	0.495
Average	26.9	2.37	0.27	1.07	1.80	0.05	0.552	0.423
All individuals	402	3.71	-	-	-	-	0.519	0.462
Restored	355	3.14	2.00	-	2.82	0.16	0.532	0.462
Natural	47	3.43	4.00	-	3.43	0.77	0.426	0.402

Table 4.3 Genetic diversity indices for *Sambucus palmensis* in La Gomera. Localities are coded as in Table 4.2. *N* = sample size; *A* = average of alleles per locus; *PA* = number of private alleles; *RA* = rare alleles (present in 4 localities or less); A_R = rarefied allelic richness; PA_R = rarefied private allelic richness, H_O = observed heterozigosity; H_E = unbiased expected heterozygosity.

presented rare alleles, which were present in four localities or less (Table S.4.2). Between restored and natural groups, the natural group displayed higher allelic richness and a greater presence of private alleles, but a lower observed heterozygosity than in the restored groups. The results of tests to detect recent bottleneck events in the natural individuals, considering them as a single population, were not significant for any of the tests implemented. Only the locus Sam_Hex2 presented evidence of null alleles in El Cedro, El Rejo and Meseta Vallehermoso.

AMOVA results were similar in the two approaches tested (Table 4.4). In both cases, the variance between individuals within groups was higher than that between groups. The variance was 2.5 % and 2.9 %, between localities and between the natural and restored groups respectively. This low variance shows the lack of differentiation between all the localities studied. As with the AMOVA results, the PCoA, with a total explanation of 62.06 % did not reveal a clear aggrupation between natural and restored individuals (Figure 4.2).

Source of variation	Degrees of freedom	Sum of squares	Variance of components	Percentage of variation	F-statistics
All localities					
Among groups	14	14.7	0.012	2.5	
Within groups	789	370.0	0.469	97.5	
Total	803	384.6	0.481		0.025***
Natural vs. Restored					
Among groups	1	2.9	0.014	2.9	
Within groups	802	381.8	0.476	97.1	
Total	803	384.6	0.490		0.029***

Table 4.4 AMOVA analysis for *Sambucus palmensis* in La Gomera. Individuals were grouped according to their locality and origin (natural or restored). ***$P < 0.001$

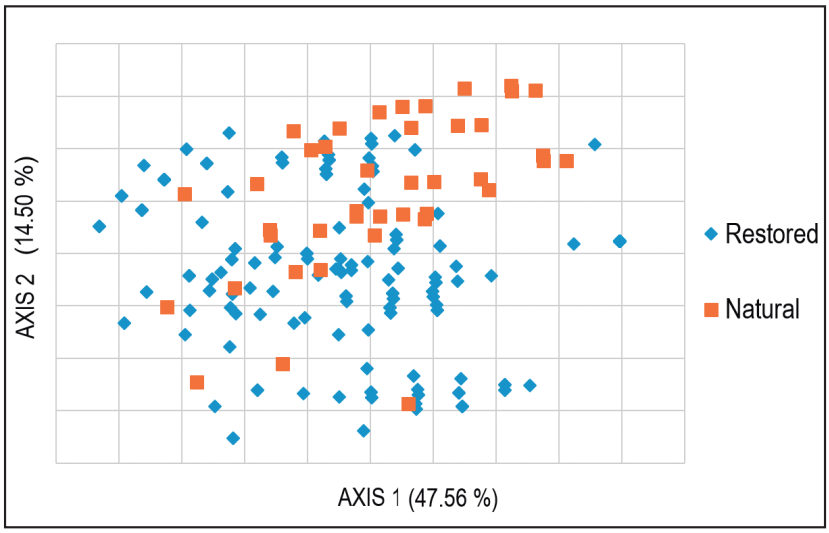


Figure 4.2 Principal coordinate analysis (PCoA) for all *Sambucus palmensis* individuals sampled in Garajonay National Park (La Gomera). The individuals were represented according to their origin (restored or natural). The first two axes explained 62.06 % of the total variation. Percentage of variation for each axis are indicated within brackets.

In the Structure analysis, two possible best values for K were found according to the ΔK and the mean of log-likelihood values (K=2 and K=5) (Figure S.4.1). Therefore, both possibilities are shown in Figure 4.3. In K=2, all individuals were admixed in the two clusters described with evident lack of genetic structure. In the representation of K=5, Liria was the only locality with a high assignation to a single cluster. The other localities presented admixture of the five clusters, except for Cañada Jorge, but in that locality, all individuals shared the same genotype (Table 4.2).

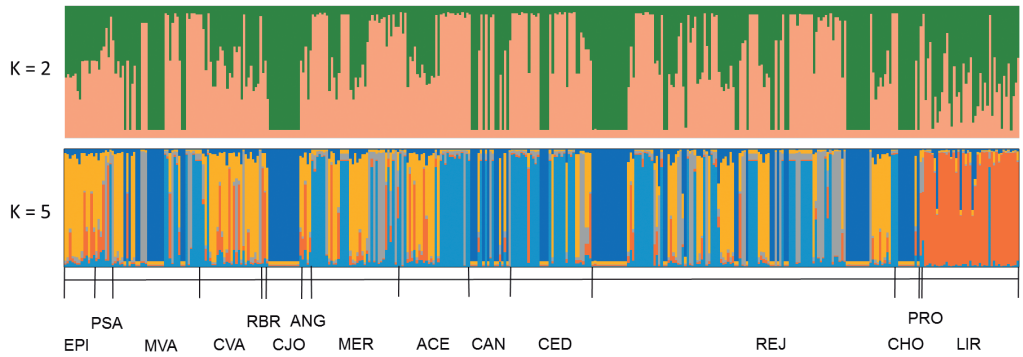


Figure 4.3 Bar plots for the proportion of coancestry inferred from Bayesian cluster analysis implemented on STRUCTURE and CLUMPP. The first plot includes all the individuals grouped in K = 2 and the second one in K = 5 following the STRUCTURE HARVESTER results shown in Figure S.4.1. Locality codes are indicated in Table 4.2

Finally, we proposed a list with the 25 most suitable individuals with which to build a conservation genetic program to enhance the genetic variability of the populations (Table S.4.3). Individuals with unique or rare genotypes, which also presented a high individual heterozygosity and private or rare alleles were considered.

4.4. DISCUSSION

Our results suggest that the restoration programs of *Sambucus palmensis* in La Gomera have greatly improved the genetic status of this species in the island. Although there is a high proportion of clonal specimens, natural regeneration has occurred in the restored sites, generating new genotypes and alleles that were not present in the original populations. Nonetheless, there are still some

major concerns in the conservation of *S. palmensis*, such as the difficulties in sexual reproduction, or the high mortality rates of young plants.

The genetic diversity estimates for La Gomera are higher than those reported by Sosa *et al.* (2010). This is an expected result due to the increase in the number of polymorphic microsatellite markers. In the aforementioned study, only five markers were included, with 80 % of polymorphism in La Gomera. Moreover, the high number of samples taken for this more detailed survey are a better representation of the real populations. Despite the new findings, the genetic diversity in La Gomera might possibly still be lower than in Tenerife and La Palma, whose populations have not yet been analyzed with the new set of markers.

Since the article by Sosa *et al.* (2010) was published, the knowledge of genetic diversity measured with microsatellite markers in oceanic endemics has significantly increased. Therefore, we are now able to confirm that *S. palmensis* presents moderate levels of genetic diversity for a rare endemic (Sosa *et al.* 2011). Overall, outcrossing species present higher diversity than selfing species (Hamrick & Godt 1996; Pérez de Paz & Caujapé-Castells 2013). In comparison with other self-incompatible species, the rare endemics *Bethencourtia hermosae* (Rodríguez-Rodríguez, under review) or *Limonium macrophyllum* (Jiménez *et al.* 2017) presented very low expected heterozygosity levels. But those species are single-island endemics and habitat-restricted. *Sambucus palmensis* is present in four islands and was probably even more widespread in the past (Beltrán *et al.* 1999; Sosa *et al.* 2010). In this respect, *S. palmensis* revealed similar heterozygosity values to the widespread dioecious palm *Phoenix canariensis* (Saro *et al.* 2015) or the laurel forest tree *Ilex canariensis* (Sosa *et al.* 2013).

4.4.1. Effects of restoration activities on genetic diversity and structure

Because of the main propagation method, by the reintroduction of cuttings, we have detected a high presence of clonal specimens. Just like its relative *S. nigra*, *S. palmensis* also presents natural vegetative reproduction (Marrero *et al.* 1998; Bañares *et al.* 2003). Therefore, natural clones can be found in Liria, which presented 17.14 % of shared genotypes. But the percentage of shared genotypes in the other populations that have been restored is much higher, due to the extensive reintroduction of clone specimens. On the other hand, natural regeneration has been detected in some of the localities, with the

appearance of new unique genotypes and private alleles in the restored sites. Nonetheless, the private alleles that are present in the restored sites could have come from natural individuals which are now dead and not included in this study. Therefore, the translocation of genotypes may be increasing the chances of sexual reproduction, as has already been detected in some of the restored sites (Marrero *et al.* 2015).

The percentages of variation found between localities are low for an outcrossing species (Hamrick & Godt 1996), and they are also lower than that found by Sosa *et al.* (2010) among populations within islands (15%). The variation values detected are also lower than those found for the relative *Viburnum treleasi*, which has low variation among populations and also presents both sexual and clonal reproduction (Moura *et al.* 2013). Therefore, there is evidence that the admixture of genotypes across populations have favored the gene flow across the habitat range of *S. palmensis* in La Gomera. In the STRUCTURE results, Liria, which represents the best-conserved natural population in La Gomera, was the only locality that presented less admixture of individuals. The high number of unique genotypes and the presence of private alleles in Liria suggest that these individuals have been rarely used as a genetic source in the restoration programs. Although we did not find a sign of recent bottleneck events in the natural source, most of the individuals came from Liria, which has naturally increased its population size since the monitoring programs started. Thus, bottleneck events in the other natural localities are difficult to infer, as only a few individuals have remained.

Overall, there was a light increase in the observed heterozygosity in the restored sites. These results, together with the high admixture found in the genetic structure, also explain the artificial gene flow implemented with the restorations. Outcrossing of inbred isolated populations is playing a major role in the genetic rescue of endangered species (Love Stowell *et al.* 2017), but a balance between genetic rescue and "outbreeding depression" must be found in the management of populations, paying attention to the particular needs in each case (Hufford & Mazer 2003). In addition, the habitat and climatic continuity of the laurel forest in La Gomera, together with the outcrossing system of the species could have favored the gene flow between the past populations of *S. palmensis*, hindering high population differentiation or local adaptation. Even if the translocations of genotypes have led to outbreeding depression, the advantages of outcrossing can be greater,

especially for self-incompatible species, as it increases the chances of finding available mates (Willi *et al.* 2007; Pickup & Young 2008). Despite the increased observed heterozygosity, the number of alleles (the average of alleles per locus and allelic richness) are still lower in the restored individuals compared to the natural ones. These results can be taken as positive, because they indicate that the restored sites can still benefit from a greater outcrossing and admixture with the natural individuals within La Gomera.

4.4.2. Recommendations for conservation actions

In conclusion, one of the main purposes of restoration ecology is to simulate the characteristics of the natural populations (Pavlik 1996) and the restored sites do not show diminished levels of genetic diversity compared to the original populations, despite the high number of clonal specimens. Nonetheless, to improve the sexual regeneration in future reintroductions, we encourage further studies of the reproductive biology of *S. palmensis*. The detection of the possible causes of self-incompatibility would help to increase the level of available mates and therefore gene flow and offspring. Also, more demographic studies such as that carried out in Meseta Vallehermoso (Marrero *et al.* 2015), will help to monitor the fitness and survival of restored sites over time. The combination of demographic and genetic studies is vital to ensure the recovery of endangered species (Oostermeijer *et al.* 2003).

As an urgent measure to continue to improve the genetic diversity of *S. palmensis* in La Gomera, we have already provided a list to the Garajonay National Park managers with the best candidates for a conservation genetic program (Table S.4.3). We also believe that it is important to avoid the genotypes detected in this study that have been extensively used in some sites. As indicated in Vergeer *et al.* (2004), we would also suggest increasing the number of unrelated seed producer individuals to create sustainable and viable populations, which would also avoid inbreeding processes. Since propagation by cuttings is the most viable way of reintroducing new individuals, the consideration of all individuals with unique genotypes for future reintroductions is also a conservation measure to be taken into account. Although it is possible that individuals from Tenerife were introduced in the past (Sosa *et al.* 2010), we consider that there is enough genetic variability in La Gomera to continue the restoration programs using the genotypes that are currently present on the island.

On a long-term basis, this case study will provide a great deal of information regarding the consequences of restoration actions in self-incompatible clonal species. Moreover, these results can serve as a guideline for the restoration programs in Tenerife, La Palma and Gran Canaria, whose island governments are also restoring *S. palmensis* populations. For example, in Gran Canaria, only two naturally occurring individuals and some cultivars were found prior to the reintroductions. Therefore, a better knowledge of the genetic background of the restored individuals would increase the success and long survival of the populations.

Table S.4.1 Genotypes per locality found for *Sambucus palmensis* in La Gomera. Shared genotypes are coded with letters from A to ZZ.

Genotypes	ACE	CAN	ANG	CJO	CVA	CED	CHO	REJ	EPI	LIR	MER	MVA	PSA	PRO	RBR	Nº localities	Nº individuals
Unique	4	1	1		6	4		18	1	29	11	3	2	2	2	13	84
A											1	2				2	3
AA						1			1							2	2
AAA	1							1								2	2
B		1						3								2	4
BB	1								1			1	1			4	4
BBB	1					2		1								3	4
C								1			2					2	3
CC						2										1	2
CCC	2					1		1								3	4
D								2								1	2
DD	1										1					2	2
DDD	1							1								2	2
E		1						2								2	3
EE	1					1					1					3	3
EEE	1				1	1										3	3
F								2								1	2
FF					1			1								2	2
FFF										2						1	2
G												2				1	2
GG								1	1							2	2
GGG										2						1	2

Genotypes	ACE	CAN	ANG	CJO	CVA	CED	CHO	REJ	EPI	LIR	MER	MVA	PSA	PRO	RBR	Nº localities	Nº individuals
H					1			1	1							3	3
HH					1			2			1					3	4
HHH								2								1	2
I								2								1	2
II								2			1					2	3
III									1						1	2	2
J	1											1				2	2
JJ		9		13		4	8	34				13				6	81
JJJ						1		1	1			2				4	5
K					1							2				2	3
KK	2								1							2	3
KKK										2						1	2
L					1			2								2	3
LL	1				1			1								3	3
M								2								1	2
MM								2				1				2	3
N			1						1		2					3	4
NN						2		2	1							3	5
O					1			1								2	2
OO	1							2								2	3
P					3			2			1					3	6
PP	1							3		1	2					4	7
Q					1			1			1					3	3

Genotypes	ACE	CAN	ANG	CJO	CVA	CED	CHO	REJ	EPI	LIR	MER	MVA	PSA	PRO	RBR	Nº localities	Nº individuals
QQ	2					1		2				1				4	6
R								1			1					2	2
RR										2						1	2
S	1					1		1				1	1			5	5
SS										2			1			2	3
T	1							2								2	3
TT	2					5		1				1				4	9
U					1	1										2	2
UU	2				1	3		6				3				5	15
V	2	3				1		14			7	3				6	30
VV						1		2								2	3
W						2		1			2					3	5
WW					1		1		1							3	3
X	1										1					2	2
XX			1					1	1			1				4	4
Y		2	1		1								1			4	5
YY	1		1		1								1			4	4
Z								1	1			1				3	3
ZZ					2			1			1					3	4
Nº of individuals	31	17	5	13	25	34	9	129	13	40	36	38	7	2	3		402
Nº of shared genotypes	21	5	4	1	16	17	2	40	12	6	15	15	5	0	1		
Total genotypes	25	6	5	1	22	21	2	58	13	35	26	18	7	2	3		

Locus	Allele	ACE	CAN	ANG	CJO	CVA	CED	CHO	REJ	EPI	LIR	MER	MVA	PSA	PRO	RBR
Sam_Tet2	**118**										**0.013**				**0.500**	
	130	0.065	0.059			0.020	0.015		0.066			0.083	0.066			
	134	0.258	0.471	0.300	0.500	0.460	0.235	0.444	0.411	0.462		0.458	0.395	0.286	0.500	0.167
	138	0.677	0.471	0.700	0.500	0.520	0.750	0.556	0.523	0.538	0.763	0.458	0.539	0.714		0.833
	146										***0.225***					
EMSn017	**202**	0.903	0.706	0.800	0.500	0.800	0.882	0.556	0.733	0.769	0.800	0.875	0.776	1.000	1.000	0.500
	204		**0.029**						**0.039**				**0.013**			
	206	0.097	0.265	0.200	0.500	0.200	0.118	0.444	0.229	0.231	0.200	0.125	0.211			0.500
Sam_Hex1	**199**	0.597	0.824	0.600	1.000	0.640	0.574	0.944	0.767	0.615	1.000	0.708	0.737	0.714	0.500	0.667
	211	0.403	0.176	0.400		0.360	0.426	0.056	0.233	0.385		0.292	0.263	0.286	0.500	0.333
Sam_Hex2	**218**	0.758	0.353	0.500		0.560	0.691	0.056	0.531	0.500	0.438	0.792	0.500	0.643	0.750	0.500
	224	0.242	0.647	0.500	1.000	0.440	0.309	0.944	0.469	0.500	0.563	0.208	0.500	0.357	0.250	0.500
Sam_tri8	**100**										***0.225***					
	106	**0.016**									**0.088**				**0.500**	
	115		**0.029**						**0.031**				**0.013**	**0.071**		
	121	0.839	0.618	0.900	0.500	0.660	0.853	0.556	0.671	0.731	0.688	0.778	0.645	0.786	0.500	0.833
	124	0.032	0.029			0.060	0.029		0.035			0.028	0.053			
	127	0.113	0.324	0.100	0.500	0.280	0.118	0.444	0.264	0.269		0.194	0.289	0.143		0.167
EMSn025	**191**	0.129	0.294	0.300	0.500	0.180	0.088	0.500	0.244	0.308		0.069	0.237	0.071		0.167
	192										***0.175***					
	195	0.871	0.706	0.700	0.500	0.820	0.912	0.500	0.756	0.692	0.825	0.917	0.763	0.929	1.000	0.833
	197											***0.014***				
EMSn003	**202**	0.145	0.353	0.300	0.500	0.140	0.088	0.500	0.202	0.269	0.375	0.097	0.237	0.214		0.500
	210	0.065	0.265	0.200	0.500	0.280	0.118	0.444	0.229	0.231	0.313	0.097	0.224	0.143		0.167
	220	0.613	0.353	0.500		0.520	0.559	0.056	0.473	0.500	0.313	0.736	0.434	0.571	1.000	0.333
	224	0.177	0.029			0.060	0.235		0.097			0.069	0.105	0.071		

Table S.4.2 Allele frequencies for the seven microsatellites studied in the *Sambucus palmensis* localities in La Gomera. Private alleles per locality are in bold and italics. Rare alleles (present in 4 localities or less) are in bold. Locality codes are indicated in Table 4.2.

ID	Locality	Genotype code	H-ind
732	ACE	25	0.500
677	CAN	B	0.625
890	REJ	8	0.500
876	REJ	9	0.750
885	REJ	18	0.625
912	REJ	21	0.625
935	REJ	41	0.625
817	LIR	3	0.750
811	LIR	50	0.500
832	LIR	72	0.500
823	LIR	74	0.625
809	LIR	75	0.500
837	LIR	76	0.500
824	LIR	77	0.500
827	LIR	78	0.750
554	LIR	79	0.750
806	LIR	80	0.625
835	LIR	81	0.625
802	LIR	83	0.500
805	LIR	84	0.500
813	LIR	KKK	0.625
775	MER	43	0.750
578	MVA	40	0.750
590	MVA	G	0.625
794	PRO	2	0.500

Table S.4.3 List of the best candidates for future restoration programs. Individuals with unique or rare genotypes, which also presented a high individual heterozygosity and private or rare alleles were considered. ID = Code assigned to each individual, Genotype code = Identification code of the genotypes found for each individual, H-ind = Individual heterozygosity. Locality codes are indicated in Table 4.2.

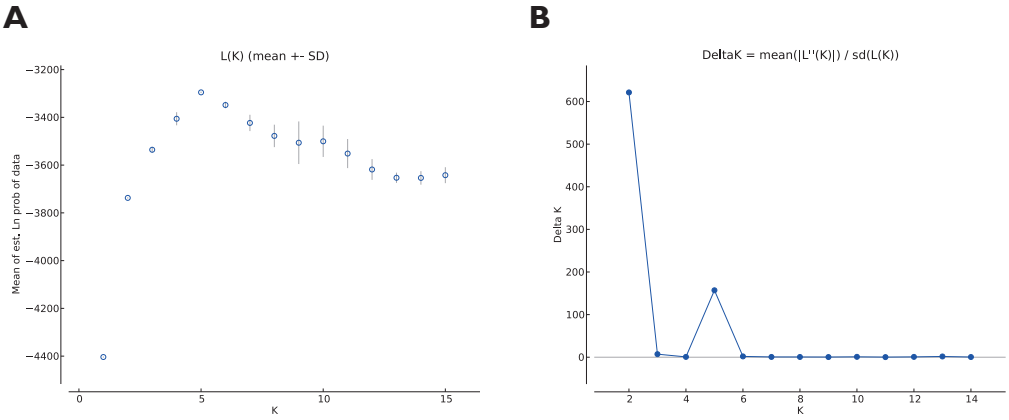


Figure S.4.1 Output results from STRUCTURE HARVESTER. (A) The mean of log-likelihood values for each value of K (1-15), (B) Ad hoc statistic based on the rate of change in the log probability of data between successive K values (ΔK, following Evanno *et al.* (2005).

THE ALPINE
Viola cheiranthifolia

5. Alpine species in dynamic insular ecosystems through time: Conservation genetics and niche shift estimates of the Teide Violet (*Viola cheiranthifolia*) in the island of Tenerife.

ABSTRACT

Properties of alpine oceanic ecosystems make their biota highly vulnerable to climate changes and disturbances. Our first goal was to estimate the past and future population genetic structure and diversity in the oceanic alpine endemic *Viola cheiranthifolia* (Violaceae). The results suggested that *V. cheiranthifolia* presents moderate levels of genetic diversity and a clear population structure divided in two main groups (Teide and Las Cañadas Wall), showing signs of recolonization dynamics after volcanic eruptions. Therefore, Las Cañadas caldera could be acting as the main barrier to gene flow. The results for demographic history showed that individuals from Teide could have originated from the oldest population in Guajara (Las Cañadas Wall). Future estimates for the distribution of the populations studied also showed that, being extremely vulnerable to climate change, the species will undergo continuous demographic fluctuation. The low dispersal capacity of this species, coupled with herbivory pressure from rabbits and mouflons will make its adaptation to future climate conditions in this fragile alpine ecosystem difficult. Thus, conservation actions should be focused on control of herbivores, population reinforcement, and surveillance of niche shifts.

5.1. INTRODUCTION

Alpine ecosystems located in oceanic islands can be considered among the rarest, most restricted and vulnerable habitats. They host a unique biota, which is especially vulnerable to climate change and disturbance due to their small and isolated distributions (Fernández-Palacios *et al.* 2014; Harter *et al.* 2015), leveraged to the extreme in the isolated summits of the highest islands. The ontology of high altitude islands confers on the biota a complex and challenging biogeographic history. This is related to episodes of colonization, volcanism, subsidence, landslides, topographical complexity and particularities of the impoverished and unbalanced specific diversity of islands (Whittaker & Fernández-Palacios 2007). Oceanic insular summits therefore constitute islands within islands, in the sense that they are scarcer, smaller, and even more isolated than the islands to which they belong. This exacerbated insularity makes them hotspots of endemic biodiversity, which can be as ephemeral as their habitats, subjected to the dynamic geology of islands.

The colonization of newly emerging summits must occur either through colonization of summit-pre-adapted continental species (through long-distance dispersal) (Nogales *et al.* 2012), as the result of dispersal from other high altitude island sites (pre-adapted to alpine environments), or as the product of in situ diversification from a lowland or mid-altitude ancestor that colonized and adapted to the ecological conditions prevailing on islands summits (Trigas *et al.* 2013). For example, Merckx *et al.* (2015) recently investigated the origins of species on a mountain in tropical Borneo, and found two striking results: first, that most species are relatively young, starting to speciate after, or at the same time as the rise of the mountain they inhabit, and second; that some of the endemics derive from distant immigrants that were pre-adapted to cool environments outside Borneo. Molecular studies also suggest that many of the Canarian endemics have diversified in the islands recently (Jones *et al.* 2014; Saro *et al.* 2015), and that most originated from the Mediterranean region (Caujapé-Castells 2011).

Apart from this, climate change is of special importance among the threats to native and endemic island biota, especially on island summits. The effects of climate change on continental alpine ecosystems have been widely studied (Gottfried *et al.* 2012; Morueta-Holme *et al.* 2015). However, little has been addressed in oceanic islands on the same terms (Caujapé-Castells *et al.* 2010;

Courchamp *et al.* 2014). In this regard, niche modelling predictions show that plant species in oceanic upland ecosystems are prone to decreasing or losing their range in the near future (Halloy & Mark 2003; Upson *et al.* 2016). Moreover, have a small range, are weakly dispersing and are in previously stable regions, but experience climatic changes, they will be at the greatest risk of extinction from anthropogenic climate change (Sandel *et al.* 2011; Dullinger *et al.* 2012). It is therefore of great importance to predict distribution changes in insular mountains species as indicators of the fate of other alpine species. However, to date, studies of the impact of climate change on the distribution of island species are scarce. Indeed, population genetic analysis and species distribution models with simulations for putative heterozygosity loss have rarely been used simultaneously to assess future population dynamics in alpine oceanic ecosystems.

The Teide mountain on the island of Tenerife (Teide; 3,718 m), as part of the complex Canarian archipelago and due to its high isolation and altitude, may be a paradigmatic example of complex biogeographic history under geological change for predicting niche shifts of plant species. Tenerife is the tenth highest island worldwide and the highest peak in the Atlantic Ocean. It is occupied by an alpine endemic dry habitat and currently presents a rapid increase in temperature at the summit of the island (with an increase of 0.14 ± 0.04 °C per decade and a significant increase of maximum and minimum temperatures and changes in insolation (Sanroma *et al.* 2010; Martín *et al.* 2011). The Teide-Pico Viejo complex is now surrounded in the south by the vertical walls of the older series of volcanoes that used to form Las Cañadas (3.5 – 0.2 Ma) (Ancochea *et al.* 1999). 200 ka ago, Las Cañadas volcano suffered a lateral landslide, with subsequent episodes of massive landslides and eruptions that changed dramatically the morphology, topography, composition and age of the area (Carracedo 2014). Later, the Teide-Pico Viejo complex was built on top of Las Cañadas caldera, whose construction culminated c. 30 ka ago, resulting in a 3,500-m-high stratovolcano with a large open crater. Finally, in recent geological times (Lavas negras; 1,150 BP ± 140) the last eruption of the Teide stratovolcano occurred, with the generation also of young peripherical lava domes (Roques Blancos and Montaña Blanca, 1,714-2,000 BP respectively) (Carracedo *et al.* 2007). Therefore, under these circumstances of rapid, violent events, the mountain insular biota must have undergone similarly dramatic biogeographical and genetic changes.

In this study, we focused on *Viola cheiranthifolia* as a model species, with a narrow distribution and short-range dispersal mechanisms, to infer its biogeographical history and to predict the impact of climate change on its genetic structure and diversity. *Viola cheiranthifolia* is a high mountain dwarf chamaephytic plant, endemic to Tenerife. It is the most dominant and structuring species within the summit vegetation of Teide. The largest populations are found around Teide and Pico Viejo stratovolcanoes at altitudes from c. 2,400 m to c. 3,700 m at the peak of Teide, although some small populations occur at the highest points of the caldera (Guajara; 2,715 m, Pasajirón; 2,531 m). The plant grows in poor soils on cinder flats amongst the volcanic rubble, mixed with pumice stones in some localities. The species is self-compatible, but cross-pollination by insects increases the seed production, with more than 20 pollinator species, especially hymenoptera species (Seguí *et al.* 2017). *Viola* species typically present diplochory with explosive ejection of seeds and posterior myrmecochory (Beattie & Lyons 1975). But this double dispersion system by ants has not been observed in *V. cheiranthifolia*, and its seeds present a very small elaiosome. It is considered as Vulnerable (VU D2) by The Red List of Spanish Vascular Flora (Moreno-Saiz 2008), and especially threatened by the presence of non-native rabbits in the National Park, extremely reducing its plant fitness and abundance (Seguí *et al.* 2017).

Viola cheiranthifolia is included within sect. *Melanium*, commonly called Pansies, which are distributed throughout frost-free regions of the world, mainly the Northern Hemisphere (Yockteng *et al.* 2003). It is known that hybridization and polyploidy have played an important role in the evolutionary history of this clade in the *Viola* genus, with high variation in chromosome numbers and different ploidy levels (Ballard *et al.* 1999; Marcussen *et al.* 2010). The sect. *Melanium* has an allopolyploid origin and is derived from two diploid lineages. Indeed, further gene duplications have occurred in *Melanium*, resulting in species with higher ploidy levels (Marcussen *et al.* 2010).

The main objectives of this study were: (i) to infer the current genetic diversity and population structure of *V. cheiranthifolia*, (ii) to track the recent demographic history using a Bayesian coalescent approach, (iii) to estimate future environmental suitability by projecting SDMs to different climatic scenarios, (iv) and to project future fluctuations in genetic diversity (H_E), as well as setting out their implications for conservation.

5.2. MATERIAL AND METHODS

5.2.1. Microsatellite development

Initially, we described the development of 16 microsatellite markers in *Viola cheiranthifolia*, and their cross amplification on the related species *V. palmensis*, indicating their effectiveness in identifying patterns of genetic diversity. The development of these markers was published in Rodríguez-Rodríguez *et al.* (2015).

Genomic DNA for the development of markers and subsequent surveys were extracted from leaf tissue using the Dellaporta *et al.* (1983) protocol.

Microsatellite loci were developed by Savannah River Ecology Laboratoy (University of Georgia) using a 454 sequencing platform. Extracted DNA was serially enriched twice for microsatellites using three probe mixes. 67 primers pairs were initially chosen, of which 16 amplified consistently and were used for initial screening. 33 natural individuals of the Montaña Blanca population of *V. cheiranthifolia* and 32 of the Pico de la Cruz population of *V. palmensis* were tested.

Each 25 µL PCR (polimerase chain reaction) contained approximately 20 ng of DNA, 10 pmol of each primer, as well as PCR Master Mix (Reddy-Mix, ABgene, Surrey, UK) which included 0.625 units of Taq DNA polymerase, 75 mM Tris-HCl, 20 mM $(NH_4)2SO_4$, 0.01% Tween20, and 0.2 mM of each dNTP. $MgCl_2$ concentrations are shown. Forward primers were colour-labelled at the 5'-end with 6-FAM, PET, NED, VIC or TAMRA.

Amplifications were carried out using the following thermal cycling conditions: 3 min denaturation at 95°C, 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 60°C (except Viodi-10 at 62.5°C), and 72°C for 1.5 min; followed by 5 min elongation at 72°C. The products were detected with an ABI 3130XL, and fragment sizes were determined using GENEMAPPER 4.0 (Applied Biosystems, Inc.). We identified allele peak profiles at each locus and assigned a genotype to each individual.

The 16 tested primer pairs amplified and were polymorphic in *V. cheiranthifolia* and 15 could be transferred to *V. palmensis*. 10 of the markers also amplified in the other species belonging to section *Melanium*, not showing transferability to the section *Viola*.

5.2.2. Sample collection and genotyping

Once the set of microsatellites was designed, we proceeded to the collection of samples from the whole distribution of *V. cheiranthifolia*. The distribution of *V. cheiranthifolia* covers extensive areas at the highest altitudes in the Teide National Park, with nine main localities described. 266 individuals covering the whole distribution of the species were sampled during the spring and summer of 2013 and 2014 (Figure 5.1). All specimens were georeferenced when possible and fresh leaves were collected and stored in silica gel.

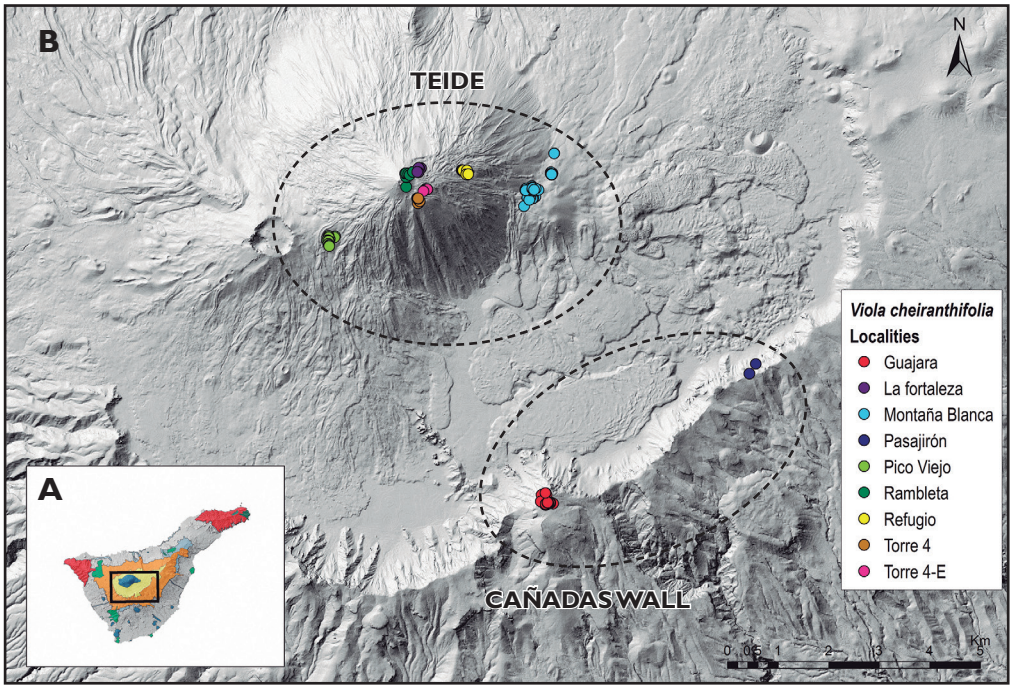


Figure 5.1 (a) Tenerife Island with the representation of natural protected areas (Teide National Park, in yellow and blue). The square indicates the extension of the study area (b) Map of the distribution of *Viola cheiranthifolia* localities sampled for this study.

To perform the genetic analysis, 14 polymorphic microsatellite markers were used to genotype all the samples collected. Although 16 primer pairs were described in Rodríguez-Rodríguez (2015), due to difficulties in the interpretation of two of the markers (VIOtri-6 and VIOtri-13) in some of the localities, they were discarded for the final genotyping. The chosen primer pairs and their characteristics are included in Table 5.1.

DNA was extracted and amplified with the same protocols implemented in the characterization of the microsatellites (described above). We identified

allele peak profiles at each locus and assigned a genotype to each individual. The polyploid status of *V. cheiranthifolia* was only determined after interpreting the peak profiles in all the populations, especially in Guajara and Pasajirón, that were genotyped after the characterization of the microsatellites was published. The Guajara and Pasajirón populations presented a clearer polyploid pattern, which led to the revision of the profiles of all the individuals for genotyping.

Locus	EMBL ID	Motif	PCR primer sequence (5'->3')	Range (bp)	Dye
VIOdi-2	HE601734	(AG)9	F: GGCGAGCAACCTATAATATC R: CACCACGTTCTTCCATACTC	139 -141	PET
VIOdi-6	HE601736	(AG)8	F: CTTGATTGCTGGAGTGTGAC R: GGCGAATCACTACTGTTGTC	167-175	VIC
VIOdi-8	HE601737	(AC)9	F: CACAGCTTCTCCATCACAAC R: TAGGAATGACTTGGCTTCTG	263-265	PET
VIOdi-10	HG916757	(AC)13	F: CTACTGATGGGTGTCGAATC R: GGAACGTGAAACTCTGTAGC	386-400	NED
VIOdi-24	HG916762	(AG)11	F: ACTTCTTGATTGAACGGAAC R: TCACATTCATCGGATCTTTC	273-279	TAMRA
VIOtri-1	HE601738	(AAC)7	F: CTTTCGCTGGAGGACTATAG R: TTAGCTGTGGTGGAGAAGTC	237-240	NED
VIOtri-4	HE601739	(ATC)8	F: GTGAGGATCGGAAACAATAG R: CTATGGCGGGTGTAGTAATC	146-177	VIC
VIOtri-7	HG916755	(AAG)8	F: CTCGGTTCGGGATATATAAG R: ATGGAAAGTATGCGAGATTC	128-154	6-FAM
VIOtri-8	HE601741	(ACT)8	F: TCGAAGGGTTCCATATAATC R: TTATCTCCGATCCTCAATTC	268-274	PET
VIOtri-9	HG916756	(ATC)10	F: TCCTTCAAATTCATGGTGAG R: CCACTCTTCAACAAGGAATG	183-215	6-FAM
VIOtri-14	HG916759	(ATC)7	F: CTTCCAGGTTTCAAAGACAG R: GTTATAGGCTGAAGGGTCAC	131-148	VIC
VIOtri-24	HG916761	(ATC)8	F: ACTGAGAGCCAATCAAAGAG R: TCACTCCCAAATCAAGAAAC	263-275	6-FAM
VIOtet-8	HE601742	(ACAT)13	F: AAACAGCCATCACCACTTAC R: ATTACAAACACGGGAAGTTG	239-307	6-FAM
VIOtet-13	HG916763	(AAAC)8	F: AGTCTAGTTTGCGCCTGTAG R: ATCTGCACAGGAGGTAAATG	156-172	ROX

Table 5.1 Characteristics of the 14 microsatellite markers developed and employed in the genotyping of *Viola cheiranthifolia* populations.

5.2.3. Population genetic analysis

SSR peak profiles showed a pattern compatible with tetraploidy since a high number of individuals for most loci presented up to four alleles. This information was used to define the statistical analysis methods, due to the difficulties in polyploids of assigning the correct allele dosage for each locus and individual (Dufresne *et al.* 2014).

Consequently, software and statistics which allow genotypic ambiguity were used to estimate the genetic diversity and the population structure of this species. The statistics used commonly assume a polysomic inheritance pattern (autopolyploids), instead of a disomic inheritance pattern (allopolyploids). Contrary to what has been cited, that sect. *Melanium* is allopolyploid (Marcussen *et al.* 2010), we did not detect either fixed heterozygosity or allele segregation in pairs of isoloci. Furthermore, the distinction between allopolyploid and autotetraploid is not absolute, as there is usually a continuum between polysomic and disomic inheritance which depends on the divergence time between the progenitors (Comai 2005). Meirmans & van Tienderen (2013) stated that some allele exchange between subgenomes is sufficient to homogenise the allele frequencies between the two subgenomes to an extent that biases associated with strict disomy are removed. Following this hypothesis, we proceeded to analyse our codominat dataset as autotetraploid with statistics that allow genotypic ambiguity.

Genetic diversity

To examine the genetic diversity of populations of *V. cheiranthifolia*, we computed the average number of alleles (*A*), the allelic richness expressed as the expected number of alleles among k gene copies (A_R; k = 11), the observed heterozygosity (H_o) and the expected heterozygosity (gene diversity) corrected for sample size (H_E) (Nei 1978) in each population using SPAGeDi 1.5 (Hardy & Vekemans 2002), a program which computes statistics and permutation tests of relatedness and differentiation among populations oforganisms of any ploidy. In addition, selfing rate estimates were also obtained from SPAGeDi with a method suited to polyploids, which is based on phenotypes and irrespective of the allele dosage (Hardy 2015). The mean numbers of private alleles per locus were calculated using Microsoft Excel(R) based on SPAGeDi allelic frequency data.

Population genetic structure

First, an allele size permutation test (Hardy *et al.* 2003) in the SPaGeDi program was used to assess if stepwise mutations on microsatellites affected genetic differentiation in our study populations. This test is based on the comparison of observed pairwise R_{ST} values with the distribution of R_{ST} values (pR_{ST}) obtained by 10,000 permutations of allele sizes among allelic states. A significant test (observed R_{ST} higher than 95% of the pR_{ST}) indicates that mutations have contributed to genetic differentiation between subpopulations.

Bayesian model-based clustering of microsatellite data was employed using the procedure implemented in STRUCTURE 2.3.4 (Pritchard *et al.* 2000) to infer genetically distinguishable clusters in the sampled populations. "Polysat 1.4-1" (Clark & Jasieniuk 2011) was used to generate the input data file for STRUCTURE computations, setting the overall ploidy to 4. The recessive alleles method was implemented to account for allele copy ambiguity in the codominant data set. (Falush *et al.* 2007). The program was run ten times from K = 1 to K = 10 with the burn-in length of 10^5 generations followed by 10^6 Monte Carlo Markov chain (MCMC) iterations, using the admixture model with correlated allele frequencies. The optimal K value for each analysis was estimated by the maximum value of ΔK following the Evanno method (Evanno *et al.* 2005), implemented in STRUCTURE HARVESTER (Earl & vonHoldt 2012). Following a hierarchical approach, we continued the exploration within clusters at the highest ΔK using the same settings. Alignment of cluster assignments across replicate analyses was then conducted in CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007).

To examine genetic similarities and relationships between individuals, we performed a principal co-ordinates analysis (PCoA) in GENALEX 6.5 (Peakall & Smouse 2012). As input for the PcOA, a pairwise distance matrix for all samples was calculated using Bruvo distances (Bruvo *et al.* 2004) as implemented in "Polysat 1.4-1" in R. This measure of genetic distance has been developed for polyploid organisms and takes distances between microsatellite alleles into account without knowledge of the allele copy number (Clark & Jasieniuk 2011). In addition, a nested analysis of molecular variance (AMOVA) (Excoffier 1992) was performed with the R packages "adegenet 2.0.1" (Jombart 2008) and "poppr 2.3.0" (Kamvar *et al.* 2014). The hierarchical level used for this analysis followed the clustering results obtained with STRUCTURE and the PCoA. Significance values were estimated over 999 permutations.

Finally, pairwise Rho statistics (Ronfort *et al.* 1998) for all population pairs were calculated to infer the degree of population differentiation between the localities studied. Rho is an inter class relatedness coefficient permitting comparison between ploidy levels and it was calculated using SPAGeDi. It has been determined that the Rho statistic is more appropriate for polyploids, being independent of the ploidy level, the selfing rate and the rate of tetrasomic inheritance (Meirmans & Van Tienderen 2013).

Fine-scale spatial genetic structure

Spatial genetic structure (SGS) of all the georeferenced individuals was assessed for the resulting Bayesian clusters from the whole database. The locality of Pasajirón was excluded from this test due the low number of georeferenced individuals. Pairwise kinship coefficients (F_{ij}) between individuals and 95% confidence intervals with jackknifing over loci were estimated at different distance classes every 20 meters. After 250 meters the pairwise comparisons were unified into a final distance class. Significant SGS was evaluated by testing the regression slope on the distance (b_{lin} and b_{log}) with 10,000 randomizations. Estimates of the *Sp* statistics (a measure of SGS strength) were obtained for each group from the slope of the regression of F_{ij} on linear and logarithmic distance (b_{lin} and b_{log}) and the mean pairwise kinship coefficient measured at the first distance class (F_1) with the formula $Sp = -b\ (1- F_1)$, following Vekemans & Hardy (2004). All SGS analysis were perfomed on SPAGeDi.

Inference of demographic history and effective population sizes

The Approximate Bayesian Computation (ABC) method (Beaumont 2010) implemented in DIYABC 2.1 (Cornuet *et al.* 2014) was used to explore the demographic history which may have generated the current genetic structure and to estimate associated demographic parameters. To simplify analysis and to respect the assumption of population isolation made by DIYABC, populations were grouped according the structure results and the Rho statistic. These groups are Teide (all localities in Teide-Pico Viejo Complex), Guajara and Pasajirón.

As DIYABC does not allow tetraploid input datasets, we simulated a diploid dataset with the same method implemented in Lepais *et al.* (2013). The function SimpleFreq in "Polysat 1.4-1" was used to calculate allele frequencies at each

locus for each of the nine sampling sites. The obtained allele frequencies were then used with the function SAMPLE in R to resample alleles and generate diploid multilocus genotypes as input data for DIYABC. These simulated populations included the same number of individuals originally sampled.

Six demographic scenarios characterized by divergence times in generations (t, t1, t2, t2a), effective population size of actual populations (N1, N2 and N3 for Teide, Guajara and Pasajirón respectively) and a putative ancestral population (Na) were compared (Figure S.5.1). The scenarios and priors were chosen for computation after initial trials. In the simplest scenario, all three populations diverged simultaneously at time t from a common ancestor. Scenarios 2 to 5 considered all dichotomous combinations of population divergence, considering Guajara or Pasajirón as the oldest populations that derived from the ancestor (of effective size Na) at time t2a. A second population took place at time (t2) and the later divergence (t1) originated a third population. Scenario 6 showed an admixture between Guajara and Pasajirón that originated the Teide population.

For each simulated scenario, priors were set as a wide Uniform distribution bounded between 10 and 20,000 individuals for N1, N2, N3 and Na and between 1 and 10,000 generations for divergence times (t1, t2, t2a) with the additional constraints $t1 < t2 \leq t2a$. Default values were used for genetic parameters, assuming a generalized stepwise mutation model (GSMM) for microsatellite mutation rate, using a bounded Uniform Prior distribution between 10^{-4} and 10^{-3}. Single nucleotide indels (SNI) were allowed at a rate between 10^{-8} and 10^{-5}.

The observed and simulated genetic datasets were summarized using mean number of alleles, mean size variance and M index for each population and for each pair of populations, mean number of alleles, mean size variance, classification index, F_{ST} and shared allele distance between populations. In total, twenty-seven summary statistics were recorded as sample statistics to estimate scenario probability and parameter confidence intervals, as well as to assess confidence level for the selection of a given scenario. $3x10^6$ simulations were run for all scenarios. The 30,000 simulations closest to the real genetic dataset were subjected to a weighted polychotomous logistic regression to estimate posterior probabilities (*P*) for each scenario. We tested the confidence our scenario choice with the estimate of a type I and II errors, in order to provide probabilities of choosing the wrong scenario.

Demographic parameters were inferred from the most likely scenario using the normalized Euclidian distance between the observed and simulated summary statistics to select the 10% datasets closest to observed data. The performance of parameters estimation was assessed with 5,000 (1%) pseudo-observed datasets. Comparisons of known and estimated demographic values were used to infer bias and precision of estimations (Cornuet *et al.* 2014) .

5.2.4. Species distribution modelling

Predictor variables

The high resolution of spatial environmental predictors improves the accuracy and performance of niche modelling procedures. For this purpose, we developed the assemble of predictor maps at the fine scale of 20 meters/pixel grid based on the LIDAR Digital Elevation Model (DEM) of the Canarian archipelago (Instituto Geográfico Nacional, Madrid, Spain), restricted to the island of Tenerife. We derived the topographic variables slope and topographic index from DEM using the "raster" package (Hijmans & van Etten 2014) implemented in R software.

We then derived layers for monthly predictors of minimum, average and maximum temperature and total precipitation with the procedure established by González Fernández de Castro (2016). The monthly data on precipitation and temperature from 275 climatic stations in the Tenerife agro-climatic network were used as response variables in a stepwise generalized additive model (GAM) using the following predictor variables: 1) for precipitation: altitude, northness, x and y coordinates; 2) for temperature: altitude, northness, slope, x and y coordinates, selecting models by AIC. The residuals of the values of meteorological stations were mapped and interpolated by fixed weighting splines in ArcGIS 10.4. The resulting 12 maps for monthly temperature variables were used to calculate bioclimatic variables following Hijmans *et al.* (2005). We developed potential evapotranspiration (PET) maps with with package "r2dRue" in R (del Barrio *et al.* 2013).

Finally, we developed, for the first time, a nival model for Tenerife following Carlson *et al.* (2015), based on snow cover data acquired either by Landsat images or aerial photography. Landsat 7 and Landsat 8 covered 8 and 5 images respectively.

Future climate variables

For each of the monthly variables of temperature and precipitation, we employed the Delta method technique REF to downscale anomalies to 20 meters resolution. Downscaling was used with "downscaleR" package in R (Santander Meteorology Group 2017). We used General Circulation Models MIROC and CSIRO, for the emissions scenarios 2.6, 6.0 and 8.5 developed by IPCCC 5 (IPCC Working Group I 2013), for years 2030, 2050 and 2080.

Species distribution modelling

We used the "BIOMOD" package (Thuiller *et al.* 2009) implemented in R software. We selected six algorithms: generalized linear models (GLM) and generalized additive models (GAM), generalized boosted regression models (GBM), random forest (RF), multiple adaptive regression splines (MARS) and artificial neural networks. We performed 10 runs for each method, randomly selecting five pseudoabsence locations in each run. 75% of the points in the data were randomly selected for model calibration, and the remaining 25 % were used for model evaluation, based on AUC and TSS statistics. Models with AUC and TSS> 0.8 were accounted for in the construction of an ensemble model based on the average suitability of each model. The ensemble map of topoclimatic suitability was converted to binary (presence-absence), by choosing the threshold maximizing TSS value. The ensemble model was finally projected to each future climate scenario combination of circulation model, year, and emissions pathways.

Genetic diversity loss projections

The "migclim" package (Engler *et al.* 2012) was used to project intermediate potential distribution areas for every year, assuming no migration limitations. Then, the suitable area for *V. cheiranthifolia* was divided in two, according to the population structure found by STRUCTURE (see results): one area comprised Las Cañadas Wall population (Guajara and Pasajirón) in the south and the other area, the localities in Teide. The microsatellite matrix was divided in two according to this split. The following simulation was then run: each iteration comprised a 5-year period. The probability of each individual in each population of becoming extinguished was 0 at present, and varied according to the variation in the potential area. The migration rate was

also used to calculate the probability of each individual migrating to the other population. With these two procedures, the resulting matrices for the populations were used again to compute allele frequencies and then expected heterozygosity as a measure of variation of genetic variation across time. The procedure was run until 2080 and repeated 5,000 times for each combination of scenarios.

5.3. RESULTS

5.3.1. Genetic diversity and population structure

The scorability of the microsatellites included in this study was high for all the localities. Estimates of genetic diversity for all the sampling sites are presented in Table 5.2. A total of 126 alleles were found for *Viola cheiranthifolia* with an average expected heterozygosity (H_E) of 0.550 and a total of 0.637. In total, the microsatellite loci revealed 9.0 alleles per locus ranging from 4 (Di-24 and Tri-1) to 30 (Tet-8).

The localities which presented the highest and lowest values for all diversity measurements were GUA and PAS respectively. Measurements of allelic richness (A_R) and expected heterozygosity (H_E) were similar for all the localities, except for PAS, the low diversity of which was notable. The mean number of alleles varied from 2.14 (PAS) to 6.93 (GUA) and the average of private alleles from 0.00 (RB, FOR, PAS) to 0.86 (GUA). Considering Las Cañadas Wall and Teide populations as a whole, Las Cañadas Wall presented higher diversity values in terms of allelic richness and heterozygosity. The results of the test for the selfing rate for every locality were quite similar and show an autogamy rate of 50% for this species (Table 5.2).

The allele permutation test with SPaGeDi suggested that stepwise mutations at microsatellite loci did not contribute significantly to genetic differentiation between localities compared with genetic drift and migration (observed R_{ST} = 0.1807, permuted pR_{ST} = 0.1055, P = 0.0735). Therefore, in our case study, allele-identity-based estimators (F_{ST}/Rho) are preferable to R_{ST} for genetic differentiation.

Including the whole set of samples, the Bayesian Structure analysis identified two genetic clusters according to ΔK (K = 2, Figure 5.2). In concordance with the PCoA, individuals are aggregated within the Teide and Las Cañadas Wall in well-assigned clusters with more than 80 % of

Locality	Population code	*N*	*NA*	*PA*	A_R	H_O	H_E	*Sr*
Pico Viejo	PV	27	5.14	0.14	3.39	0.655	0.556	0.521
La Fortaleza	FOR	19	4.00	0.00	3.03	0.461	0.522	0.533
La Rambleta	RB	14	4.07	0.00	3.09	0.54	0.547	0.536
Torre 4	T4	30	5.57	0.07	3.32	0.56	0.566	0.527
Torre 4 - Este	T4-E	10	4.29	0.14	3.36	0.551	0.554	0.553
El Refugio	REF	28	5.21	0.21	3.32	0.458	0.557	0.519
Montaña Blanca	MB	80	6.64	0.21	3.50	0.577	0.592	0.530
Guajara	GUA	48	6.93	0.86	3.92	0.524	0.666	0.541
Pasajirón	PAS	10	2.14	0.00	2.08	0.471	0.391	0.553
Total		266	9.00	1.64	3.90	0.557	0.637	
Teide		208	8.07	-	6.65	0.538	0.587	0.527
Cañadas Wall		58	7.00	0.93	6.89	0.624	0.645	0.543

Table 5.2 Genetic diversity estimates for *Viola cheiranthifolia* populations. *N* = sample size, *NA* = Mean number of alleles per locus, *PA* = Mean number of private alleles per locus, A_R = allelic richness based on the expected number of alleles among 11 gene copies, H_E = Nei's (1978) Gene diversity, *Sr* = Selfing rate (0-1) values based on phenotypes and irrespective of allele dosage.

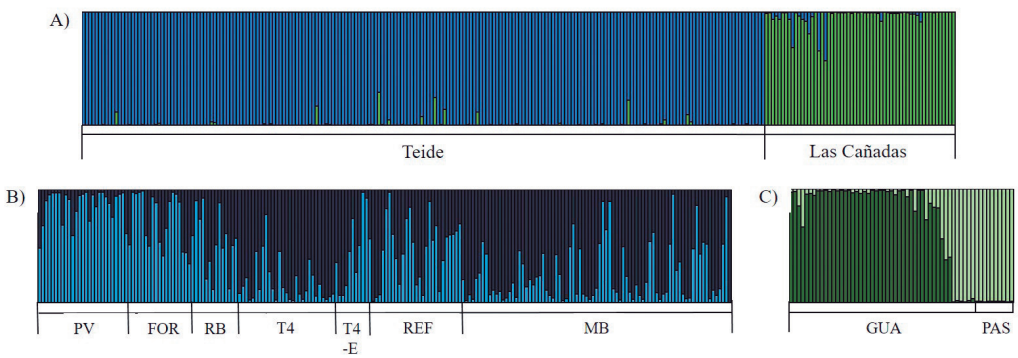


Figure 5.2 Bar plots for the proportion of coancestry inferred from Bayesian cluster analysis implemented on STRUCTURE and Clumpp. (A) The whole set of sampled individuals and localities (K = 2). (B) The individuals within Teide. (C) The individuals within Las Cañadas Wall.

assignation. Following a hierarchical approach, in the analysis carried out with the localities only from Teide, the highest ΔK was found for K=2. Pico Viejo presented a higher assignation to one of the cluster together with individuals from other localities, resulting in a high homogenization within Teide. However, it appears that there is more aggrupation among individuals on the west and east sides of Teide respectively. In the third analysis, for Las Cañadas Wall, the highest ΔK was also found for K=2. In this case, there is a clear separation of Pasajirón individuals into one cluster together with six individuals from the northern part of Guajara. All results from STRUCTURE-harvester for all the analyses are included in Figure S.5.2.

The PCoA revealed two well differentiated groups (Teide and Las Cañadas Wall) (Figure 5.3). The AMOVA indicated that most of variation was found within localities (83.9 %) (Table 5.3). The variation between Teide and Las Cañadas Wall (13.4 %) was higher than the variation between the sampled localities (2.7 % and 83.9 % respectively). Rho statistic pairwise values (Table 5.4) ranged from 0.015 (MB-T4) to 0.558 (PAS-RB). All values were significant ($P < 0.001$) apart from MB-T4 ($P < 0.05$). The most differentiated population according to the Rho statistic was PAS, with the highest values (from 0.232 to 0.588). This is followed by GUA, with similar values in all the pairwise comparisons (from 0.232 to 0.291). All the localities within Teide showed lower values (from 0.015 to 0.119), with a higher degree of admixture.

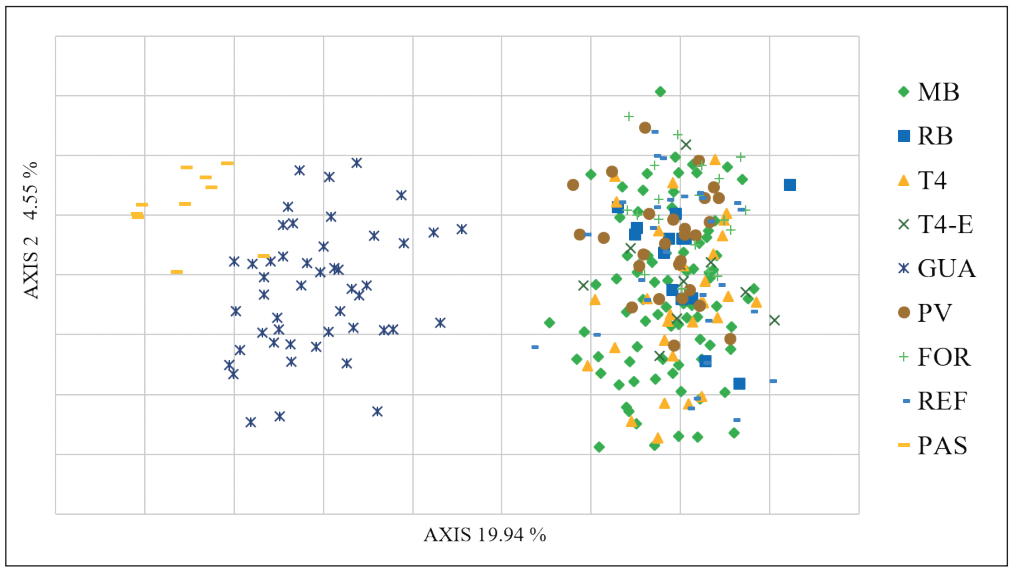


Figure 5.3 Principal coordinate analysis (PCoA) for all *Viola cheiranthifolia* localities sampled in Teide National Park. Population codes are indicated in Table 5.2.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	Φ - statistics
Teide vs. Las Cañadas Wall	1	90.67	0.86	13.4	ΦCT = 0.134*
Between localities within populations	7	68.59	0.17	2.7	ΦSC = 0.031**
Within localities	257	1386.43	5.39	83.9	ΦST = 0.160**
Total	265	1545.69	6.43	100.0	

Table 5.3 AMOVA analysis for *Viola cheiranthifolia*. Hierarchical levels were implemented based on the clustering found by STRUCTURE (Teide vs Las Cañadas Wall). *P < 0.05; **P < 0.01

	PV	FOR	RB	T4	T4-E	REF	MB	GUA
PV								
FOR	**0.088**							
RB	**0.091**	**0.076**						
T4	**0.092**	**0.119**	**0.078**					
T4-E	**0.112**	**0.118**	**0.146**	**0.090**				
REF	**0.073**	**0.083**	**0.104**	**0.083**	**0.065**			
MB	**0.079**	**0.086**	**0.072**	**0.015**	**0.096**	**0.059**		
GUA	**0.265**	**0.266**	**0.274**	**0.274**	**0.291**	**0.263**	**0.253**	
PAS	**0.492**	**0.536**	**0.558**	**0.526**	**0.609**	**0.510**	**0.474**	**0.232**

Table 5.4 Pairwise Rho's statistics for all the population comparisons. Bold values: P < 0.001.

The analysis of SGS in the populations analyzed showed a negative linear relationship between kinship coefficients and the logarithm of distance (Figure 5.5). The *Sp* statistics indicated signs of SGS in both analysis, but with higher values for Guajara than Teide (Sp_{log} = 0.0112; 0.0063, respectively) (Table 5.5). Permutation tests showed that regression slopes were significantly different from zero in both cases.

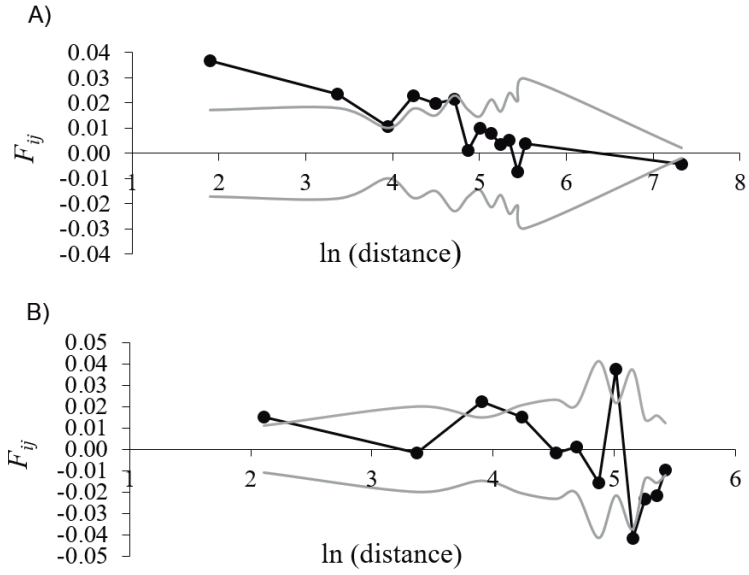


Figure 5.5 Average kinship coefficients F_{ij} between pairs of individuals at geographical distances intervals every 20 meters (log scale) in Teide (A) and Guajara (B). Grey lines show the 95 % confidence intervals and the dots that are out of these margins indicate a significant deviation of the random spatial distribution ($P < 0.05$).

	F_1	b_{lin}	b_{log}	Sp_{lin}	Sp_{log}
Teide	0.0366 *** ± 0.0086	-0.0000051 *** ± 0.0000011	-0.0061 *** ± 0.0012	0.0000052 ± 0.0000011	0.0063 ± 0.0013
Guajara	0.0149 * ± 0.0055	-0.0001746 *** ± 0.0000429	-0.0113 *** ± 0.0026	0.0002000 ± -0.0000431	0.0112 ± -0.0026

Table 5.5 Spatial genetic structure (SGS) at a fine scale, analyzed with 14 microsatellite markers in the two main groups detected with STRUCTURE: Teide and Guajara. F_1 : mean coancestry coefficients (F_{ij}) in the first distance class; b_{lin} and b_{log}: regression slopes of F_{ij} values over the linear and logarithmic spatial distances, respectively; *Sp* statistics (Sp_{lin} and Sp_{log}), indicators of the SGS strength, calculated with the formula $-b\ (1 - F_1)$, and using both b_{lin} and b_{log}. *** $P < 0.001$; * $P < 0.05$.

5.3.2. Demographic history and effective population sizes

Among the six tested scenarios, the scenario 2 gave the highest posterior probability from the data ($P = 0.669$ [0.583; 0.754]) (Figure 5.6). However, confidence tests revealed moderate error probabilities for type I ($P = 0.518$) and type II ($P = 0.486$) calculated with the logistic regression method. This uncertainty could be due to the difficulties in distinguishing between scenario

2 and 3, as both scenarios show and earlier divergence between the Las Cañadas Wall and Teide populations. Therefore, demographic parameters were calculated under scenario 2. In that scenario, the divergence between Las Cañadas Wall and Teide was again highlighted. The oldest current population was Guajara which derived from an unknown ancestral population at the event t2a (4,610 [2,160; 19,100] generations; mode and 2.5-97.5% quantiles of the posterior distribution respectively). From Guajara, two divergence times are presented, the earliest giving place to Teide in t2 (857 [362; 4,630] generations) and the latest one to Pasajirón in t1 (134 [53.6; 657] generations) (Table 5.6). In general, mean bias estimates for the divergence times were low, with high factor 2 values (Table S.5.1). Following other estimates of perennial violet lifespans, we have considered that *V. cheiranthifolia* may live up to 25 years (Solbrig 1981; Batista & Sosa 2002; Culley 2002; Herrera & Bazaga 2010). Given that generation time, Guajara would have derived from the ancestral population 115,250 [54,000; 477,500] years ago. The divergence times between GUA-TEIDE and GUA-PAS would be 21,425 [9,050; 115,750] and 3,350 [1,340; 16,425] years ago respectively. Nonetheless, the estimate of the generation time in *V. cheiranthifolia* is still uncertain, due to the difficulties in tracing the regeneration of the vegetative part of the plants each year.

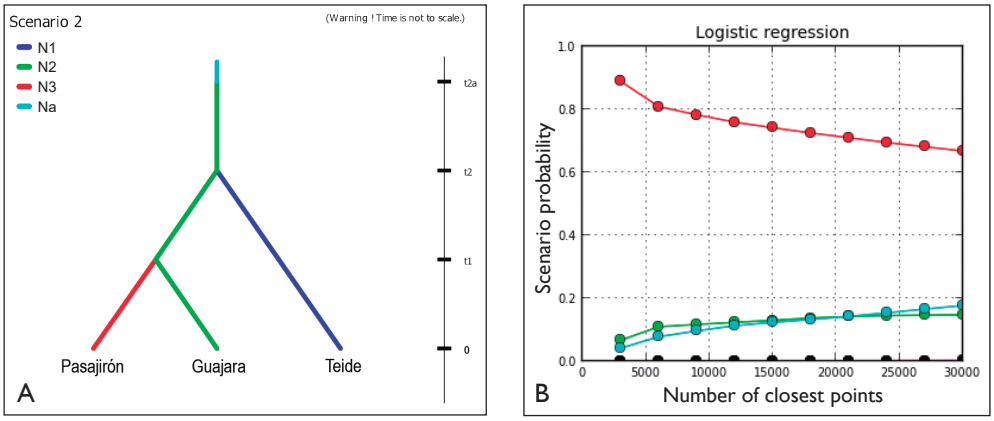


Figure 5.6 Demographic analysis of *Viola cheiranthifolia* populations using the ABC method implemented in DIYABC (Cornuet *et al.* 2014). A) Representation of the most probable scenario (Scenario 2); the populations studied were inferred from the STRUCTURE and the Rho pairwise distances results. t1, t2, t2a: time scale of divergence times, measured in generations, since present (t=0). N1, N2, N3 and Na refer to effective population sizes respectively of standing populations (Teide, Guajara and Pasajirón) and from a non-sampled ancestral population. B) Probability of each scenario over the 10 % of the closest simulated and observed data using the logistic regression method implemented in DIYABC. The 'red line' represents the scenario 2, while the others represent the other 5 scenarios. See the tested demographic scenarios in the Figure S.5.1.

Demographic Parameter	Mean	Median	Mode	Quantile 2.5 %	Quantile 97.5 %
Effective population size					
N1 (Teide)	6,370	6,470	6,820	2,420	9,680
N2 (Guajara)	4,690	4,470	4,040	1,290	9,160
N3 (Pasajirón)	283	166	115	40	1,310
Na	7,090	7,900	9,810	938	9,920
Time scale in generations					
t1	244	208	134	54	657
t2	1,530	1,240	857	362	4,630
t2a	9,460	8,740	4,610	2,160	19,100

Table 5.6 ABC demographic parameters estimates for scenario 2. The mean, median and mode for each parameter are given, along with 95 % credibility intervals. N1, N2, N3 and Na refer to effective population sizes in number of diploid genomes respectively of standing populations (Teide, Guajara and Pasajirón) and from a non-sampled ancestral population respectively, as modelled in the most-likely of the six tested scenarios (Figure S.5.1). t1, t2, t2a: time scale of divergence times, measured in generations, since present (t=0).

Estimates of effective population size were contrasted across populations with much bigger sizes in Teide (N1: 6,820 [2,420; 9,680] diploid genomes), mode and 2.5-97.5 % quantiles of the posterior distribution respectively, compared to Guajara (4,040 [1,290; 9,160] diploid genomes) and Pasajirón (N3: 115 [40.4; 1,310] diploid genomes] (Table 5.6). The ancestor population showed a higher effective population size (Na: 9,810 [938; 9,920] diploid genomes), although this last estimate is unreliable due to the high bias and error and low factor 2 (Table S.5.1).

5.3.3. Species distribution modeling

All algorithms performed well for the majority of runs and with an average of scoring over 0.8 for both evaluation metrics (Figure S.5.3). Snow cover was the most important variable in all models except GBM, followed by annual precipitation (Bio 12), mean annual temperature (Bio01) and slope (supplementary table S.5.2). Conversely topographic position and TPI showed very low values.

The total current present area encompassed 5,267 km2 (Figure 5.6), therefore the realized distribution is only 1.91% of the total designated area. Climate suitability was declared exclusively within Teide stratovolcano and the top of the Las Cañadas Wall area, with no suitable areas between them. Climate change scenarios combinations showed a general decrease in the species suitability (Figure 5.6) but with strong variations between GCMs, RCPs and years (Figure 5.7, Table S.5.3). For instance, in the MIROC model under RCPs 2.6 and 6.0 climate suitability showed an increase in the suitability in 2080 but a sharp decrease under the 8.5 RCP scenario. As shown in Figure 5.6, under climate change, suitable areas are prone to diminish in Guajara-Pasajirón and in the lower areas of the stratovolcano. Under the most pessimistic scenario (MIROC, RCP 8.5 year 2080), the remaining suitable areas persisted only at the summit of Teide stratovolcano.

With respect to simulations of heterozygosity loss, the Las Cañadas Wall cluster initially showed higher heterozygosity values than the Teide cluster (Figure 5.8). Under all the combinations of scenarios, simulations of heterozygosity loss behaved in almost the same manner: values increased slightly for both genetic clusters, until the year 2060, when values dropped sharply. In all scenarios except MIROC RCP=8.5 and CSIRO RCP=6.0 the decrease in heterozygosity was somewhat higher in the Las Cañadas Wall than in the Teide cluster. Both year and RCP had a significant impact on the variation in heterozygosity in the simulations, but not model or interactions between factors (Year: F=257.885, P<0.001, Df=1, RCP, F=39.562, P<0.001, Df=8).

a) Current niche suitability

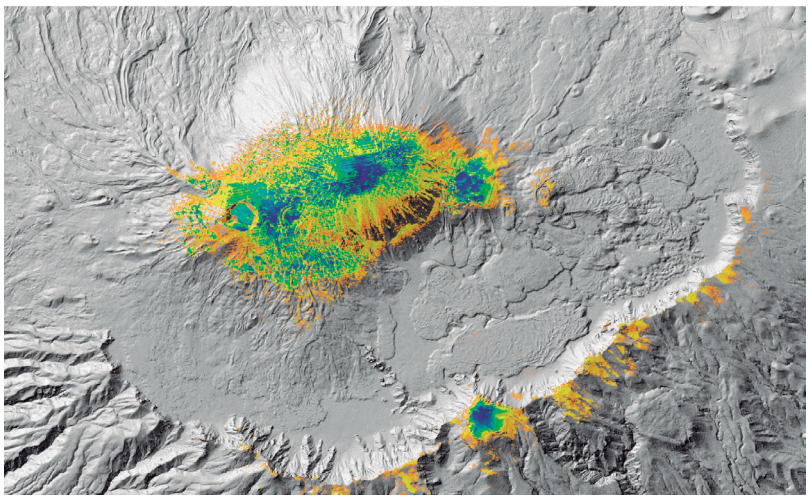


Figure 5.6 a) Current topoclimatic suitability of *Viola cheiranthifolia*.

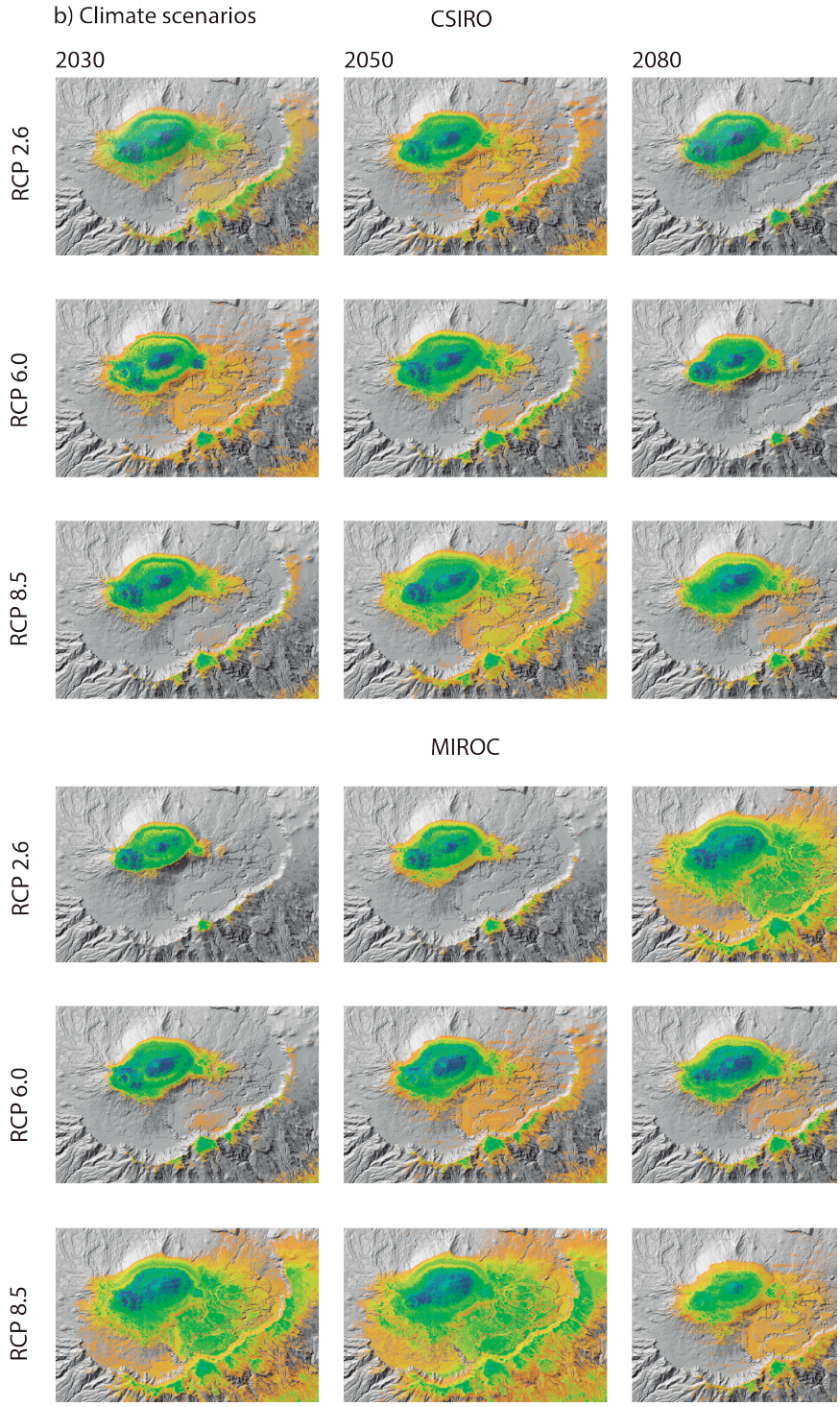


Figure 5.6 (continued) b) Projections of topoclimatic suitability under climate change scenarios. Suitability is displayed in a continuous range for the present, and in presence/absence for climate change scenarios for a threshold of 650 according to the TSS score (see results).

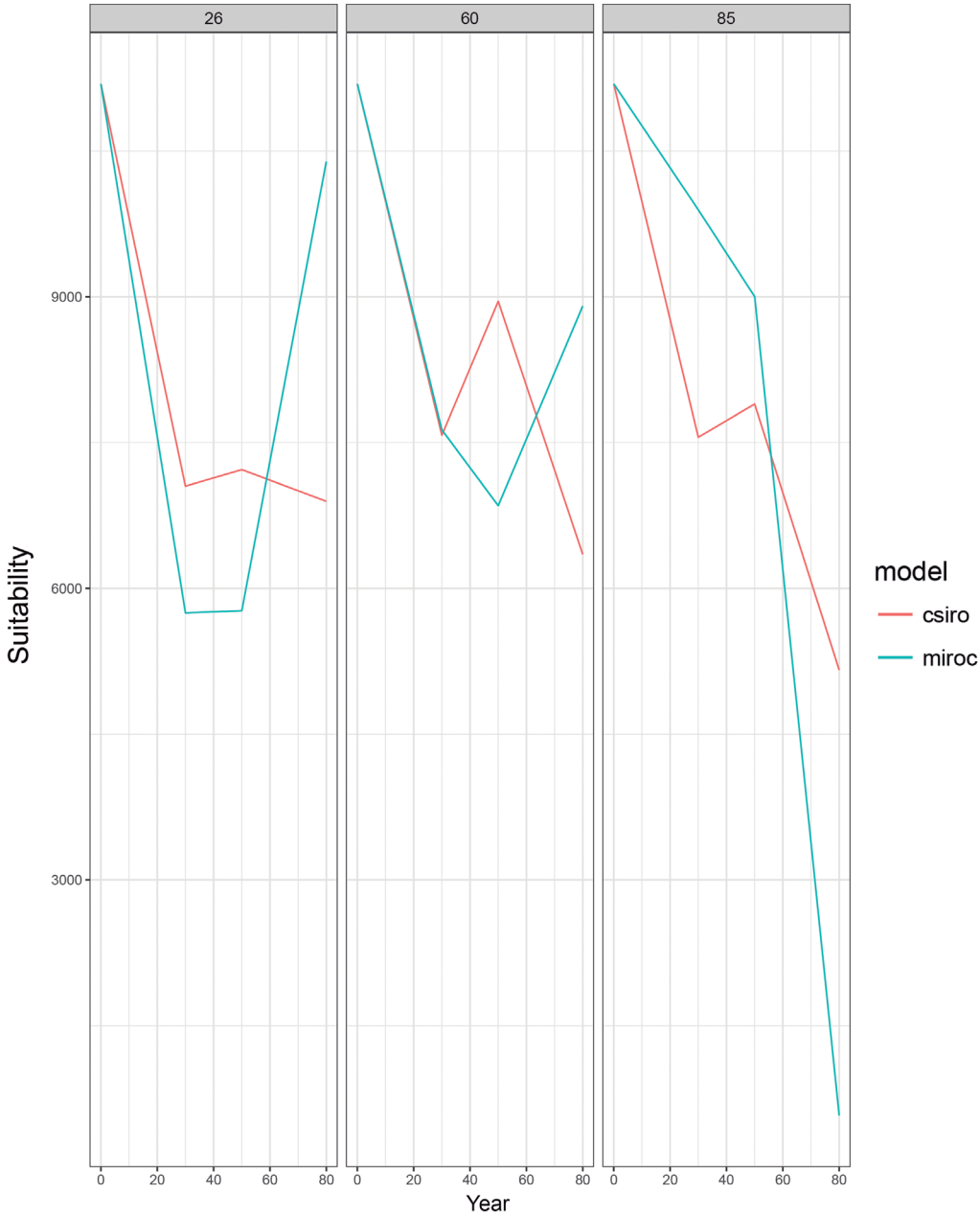


Figure 5.7 Number of suitable cells of *Viola cheiranthifolia* (suitability > 650) for each climate change scenario.

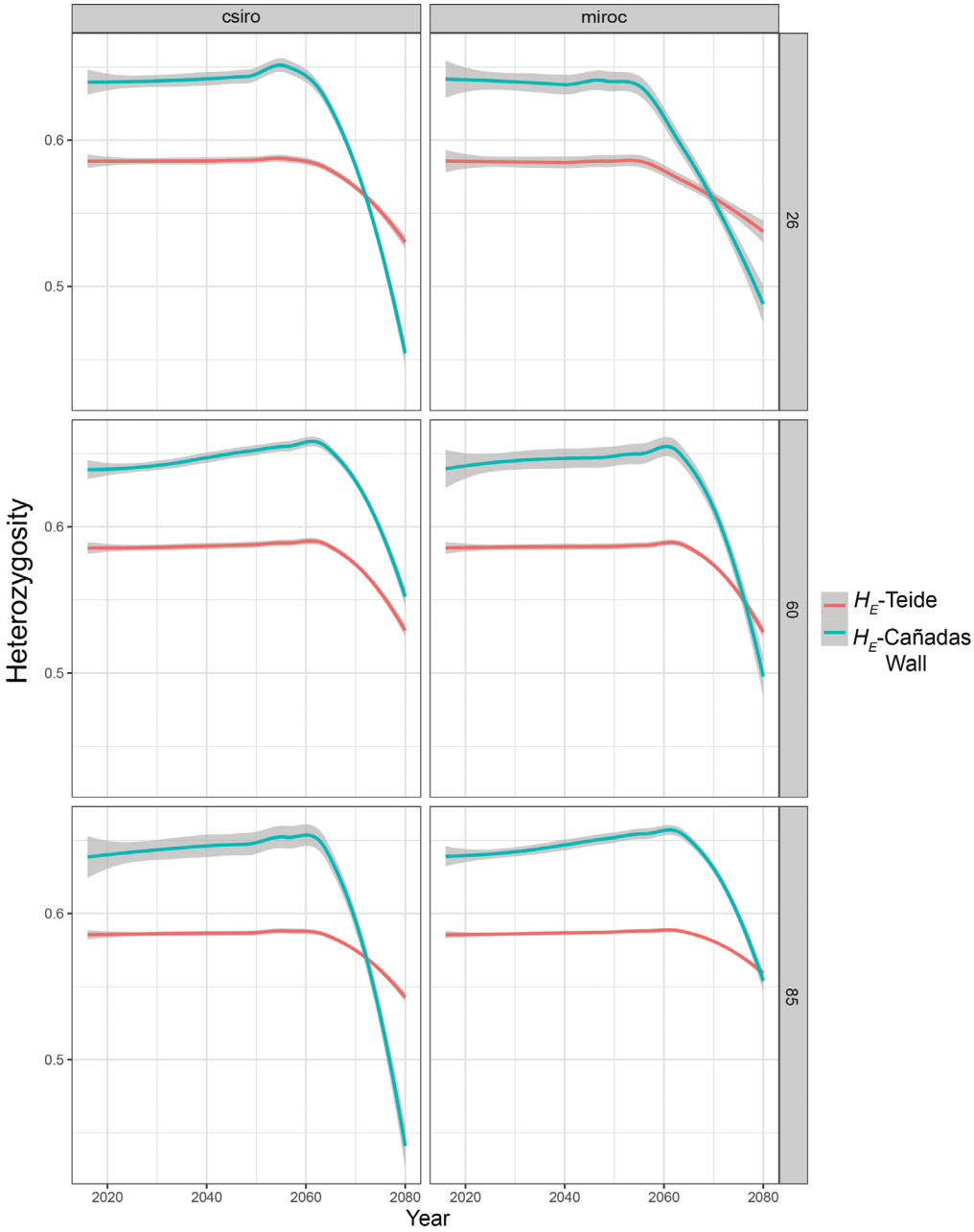


Figure 5.8 Simulated levels of heterozygosity across time for the combinations of RCP and GCM for both populations, Las Cañadas Wall and Teide.

5.4. DISCUSSION

5.4.1. Genetic diversity of an endemic alpine polyploid

The tetraploid pattern found in *Viola cheiranthifolia* agrees with other studies of species of polyploid origin in the sect. *Melanium* (Marcussen *et al.* 2010). Although we found a maximum of four alleles per sample and locus, a detailed chromosome count and exact estimation of the ploidy level would be of interest for the evolutionary history of the species within sect. *Melanium*. Moreover, the high selfing rate estimated (around 50%) in this study agrees with the values of autonomous selfing obtained in a previous study (Seguí *et al.* 2017). Nonetheless, they detected a significant increase in self-fertility with elevation and with rabbit herbivory.

Despite being an isolated endemic species with self-fertility, *V. cheiranthifolia* did not show diminished levels of genetic diversity, just like its relative *V. palmensis* at the summit of La Palma (Batista & Sosa 2002). Furthermore, the expected heterozygosity levels detected in *V. cheiranthifolia* (average per population = 0.550; total = 0.637) were similar or even higher than those ones in diploid violets studied with microsatellites (Culley 2005; Hirai *et al.* 2012; Kang *et al.* 2016, 2017). In the Canary Islands, taxa with either lower or higher genetic variation than *V. cheiranthifolia* can be found (Sosa *et al.* 2011). For example, another Teide endemic *Silene nocteolens* (Sosa *et al.* 2011), the Canary olive tree *Olea europaea* ssp. *guanchica* (García-Verdugo *et al.* 2010) and the narrow-ranged *Ruta oreojasme* (Meloni *et al.* 2015) presented surprisingly high genetic variation values. On the other hand, the species within the endemic genus *Bethencourtia* (Rodríguez – Rodríguez, in review), *Ilex perado* ssp. *lopezlilloi* (Sosa *et al.* 2011) or *Limonium macrophyllum* (Jiménez *et al.* 2017) presented very low levels of diversity, typical of rare endemic species (Cole 2003). However, the generalization that endemic species lack variability is not always certain, as in many cases their genetic diversity equals that of their widespread congeners (Gitzendanner & Soltis 2000). It has also been pointed out that endemic plants in the Canary Islands show higher levels of genetic diversity than expected for oceanic archipelagos (Francisco-Ortega *et al.* 2000), possibly due to their close proximity to the continent.

High genetic diversity levels present in rare species can also be an indicator of a wider distribution in the past (Ellstrand & Elam 1993; Sosa *et al.* 2010), when all invasive herbivores were absent and the climate was more favorable.

The estimations of effective population sizes did not show a notable decline over time, although these estimates cannot be accurate due to the lack of prior knowledge of *V. cheiranthifolia*, and the results presented broad confidence intervals. Moreover, the species' biology makes demographic censuses difficult, as the emerging individuals depend highly on the annual precipitation.

Therefore, the rapid radiation taking place in the section *Melanium* coupled with polyploidization events (Yockteng *et al.* 2003), could be the most feasible factors to have enhanced a higher genetic diversity. It can be concluded that *V. cheiranthifolia* shows moderate levels of genetic variation possibly favored by its polyploid origin, and counteracted by its isolated condition and high levels of self-fertility.

5.4.2. Genetic structure and demographic history of populations

The population differentiation analysis (STRUCTURE, PCoA, Rho statistic and AMOVA) showed a clear differentiation between the populations in Teide and the ones in Las Cañadas Wall. This genetic barrier was expected since the groups are separated by more than 6 km, and it is well known that solitary bees tend to forage within 2.0 km of their hive if there are attractive floral resources in the vicinity (Zurbuchen *et al.* 2010). Nonetheless, periods of strong winds could promote the accidental displacement of bees, opening temporary windows for gene flow. The Species Distribution Modelling approach also supports the existence of a geographical barrier. Since the models do not declare this central area as suitable, the hypothesis of the of a continuous population can be discarded. However, we did not detect a phylogeographic signal with the permutation tests described in Hardy *et al.* (2003). Lack of signification suggests high migration rates between populations and/or a relatively low number of generations in isolation (Hardy *et al.* 2003). The second option is highly plausible in this case, due to the young age of the Teide-Pico Viejo complex and the perennial trait of *V. cheiranthifolia*.

Although there is an apparent segregation within populations in the STRUCTURE results, the low percentage of variation between localities within groups shows evident gene flow within Teide. Between Guajara and Pasajirón this subpopulation structure is more evident due to the greater geographical distance. Besides this, there was admixture found between the two clusters

in Las Cañadas Wall, with some individuals on the north side of Guajara being clustered together with Pasajirón individuals. Review analysis of genetic variation in plants has shown that long-lived, outcrossing, late successional taxa retain most of their genetic variability within populations (Hamrick & Godt 1990; Nybom 2004). The significant SGS found was also congruent with the subpopulation structure findings and the high percentage of variation within localities, as the short-distance dispersal ability of *V. cheiranthifolia* seeds and the high levels of self-fertilization are promoting the genetic spatial structure among individuals. Nevertheless, following the review made by Vekemans and Hardy (2004), our values for the *Sp* statistics are lower than their estimations according to the biotic traits of *V. cheiranthifolia*. *Sp* statistics are expected to be inversely proportional to the density (Heywood 1991), and it has been found that the damage caused by rabbits in *V. cheiranthifolia* significantly decreases the population densities (Seguí *et al.* 2017). Moreover, pollen dispersal by insects may be promoting higher outcrossing among individuals within populations, compensating for the effects of self-fertility on the SGS. If the strong herbivory by rabbits continues and the species tends to shift upwards as expected, it could lead to an increase of selfing, and, therefore, a change in the spatial genetic structure of the populations.

According to the ABC estimates (115,250 yrs ago), the species was present in Guajara after the lateral collapse of northern flank of the island of Tenerife, which formed Las Cañadas Caldera. The hypothesis that the individuals in the Teide group were colonized from the older parts of Guajara was supported by the ABC estimates (21,425 yrs ago), since Teide stratovolcano reached its maximum size 30 ka BP . Moreover, Guajara showed higher levels of variation than Teide, which supports the idea of a founder effect from an older population (Frankham *et al.* 2002). Eruptive activity of the Teide cone seems to have declined over the past 30 ka, with only some eruptions of peripheral lava domes (10-2 ka) and the last prehistoric eruption of Lavas Negras (1,150 yrs BP) in the Teide cone (Carracedo *et al.* 2007).

5.4.3. The fate of *Viola cheiranthifolia* populations and implications for conservation.

Projections of the fate of alpine species under climate change are considered among the most pessimistic due to the spatial limitation for upward migration to meet suitable climate conditions especially accounting for the limited migration

ability of most of these species (IPCC Working Group I 2013). In this paper, we used an accurate spatial scale to reflect the interplay between topographic and climate constraints, also allowing us to minimise the risk of overfitting models. SDMs and their use to simulate heterozygosity loss allows us to draw two conclusions for the conservation strategy of the species: one is the persistence of suitable areas and the second, the extent of heterozygosity loss.

Regarding the first, suitable habitats in the future will allow in situ conservation of the species. Contrary to the generalized expectation of extinction of mountain species, projections show that the climatic niche of *V. cheiranthifolia* will be able to persist under any scenario, thus facilitating the conservation approach for the species. Furthermore, Seguí *et al.* (2017) found that a lack of snow can facilitate reproductive success. Therefore, demographic parameters for *V. cheiranthifolia* can improve under warmer scenarios. We found, nevertheless, a great uncertainty linked to the GCM used for climate change scenarios. We warn that three assumptions made in the modelling approach must be met to validate these forecasts: 1) reproductive success is high enough to maintain demographic viability of the species. Therefore, the conservation strategy for the species has managed to prevent herbivory by introduced rabbits as warned by Seguí *et al.* 2017; 2) *Viola cheiranthifolia* is fully able to migrate to new suitable areas. Seed dispersal occurs mostly by diplochory, which does hinder species' ability to adapt to climate change which indeed hinder species to keep with climate change. SDMs show that in most scenarios the climate suitability does not migrate towards the Teide summit, but rather the current distribution decreases in peripheral areas. Therefore, conservation strategies may not need to take into account measures to facilitate seed dispersal, as populations are likely to be sufficiently static. Monitoring of distribution shifts must still be performed, as part of the conservation strategy; and 3) no other environmental constraints related to human land occupation or soil properties are preventing suitable areas hosting *V. cheiranthifolia* populations. Regarding anthropogenic disturbances, since the summit of Teide Tenerife has the maximum conservation status, further anthropogenic constraints affecting the distribution are unlikely.

The second conclusion, regarding the extent of heterozygosity loss, allows the linkage of genetic markers with climate change scenarios, showing how heterozygosity values will only drop by year 2060. This means that genetic diversity levels will remain stable for a long period before showing a

decline consistent with the suitability loss forecast for the last time period. Whilst comparison with other organisms remains difficult given the number of factors affecting genetic diversity, our results were consistent with other papers predicting a loss of genetic diversity proportional to habitat loss (Alsos *et al.* 2012; Blanco-Pastor *et al.* 2013; Chala *et al.* 2016). The main reason for concern is the loss of heterozygosity in the Las Cañadas Wall population, which needs to be supported in situ and ex situ, possibly by reinforcing/enriching populations.

In summary, we have clarified the current genetic status of *V. cheiranthifolia*, together with its demographic history and future climate suitability. Overall, it is a resilient alpine species with moderate to high genetic diversity. However, herbivory pressure is seriously affecting its populations by reducing fitness and population densities, increasing self-fertilization and reducing their generation time (Seguí *et al.* 2017). Although the estimations show that the species will be able to sustain itself under climate change, there is a tendency to a shift in altitude of habitat and the heterozygosity loss will increase by year 2060. Populations in Las Cañadas Wall may be the most affected of all, as an upward migration is not possible. On the one hand, Pasajirón presents a small population size and variability. On the other, Guajara holds an important genetic source for the species. Therefore, the implementation of fenced areas, as well as an exhaustive control of herbivores (specially rabbits) in every locality of the National Park, are urgent measures for the long survival of *V. cheiranthifolia*. Moreover, monitoring of the upward shift in Las Cañadas Wall and Teide, would help detect the decline in populations over time. Finally, this is the first study which combined population genetics with niche modelling and heterozygosity loss estimations in an alpine oceanic endemic. More studies like this could help to understand population dynamics and patterns of distribution of alpine plant species under current climate change conditions.

5.5. SUPPORTING INFORMATION

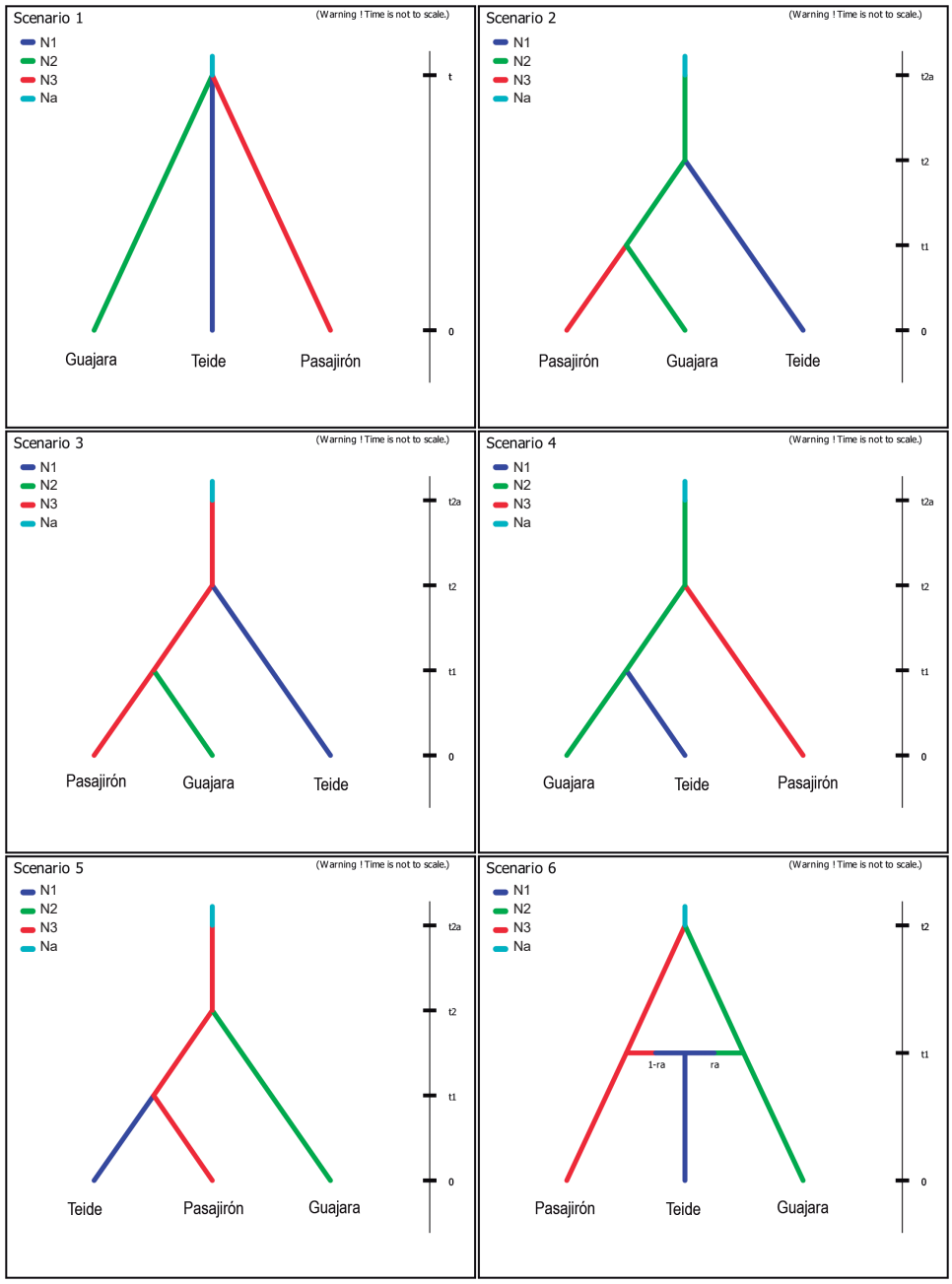
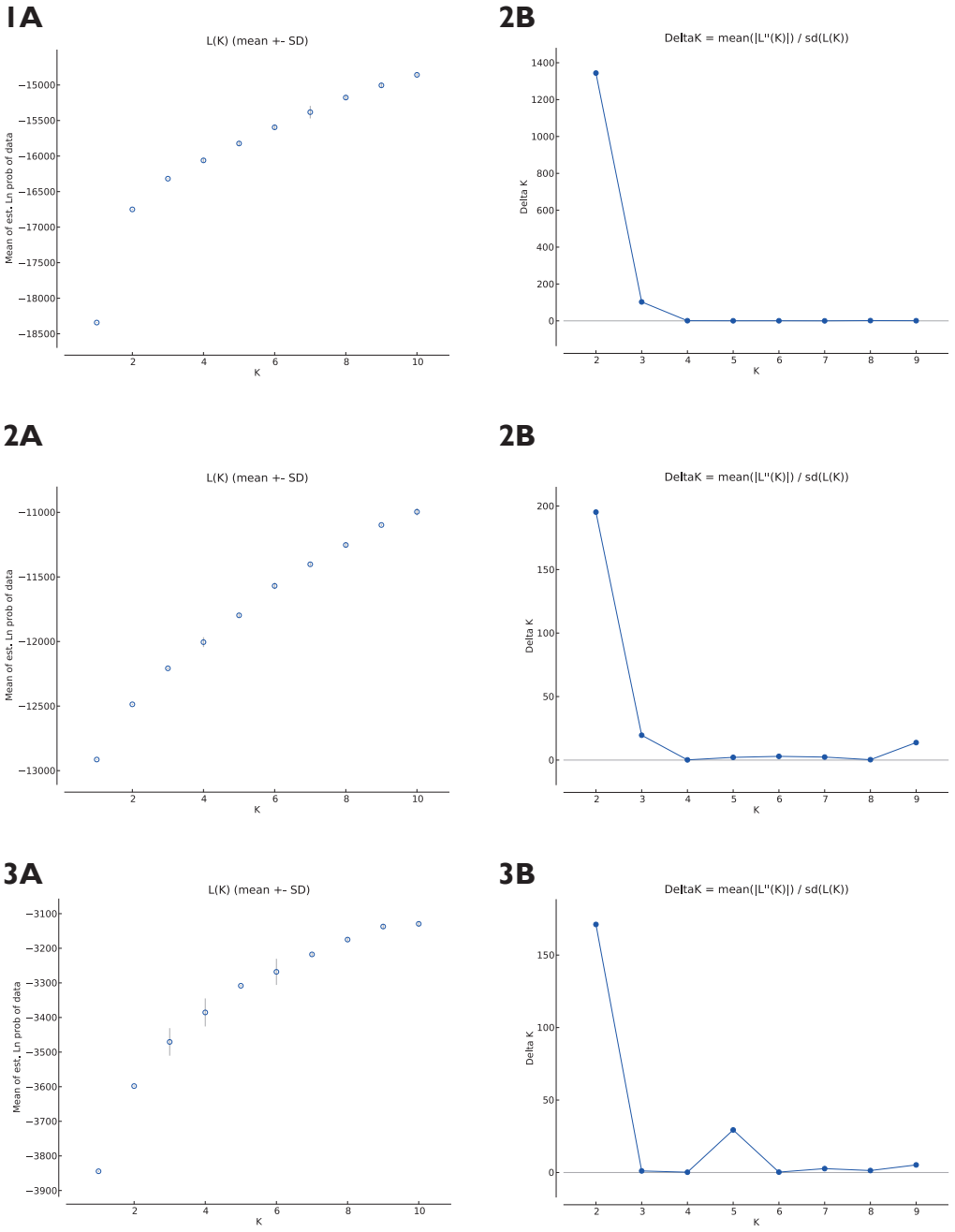


Figure S.5.1 The six scenarios of population demography of *Viola cheiranthifolia* examined by ABC analysis as implemented in DIYABC (Cornuet *et al.* 2014). Teide (Pop1), Guajara (Pop2) and Pasajirón (Pop3) were considered in the scenarios following the groups obtained by the STRUCTURE and pairwise Rho distance results. t1, t2, t2a: time scale of divergence times, measured in generations since present (t=0). N1, N2, N3 and Na: population effective size of current populations (Teide, Guajara and Pasajirón) and a non-sampled ancestral population respectively.

Figure S.5.2 Output results from STRUCTURE HARVESTER. (A) The mean of log-likelihood values for each value of K (1-15), (B) Ad hoc statistic based on the rate of change in the log probability of data between successive K values (ΔK, following Evanno *et al.* (2005). 1A: Log-likelihood for each value of K (1-15) for the whole dataset. 1B: ΔK values for each K for the whole dataset. 2A: Log-likelihood for each value of K (1-10) for the Teide population. 2B: ΔK values for each K for the Teide population. 3A: Log-likelihood for each value of K (1-10) for the Las Cañadas Wall population. 3B: ΔK values for each K for the Las Cañadas Wall population.



	Prior distribution			Posterior distribution					Confidence in parameter estimation (mode)				
Demographic parameter	Family	min	max	mean	median	mode	Q0.025	Q0.975	Bias	RMSE	50 % coverage	95 % coverage	Factor 2
N1	U	10	20,000	6,370	6,470	6,820	2,420	9,680	0.0099	0.0099	0.506	0.964	0.956
N2	U	10	20,000	4,690	4,470	4,040	1,290	9,160	-0.0109	-0.0109	0.518	0.968	0.944
N3	U	10	20,000	283	166	115	40	1,310	-0.0151	-0.0151	0.500	0.974	0.902
Na	U	10	20,000	7,090	7,900	9,810	938	9,920	17.264	17.264	0.456	0.952	0.416
t1	U	1	10,000	244	208	134	54	657	-0.1846	-0.1846	0.504	0.966	0.784
t2	U	1	10,000	1,530	1,240	857	362	4,630	-0.0454	-0.0454	0.476	0.956	0.902
t2a	U	1	10,000	9,460	8,740	4,610	2,160	19,100	0.2744	0.2744	0.498	0.954	0.912

Table S.5.1 All simulations were run in DIYABC v2.0 (Cornuet *et al.* 2014). Description of demographic parameters and tested population history scenarios are detailed in Figure S.5.1 and Table 5.6. Bias: mean relative bias (distance between mode of the mode (global) and true value); RMSE: relative mean square error (precision, distance between each estimates ant the true value); 50% and 95% coverage: proportion of simulations in which the true value lies within the 50% and 95% credible interval around the estimate; Factor 2: proportion of estimated values falling within the interval between 50% and 200% of the true value. The performance of parameter estimation was assessed by simulating 5,000 pseudo-observed datasets generated using known demographic parameter values drawn from prior distributions. Comparisons of known and estimated demographic values were used to infer bias and precision of estimations (Cornuet *et al.* 2014).

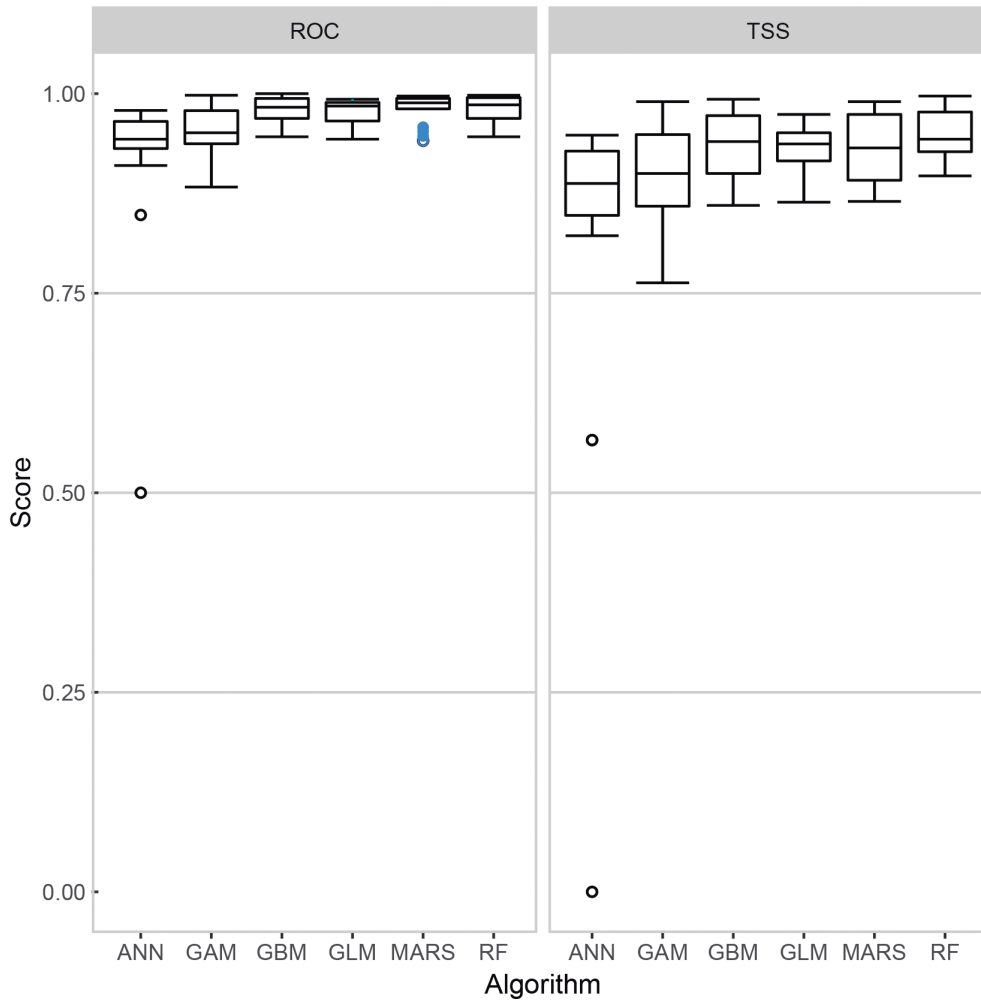


Figure S.5.3 Boxplots of the scores of the two evaluation metrics used in SDMs, TSS and ROC, for the ensemble of runs of each algorithm.

Variable	Ensemble	ANN	GAM	GBM	GLM	MARS	RF
Slope	0.226	0.119	0.315	0.319	0.163	0.120	0.261
TPI	0.020	0.008	0.152	0.003	0.025	0.030	0.005
PET	0.032	0.109	0.208	0.026	0.064	0.012	0.009
Bio01	0.238	0.307	0.495	0.692	0.204	0.326	0.214
Bio12	0.374	0.560	0.656	0.025	0.449	0.607	0.035
Snow cover	1.000	1.000	0.980	0.252	1.000	0.995	0.074

Table S.5.2 Variables importance for the individual and ensemble models.

Model	rcp	year	Suitable area
current	current	current	13,167
miroc	26	30	5,748
miroc	60	30	7,632
miroc	85	30	9,896
csiro	26	30	7,052
csiro	60	30	7,576
csiro	85	30	7,555
miroc	26	50	5,770
miroc	60	50	6,851
miroc	85	50	9,003
csiro	26	50	7,222
csiro	60	50	8,954
csiro	85	50	7,897
miroc	26	80	10,394
miroc	60	80	8,906
miroc	85	80	576
csiro	26	80	6,896
csiro	60	80	6,349
csiro	85	80	5,160

Table S.5.3 Number of suitable cells for each combination of model, emissions scenario and temporal horizon.

Foto: Pedro Sosa

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