PROCESOS ECOLÓGICOS Y EVOLUTIVOS EN INSECTOS DE LA MACARONESIA

TESIS DOCTORAL

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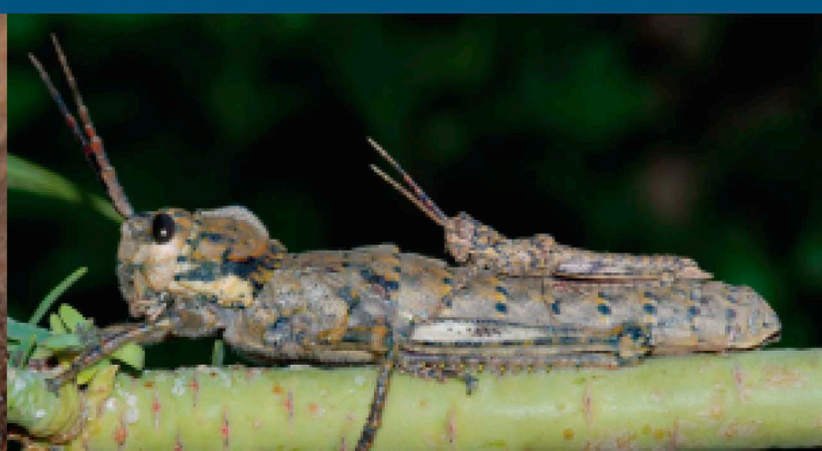

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Universidad de La Laguna

Departamento de Biología Animal y Edafología y Geología

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El Dr. Pedro Oromí Masoliver, Catedrático de Zoología del Departamento de Biología Animal y Edafología y Geología de la Universidad de La Laguna, y el Dr. Heriberto D. López Hernández, Investigador del Instituto de Productos Naturales y Agrobiología (IPNA-CSIC)

CERTIFICAN

Que la tesis doctoral titulada "Procesos ecológicos y evolutivos en insectos de la Macaronesia" presentada por el Licenciado David Jesús Hernández Teixidor, ha sido realizada bajo su dirección, y consideran que reúne las condiciones necesarias de calidad y rigor científico, autorizando su presentación y defensa para optar al grado de Doctor en Biología.

y para que conste a los efectos oportunos, firman la presente en La Laguna, a 25 de noviembre de 2015.

Fdo. Pedro Oromí Masoliver

Fdo. Heriberto D. López Hernández

A mi familia

y a Vanessa

Agradecimientos

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1. Introducción general

INTRODUCCIÓN GENERAL

ISLAS Y EVOLUCIÓN

Desde la época de Darwin, las islas han atraído a científicos y naturalistas debido a sus características geográficas únicas, y a la diversidad de especies y hábitats que albergan (Mayr 1967, Emerson 2002). Además, la simplicidad de los sistemas insulares respecto a los continentales los ha hecho particularmente útiles para la realización de estudios ecológicos y evolutivos (Warren *et al.* 2015).

Gillespie y Roderick (2002) clasifican las islas en tres categorías: las islas darwinianas, originadas *de novo* en el mar por la actividad volcánica; los fragmentos insulares, islas constituidas al separarse del continente, y por tanto con una historia previa; y las islas mixtas, con un origen continental y volcánico. El ejemplo más claro de **islas darwinianas** son las islas oceánicas, desprovistas inicialmente de vida tras su formación, por lo que las especies que se encuentren en ellas son aquellas que han sido capaces de colonizarlas.

La característica común de todos los sistemas insulares es el aislamiento, el cual puede originar una biota única. El número de especies presentes en una isla depende del grado de aislamiento (lejanía al continente o de la fuente de migrantes) y del tiempo (Gillespie & Roderick 2002), así como de la capacidad de dispersión y establecimiento de cada especie, y la presencia o no en la isla de unas condiciones que satisfagan los requerimientos de dichas especies (Whittaker & Fernández-Palacios 2007). Además, este número también se ve influido por la dirección de los vientos y corrientes oceánicas predominantes (Cook & Crisp 2005). Estas características hacen que en islas haya un menor número de especies que en un ecosistema comparable de igual área en el continente (empobrecimiento), y que estén totalmente ausentes determinados grupos taxonómicos (desarmonía). Estos hechos

implican que en los ecosistemas insulares existan nichos vacíos, o lo que es lo mismo, recursos espaciales, temporales o alimenticios sin explotar (Fernández-Palacios 2004). Los individuos que llegan a una isla tienden a ocupar los nichos propios de su especie en el lugar de origen, y si hay otros vacíos, con el tiempo pueden intentar ocuparlos, surgiendo así diferencias ecológicas con respecto a sus parientes continentales. Así, las especies que consiguen establecerse en una isla se ven sometidas a procesos ecológicos y evolutivos que las modifican con el paso del tiempo, pudiendo originar nuevos taxones.

PROCESOS ECOLÓGICOS

Son aquellos que tienen lugar en pocas generaciones, y afectan a miembros o poblaciones de una misma especie sin implicar el cambio taxonómico de sus miembros. Estos procesos tienen lugar debido a la menor intensidad competitiva interespecífica que existe en islas respecto al continente, causada por el empobrecimiento y la desarmonía que hay en ellas. Entre los procesos más importantes destacan: i) la ampliación del nicho trófico: aumento en el rango de hábitats o recursos utilizados por una población, proceso que puede ocurrir cuando un competidor potencial está ausente; ii) la sobrecompensación de densidades: aumento de densidades y tamaños poblacionales de especies colonizadoras al estar ausentes sus competidores continentales; iii) el desplazamiento de caracteres: cambios en algún carácter usado para la explotación de un nuevo recurso; iv) el cambio de dieta: sustitución de un recurso trófico por otro ante la ausencia del usado en el continente; v) la sustitución de polinizadores o dispersores: la ausencia de polinizadores continentales comunes (abejas, avispas, colibríes, etc.) o dispersores (algunas aves terrestres y mamíferos no voladores) en islas relativamente lejanas ha permitido que animales que normalmente no

actúan como polinizadores ni dispersores, adquieran o amplíen estas funciones (Whittaker & Fernández-Palacios 2007).

Estos procesos pueden ser resultado de la plasticidad fenotípica, es decir, la potencialidad de un genotipo para producir múltiples fenotipos en respuesta a la variación del ambiente (Thompson 1991, Agrawal 2001, Pfennig *et al.* 2010). Se ha propuesto que la plasticidad fenotípica puede inducir mejoras en el apareamiento selectivo y restringir el flujo de genes entre poblaciones, llevando eventualmente a la especiación (Whitman & Agrawal 2009). Esto ha sido sugerido en particular para especies de insectos herbívoros, en los que la plasticidad fenotípica puede facilitar la especiación antes del desarrollo de las barreras de aislamiento reproductivo (Görür 2005).

PROCESOS EVOLUTIVOS

Son aquellos que tienen lugar a largo plazo, dando lugar a especies distintas a las de origen. Entre los procesos evolutivos más importantes en islas, Grant (1998) destaca la reducción de la capacidad de dispersión, los cambios de tamaño corporal o de algunas partes del cuerpo, y la hibridación. Posteriormente, Fernández-Palacios (2004) señala además como procesos evolutivos la especialización (reducción del nicho fundamental), la radiación adaptativa o no adaptativa, y la especiación (procesos que conducen a la formación de una o varias especies nuevas (neoendemismos)).

El proceso evolutivo más importante en islas, tanto desde el punto de vista ecológico como genético, es la **especiación** (Williamson 1981). Las especies no son entidades fijas, sino que sus características pueden cambiar a través de generaciones (Grant & Grant 2011). Las poblaciones pertenecientes a una especie se van diferenciando entre sí mediante selección o

mutación y deriva génica, pudiendo llegar con el tiempo a divergir, y eventualmente originar especies nuevas (Hey 2009). La especiación puede dividirse en etapas: la primera comienza con una sola población en la que existe variación entre los individuos; la segunda implica una población dividida en subpoblaciones fácilmente distinguibles que, sin embargo, todavía se entrecruzan; la tercera cuenta con distintas poblaciones con cruzamiento limitado; la especiación termina con distintas poblaciones, en las cuales el aislamiento reproductivo es irreversible (Hendry *et al.* 2009). Al ser un proceso continuo, una vez finalizada la última etapa el proceso volverá a empezar. Cada especie o población se encuentra en alguna de estas etapas, por lo que al realizar el estudio de una especie/población detectaremos una mayor o menor divergencia entre sus poblaciones en función de la etapa en que se encuentre. El conjunto de caracteres visibles que presenta un individuo (fenotipo) es el resultado de la interacción entre su genotipo y el ambiente en el que se ha desarrollado (Mazer & Damuth 2001). Dentro de ese conjunto de caracteres podemos diferenciar los caracteres taxonómicos, que se definen como cualquier estructura física (macroscópica, microscópica o molecular) o sistema de comportamiento que pueda tener más de una forma (estado del carácter), variación que potencialmente proporciona información filogenética (Quicke 1993). Además, Mayr (1969) los define como cualquier atributo de un miembro de un taxón por el cual se diferencia o pueda diferenciarse de un miembro de un taxón diferente. Los caracteres taxonómicos pueden ser morfológicos, químicos, fisiológicos, de comportamiento, genéticos, reproductivos, o ecológicos (Winston 1999).

Existen diversos criterios para diferenciar los tipos de especiación. El más empleado es el espacial, el cual clasifica los procesos de especiación en función de los patrones de separación geográfica de las poblaciones en vías de divergencia. Sin embargo, en los últimos

años ha cambiado la tendencia en la clasificación de los modelos de especiación, desde los clásicos basados solamente en los tipos de divergencia geográfica, a los basados en los procesos evolutivos que conducen a la divergencia genética (es decir, los "mecanismos" de especiación) (Fitzpatrick *et al.* 2008, Mallet *et al.* 2009, Gavrilets 2014). En la presente tesis doctoral se utilizará una combinación de ambas, basándose en una clasificación geográfica y dentro de ésta en función de los mecanismos que conducen a la especiación.

ESPECIACIÓN ALOPÁTRICA

Este tipo de especiación tiene lugar debido al aislamiento geográfico. Las poblaciones separadas tienen acervos genéticos distintos que con el paso de generaciones darán lugar a taxones distintos. Esto es debido a procesos como la deriva genética, la mutación y la selección natural que hacen que las poblaciones diverjan, alcanzando finalmente el aislamiento reproductivo al interrumpirse el flujo génico (Mayr 1963, Bush 1975, Bergstrom & Dugatkin 2012). La idea de que las especies pueden originarse por separación geográfica ya era defendida en el siglo XIX, siendo Moritz Wagner su mayor valedor (Mayr 1976). Actualmente este tipo de especiación está ampliamente aceptado y además considerado por muchos como el más frecuente en animales (Futuyma 2005, Santini *et al.* 2012).

Los casos más conocidos son:

Especiación por subdivisión o efecto vicariante

Una población inicialmente grande se subdivide en nuevas poblaciones aún relativamente grandes, quedando geográficamente separadas. Este aislamiento provocado interrumpe el flujo génico, comenzándose a acumular diferencias genéticas entre las poblaciones aisladas

que darán lugar a especies nuevas (Bush 1975, Bergstrom & Dugatkin 2012). Es el caso, por ejemplo, de la escisión de un fragmento continental o también el de las poblaciones localizadas en los extremos de una distribución continúa original, tras la extinción de las poblaciones intermedias (Fernández-Palacios 2004).

Especiación por efecto fundador

El establecimiento en un nuevo lugar de una nueva población a partir de un pequeño número de individuos (fundadores) provenientes de una población grande. Las poblaciones que están geográficamente aisladas difieren en tamaño, con una gran población (la de origen) y una o varias poblaciones pequeñas (Bush 1975). Este tipo de especiación implica que sólo una fracción de la variabilidad genética de la población de origen va a estar presente en el comienzo de la/s nueva/s población/es, haciendo que la deriva genética acelere la divergencia de la población. Un ejemplo clásico es el poblamiento de islas oceánicas a partir de individuos de una población continental tras atravesar un brazo de mar (Bergstrom & Dugatkin 2012).

ESPECIACIÓN PARAPÁTRICA

La especiación alcanza sin una separación geográfica completa de las poblaciones. Sin embargo, la existencia de una clina (gradiente espacial en la frecuencia de fenotipos o genotipos) provoca que el apareamiento no sea aleatorio, y por tanto que las poblaciones diverjan (Bush 1975, Bergstrom & Dugatkin 2012). Esta divergencia puede ser debida a una disminución del flujo génico dentro de la población, o a presiones selectivas que varían a lo largo del área de distribución de la población. Las dos poblaciones que están divergiendo son

contiguas en el espacio y presentan una zona híbrida en el área de contacto, la cual acabará desapareciendo completando el proceso de especiación (Barton & Hewitt 1985). En general, este tipo de especiación es difícilmente distinguible de la especiación alopátrica seguida de un contacto secundario posterior, lo cual ha conducido a amplios debates acerca de la importancia y validez de este tipo de especiación (Perfectti 2002).

ESPECIACIÓN SIMPÁTRICA

Engloba los procesos de especiación en los que las poblaciones divergen sin haber aislamiento geográfico (Fitzpatrick *et al.* 2008), por lo que son otros los mecanismos responsables de que una especie se separe en dos. La especiación se produce a pesar de la existencia de flujo génico sin restricciones o de cruzamientos aleatorios, por lo menos en el inicio de la divergencia del linaje (Bird *et al.* 2012). Los escenarios más comunes implican selección disruptiva, es decir, que la selección natural conduce a una población en dos direcciones diferentes a la vez (Coyne 2007). La idea de que la selección natural puede llevar a la divergencia y especiación de las poblaciones simpátricas se remonta a la época de Darwin, en la que este autor ya hacía referencia a esta posibilidad (Darwin 1859). Sin embargo, este modelo ha generado gran controversia durante varias generaciones de biólogos evolutivos, siendo Mayr uno de los más escépticos y críticos con esta idea en sus trabajos (Mayr 1963).

En las últimas décadas han aumentado enormemente los estudios publicados sobre la especiación simpátrica (p. ej. Via 2001, Berlocher & Feder 2002, Bird *et al.* 2012), al mismo tiempo que se afianzaba la idea de la importancia que puede jugar la ecología en el inicio de la especiación (Rundle & Nosil 2005).

Los factores más importantes que conducen a la especiación simpátrica son:

Adaptación a ambientes divergentes

Rasgos sometidos a la adaptación diferencial en hábitats ecológicamente diferentes a menudo pueden actuar como barrera inicial para el flujo génico entre especies incipientes. De este modo las barreras al flujo génico serían ecológicas y no geográficas. Este sería el caso de la **Especiación ecológica**, que ocurre cuando la adaptación a entornos divergentes, como diferentes recursos o hábitats, conduce al aislamiento reproductivo (Schluter 2000, Nosil 2012). Sin embargo, para que se complete el proceso de especiación, la selección divergente (o disruptiva) entre entornos o recursos debe causar divergencia adaptativa de las poblaciones (Elias *et al.* 2012). Los agentes de selección divergente durante la especiación ecológica son extrínsecos y pueden incluir factores abióticos y bióticos, como los recursos tróficos, el clima, el hábitat, e interacciones entre especies tales como la enfermedad, la competencia, y el comportamiento (Nosil 2012). Un ejemplo fácil de entender y aceptar es la especiación simpátrica debido a cambios de hospedador, ya que en este caso se produciría directamente el aislamiento reproductivo si el apareamiento tiene lugar en el hospedador (Bolnick & Fitzpatrick 2007).

Selección sexual

La selección sexual es una poderosa fuerza evolutiva que modela la elección de pareja. En especies con reproducción sexual cruzada, la reproducción exitosa siempre implica encontrar y elegir a una pareja, lo que genera la oportunidad para que la selección sexual actúe en fenotipos que participan en el apareamiento. Rasgos que influyen en la elección de

una pareja pueden conducir al apareamiento selectivo. Cuando el apareamiento selectivo es lo suficientemente fuerte, puede contribuir al aislamiento reproductivo entre las poblaciones y por lo tanto a la especiación (Grace & Shaw 2011, Herron & Freeman 2014). Actualmente se debate si la selección sexual puede conducir a la especiación por sí sola. Algunos autores creen que es poco probable, aunque puede ser un poderoso motor de la especiación en combinación con la selección natural (Servedio & Kopp 2012). La selección natural llevaría a la divergencia en las preferencias de apareamiento, ya sea por mecanismos ecológicos (especiación ecológica) o mutacionales (especiación por orden de mutación) (Schluter 2009).

Mutaciones, hibridación y poliploidía

La hibridación tiene muchos y variados impactos en el proceso de especiación. Por un lado, puede ralentizar o revertir la diferenciación al permitir el flujo génico y la recombinación (Abbott *et al.* 2013). Por otro, la hibridación puede acelerar la especiación ya sea mediante la transferencia de rasgos adaptativos a través de introgresión, mediante el establecimiento de formas recombinantes (especiación híbrida homoploide), o a través de alopoliploidización, que puede conducir a la especiación en pocas generaciones (Soltis 2013). La especiación por hibridación puede generarse a través de varios procesos:

i) La especiación híbrida homoploide es aquella que da lugar a un nuevo taxón, distinto de ambas especies parentales pero sin aumento de ploidía. Combinaciones novedosas de alelos parentales deben haber contribuido a la creación y persistencia de una nueva población que mantiene su distinción por medio de barreras reproductivas con ambos padres (Abbott *et al.* 2013).

ii) La poliploidía, la cual da lugar a especies que contienen tres o más conjuntos de cromosomas homólogos en lugar de los dos de sus antepasados diploides, es un mecanismo importante en la especiación híbrida, ya que crea una fuerte, aunque a menudo incompleta, barrera reproductiva postcigótica entre un híbrido y sus padres. Existen dos tipos de poliploides reconocidos: *autopoliploides* en los que los conjuntos de cromosomas derivan de la misma especie y *alopoliploides* que contienen conjuntos de cromosomas de diferentes especies como consecuencia de la hibridación interespecífica (Abbott *et al.* 2013). La alopoliploidía se considera que es más común en la naturaleza que autopoliploidía (Coyne & Orr 2004). Las mutaciones que provocan la poliploidización pueden producir aislamiento reproductivo instantáneo entre la población parental e hija (Bolnick & Fitzpatrick 2007). Aunque este tipo de especiación es mucho más común en plantas, se han documentado algunos casos en vertebrados (Herron & Freeman 2014). La hibridación y la poliploidización generan novedades genéticas que puede manifestarse como adaptaciones ecológicas, tales como la capacidad de ocupar nichos separados de los de las especies parentales. Es posible, por tanto, que la divergencia ecológica sea un importante motor de creación de alopoliploidía, permitiendo escapar de situaciones de desventaja en los hábitats de los padres (Soltis 2013).

iii) La introgresión adaptativa es la transferencia de una población a otra de alelos favorecidos por selección. Como resultado, el flujo génico entre las especies permite un intercambio de variables adaptativas de manera más rápida que en eventos de mutación. Esto puede reunir nuevas combinaciones de alelos adaptativos, que surgieron en diferentes poblaciones (Abbott *et al.* 2013).

Por otro lado, existe un modelo de especiación que combina mutaciones y selección conocido como especiación por orden de mutación. En este modelo el aislamiento reproductivo se alcanza mediante la fijación de diferentes mutaciones ventajosas en poblaciones separadas que experimentan presiones de selección similares (selección uniforme) (Mani & Clarke 1990, Schluter 2009). En esencia, diferentes poblaciones encuentran diferentes soluciones genéticas al mismo problema selectivo. A su vez, las diferentes soluciones genéticas (mutaciones) son incompatibles entre sí, provocando el aislamiento reproductivo. Las poblaciones no adquieren las mismas mutaciones o no las fijan en el mismo orden, por lo tanto la divergencia es estocástica pero el proceso implica selección, y por lo tanto es distinto de la deriva genética (Mani & Clarke 1990). La selección sexual puede causar esta especiación si el aislamiento reproductivo se desarrolla por la fijación de mutaciones alternativas ventajosas - por ejemplo, las que aumentan el atractivo del individuo - en diferentes poblaciones que viven en ambientes ecológicos similares (Nosil 2012).

PLANTEAMIENTO DE LA TESIS DOCTORAL

En todo este contexto geográfico, ecológico y evolutivo, algunos procesos en islas permanecen poco documentados. En esta tesis doctoral se intentará mejorar el conocimiento que se tiene de estos procesos mediante el estudio de especies insulares que los han experimentado. Las preguntas que queremos resolver son las siguientes:

1. **¿Qué influye más en la diversificación de un linaje insular, los factores geográficos o los ecológicos?**

En la literatura consultada ambos factores suelen ser importantes en el proceso de diversificación. Wang y colaboradores (2013) han demostrado que en *Anolis*, aunque ambos procesos contribuyen significativamente, el aislamiento geográfico explica sustancialmente más divergencia genética que el ecológico. Ello sugiere que los factores no ecológicos desempeñan un papel dominante en la evolución de la divergencia genética espacial de *Anolis*. Un resultado similar ha sido obtenido por Frey (2010) en la diversificación del género de caracoles marinos *Nerita*, la cual parece estar caracterizada por transiciones ocasionales a nuevos hábitats, junto con la predominante especiación alopátrica. Sin embargo, Sexton y colaboradores (2014), tras revisar 110 estudios de especies en poblaciones naturales, concluyen que el aislamiento ambiental o ecológico es más común que el producido por la distancia o geográfico, sobre todo en animales. En el segundo capítulo de esta tesis doctoral estudiaremos los factores que afectan a la diversificación de un linaje insular, y trataremos de descubrir qué factores son más importantes en cada caso concreto.

2. La adaptación a entornos divergentes, como recursos o hábitats distintos, ¿conduce a la especiación incipiente? Si es así, ¿qué características se ven afectadas?

La adaptación a recursos o hábitats diferentes puede conducir a la especiación, como así se ha detectado en varios grupos de animales y plantas. Algunos de los mejores ejemplos de especiación ecológica en animales son: a) insectos fitófagos en diferentes plantas hospedadoras, como los pulgones *Acyrthosiphon*, las moscas de la fruta *Rhagoletis*, los escarabajos *Neochlamisus* o los insectos palo *Timema*; b) peces con poblaciones diferenciadas por tipo de sustrato, salinidad, predación, etc, como

Gambusia, *Gasterosteus*, *Halichoeres*, etc; c) aves que se alimentan de diferentes tipos de semillas, como los pinzones de Darwin o de Tristan da Cunha; d) lagartos en diferentes hábitats como los *Anolis*; y e) caracoles *Littorina* con poblaciones a distintas alturas de marea (Hendry 2009).

Aunque ya se han detectado casos de este fenómeno en especies insulares, en el tercer capítulo intentaremos descifrar si este fenómeno de especiación tiene lugar en especies presentes en los archipiélagos de la Macaronesia, en particular Canarias, Madeira y Azores.

3. **¿Pueden ocurrir fenómenos de especiación en islas sin un aparente aislamiento geográfico ni ecológico? Si es así, ¿cómo tienen lugar estos fenómenos?**

Para intentar responder a estas tres preguntas de la mejor forma posible se han utilizado como modelos diferentes organismos presentes en la Macaronesia, como es el caso de los coleópteros del género *Rhopalomesites* presente en varios archipiélagos, y los ortópteros panfágidos de Canarias.

LA MACARONESIA

Es una región biogeográfica que incluye todos los archipiélagos de origen oceánico del Atlántico nororiental que están frente a las costas europeas y norteafricanas, es decir, los archipiélagos de Azores, Madeira y las Islas Salvajes, Canarias y Cabo Verde (Báez & Sánchez-Pinto 1983). Esta región está formada por 39 islas mayores de 1 km^2 y más de un centenar de islotes y rocas que no llegan a ese tamaño, distribuidas entre 14,8º N (Brava, Cabo Verde)

y 39,7º N (Corvo, Azores), y entre 13,4° W (Roque del Este, Canarias) y 30,9º W (Flores, Azores) (Fernández-Palacios, 2011).

Estas islas han surgido del mar por actividad volcánica dentro de la placa africana, salvo el archipiélago de Azores originado a partir de la actividad volcánica procedente de la dorsal oceánica mesoatlántica (Whittaker & Fernández-Palacios 2007). Sin embargo, el vulcanismo sigue activo solamente en los archipiélagos de Azores, Canarias y Cabo Verde (Fernández-Palacios 2011). Las edades de emersión de las islas de los archipiélagos macaronésicos varían desde los 29 Ma (Salvaje Pequeña, Islas Salvajes) hasta los 0,25 Ma (Pico, Azores), por lo que las más antiguas son las Salvajes (29,5 a 24,2 Ma), luego Canarias (22 a 1,2 Ma) y Cabo Verde (22 a 1 Ma), y finalmente Madeira (14 a 0,7 Ma) y Azores (8,12 a 0,25 Ma) [edades extraídas de Geldmacher *et al.* (2000; 2001), Carracedo & Day (2002), Azevedo & Ferreira (2006), y Dyhr & Holm (2010)].

Además de los cinco archipiélagos que actualmente conforman la Macaronesia, en el Atlántico Norte oriental hay una serie de montes submarinos, conociéndose el conjunto de ambos como la Paleo-Macaronesia (Figura 1.1, Fernández-Palacios *et al.* 2011). Dichos montes submarinos estuvieron emergidos en distintas épocas, millones de años atrás, estando datado el más antiguo en 60 Ma (Geldmacher *et al.* 2001). La emersión de estos grupos de islas entre los archipiélagos actuales, y entre ellos y los continentes europeo y africano, ha podido ser utilizada como puntos intermedios en el movimiento de poblaciones, facilitando la dispersión de especies a través del océano, fenómeno conocido como *stepping stone* (Kimura & Weiss 1964). Este fenómeno ha estado favorecido a lo largo del tiempo no solo por la formación de nuevas islas, sino también por la emersión durante las glaciaciones de montes submarinos sumergidos a menos de 120 m (nivel máximo de bajada del nivel del

mar respecto al actual), disminuyendo la distancia entre las islas actuales y el continente (Fernández-Palacios *et al.* 2011, Rijsdijk *et al.* 2014).

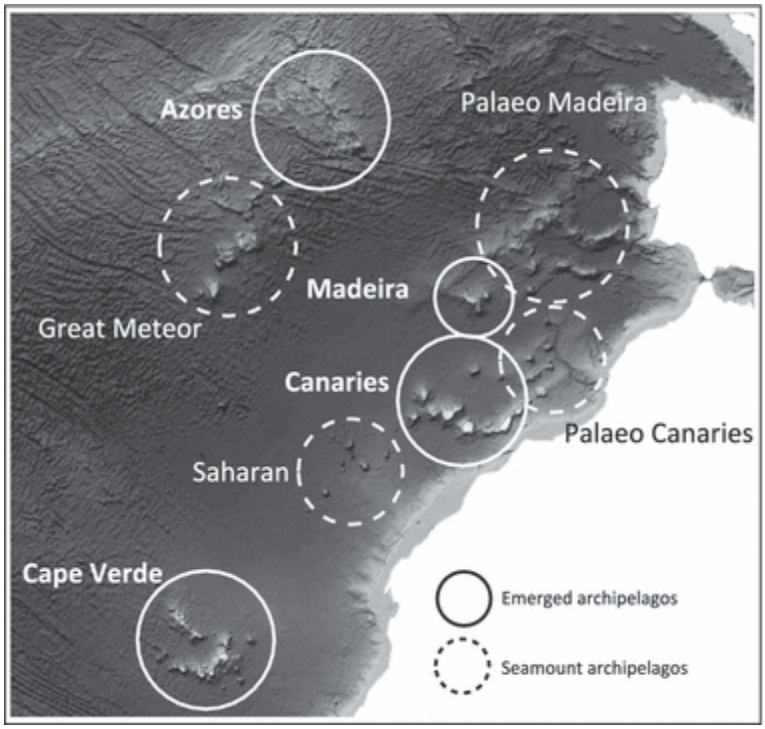

Figura 1.1. Mapa batimétrico de la región noreste del Océano Atlántico, donde se resaltan los archipiélagos emergidos actualmente y los montes submarinos, que en conjunto conforman la Paleo-Macaronesia. Figura extraída de Fernández-Palacios *et al.* 2011.

En la Macaronesia actual, al ser una región tan amplia, las distancias al continente varían enormemente, desde 96 km entre Fuerteventura (Canarias) y el Sáhara Occidental, a los 1.370 km entre São Miguel (Azores) y Lisboa (Whittaker & Fernández-Palacios 2007). Las importantes diferencias latitudinales existentes entre los archipiélagos de la Macaronesia hacen que existan diferencias climáticas significativas entre ellos. En Madeira y Canarias predomina un clima mediterráneo (inviernos húmedos y frescos, y veranos cálidos y secos), mientras que en Azores hay un clima oceánico templado y húmedo, y en Cabo Verde un clima cálido y seco con influencia del monzón tropical en verano (de Nicolás *et al.* 1989). Estas diferencias climáticas hacen que el periodo de lluvias en cada archipiélago también

varíe, de modo que en Azores se extiende a todo el año, mientras que en Madeira y Canarias se produce entre el otoño y la primavera (con el verano como estación seca), y en Cabo Verde solamente en verano debido a la influencia del monzón tropical. Respecto a las temperaturas, éstas siguen un gradiente latitudinal, disminuyendo hacia el Norte y uno altitudinal, disminuyendo hacia las cumbres (Fernández-Palacios 2011).

ESPECIES OBJETO DE ESTUDIO

Los coleópteros del género *Rhopalomesites* (F. Curculionidae, Subfam. Cossoninae) son endémicos de los archipiélagos macaronésicos de Madeira y Canarias excepto *Rhopalomesites tardyi* (Curtis, 1825), que se distribuye en parte de la Europa atlántica occidental y Azores (Löbl & Smetana 2013). Todas las especies presentan larvas xilófagas. Hasta la fecha se conocen siete especies pertenecientes al género *Rhopalomesites*, cuatro de ellas presentes en Canarias, tres en Madeira, y una en Azores y parte de Europa.

Rhopalomesites euphorbiae (Wollaston, 1854), descrita originalmente de Madeira, está citada además en Porto Santo y en las islas canarias de Tenerife, La Palma y La Gomera (Israelson *et al.* 1982, Oromí *et al*. 2010). Esta especie vive ligada a su principal planta hospedadora, la tabaiba de monte *Euphorbia mellifera*, en zonas de laurisilva de ambos archipiélagos. Además, en zonas bajas no forestales de Madeira y Porto Santo se alimenta de *Euphorbia piscatoria* Ait.. Sin embargo, en Canarias las *Euphorbia* de zonas xerófilas tienen ya sus propias especies de *Mesites* (Folwaczny 1984) y nunca albergan *Rhopalomesites*. La existencia de *Rh. euphorbiae* en Canarias fue descubierta tan sólo hace unos decenios (Israelson *et al*. 1982), y parecía haberse encontrado exclusivamente en *E. mellifera*. *Rhopalomesites palmi* (Folwaczny, 1979) está considerada como especie endémica de Porto

Santo, donde convive con *Rh. euphorbiae* en el matorral xerófilo de *E. piscatoria*. *Rhopalomesites proximus* (Wollaston, 1861) fue descrito del noreste de Tenerife sin especificar la planta huésped en la que se encontraba, y posteriormente recolectado por Lindberg (1958) en madera muerta de laureles. La especie *Rh. tardyi*, de Europa Occidental y Azores, se alimenta de la madera en descomposición de varios árboles forestales (Morris, 2002) y de acuerdo con Stüben (2005) raramente de *Euphorbia stygiana*. Las especies restantes son propias de la laurisilva: *Rhopalomesites maderensis* (Wollaston, 1854) de Madeira, *Rh. persimilis* (Wollaston, 1861) de Tenerife y La Gomera, y *Rh. complanatus* (Wollaston, 1861) de La Palma, donde se alimentan de madera de distintos árboles de laurisilva, principalmente laurel (*Laurus azorica*).

La distribución de las especies de este género y las diferentes plantas hospedadoras y hábitats en los que se encuentran parecen un inmejorable marco para estudiar la diversificación de un linaje insular. Además, permitirá testar qué ha tenido más peso en el proceso de diversificación del grupo, si los factores geográficos relacionados con la distancia entre poblaciones o los factores ecológicos como el hábitat que ocupan o sus plantas huésped.

La familia Pamphagidae (Orthoptera) está representada en Canarias por dos géneros exclusivos que incluyen cinco endemismos locales: *Purpuraria erna* Enderlein, 1929 de Fuerteventura, Lanzarote e islotes adyacentes, *Acrostira tamarani* Báez, 1984 de Gran Canaria, *A. tenerifae* Pérez & López, 2005 de Tenerife, *A. bellamyi* (Uvarov, 1922) de La Gomera y *A. euphorbiae* García & Oromí 1992 de La Palma (López *et al*. 2004, 2007a). Todas estas especies son habitantes de zonas abiertas con vegetación arbustiva xerófila, y parecen depender sobre todo de plantas del género *Euphorbia*, de cuyas hojas y brotes tiernos se

alimentan (López *et al*. 2005, 2007b). Son especies ápteras, con hembras de gran tamaño y machos mucho menores, que viven prácticamente todo el tiempo sobre la vegetación de la que se alimentan y usan para ocultarse, y rara vez bajan al suelo, salvo para cambiar de arbusto o cuando las hembras descienden para enterrar las puestas.

Los panfágidos canarios responden a unos patrones ecológicos y de distribución insular bastante uniformes salvo en dos de las especies. La primera es el endemismo gomero *Acrostira bellamyi*, que además de contar con poblaciones en los tabaibales como el resto de sus congéneres, tiene poblaciones en la laurisilva, un hábitat completamente distinto al tabaibal, dentro del cual las euforbias son extremadamente escasas. Casi todas las observaciones de esta especie en la laurisilva son de hembras que han descendido de la vegetación para hacer sus puestas en el suelo. Los machos probablemente permanecen casi siempre en el dosel vegetal, dados los escasos avistamientos de éstos que se han producido. En la laurisilva las observaciones de ejemplares de *A. bellamyi* sobre la vegetación habían sido muy escasas, y se desconocía sobre qué árboles o arbustos vive y cuáles son sus exigencias tróficas en estas poblaciones forestales tan inusuales en este grupo. Dado que han sido capaces de colonizar un nuevo hábitat con unas condiciones y recursos tróficos totalmente distintos, su adaptación al mismo ¿los habrá conducido a la especiación o lo habrán conseguido gracias a su plasticidad fenotípica?

La segunda es *Purpuraria erna*, especie con una distribución atípica (Lanzarote, Montaña Clara, Lobos y Fuerteventura) respecto al resto de panfágidos canarios que son siempre de distribución monoinsular y cuentan únicamente con una especie por isla. No se ha observado que las poblaciones de las diferentes islas e islotes presenten diferencias tróficas o de hábitat entre ellas. Sin embargo, el aislamiento sufrido por las poblaciones de

cada isla ¿ha podido llevar a cabo fenómenos de especiación o diversificación entre las mismas? O incluso, ¿han podido ocurrir fenómenos de especiación dentro de una misma isla sin la presencia de barreras geográficas o ecológicas visibles actualmente?

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1. Introducción general

Capítulo 2

OBJETIVOS

1. Determinar qué factores son más importantes, los geográficos o los ecológicos, en la diversificación dentro del género de coleópteros macaronésico *Rhopalomesites*.

2. Comprobar si la adaptación a entornos muy divergentes, como recursos o hábitats diferentes, conduce a la especiación incipiente en el caso del saltamontes *Acrostira bellamyi.*

3. Descifrar cómo ha tenido lugar la diversificación del género de saltamontes *Purpuraria,* sin aparente aislamiento geográfico ni ecológico.

Capítulo 3

Capítulo enviado:

Hernández-Teixidor, D., López, H., Pons, J., Juan, C. & Oromí, P. Host plant associations and geographical factors in the diversification of the macaronesian *Rhopalomesites* beetles (Coleoptera: Curculionidae).

HOST PLANT ASSOCIATIONS AND GEOGRAPHICAL FACTORS IN THE DIVERSIFICATION OF THE MACARONESIAN RHOPALOMESITES BEETLES (COLEOPTERA: CURCULIONIDAE)

ABSTRACT

The *Rhopalomesites* beetles are an attractive study-system to explore the extent and importance of geographical versus ecological barriers, among volcanic islands of both the same or different island groups, or even within the same island. The main aim was to obtain nuclear and mitochondrial phylogenies of Macaronesian *Rhopalomesites* weevils and test the monophyly and time of origin of two species groups feeding on Euphorbiaceae and other plants. Additionally, we aimed to investigate the population structure within species, and its associations with geographic isolation versus trophic selection. Maximum Likelihood and Bayesian phylogenetic analyses were undertaken using mitochondrial (*cox1* and *cytb*) and nuclear (ITS-2 and 28S RNA) genomic sequences. Ancestral *Rhopalomesites* host plant associations and divergence times were inferred from Bayesian analyses and population data. Evidence was found for two *Rhopalomesites* monophyletic lineages. One was associated with *Euphorbia* host plants, having vicariant species in the Madeira and Canary archipelagos. In this lineage, an ancestral association with *Euphorbia mellifera* in the two island groups was deduced, which has subsequently undergone shifts to related host plant species in marginal areas. A second ecologically generalist lineage—exploiting decaying wood from the Lauraceae or other forest trees—is also present on such islands along with the Azores and parts of Atlantic Western Europe. The results point to a quasi-parallel colonization of the two ecologically distinct lineages in Macaronesia, dating to the early

Pliocene, followed by allopatric isolation according to the presence of suitable habitats on particular islands in more recent times.

INTRODUCTION

Phytophagous insect lineages on oceanic islands provide unprecedented opportunities to explore the roles of adaptive radiation and niche conservatism in community assemblies (Hembry *et al.* 2012). Speciation on islands can be promoted by the initial presence of empty ecological niches and subsequent ecological shifts. These factors are associated with the geological origin and topographic and climate variations characteristic of such archipelagos, combined with recurrent geographical isolation events occurring among and within oceanic islands during their complex volcanic evolution (Emerson 2002).

The highly diversified endemic woody herb and shrub flora of Macaronesia (Azores, Madeira, Canary and Cape Verde archipelagos) is an important resource for herbivorous insects (Jordal 2006). On the Macaronesian Islands, several bark beetle lineages (Curculionidae: Scolytinae) that feed and complete their development on dead wood of dendroid *Euphorbia* plants have undergone niche shifts and radiations (Jordal *et al. 2*004, Jordal & Hewitt 2004). Within the scolytine bark beetle genus *Liparthrum,* species associated with the plant genus *Euphorbia* constitute a relatively old clade in which a scenario of cryptic species using specific *Euphorbia* host associations was uncovered (Jordal *et al.* 2004). In *Aphanarthrum* scolytine beetles, geographical isolation has been more important than host switching in favouring diversification (Jordal & Hewitt 2004).

Another promising phylogenetically unexplored Macaronesian lineage is that of the xylophagous *Rhopalomesite*s weevils (Cossoninae). Most of the species of this genus are

endemic to the Madeira and Canary archipelagos except for *Rhopalomesites tardyi* (Curtis, 1825), which is distributed over the Azores and Atlantic Western Europe (Löbl & Smetana 2013). The genus is noticeably associated with the Macaronesian laurel forest, a biome sustained by a cloud layer produced by highly humid trade winds blowing from the northeast, considered to be a "Tertiary relict" vegetation type (Fernández-Palacios *et al.* 2011). However, recent studies point to a much more recent origin for many of the Macaronesian laurel forest taxa, in the Pliocene/Pleistocene (Kondraskov *et al.* 2015 and references therein). Laurel forests in Macaronesia suffered repeated expansion–reduction cycles during the Pleistocene and their more recent progressive fragmentation and patchy distribution could be associated with the arrival of the first human settlers in the area (Fernández-Palacios *et al.* 2011, Kondraskov *et al.* 2015). Moreover, areas formerly covered by laurel forest have been replaced progressively by trees, or even shrubs, considered as marginal species (Moya *et al.* 2004). Species of *Rhopalomesites* are known to feed on several *Euphorbia* dendroid spurges, either in the laurel forests or in open marginal areas of the islands of Madeira, Porto Santo, Tenerife, La Palma, and La Gomera. However, other *Rhopalomesites* taxa are associated with Lauraceae and other trees in humid forests (Hernández-Teixidor *et al.* 2012).

Here, we aimed to analyse the role of host shifts versus niche conservation or generalist host associations in the Macaronesian *Rhopalomesites* taxa, and to determine the age and origin of these insect–plant associations. We sampled *Rhopalomesites* species from their complete area of distribution and used a nuclear and mitochondrial multigene approach to obtain a robust molecular phylogeny of the group. Evidence of two *Rhopalomesites* clades has been obtained: one consisting of specialist species ancestrally

associated with *Euphorbia mellifera,* while the other clade is a feeding generalist. Both groups originated in the Pliocene, consistent with age estimations recently calculated for several Macaronesian laurel forest taxa, including *Euphorbia* (Kondraskov *et al.* 2015).

MATERIAL AND METHODS

SAMPLING AND DNA DATA

Specimens were collected from the different host plants in their known geographical distribution on the Macaronesian islands, Great Britain and Ireland and stored in 100% ethanol at 4 ºC (see Table S1a in Appendix S1 in Supporting Information & Fig. 3.1 for the list of species, localities and host plants). After a careful revision of specimens in collection and our own observations, we concluded that *Rh. euphorbiae* is present in the Madeira archipelago but not in the Canaries, being the only species occurring on Porto Santo; while *Rh. proximus* is the only species feeding on *E. mellifera* in the Canary Islands (see Table S1a in Appendix S1 for details). Individuals of the related genera *Mesites*, *Pselactus, Amaurorhinus* and *Rhyncolus* in the subfamily Cossoninae were tentatively used as outgroups. *Mesites* spp., the morphologically more similar genus to *Rhopalomesites*, proved to be the phylogenetically closest taxa*,* so subsequent analyses were carried out using exclusively species of this genus as outgroup.

A DNA fragment of mitochondrial cytochrome oxidase I gene (*cox1*) and one of cytochrome b (*cytb*) were amplified along with regions of the nuclear ribosomal internal transcribed spacer 2 (ITS-2) and the nuclear large ribosomal subunit (28S RNA) (See Appendix S2 in Supporting Information for details of DNA extraction and sequencing).

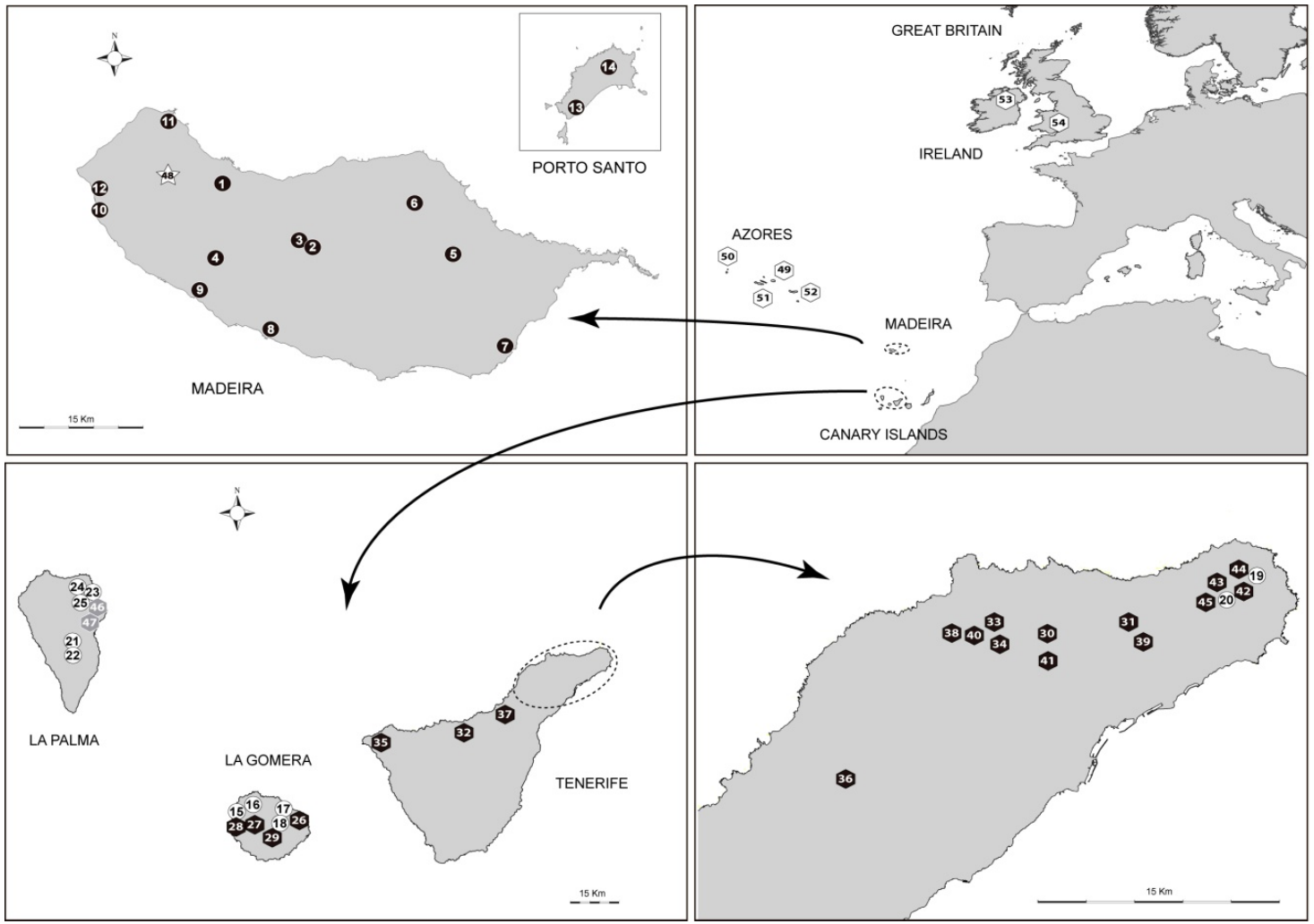


Figure 3.1. *Rhopalomesites* sampling sites in Macaronesia and Western Europe. Locality numbers as in Table S1a in Appendix S1 of Supporting Information. *Rhopalomesites euphorbiae* populations indicated with black circles; *Rh. proximus* white circles; *Rh. persimilis* black hexagons; *Rh. complanatus* grey hexagons; *Rh. tardyi* white hexagons, *Rh. maderensis* white stars.

SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSES

DNA sequences were aligned with MAFFT v.7 considering the secondary structures for 28S RNA and ITS-2 sequences. Two gap positions of one nucleotide were obtained for ingroup 28S RNA sequences and the alignment was straightforward for this marker. For ITS2, seven gaps (between 1 - 14 nuclotides (nts) long, with a total of 32 nt positions included in gaps from a total of 575) were obtained for the ingroup dataset but they could be aligned unambiguously. Both alignments have been deposited in the TreeBASE Repository (Accession number TB2:S18466). The nuclear genotypes from heterozygous individuals were manually reconstructed in order to identify their gametic phases (29 for ITS2 and 16 for 28S).

The program MrAIC (Nylander *et al.* 2004) was used to select the best nucleotide substitution model for each data partition of ingroup sequences according to the Bayesian Information Criterion (BIC). We explored seven different partitioning strategies performing Maximum Likelihood (ML) analyses in RaxML v. 7.2.8 (Stamatakis 2006) and compared them using BIC: 1) a single partition; 2) one partition for each gene; 3) mitochondrial + nuclear gene partitions, 4) mitochondrial codon position (MCP) 1 plus 2 + MCP 3 + nuclear; 5) MCP 1 + MCP 2 + MCP 3 + nuclear; 6) MCP 1+2 + MCP 3 + ITS2 + 28S, and finally 7) MCP 1 + MCP 2 + MCP 3 + ITS2 + 28S. Bayesian phylogenetic analyses were conducted with MrBayes 3.2.2 (Huelsenbeck & Ronquist 2001). Two independent runs were performed for each Bayesian search with default prior values, random trees and three heated and one cold Markov chains running for 20 million generations and sampled at intervals of 1000 generations. All parameters were unlinked and rates were allowed to vary freely over partitions. Convergence and accuracy of all parameters in the two independent runs were also assessed using Tracer v. 1.5 (Rambaut & Drummond 2007). After the 25% burn-in samples, the

remaining trees were combined into a single majority consensus topology, and the frequencies of the nodes in the majority rule tree were taken as the posterior probabilities (Ronquist & Huelsenbeck 2003). ML analyses were implemented in RaxML v. 7.2.8 (Stamatakis 2006) and support estimated by one 1000 fast bootstrapping replicates. Incongruence among genes was explored using Partition Bremer Support (PBS) values estimated with TreeRot v. 3 (Sorenson & Franzosa 2007). Likelihoods obtained through the Shimodaira–Hasegawa test (Shimodaira & Hasegawa 1999) were also used to test if the topology obtained by each gene partition was statistically different than the ones deduced from the other three markers in RAxML. Pairwise genetic distances for Tajima-Nei model with the gamma distribution were calculated between sequences, using MEGA v.6.0 (Tamura *et al.* 2013).

ESTIMATION OF DIVERGENCE TIMES

Node age estimates were obtained with Beast 1.7.5 (Drummond & Rambaut 2007), enforcing a relaxed or fixed molecular clock and a Yule speciation model. The expected long-term substitution rate at above the species level base of the tree is expected to be slower than the intraspecific diversification (coalescence), resulting in time estimates with broad confidence intervals if both species- and population-level sampling are mixed in the estimation of divergence time (Ho *et al.* 2005). To avoid this we used a reduced dataset including one specimen per species and island. We selected for this analysis the sequence that displayed the longest branch distance within species (i.e. the closest to the MRCA of the sequences sampled) to estimate maximum age estimates. We compared clock models using Bayes Factors estimated from marginal likelihoods based on stepping stone sampling in

BEAST (Baele *et al.* 2013). Five independent nucleotide substitution models were assumed as described above along with two independent clocks: one for mitochondrial protein coding genes, and another for the nuclear genes. As there is an absence of fossil data for *Rhopalomesites,* and using an internal calibration such as the age emergence of islands can be problematic (see Baldwin & Sanderson 1998, Emerson 2002) we have used an external rate to calibrate the clock. Pons *et al. (*2010) obtained an average nucleotide substitution rate for the set of mitochondrial protein coding genes of 2.6% My^{-1} per lineage for the four coleopteran suborders, an estimate that is close to the standard 2.3% of Brower (1994) and not dissimilar to the rates obtained for particular coleopteran groups (e.g. Papadopoulou *et al.* 2010, Ribera *et al.* 2010, Andújar *et al.* 2012). We used a lognormal distribution with a median of 1.30 % My^{-1} per lineage (95% HPDs 0.88–1.89) for the mitochondrial clock prior, setting a soft prior (a uniform distribution from 0 to 100) for the nuclear clock. Beast analyses were run for 50 million generations, sampling every 1000 generations. Convergence of parameters and node ages were analysed with Tracer 1.5 and TreeAnnotator 1.6.0 (Drummond & Rambaut 2007), discarding the first 5 million generations as burn-in.

RECONSTRUCTION OF ANCESTRAL HOST PLANT-ASSOCIATIONS

Bayesian reconstruction of ancestral plant-associations was performed on a sequence dataset obtained from individuals of *Rh. euphorbiae* and *Rh. proximus* that were found feeding on three different *Euphorbia* plants of the *Esula* subgenus (Riina *et al.* 2013): *Euphorbia bourgaeana*, *E. mellifera*, and *E. piscatoria*. All other species feed on Lauraceae except *Rh. tardyi*, which is polyphagous (Morris 2002). We implemented the preferred partition scheme and applied the same nucleotide substitution and clock model as above in

Beast using a coalescent model of diversification with constant population size, since this dataset includes population information. The plant feeding character was defined as three possible states on which we implemented a symmetric substitution model and a strict molecular clock. The ancestral plant association was also reconstructed from mitochondrial and nuclear partitions separately. Beast analyses were run for 100 million generations, sampling every 1000 generations, and ancestral state credibility was calculated in TreeAnnotator 1.6.0 (Drummond & Rambaut 2007) after discarding as burn-in the first 10 million generations.

HAPLOTYPE NETWORKS AND POPULATION STRUCTURE ANALYSES

Median-joining networks (Bandelt *et al.* 1999) were calculated with NETWORK v. 4.612 (http://www.fluxusengineering.com) keeping parameter e = 0, starting with minimum spanning trees combined within a single network, and then adding median vectors (consensus sequences) to reduce tree length. Estimates of nucleotide and haplotype diversity values were obtained with DnaSP v. 5.10.1 (Librado & Rozas 2009). Spatial analysis of molecular variance implemented in SAMOVA v. 1.0 (Dupanloup *et al.* 2002) was used to explore the genetic structure of the Macaronesian *Rhopalomesites* species, identifying geographical groupings that maximized genetic variance between groups of populations (FSC). The program was run with 100 random initial conditions and 10,000 iterations, using an *a priori* number of groupings K = 2 to 10. Differences between sampling sites nested within geographical groupings in the same island and differences between groups were tested using AMOVA analyses on the mtDNA dataset with Arlequin v. 3.5.1.3 (Excoffier *et al.* 2005). The significance of the variance components was evaluated by a non-parametric

permutation test using 10,000 permutations. For the species occurring in two different host plants (*Rhopalomesites euphorbiae* on Madeira and *Rh. proximus* on La Gomera), their populations were grouped either by host plant or by sampling localities. The grouping that maximized the geographical subdivision was assumed as the most plausible within those supported by significant variation among regions.

RESULTS

MITOCHONDRIAL AND NUCLEAR SEQUENCE VARIATION

We sampled 310 *Rhopalomesites* individuals from the species present on the Macaronesian Islands, and also from Great Britain and Ireland (see Table S1a in Appendix S1 and Fig. 3.1). Information on the number of individuals sequenced for each genetic marker, number of haplotypes, nucleotide and haplotype diversity, polymorphic sites and number of parsimony informative sites for the four gene fragments is presented in Tables S3a–d in Appendix S3 of Supporting Information. The haplotype sequences obtained have been deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under accession numbers LN835529–LN835767. A total of 45 individuals were heterozygous for the nuclear sequences, and haplotypes were inferred manually from forward and reverse sequence chromatograms following Flot *et al.* (2006). The maximum sequence divergences between species for the mitochondrial markers were 12.6% and 14.3% for *cox1* and *cytb*, respectively. The nuclear markers were less variable, with maximum divergences of 1.4% and 3.4% for 28S RNA and ITS-2, respectively (see Tables e–h in Appendix S3 for details on sequence divergences). The concatenated matrix consisted of 2992 bp, 1182 bp of which corresponded to the mitochondrial genes and 1810 bp to nuclear genomic fragments. The best evolutionary

substitution model for mitochondrial DNA first codon positions and 28S RNA was the Hasegawa, Kishino and Yano (HKY + I), for second positions it was the Felsenstein-1981 model (F81) (although we applied HKY because F81 cannot be implemented easily in BEAST), while the Generalised Time-Reversible (GTR) model was selected for third codon positions. The Kimura 2-Parameter model (K2P) was selected for the ITS-2 ribosomal fragment. The preferred partitioning scheme among the seven tested was as follows: first, second and third codon positions of mitochondrial DNA as separate partitions, plus the two nuclear sequences (28S RNA and ITS-2), giving five independent partitions.

PHYLOGENETIC ANALYSES

The PBS analysis and the comparison of phylogenetic topologies based on single markers and of nuclear versus mitochondrial trees revealed no major incongruences between the tree-topologies (see Figs S3i,j in Appendix S3 for details). Moreover, Shimodaira–Hasegawa tests indicated that the tree obtained by nuclear markers was not significantly different from the topologies inferred from the mitochondrial genes (Diff –lnL = 10.70; $P > 0.05$). All Bayesian and ML phylogenetic analyses clearly showed that the species feeding on Euphorbiaceae (henceforth the "Euphorb-feeding group") and those associated with Lauraceae and other trees (the "polyphagous group") are reciprocally monophyletic (Fig. 3.2). The sequences obtained from *Rh. euphorbiae* from Madeira and Porto Santo constituted a sister clade to the Canarian *Rh. proximus*. The latter is subsequently divided into the Tenerife, La Gomera and La Palma clades, the Tenerife lineage being sister to the other two island clades. In addition, the polyphagous group includes three main supported clades: *Rh. tardyi* (with sequences from the Azores, Great Britain and Ireland), the two sister

species *Rh. maderensis* (Madeira) and *Rh. complanatus* (La Palma), and finally *Rh. persimilis* divided into two subclades, from Tenerife and La Gomera (Fig. 3.2).

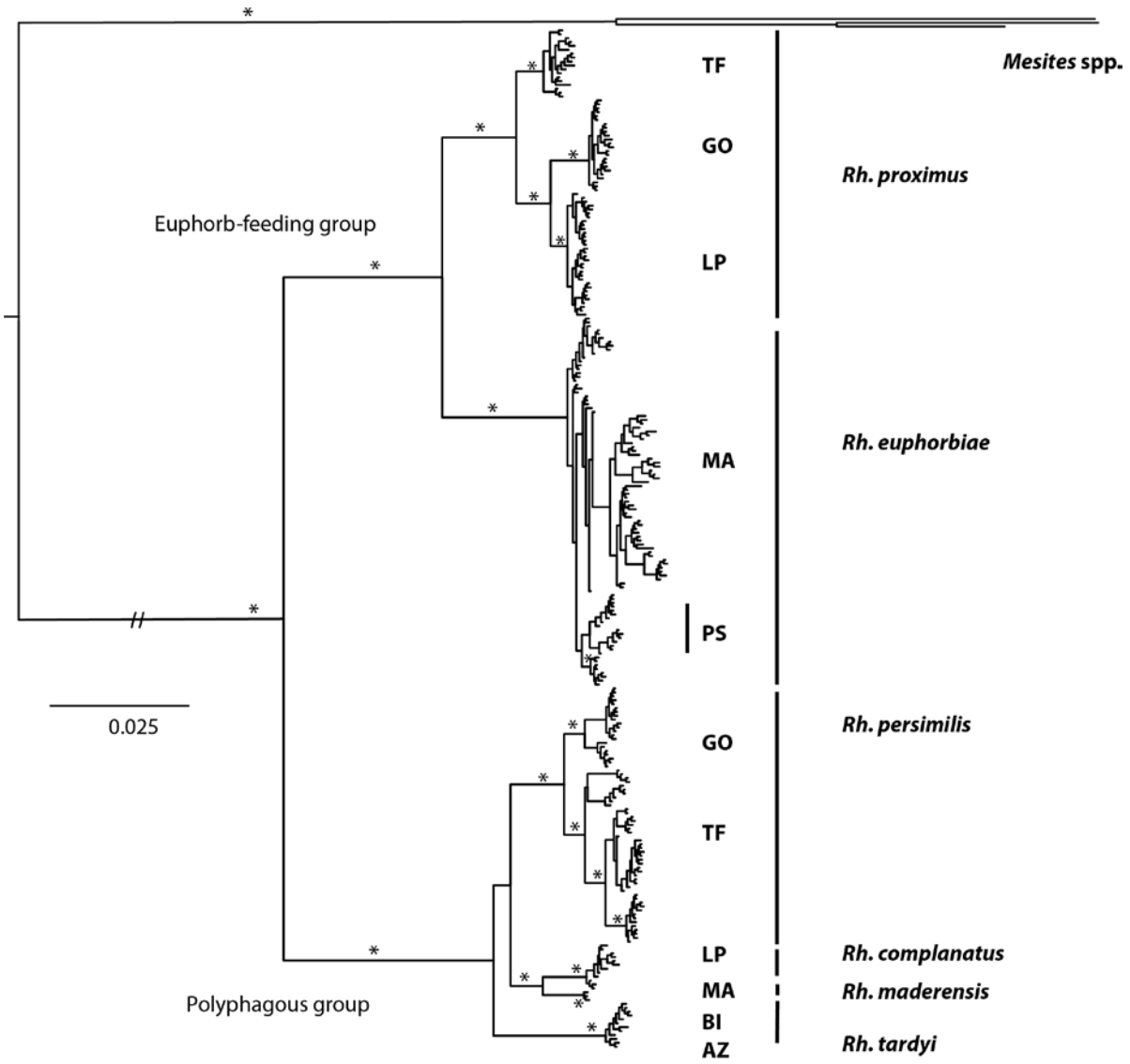


Figure 3.2. Bayesian phylogeny of *Rhopalomesites* species based on the combined mitochondrial-nuclear data set from a sample of 310 *Rhopalomesites* individuals and 2992 bp DNA sequence. Asterisks identify nodes with posterior probability values > 0.95. TF: Tenerife, GO: La Gomera, LP: La Palma, MA: Madeira, PS: Porto Santo, BI: British Isles, AZ: Azores. Scale bar represents the number of nucleotide substitutions per site.

ESTIMATION OF DIVERGENCE TIMES

The hypothesis of a strict versus a relaxed clock was rejected on the reduced dataset (marginal likelihood –8292.405). The posterior nucleotide substitution rate estimated for the combined nuclear markers was about four times slower than the rate estimated for the mitochondrial ones (nuclear markers 0.36% My^{-1} per lineage, 95% HPDs 0.14–0.68) in the range estimated for the same gene fragments in carabid beetles (Andújar *et al.* 2012). Age estimates based on a relaxed log-normal molecular clock tree obtained with the reduced dataset indicate that divergence between the Euphorb-feeding and the polyphagous group occurred during the Miocene-to-Pliocene transition at about 5.3 Ma (95% high posterior densities, HPDs 3.2–7.6 Ma) (Fig. 3.3). The initial divergence of the Euphorb-feeding species can be estimated to have occurred during the early Pleistocene, with the divergences within Canarian and Madeiran species deduced to be in the late Pleistocene. Similar age estimates can be deduced for the initial divergence within the polyphagous group, although the estimates were somewhat younger.

RECONSTRUCTION OF ANCESTRAL HOST PLANT-ASSOCIATIONS

The Bayesian reconstruction of ancestral plant associations, using mitochondrial, nuclear or a combination of both sequence datasets, revealed that the laurel forest *E. mellifera* is the ancestral host plant of the Euphorb-feeding group (Fig.3.4). The *Rhopalomesites euphorbiae* populations from inland Madeira retain this ancestral host plant association. Within the Madeiran clade, a population from coastal northwest Madeira appears to have shifted recently to feed on *E. piscatoria*. In addition, a transition to an older *E. piscatoria* association from the ancestral *E. mellifera* took place in all southern coastal *Rh. euphorbiae* Madeiran

populations plus those from Porto Santo. In the case of *Rh. proximus* from the Canary Islands, it appears that host plant shifts from the ancestral *E. mellifera* association have been restricted to La Gomera, where the western populations have adapted to the forest margin *E. bourgaeana* populations instead of *E. mellifera*, in two independent events.

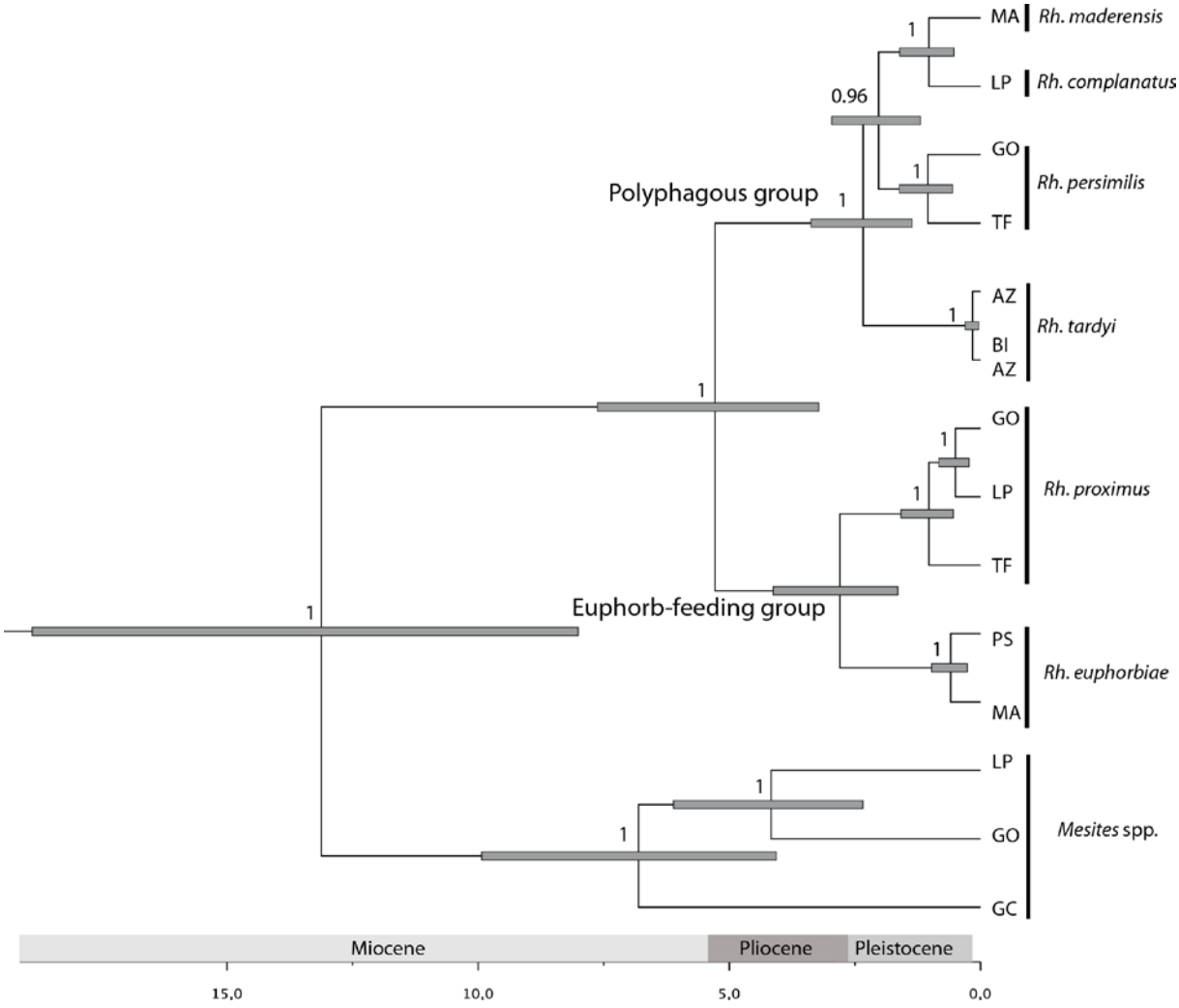


Figure 3.3. Ultrametric tree with estimation of divergence times (chronogram) enforcing a relaxed molecular clock and a Yule speciation model on a reduced dataset including one specimen per species and island (14 individuals; 2862 bp DNA sequence). The scale bar corresponds to million years and horizontal bars across nodes represent the 95% highest probability density intervals of the estimated age for each node. Values above nodes correspond to posterior probability values.

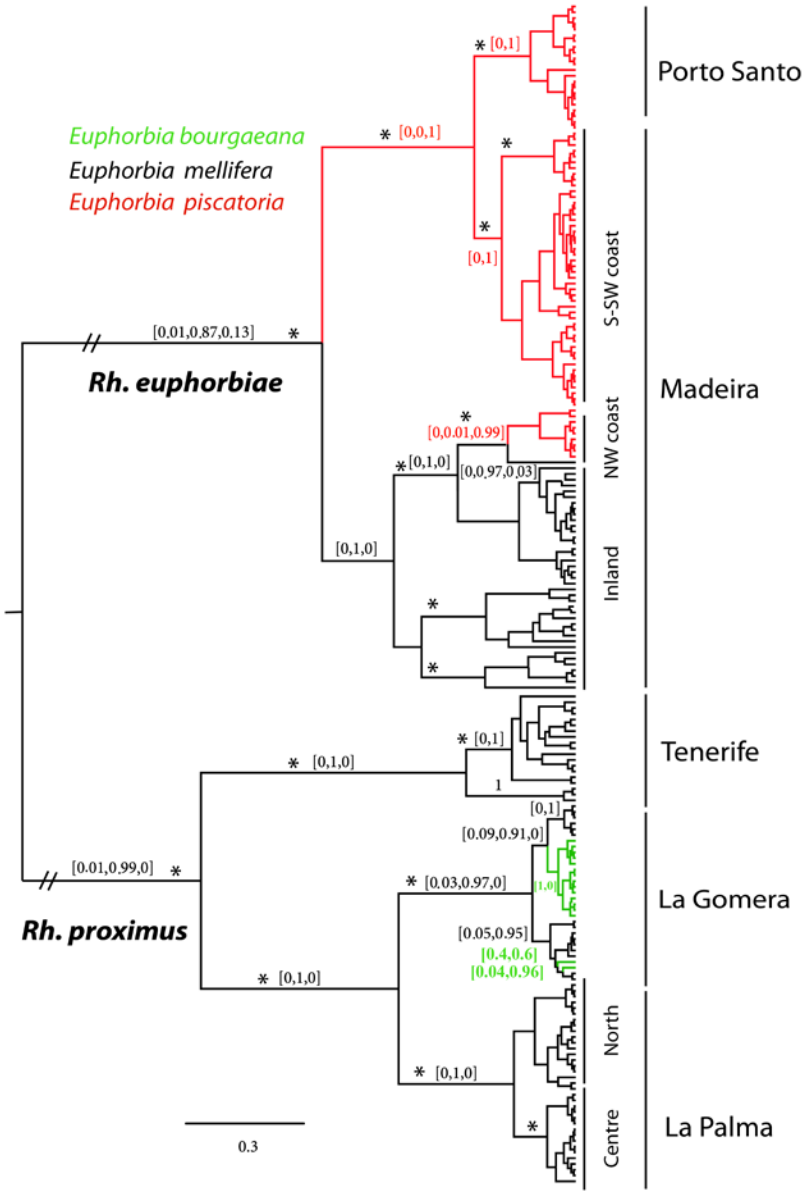


Figure 3.4. Ancestral character reconstruction of plant-associations for *Rh. proximus* and *Rh. euphorbiae* based on 204 DNA sequences of 2822 bp. Green, black and red colours define populations feeding on *Euphorbia bourgaeana, E. mellifera* and *E. piscatoria*, respectively. Asterisks show nodes with posterior probability values > 0.95, and numbers in brackets are the posterior probability for each ancestral character at different branches in the tree, corresponding to *E. bourgaeana* (first number), *E. mellifera* (second number) and *E. piscatoria* (third number) plant-associations as mentioned. The scale bar represents the number of nucleotide substitutions per nucleotide site.

GENETIC DIFFERENTIATION IN *RHOPALOMESITES* SPECIES ASSOCIATED WITH THE EUPHORBIACEAE

Rhopalomesites euphorbiae specimens were sampled on Madeira and Porto Santo, with haplotypes from the latter appearing to be sister to those from southern Madeira (Fig. 3.4). Mitochondrial networks revealed a remarkable geographical structure in Madeira (Fig. 3.5). SAMOVA analysis for *Rh. euphorbiae* mitochondrial sequences suggests that 10 groupings maximize the variation among groups (F_{CT}). Analyses of molecular variance showed that mitochondrial haplotypes grouped by geographical regions explained a higher percentage of genetic variation between groups (64.78%) than when being grouped by host plants (34.75%) (see Tables l,m in Appendix S3 for details on SAMOVA analyses of *Rh. euphorbiae*).

The phylogenetic relationships in the Canarian *Rhopalomesites proximus* show relatively low within-island genetic divergences but clear-cut island clades (Figs 3.2 & 3.4). Thus, sequences from La Gomera and La Palma are reciprocally monophyletic, and together constitute a sister group to that of Tenerife, suggesting a dispersion from this island towards the west (Figs 3.4 & 3.6). Within each island, only populations from the north and centre of La Palma displayed monophyletic haplotypes. The SAMOVA analysis for the La Gomera *Rh. proximus* mitochondrial sequences showed that three groupings maximize the variation among groups: one including the two localities with an *E. mellifera* association, and two groups for each of the localities in the northwest where *Rh. proximus* is associated with *E. bourgaeana*. Analyses of molecular variance showed that mitochondrial haplotypes grouped by geographical regions explained a limited but higher percentage of genetic variation among groups (15.16%) than when being grouped according to host plant use (6.92%) (see Tables n,o in Appendix S3 for details on SAMOVA analyses of *Rh. proximus*). However, most of the variation is explained by variation within populations in the two cases (82.94%

considering geographical regions, and 83.12% for host plant).

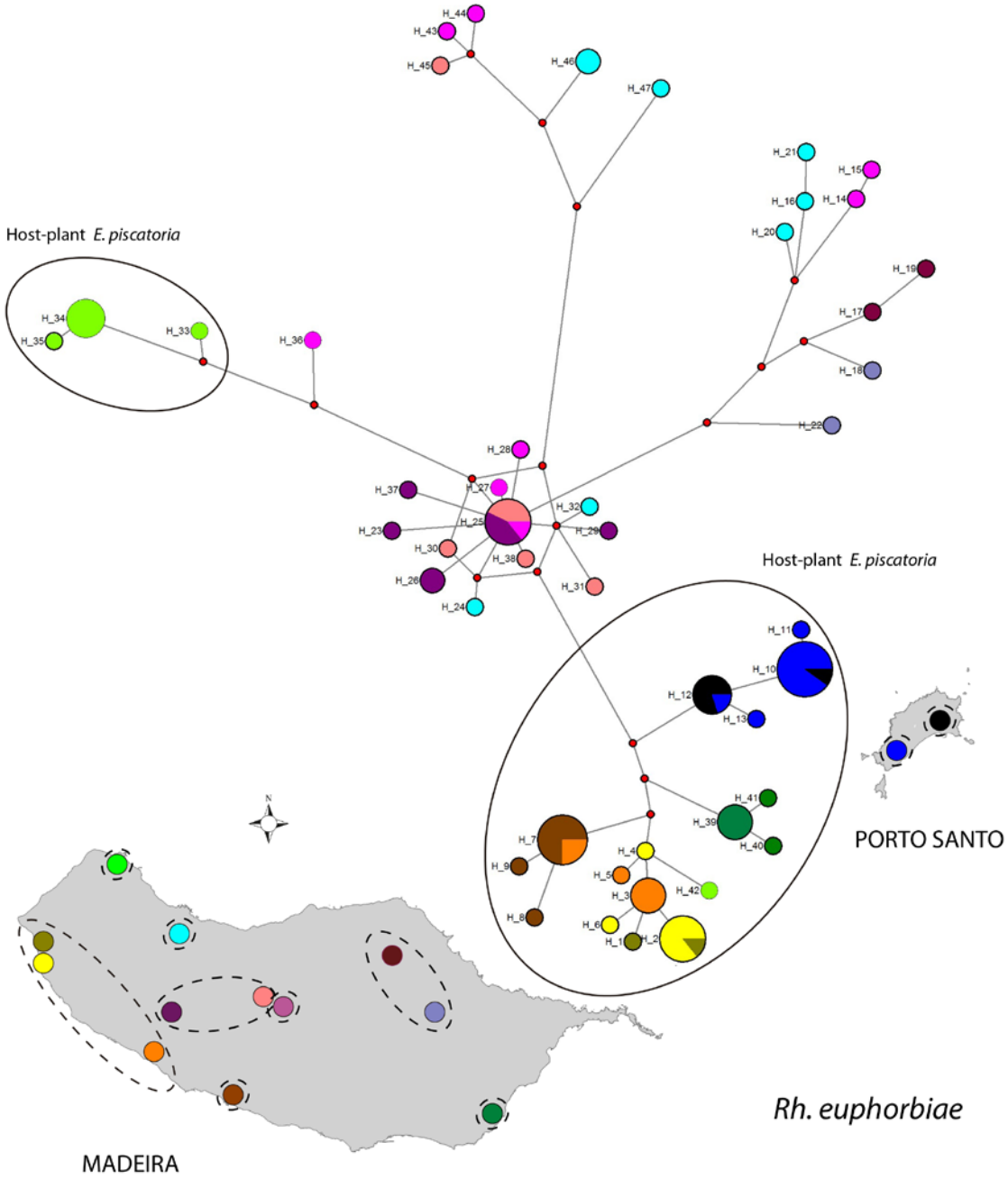


Figure 3.5. Median joining network obtained from mitochondrial sequences of *Rh. euphorbiae* (91 haplotypes; 1182 bp DNA sequence). Circle sizes and branch lengths are proportional to haplotype frequencies and to the number of nucleotide changes, respectively. Red dot vertices are median vectors representing missing haplotypes. Ovals with discontinuous lines indicate the optimum population grouping obtained by SAMOVA, and continuous line ovals indicate the host-plant *E. piscatoria*.

GENETIC DIFFERENTIATION BETWEEN *RHOPALOMESITES* SPECIES IN THE POLYPHAGOUS GROUP

The *Rhopalomesites tardyi* sequences from Great Britain and Ireland are closely related to those obtained from Terceira and Flores in the Azores. The prevalent *cox1* haplotype from the British Isles is in fact identical to one found in individuals from Terceira and Flores. Similar results are obtained considering *cytb* and the nuclear sequences. On the whole, the haplotype and nucleotide diversity found in the Azores populations is remarkably higher than that of the British populations (see Tables a–d and Fig. S3k in Appendix S3).

Rhopalomesites maderensis (Madeira) and *Rh. complanatus* (La Palma) are clearly sister species. The latter shows a strong geographical structure, with haplotypes of the two nearby north-eastern localities being reciprocally monophyletic (see Tables S3a–d) in Appendix S3; species tree not shown). Finally, *Rh. persimilis* on La Gomera shows a genetic structure divided into two genetic groupings with a clear east-to-west differentiation in the Garajonay laurel forest. On Tenerife, there is a division between the northeast Anaga massif (in turn divided into two subgroups: West and East Anaga), and the Teno massif to the northwest (Fig. 3.6). SAMOVA analyses produced an alternative but similar subdivision, although within the West Anaga group there was a single westernmost locality, a large middle-western group and another three localities to the east (see Fig. S3p in Appendix S3).

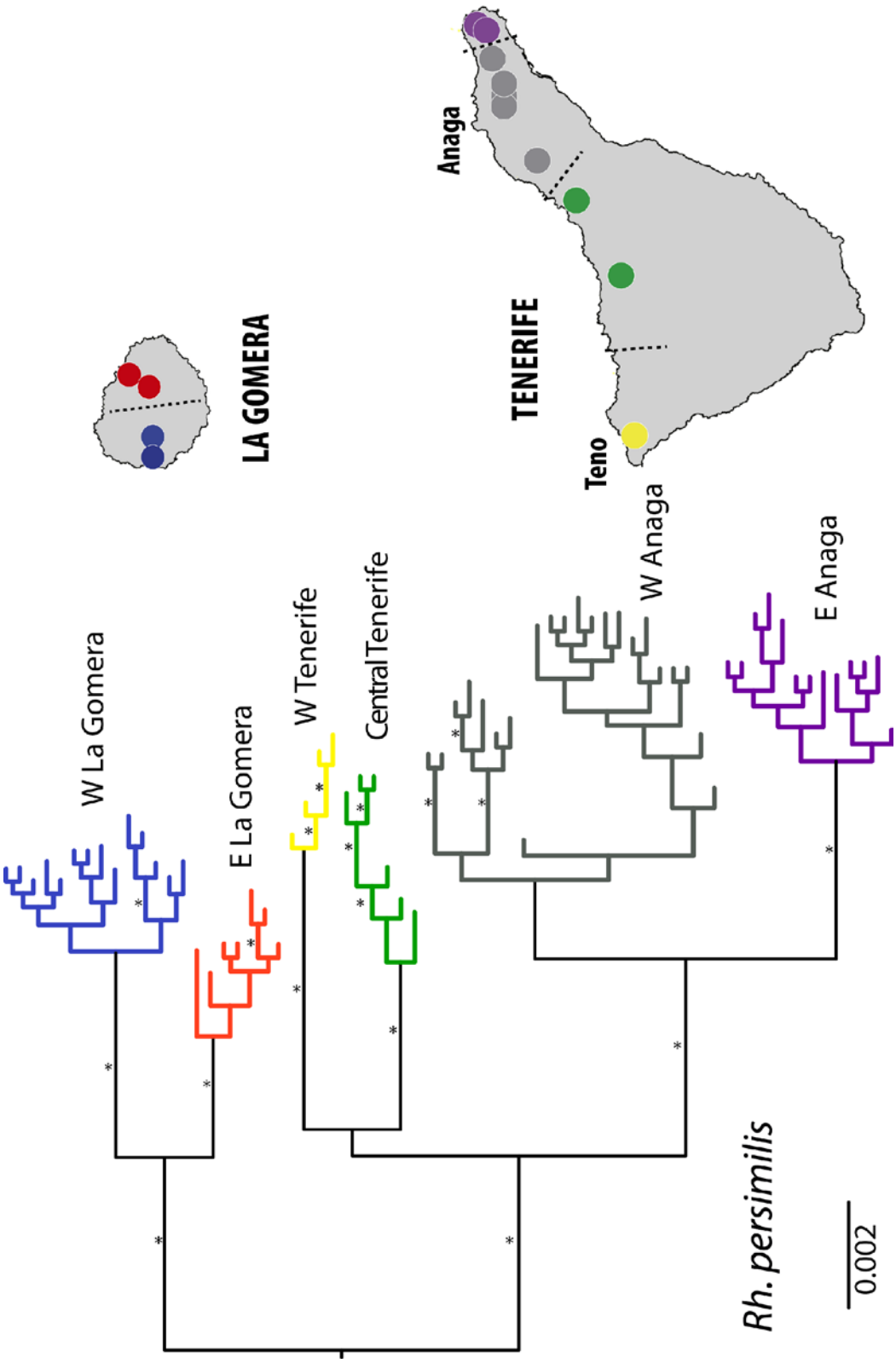


Figure 3.6. Bayesian phylogenetic tree of *Rh. persimilis* based on the combined mitochondrial-nuclear data set (108 haplotypes; 2827 bp DNA sequence). Asterisks show nodes with posterior probability values > 0.95.

DISCUSSION

SYSTEMATICS AND PLANT ASSOCIATIONS IN *RHOPALOMESITES*

Two *Rhopalomesites* lineages can be recognized according to their trophic alliances: a specialist lineage strictly associated with *Euphorbia* species in the Canary and Madeira archipelagos, and another group composed of species feeding on a variety of trees, and occasionally on *Euphorbia*. This polyphagous lineage occurs in humid forests of the Canary, Madeira and Azores archipelagos: one species is also present in Atlantic Western Europe. The mitochondrial and nuclear phylogenies clearly show that the two species lineages are reciprocally monophyletic clades that can be traced to an inferred early host plant divergence during the early Pliocene. As expected based on morphological characteristics, the genus *Mesites* appeared to be the most closely related to both lineages in our sampling of potential continental relatives, although we cannot rule out the possibility that extinctions of ancestral taxa in the laurel forests of Western Europe and North Africa during the Tertiary have extirpated traces of multiple colonizations (Emerson 2002, Amorim *et al.* 2012). Regarding the Euphorb-feeding clade, *Rhopalomesites euphorbiae* is present on both Madeira and Porto Santo. On the former island it occurs in the laurel forest feeding on decaying *Euphorbia mellifera* wood, and in open xeric shrubland it feeds on the more abundant *E. piscatoria*. On Porto Santo it feeds exclusively on *E. piscatoria*, as *E. mellifera* is absent there. This suggests that it exploits the most abundant arborescent *Euphorbia* resource in each habitat. *Rhopalomesites palmi* had been reported from Porto Santo, but after our study we conclude that this species is a junior synonym of *Rh. euphorbiae* (see text b in Appendix S1 for details on the taxonomic revision). Also within this group, the Canary endemic *Rh. proximus* feeds on the only spurge (*E. mellifera*) present in the laurel forest of

three islands, but on La Gomera two nearby populations have been found in more open, marginal areas feeding on the more abundant *E. bourgaeana*. Within the polyphagous lineage the three endemic forms, *Rh. maderensis* (Madeira), *Rh. persimilis* (Tenerife and La Gomera), and *Rh. complanatus* (La Palma) have been collected mostly from native laurels (*Laurus azorica*). Larvae of *Rh. tardyi* from Ireland and Britain feed on the dead wood of a variety of tree genera (see text c in Appendix S1 for details on particular genera) (Morris, 2002; R. Anderson pers. comm.). However, in the Azores, this species feeds on *Morella faya* (Myricaceae), *Picconia azorica* (Oleaceae), *Laurus azorica* (Lauraceae), *Acacia* spp. (Fabaceae), and more rarely on *Euphorbia stygiana* (Stüben 2005, P. Borges pers. comm.). The capacity of *Rh. tardyi* to adapt to feeding on many different plant species (and even families), either native or introduced, is remarkable compared with the other *Rhopalomesites* weevils, which are strictly associated with one or two plant species.

PHYLOGENETIC RELATIONSHIPS AMONG THE *RHOPALOMESITES* OF THE EUPHORB-FEEDING GROUP

Divergence between the two groups associated with *Euphorbia* shrubs, *Rh. proximus* and *Rh. euphorbiae* can be estimated as having occurred during the late Pliocene, much later than the maximum subaerial datings of the islands they inhabit: 11–12 Ma for Tenerife and 14 Ma for Porto Santo (Geldmacher *et al.* 2005), and consistent with the estimated ages of many Macaronesian laurel forest taxa (Kondraskov *et al.* 2015), producing island clades that underwent subsequent intra-island diversifications. Reconstructions of ancestral host plant associations show that *Euphorbia mellifera,* a member of the subgenus *Esula,* included in the section *Helioscopia* (Riina *et al.* 2013), is the ancestral host plant in both weevil species, with recent host range expansions in some populations on Madeira to *E. piscatoria,* also in the

same subgenus but in the section *Aphyllis* (Riina *et al.* 2013). These shifts appear to be related to an expansion from the laurel forest to marginal habitats in which the more abundant *Euphorbia* food resource is substituted by *E. piscatoria*. In Porto Santo, the ancestral host plant is absent and *Rh. euphorbiae* also exploits *E. piscatoria,* probably as a result of colonization from Madeira (although given the tree topology obtained we cannot completely discard the possibility that Madeiran *E. piscatoria*-feeding populations derive from Porto Santo). A similar pattern can be deduced for *Rh. proximus* on La Gomera, where a host range expansion to *E. bourgaeana* (interestingly also included in the subgenus *Esula,* section *Aphyllis,* Riina *et al.* 2013) is seen in recently colonized habitats. In these two cases, the intra-island phylogeographies strongly suggest that geographic isolation has been significantly more important than host plants in shaping genetic diversification within species.

PHYLOGENETIC RELATIONSHIPS BETWEEN *RHOPALOMESITES* IN THE POLYPHAGOUS GROUP

The phylogeny we obtained does not resolve the relationship between the polyphagous *Rh. tardyi* and the laurel-feeding species of this clade (*Rh. maderensis, Rh. complanatus* and *Rh. persimilis*). Divergence between these two groups can be estimated as having occurred in the Pleistocene, during which most of the islands in the three studied archipelagos had already emerged (Nunes 2014, Geldmacher *et al.* 2005). The diversification of *Rh. tardyi* appears to be much more recent; the specimens sampled in the British Isles displayed a much lower genetic diversity than did populations from the Azores. This suggests a recent introduction into Western Europe from the latter region, although the lack of support for some of the nodes linking the Azorean and British Isles *Rh. tardyi* DNA sequences precludes

any firm conclusion. This possible introduction from the Azores should be confirmed with more comprehensive sampling and additional DNA sequence information to provide a robust phylogenetic framework. We suggest that this introduction could have been associated with timber commerce after the arrival of the first settlers in the Azores (1420–1430). This explanation is feasible, given the remarkable plasticity of this species in adapting to feeding on many different plants. It easily could have changed from feeding on local trees (and a spurge) in the Azores to consuming both native and introduced trees of different families in Atlantic Europe. A recent introduction in reverse to the Azores by the coleopteran dytiscid *Agabus bipustulatus* was suggested, as the analysed specimens sequenced from these islands were deeply nested within the continental *A. bipustulatus* clade (Drotz 2003).

GENETIC VARIATION AND GEOGRAPHICAL STRUCTURING WITHIN *RHOPALOMESITES* TAXA

In both *Rhopalomesites euphorbiae* and *Rh. proximus*, genetic variation is structured following an allopatric pattern because of the sequential colonization and isolation typical of oceanic archipelagos (Emerson 2002). In *Rh. euphorbiae* there is also a considerable within-island population structure that can be explained by geographic isolation and plant consumption. The Tenerife *Rh. proximus* populations are genetically more diverse than those of La Gomera or La Palma, despite inhabiting two small neighbouring habitat fragments. This could suggest that the current lineages represent survivors of an older and wider diversification on Tenerife, which could be more recent on the two other islands. Within these three Canary islands, *Rh. proximus* populations are considerably fragmented and occur in remnant habitat patches with the presence of *E. mellifera* (and rarely *E. bourgaeana*), a scenario that would link the reduction in genetic diversity in this species to

habitat reduction and loss. *Rhopalomesites persimilis* populations are well differentiated between the two islands on which they occur, showing a remarkable genetic within-island structure closely linked to the laurel forest. On La Gomera, this species shows a genetic structure divided into two groups, with a clear east-to-west differentiation in the Garajonay laurel forest. However, on Tenerife only 10% of the original vast continuous laurel forest remains (Arévalo *et al.* 2012), resulting in a patchy distribution practically matching that of *Rh. persimilis*: the Teno massif to the northwest, two well-separated localities on the northern side of the island in the middle and the Anaga massif to the northeast. A well-known repeated phylogeographical pattern has been associated with Tenerife's northern two precursor islands (Teno and Anaga) for many species (Gübitz *et al.* 2000, Brown *et al.* 2000, Thorpe *et al.* 1996, Juan *et al.* 1996, Contreras-Díaz *et al.* 2003). However, given the recent origin of the diversification of *Rh. persimilis* and the abundance and distribution of its host plant, fragmentation produced by habitat loss seems to be the most likely explanation for its population structure. Moreover, the Anaga populations are clustered into two main West and East Anaga groups that can be further subdivided in the former area (Fig. 3.6). Similar genetic differentiation in populations on Anaga has also been reported for ground beetles and a spider species (Emerson *et al.* 1999, Moya *et al.* 2004, Macías-Hernández *et al.* 2013).

CONCLUSION

Plant–beetle associations in the Macaronesian Islands have formed an interesting platform to study the ecological phylogenetic and temporal factors of host use and host shifts at the species level (Jordal *et al.* 2004, Jordal & Hewitt 2004, Jordal 2006). In the Macaronesian

Rhopalomesites weevils, an initial split during the Miocene-to-Pliocene transition produced two distinct lineages that have diversified independently in a quasi-parallel fashion in the Canary and Madeiran archipelagos. First, the specialist species feeding on Euphorbiaceae have two vicariant species, one occurring on Madeira–Porto Santo and another occurring on three of the western Canary Islands. Second, the polyphagous species have radiated into several taxa present on the same three islands in the Canaries plus Madeira and the Azores. Host shifts have occurred only at within-species and recent time-scale levels and might be related to the host plant abundance and/or availability in a particular habitat. In summary, in the Macaronesian *Rhopalomesites* weevils, factors related to geography such as allopatric divergence seem to have been more important than host shifts or host range expansions.

SUPPORTING INFORMATION

APPENDIX S1 ADDITIONAL MATERIALS

Appendix S1 Table a: Table listing sampling localities for each species with geographical coordinates of each location and information on host plants. LC: locality codes.

Taxa	Island	Locality	LC	latitude	longitude	Host plant
Rh. euphorbiae						
	Madeira	Chão da Ribeira	1	32.802.447	-17.115.936	*E. mellifera*
		Encumeada	2	32.752.488	-17.017.498	*E. mellifera*
		Folhadal	3	32.753.755	-17.033.385	*E. mellifera*
		Serra dos Canhas	4	32.734.349	-17.128.345	*E. mellifera*
		Ribeiro Frio	5	32.737.216	-16.886.917	*E. mellifera*
		Queimadas	6	32.780.721	-16.917.248	*E. mellifera*
		Porto Novo	7	32.660.806	-16.816.850	*E. piscatoria*
		Ribeira Brava	8	32.669.388	-17.063.410	*E. piscatoria*
		Moledos	9	32.706.770	-17.140.618	*E. piscatoria*
		Paul do Mar	10	32.766.372	-17.235.490	*E. piscatoria*
		Ribeira da Janela	11	32.854.133	-17.153.820	*E. piscatoria*
		Fajã da Ovelha	12	32.772.722	-17.238.600	*E. piscatoria*
	Porto	Pico Ana Ferreira	13	33.037.026	-16.367.032	*E. piscatoria*

	Santo					
		Pico do Castelo	14	33.077.711	-16.336.304	*E. piscatoria*
Rh. proximus						
	La Gomera	Andenes de Alojera	15	28.162.383	-17.302.300	*E. bourgeauana*
		Epina Alta	16	28.162.972	-17.300.585	*E. bourgeauana*
		Degollada de Archejo	17	28.140.400	-17.180.700	*E. mellifera*
		Topo del Negrillo	18	28.129.136	-17.196.000	*E. mellifera*
	Tenerife	Cabezo del Tejo	19	28.565.700	-16.167.800	*E. mellifera*
		La Ensillada	20	28.556.012	-16.179.712	*E. mellifera*
	La Palma	Lomo Espiñel	21	28.648.339	-17.818.900	*E. mellifera*
		Lomo Espiñel 2	22	28.646.474	-17.819.195	*E. mellifera*
		Espigón Atravesado	23	28.782.012	-17.816.300	*E. mellifera*
		Pie Espigón Atravesad	24	28.781.295	-17.811.809	*E. mellifera*
		Caseta de la Galería	25	28.784.096	-17.807.200	*E. mellifera*
Rh. persimilis						
	La Gomera	Degollada de Archejo	26	28.140.500	-17.179.986	Lauraceae
		Jardín de las Creces	27	28.137.510	-17.287.551	Lauraceae
		Cañada de Jorge	28	28.149.549	-17.296.063	Lauraceae
		Reventón Oscuro	29	28.124.657	-17.216.470	Lauraceae
	Tenerife	Pista de las Hiedras	30	28.540.300	-16.273.700	Lauraceae
		Vueltas de Taganana	31	28.542.444	-16.228.308	Lauraceae
		Palo Blanco	32	28.357.941	-16.586.559	Lauraceae
		El Batán	33	28.535.320	-16.296.801	Lauraceae
		Zapata	34	28.531.968	-16.290.587	Lauraceae
		Monte del Agua	35	28.323.773	-16.817.247	Lauraceae
		Agua García	36	28.458.594	-16.403.473	Lauraceae
		Galería de Benza	37	28.405.291	-16.461.881	Lauraceae
		Barranco de Nieto	38	28.534.071	-16.316.247	Lauraceae
		Aguas Negras	39	28.540.000	-16.225.111	Lauraceae
		El Moquinal	40	28.537.356	-16.309.377	Lauraceae
		Monte Aguirre	41	28.529.539	-16.268.303	Lauraceae
		Hoya de Ijuana	42	28.560.191	-16.169.199	Lauraceae
		Chinobre	43	28.559.306	-16.173.222	Lauraceae
		Cabezo del Tejo	44	28.562.028	-16.171.389	Lauraceae
		El Pijaral	45	28.551.972	-16.189.222	Lauraceae
Rh. complanatus						
	La Palma	Los Tiles	46	28.790.176	-17.801.843	Lauraceae
		Cubo de la Galga	47	28.760.908	-17.776.088	Lauraceae
Rh. maderensis						
	Madeira	Fanal	48	32.806.497	-17.140.806	Lauraceae
Rh. tardyi						
	Azores	Terceira	49	38.739.081	-27.291.109	several trees
		Flores	50	39.507.422	-31.201.718	several trees
		Pico	51	38.484.172	-28.274.029	several trees
		São Miguel	52	37.798.372	-25.184.365	several trees

	Ireland	County Down	53	54.223.154	-5.875.526	several trees
	Great Britain	Cornwall	54	50.352.198	-4.173.929	several trees
***Mesites* spp.**						
	La Gomera	Andenes de Alojera		28.162.383	-17.302.303	*E. bourgeauana*
	Gran Canaria	Andén Verde		28.035.188	-15.746.346	*E. regis-jubae*
	La Palma	Hornito-Tamanca		28.556.313	-17.878.537	*E. lamarckii*
***Pselactus* spp.**						
	Tenerife	Agua García		28.458.594	-16.403.473	Lauraceae
	Madeira	Fanal		32.806.497	-17.140.806	Lauraceae
***Amaurorhinus* sp.**						
	Montaña Clara	La Caldera		29.300.516	-13.534.660	unknown
Rhyncolus crassicornis						
	Tenerife	Pista del Rayo		28.423.067	-16.433.895	unknown

Appendix S1 Text b: Text with the revision of the *Rhopalomesites* species present in Maderian and Canary archipelagos.

Before our study: i) *Rhopalomesites euphorbiae* (Wollaston, 1854) was originally described from Madeira, where larvae is feeding on the dendroid spurges *Euphorbia mellifera* and *E. piscatoria*; ii) on Porto Santo both *Rh. euphorbiae* and *Rh. palmi* (Folwaczny, 1979) had been reported by Folwaczny (1979) as feeding on *E. piscatoria*; iii) in the laurel forest of the Canary Islands of Tenerife, La Palma and La Gomera the presence of *Rh. euphorbiae* had been registered, feeding on *E. mellifera* (Israelson *et al.,* 1982); iv) *Rhopalomesites proximus* (Wollaston, 1861) was described in the northeast of Tenerife without specifying the host plant, and later collected by Lindberg (1958) on that island in decaying laurel trees; and v) *Rhopalomesites maderensis* (Wollaston, 1854) from Madeira, *Rh. persimilis* (Wollaston, 1861) from Tenerife and La Gomera, *Rh. complanatus* (Wollaston, 1861) from La Palma, and *Rh. tardyi* from the Azores and Western Europe, form a group of species with larvae feeding on the decaying wood of several forest trees (Morris, 2002) and very rarely on *Euphorbia stygiana* (Stüben, 2005). After studying a large series of individuals of *Rhopalomesites* and

according to previous results (Hernández-Teixidor *et al.* 2012), we have noted that: i) individuals initially described as *Rh. palmi* are just small specimens of *Rh. euphorbiae*, ii) all previous records of *Rh. euphorbiae* from the Canary Islands are misidentified specimens of *Rh. proximus*, iii) individuals of *Rh. proximus* collected by Lindberg (1958) feeding on laurel wood on Tenerife are misidentified specimens of *Rh. persimilis*. So henceforth we consider that *Rh. euphorbiae* is only present in the Madeira archipelago, being the only species occurring on Porto Santo; and *Rh. proximus* is the only species feeding on *E. mellifera* in the Canary Islands.

Appendix S1 Text c: Text listing the trees in which *Rhopalomesites tardyi* larvae have been found feeding on dead wood (Morris, 2002; R. Anderson pers. comm.)

Individuals of *Rh. tardyi* from Ireland and Britain feed on the dead wood of a variety of trees of the following species:

Fagus sylvatica (Fagaceae), *Ilex aquifolium* (Aquifoliaceae), *Fraxinus excelsior* (Oleaceae), *Acer pseudoplatanus* (Aceraceae), *Quercus petraea* (Fagaceae), *Ulmus glabra* (Ulmaceae), *Alnus glutinosa* (Betulaceae), *Aesulus hippocastanum* (Sapindaceae), *Betula pubescens* (Betulaceae), *Salix caprea* (Salicaceae), *Sorbus aucuparia* (Rosaceae), *Corylus avellana* (Betulaceae), *Picea abies* (Pinaceae), *Crataegus* sp. (Rosaceae) and *Rhododendron* sp. (Ericaceae) (Morris, 2002; R. Anderson pers. comm.).

APPENDIX S2 ADDITIONAL METHODS

Appendix S2: Details on DNA extraction, PCR and sequencing

DNA extraction was performed using the QIAGEN DNeasy extraction Kit (QIAGEN). Voucher specimens were conserved in the Department of Animal Biology, University of La Laguna

(Canary Islands, Spain). Two mitochondrial DNA fragments were amplified: the 3' region of cytochrome oxidase I gene (*cox1*) of 825 bp and a 357 bp portion of cytochrome b (cyt b) using the Pat and Jerry (Simon *et al.,* 1994) and CB3 and CB4 (Barraclough *et al.,* 1999) primer pairs, respectively. In addition, the primers ITS3 and ITS4 (White *et al.,* 1990) were used to amplify a fragment of about 600 bp of the nuclear ribosomal internal transcribed spacer 2 (ITS-2) and primers LSU-D1-2-fw1 and LSU-D1-2-rev1 (Sonnenberg *et al.,* 2007) to amplify a fragment of about 1070 bp of the nuclear large ribosomal subunit (28S RNA). PCRs were performed using a reaction volume of 25 µl, with the following amplification conditions: 94°C for 2 min and then 40 cycles of 94°C for 30 s, 45-55°C for 30 s and 72°C for 1 min, followed by a final incubation step at 72°C for 10 min. The fragments were sequenced in both directions using the PCR primers and the BigDye Terminator v3.1 Cycle Sequencing kit and electrophoresed and detected on an ABI 3100 automated sequencer (Applied Biosystems, Foster City, USA). Estimates of nucleotide and haplotype diversity values, polymorphic and parsimony informative positions were obtained with DnaSP v. 5.10.1 (Librado & Rozas, 2009).

APPENDIX S3 ADDITIONAL RESULTS

Appendix S3 Table a: Table summarising details of the 825 bp long *cox1* sequences obtained. LC: Locality codes as in Fig. 3.1. Numbers in parenthesis indicate individuals displaying the same haplotype. Haplotype and nucleotide diversity values estimated for each species and island are shown in boldface.

Taxa Island	**LC**	**Haplotypes**	**No individuals**	**Haplotype diversity**	**Nucleotide diversity**	**No polymorphic nucleotide positions**	**No of parsimony informative**
Rh. euphorbiae			**92**	0,95604	0,01038	51	34
Madeira				**0,95779**	**0,01103**		
	1	eup14, eup18, eup19, eup22, eup30, eup41 (2), eup42	8	0,96429	0,00841		
	2	eup12, eup13, eup23, eup25, eup26, eup34, eup39, eup40	8	1,00000	0,00947		
	3	eup23 (4), eup28, eup29, eup39	7	0,71429	0,00368		
	4	eup21, eup23 (4), eup24 (2), eup27	8	0,75000	0,00178		
	5	eup16, eup20	2	1,00000	0,00623		
	6	eup15, eup17	2	1,00000	0,00125		
	7	eup35 (4), eup36, eup37	6	0,60000	0,00083		
	8	eup6 (6), eup7, eup8	8	0,46429	0,00093		
	9	eup1(4), eup4, eup6(2),	7	0,66667	0,00225		
	10	eup2 (6), eup3, eup5	8	0,46429	0,00116		
	11	eup31, eup32 (5), eup33, eup38	8	0,64286	0,00627		
	12	eup1, eup2	2	1,00000	0,00125		
Porto Santo				**0,54248**	**0,00072**		
	13	eup9 (10), eup10, eup11 (2)	13	0,41026	0,00054		
	14	eup9, eup11 (4)	5	0,40000	0,00050		
Rh. proximus			**69**	0,87042	0,01041	32	24

La Gomera				**0,42118**	**0,00087**		
	15	pro6 (3), pro7 (3)	6	0,60000	0,00100		
	16	pro6 (6), pro8, pro10	8	0,46429	0,00125		
	17	pro6 (6), pro9 (2)	8	0,42857	0,00072		
	18	pro6 (7)	7	0,00000	0,00000		
Tenerife				**0,78205**	**0,00455**		
	19	pro2, pro3 (4),	5	0,40000	0,00201		
	20	pro1, pro2, pro3 (2), pro4, pro5 (2), pro18	8	0,92857	0,00597		
La Palma				**0,85185**	**0,00312**		
	21	pro11 (5), pro14 (3)	8	0,53571	0,00090		
	22	pro14 (5)	5	0,33333	0,00056		
	23	pro12 (2), pro13 (2), pro15 (4)	8	0,71429	0,00167		
	24	pro16 (4), pro17	5	0,40000	0,00201		
	25	pro19	1				
Rh. persimilis			**107**	0,92858	0,01237	42	36
La Gomera				**0,80072**	**0,00799**		
	26	per5 (4), per6 (3), per7	8	0,67857	0,00122		
	27	per1 (8)	8	0,00000	0,00000		
	28	per1, per2 (5), per3	7	0,52381	0,00085		
	29	per4	1				
Tenerife				**0,89715**	**0,00875**		
	30	per19 (2), per20 (4)	6	0,53333	0,00079		
	31	per15 (3), per17 (4), per18	8	0,67857	0,00275		
	32	per12, per14	2	1,00000	0,00296		
	33	per20 (3)	3	0,00000	0,00000		
	34	per19, per20	2	1,00000	0,00148		
	35	per8 (5), per9, per10	7	0,52381	0,00113		
	36	per16 (6)	6	0,00000	0,00000		

	37	per11 (2), per13 (2)	4	0,66667	0,00099		
	38	per19, per20 (5)	6	0,33333	0,00049		
	39	per21 (6)	6	0,00000	0,00000		
	40	per19, per20 (5)	6	0,33333	0,00049		
	41	per16 (6)	6	0,00000	0,00000		
	42	per22 (4), per26	5	0,40000	0,00059		
	43	per22 (3), per25, per27	5	0,70000	0,00119		
	44	per22 (5)	5	0,00000	0,00000		
	45	per17, per22, per23 (3), per24	6	0,80000	0,00474		
Rh. complanatus			**16**	0,85000	0,00333	9	7
La Palma							
	46	com1, com2 (2), com3, com4 (2), com5, com6 (2), com7	10	0,93333	0,00429		
	47	com8 (6)	6	0,00000	0,00000		
Rh. maderensis			**4**	0,00000	0,00000	0	0
Madeira							
	48	mad1 (4)	4	0,00000	0,00000		
Rh. tardyi			**16**	0,69167	0,00207	7	2
Azores				**0,88889**	**0,00263**		
	49	tar1, tar6, tar7 (2)	4	0,83333	0,00182		
	50	tar1 (2), tar5	3	0,66667	0,00242		
	51	tar3	1				
	52	tar4	1				
British isles				**0,28571**	**0,00035**		
Ireland	53	tar1 (5), tar2	6	0,33333	0,00040		
Great Britain	54	tar1	1				

Appendix S3 Table b: Table summarising details of the 357 bp long *cytb* sequences obtained. LC: Locality codes as in Fig. 3.1. Numbers in parenthesis indicate individuals displaying the same haplotype. Haplotype and nucleotide diversity values estimated for each species and island are shown in boldface.

Taxa Island	**LC**	**Haplotypes**	**No individuals**	**Haplotype diversity**	**Nucleotide diversity**	**No polymorphic nucleotide positions**	**No of parsimony informative**
Rh. euphorbiae			**94**	0,92038	0,00903	27	16
Madeira				**0,90246**	**0,00897**		
	1	eup8, eup9, eup10, eup13, eup15, eup26 (2), eup27	8	0,96429	0,01451		
	2	eup7 (2), eup13 (3), eup18, eup23, eup24	8	0,85714	0,01170		
	3	eup13 (4), eup14, eup20, eup25	7	0,71429	0,00640		
	4	eup12, eup13 (6), eup19	8	0,46429	0,00210		
	5	eup8, eup11	2	1,00000	0,00280		
	6	eup8 (2)	2	0,00000	0,00000		
	7	eup21 (8)	8	0,00000	0,00000		
	8	eup3 (8)	8	0,00000	0,00000		
	9	eup2 (5), eup3 (2)	7	0,47619	0,00133		
	10	eup2 (8)	8	0,00000	0,00000		
	11	eup16, eup17 (6), eup22	8	0,46429	0,00470		
	12	eup1, eup2	2	1,00000	0,00280		
Porto Santo				**0,54248**	**0,00172**		
	13	eup4 (10), eup5, eup6	12	0,31818	0,00132		
	14	eup4, eup5 (5)	6	0,33333	0,00093		

Rh. proximus			**61**	0,79672	0,01123	13	8
La Gomera				**0,34769**	**0,00104**		
	15	pro8 (5)	5	0,00000	0,00000		
	16	pro8 (5), pro10	6	0,33333	0,00093		
	17	pro8 (4), pro9 (2), pro11 (2)	8	0,71429	0,00240		
	18	pro8 (7)	7	0,00000	0,00000		
Tenerife				**0,92308**	**0,00661**		
	19	pro2 (2), pro3, pro5, pro17	5	0,90000	0,00504		
	20	pro1, pro2, pro4, pro5 (2), pro6, pro7, pro16	8	0,96429	0,00770		
La Palma				**0,33333**	**0,00099**		
	21	pro12 (3), pro14 (2), pro15	6	0,73333	0,00243		
	22	pro12 (4)	4	0,00000	0,00000		
	23	pro12 (7), pro13	8	0,25000	0,00070		
	24	pro12 (4)	4	0,00000	0,00000		
	25	-	-				
Rh. persimilis			**107**			25	25
La Gomera				**0,56000**	**0,00814**		
	26	per3 (4), per4 (4)	8	0,57143	0,00169		
	27	per1 (8)	8	0,00000	0,00000		
	28	per1 (8)	8	0,00000	0,00000		
	29	per2	1	0,00000	0,00000		
Tenerife				**0,74977**	**0,00987**		
	30	per12 (6)	6	0,00000	0,00000		
	31	per9 (3), per11 (5)	8	0,53571	0,00474		
	32	per7 (2)	2	0,00000	0,00000		
	33	per12 (3)	3	0,00000	0,00000		
	34	per12 (2)	2	0,00000	0,00000		

	35	per5 (5), per6 (2)	7	0,47619	0,00140		
	36	per10 (5)	5	0,00000	0,00000		
	37	per7 (2), per8 (2)	4	0,66667	0,00197		
	38	per12 (6)	6	0,00000	0,00000		
	39	per12 (6)	6	0,00000	0,00000		
	40	per12 (6)	6	0,00000	0,00000		
	41	per12 (6)	6	0,00000	0,00000		
	42	per13 (5)	5	0,00000	0,00000		
	43	per13 (5)	5	0,00000	0,00000		
	44	per13 (5)	5	0,00000	0,00000		
	45	per11, per13 (5)	6	0,33333	0,00197		
Rh. complanatus			**16**	0,64167	0,00350	4	2
La Palma							
	46	com1, com2 (8), com3	10	0,37778	0,00112		
	47	com4 (6)	6	0,00000	0,00000		
Rh. maderensis			**4**				
Madeira							
	48	mad1, mad2 (2), mad3	4	0,833	0,00280	2	0
Rh. tardyi			**15**			3	2
Azores				**0,82143**	**0,00310**		
	49	tar2, tar5 (2)	3	0,66667	0,00187		
	50	tar2, tar4 (2)	3	0,66667	0,00187		
	51	tar2	1				
	52	tar3	1				
British isles				**0,57143**	**0,00160**		
Ireland	53	tar1 (4), tar2 (2)	6	0,53333	0,00149		

Great Britain	54	tar2	1		

Appendix S3 Table c: Table summarising details of the RNA 28S sequences obtained, with a maximum length of 1070 bp. LC: Locality codes as in Fig. 3.1 of main text. Numbers in parenthesis indicate individuals displaying the same haplotype. Haplotype and nucleotide diversity values estimated for each species and island are shown in boldface.

Taxa Island	**LC**	**Haplotypes**	**No individuals**	**Haplotype diversity**	**Nucleotide diversity**	**No polymorphic nucleotide positions**	**No of parsimony informative**
Rh. euphorbiae			**80**	0,35853	0,00036	0	0
Madeira				**0,33284**	**0,00033**		
	1	eup1 (6)	6	0,00000	0,00000		
	2	eup1 (5), eup2 (3)	8	0,53571	0,00052		
	3	eup1 (6)	6	0,00000	0,00000		
	4	eup1 (5), eup2	6	0,33333	0,00032		
	5	eup1	1				
	6	eup1 (2)	2	0,00000	0,00000		
	7	eup1 (7)	7	0,00000	0,00000		
	8	eup1 (7), eup2, eup3	8	0,41667	0,00043		
	9	eup1 (4), eup2 (6)	6	0,53333	0,00051		
	10	eup1 (7), eup2 (2)	7	0,38889	0,00037		
	11	eup1 (7), eup2	7	0,25000	0,00024		
	12	eup1 (2)	2	0,00000	0,00000		
Porto Santo				**0,41905**	**0,00040**		
	13	eup1 (8), eup4 (1)	9	0,22222	0,00021		
	14	eup1 (3), eup4 (3)	5	0,60000	0,00058		

Rh. proximus			**63**	0,71014	0,00379	8	8
La Gomera				**0,00000**	**0,00000**		
	15	pro4 (7)	7	0,00000	0,00000		
	16	pro4 (8)	8	0,00000	0,00000		
	17	pro4 (7)	7	0,00000	0,00000		
	18	pro4 (7)	7	0,00000	0,00000		
Tenerife				**0,25714**	**0,00025**		
	19	pro2 (5)	5	0,00000	0,00000		
	20	pro1, pro2 (8), pro3	8	0,37778	0,00038		
La Palma				**0,34667**	**0,00034**		
	21	pro5 (7), pro6, pro7	7	0,41667	0,00042		
	22	pro5 (4)	4	0,00000	0,00000		
	23	pro5 (6), pro7 (2)	7	0,42857	0,00041		
	24	pro5 (3) , pro7	3	0,50000	0,00048		
	25	-	-				
Rh. persimilis			**107**	0,47223	0,00054	4	3
La Gomera				**0,00000**	**0,00000**		
	26	per1 8)	8	0,00000	0,00000		
	27	per1 (8)	8	0,00000	0,00000		
	28	per1 (8)	8	0,00000	0,00000		
	29	per1	1				
Tenerife				**0,54291**	**0,00062**		
	30	per1 (6)	6	0,00000	0,00000		
	31	per3 (6), per5 (2)	8	0,42857	0,00043		
	32	per1 (2)	2	0,00000	0,00000		
	33	per1 (2), per2	3	0,66667	0,00067		
	34	per1 (2)	2	0,00000	0,00000		
	35	per1 (7)	7	0,00000	0,00000		

	36	per1 (6)	6	0,00000	0,00000		
	37	per1 (4)	4	0,00000	0,00000		
	38	per1 (6)	6	0,00000	0,00000		
	39	per3 (6)	6	0,00000	0,00000		
	40	per1 (6)	6	0,00000	0,00000		
	41	per1 (6)	6	0,00000	0,00000		
	42	per3 (3), per4 (2)	5	0,60000	0,00061		
	43	per3 (4)	4	0,00000	0,00000		
	44	per3 (5)	5	0,00000	0,00000		
	45	per3 (6)	6	0,00000	0,00000		
Rh. complanatus			**15**			0	0
La Palma				0,24762	0,00023		
	46	com1 (10)	10	0,00000	0,00000		
	47	com1 (3), com2 (2)	5	0,60000	0,00057		
Rh. maderensis			**4**	0,00000	0,00000	0	0
Madeira							
	48	mad1 (4)	4	0,00000	0,00000		
Rh. tardyi			**7**	0,46429	0,00063	1	0
Azores				0,52381	0,00071		
	49	tar1	1				
	50	tar1 (2), tar2, tar3	3	0,83333	0,00109		
	51	tar1	1				
	52	tar1	1				
British isles							
Ireland	53	-	-				

Great Britain	54	tar1	1

Appendix S3 Table d: Table summarising details of the ITS-2 sequences obtained, with a maximum length of 600 bp. LC: Locality codes as in Fig. 3.1 of main text. In parentheses it is indicated the number of individuals of each haplotype greater than one. Haplotype and nucleotide diversity of each species for each island is represented in boldface.

Taxa Island	**LC**	**Haplotypes**	**No individuals**	**Haplotype diversity**	**Nucleotide diversity**	**No polymorphic nucleotide positions**	**No of parsimony informative**
Rh. euphorbiae			**87**	0,81232	0,00390	3	1
Madeira				**0,77608**	**0,00350**		
	1	eup1, eup5, eup7, eup9 (3), eup11, eup15 (2), eup16	8	0,91111	0,00423		
	2	eup1 (3), eup7, eup9 (2), eup9, eup12	7	0,78571	0,00350		
	3	eup1, eup7 (2), eup9 (3), eup15	7	0,80952	0,00352		
	4	eup1 (2), eup9 (5), eup13	7	0,60714	0,00297		
	5	eup7, eup9	2	1,00000	0,00555		
	6	eup9 (2)	2	0,66667	0,00370		
	7	eup2, eup7 (6), eup8, eup9 (2)	8	0,64444	0,00275		
	8	eup1 (4), eup4, eup9 (3), eup12	7	0,75000	0,00288		
	9	eup9, eup12 (5)	6	0,33333	0,00123		
	10	eup1 (3), eup3, eup4, eup9 (4), eup14,	8	0,80000	0,00271		
	11	eup1 (2), eup9 (4), eup9, eup10, eup11	8	0,69444	0,00257		
	12	eup9 (2)	2	0,66667	0,00246		
Porto Santo			10	**0,63971**	**0,00481**		

	13	eup1, eup6, eup7 (8), eup11 (2)	5	0,56061	0,00319		
	14	eup11 (5)	8	0,00000	0,00000		
Rh. proximus			**70**	0,64557	0,00175	5	1
La Gomera				**0,29892**	**0,00067**		
	15	pro2 (6), pro6	7	0,28571	0,00060		
	16	pro2 (7), pro4, pro6	8	0,41667	0,00093		
	17	pro2 (6), pro13, pro14	8	0,25000	0,00053		
	18	pro2 (6), pro5	7	0,28571	0,00060		
Tenerife				**0,55882**	**0,00133**		
	19	pro1 (2), pro2 (4), pro3	5	0,66667	0,00160		
	20	pro1 (8), pro2 (2)	8	0,35556	0,00075		
La Palma				**0,35484**	**0,00094**		
	21	pro1 (8), pro8	8	0,22222	0,00047		
	22	pro1 (5)	5	0,00000	0,00000		
	23	pro1 (7), pro10, pro11	8	0,41667	0,00128		
	24	pro1 (5), pro7, pro12	5	0,52381	0,00120		
	25	pro9	1				
Rh. persimilis			**106**	0,58896	0,00346	4	4
La Gomera				**0,59852**	**0,00230**		
	26	per1 (8), per4	8	0,22222	0,00043		
	27	per1 (5), per3 (3), per9	8	0,63889	0,00227		
	28	per1 (5), per2 (2), per5, per9 (3)	8	0,74545	0,00289		
	29	per1	1				
Tenerife				**0,28666**	**0,00089**		
	30	per8 (6)	6	0,00000	0,00000		
	31	per8 (8)	8	0,00000	0,00000		
	32	per5, per6 (2)	2	0,66667	0,00129		

	33	per8 (3)	3	0,00000	0,00000		
	34	per8 (2)	2	0,00000	0,00000		
	35	per6 (3), per7 (3)	6	0,60000	0,00117		
	36	per8 (6)	6	0,00000	0,00000		
	37	per5 (4)	4	0,00000	0,00000		
	38	per8 (6)	6	0,00000	0,00000		
	39	per8 (6)	6	0,00000	0,00000		
	40	per8 (6)	6	0,00000	0,00000		
	41	per8 (6)	6	0,00000	0,00000		
	42	per8(5)	5	0,00000	0,00000		
	43	per8 (4)	4	0,00000	0,00000		
	44	per8 (5)	5	0,00000	0,00000		
	45	per8 (6)	6	0,00000	0,00000		
Rh. complanatus			**15**	0,53333	0,00103	0	0
La Palma							
	46	com1 (8), com2 (3)	10	0,43636	0,00084		
	47	com2 (5)	5	0,00000	0,00000		
Rh. maderensis			**4**	0,00000	0,00000	0	0
Madeira							
	48	mad1 (4)	4	0,00000	0,00000		
Rh. tardyi			**13**	0,28205	0,00052	1	1
Azores				**0,53333**	**0,00098**		
	49	tar1	1				
	50	tar1, tar2 (2)	3	0,66667	0,00123		
	51	tar1	1				

	52	tar1	1		
British isles				**0,00000**	**0,00000**
Ireland	53	tar1 (6)	6	0,00000	0,00000
Great Britain	54	tar1	1		

Appendix S3 Table e: Table showing maximum pairwise genetic distances (%) between *Rhopalomesites* species estimated from *cox1* sequence data using Tajima-Nei model with the gamma distribution calculated between sequences, using MEGA v.6.0 (Tamura *et al.*, 2013). 1 *Rh. proximus*; 2 *Rh. euphorbiae*; 3 *Rh. complanatus*; 4 *Rh. persimilis*; 5 *Rh. maderensis*; 6 *Rh. tardyi*. Values in boldface indicate the maximum value found for sequences of the same species.

	1	**2**	**3**	**4**	**5**	**6**
1	**2.8**	7.6	10.8	11.9	12.1	12.6
2		**2.4**	11.5	12.4	12.6	12.4
3			**0.7**	4.3	3.0	5.8
4				**2.6**	4.8	5.8
5					**0**	6.0
6						**0.7**

Appendix S3 Table f: Maximum pairwise genetic distances (%) between *Rhopalomesites* species estimated from *cytb* sequence data. Numbers indicate species as in Table e and boldface maximum value found for sequences of the same species.

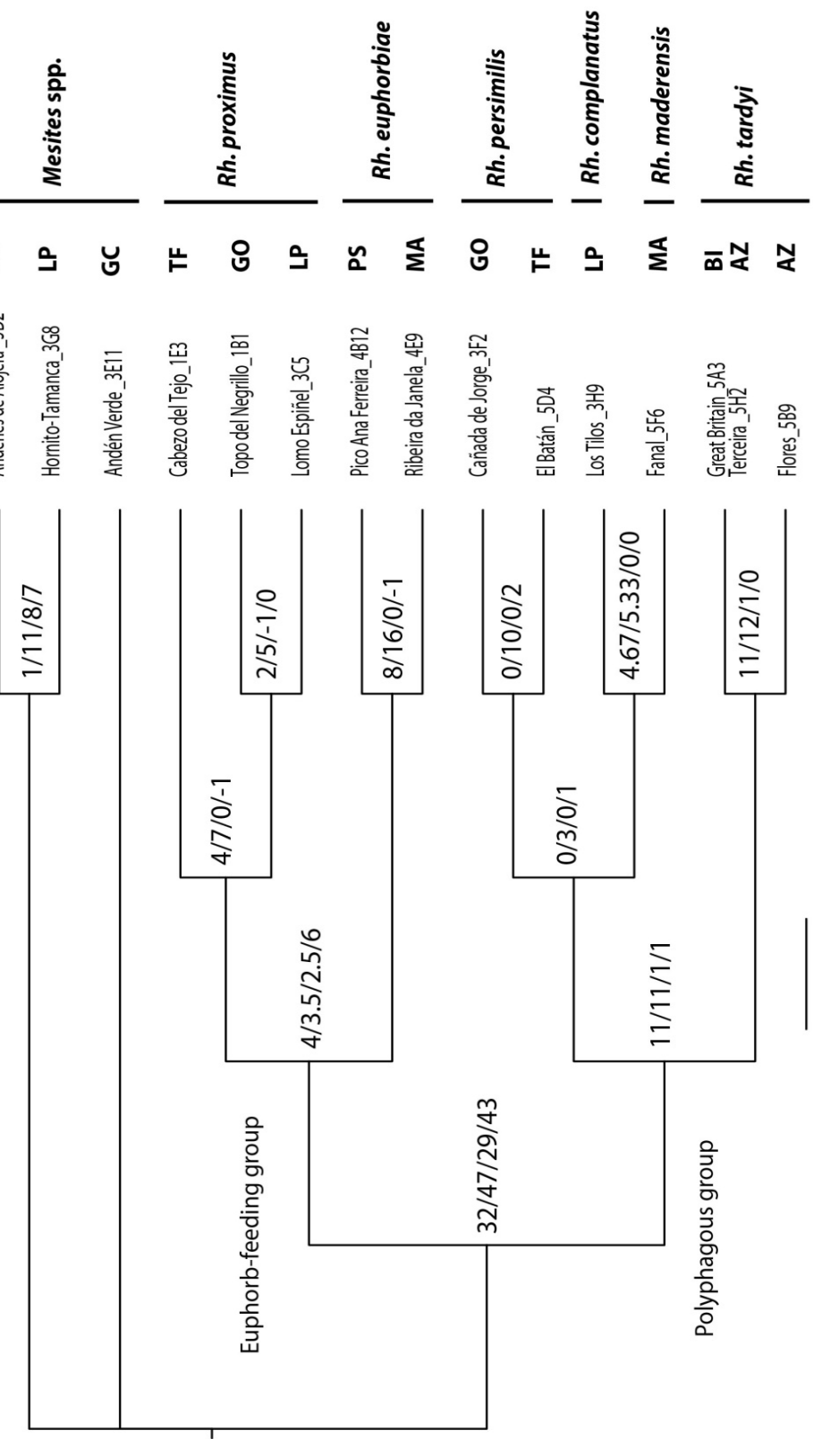
	1	2	3	4	5	6
1	**1.6**	8.2	10.1	13.5	11.8	13.1
2		**7.8**	13.5	14.3	13.9	13.5
3			**0.9**	6.0	2.2	7.4
4				**4.2**	7.4	9.4
5					**0.6**	6.3
6						**0.6**

Appendix S3 Table g: Maximum pairwise genetic distances (%) between *Rhopalomesites* species estimated from ITS-2 sequence data. Numbers indicate species as in Table e and boldface maximum value found for sequences of the same species.

	1	2	3	4	5	6
1	**0.5**	0.5	3.1	3.4	2.9	3.1
2		**0.5**	3.1	3.4	2.9	3.1
3			**0**	1.2	0.7	1.4
4				**0.5**	0.5	1.2
5					**0**	0.7
6						**0.2**

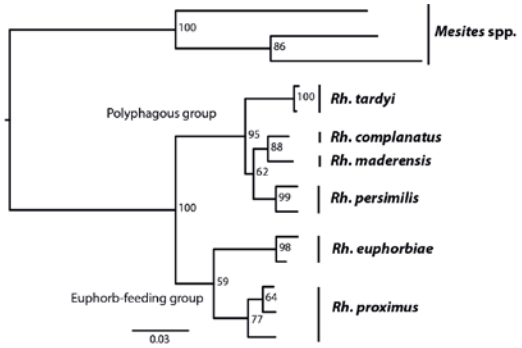
Appendix S3 Table h: Maximum pairwise genetic distances (%) between *Rhopalomesites* species estimated from 28S sequence data. Numbers indicate species as in Table e and boldface maximum value found for sequences of the same species.

	1	2	3	4	5	6
1	**0.7**	0.6	1.2	1.4	1.1	1.4
2		**0**	0.7	0.9	0.6	0.9
3			**0**	0.2	0.1	0.2
4				**0.3**	0.3	0.4
5					**0**	0.3
6						**0.1**

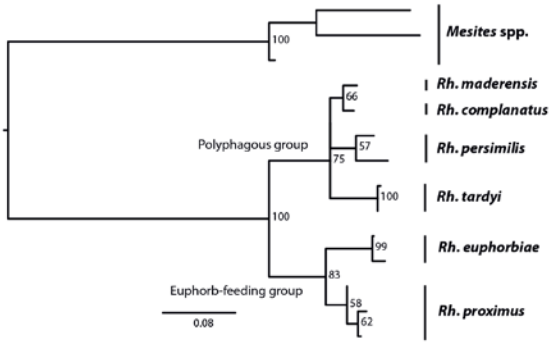


Appendix S3 Figure i: Results of the Partion Bremer Support analysis. The contribution of the specified gene to the total Bremer support calculated in the combined molecular data phylogeny using the reduce dataset are indicated at each node as follows: *cytb*, *cox1*, 28S, ITS-2.

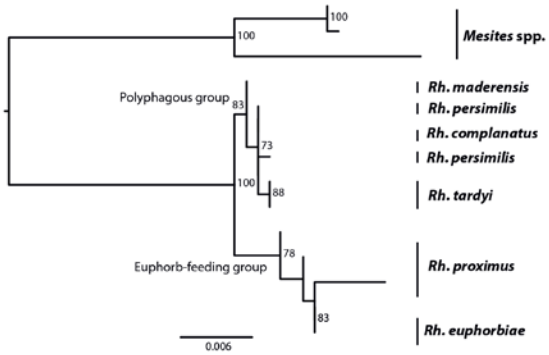
Appendix S3 Figure j: Bayesian phylogenetic trees obtained for each marker (either mitochondrial or nuclear) plus the ones deduced from the mitochondrial or nuclear gene fragments combined.



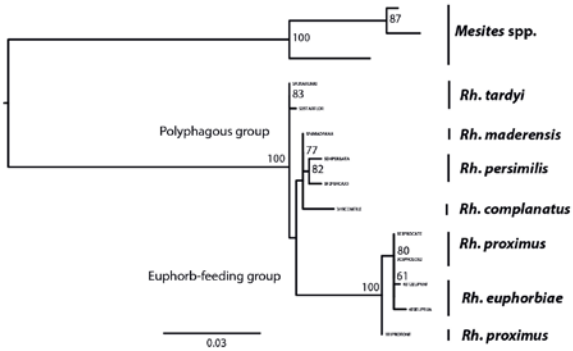
cox1



cyt b



28S



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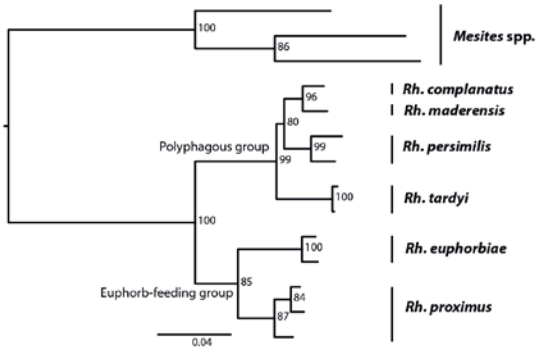
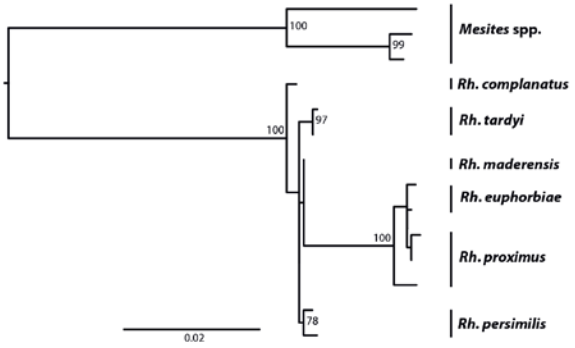
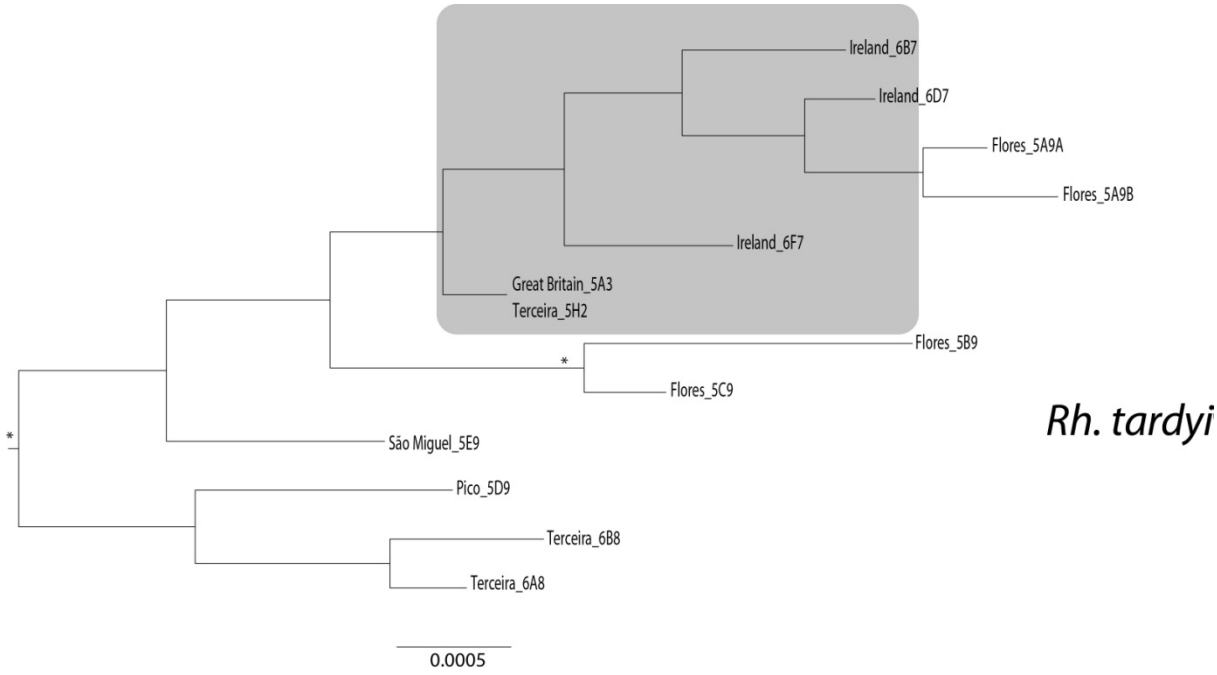


Michocondrial



Nuclear

Appendix S3 Figure k: Figure showing the Bayesian phylogenetic tree of *Rh. tardyi* based on the combined mitochondrial-nuclear data set (12 individuals; 2870 bp DNA sequence information). Asterisks show nodes with posterior probability values > 0.95. The gray rectangle includes haplotypes from the British Isles.



Appendix S3 Table l: Table showing the analysis of molecular variance (AMOVA) using mitochondrial sequence data for populations of *Rhopalomesites euphorbiae* grouped by geographic region.

Source of variation	d. f.	Sum of squares	Variance components	Percentage of variation	Φ statistics
Among groups (geographic regions)	9	360.410	4.06165	64.78	0.64780
Among populations within groups	4	10.354	0.09126	1.46	0.04132
Within populations	80	169.364	2.11705	33.76	0.66235

Appendix S3 Table m: Table showing the analysis of molecular variance (AMOVA) using mitochondrial sequence data for *Rhopalomesites euphorbiae* populations grouped by host plant taxa.

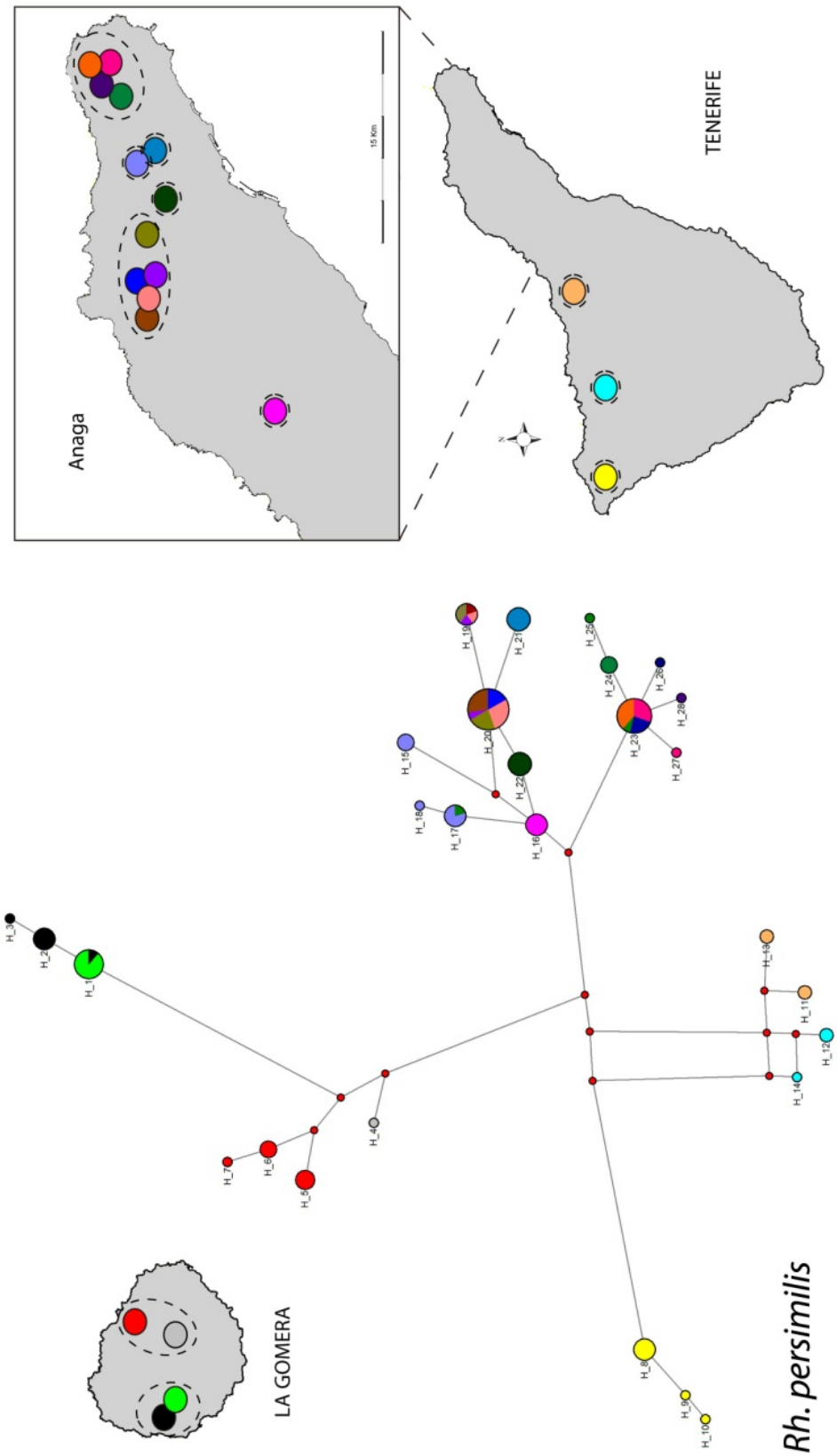
Source of variation	d. f.	Sum of squares	Variance components	Percentage of variation	Φ statistics
Among groups (host plant)	1	136.024	2.55069	34.75	0.34754
Among populations within groups	12	234.740	2.67158	36.40	0.55790
Within populations	80	169.364	2.11705	28.85	0.71155

Appendix S3 Table n: Table showing the analysis of molecular variance (AMOVA) using mitochondrial sequence data for populations of *Rhopalomesites proximus* grouped by geographic region.

Source of variation	d. f.	Sum of squares	Variance components	Percentage of variation	Φ statistics
Among groups (geographic regions)	2	3.060	0.10157	15.16	0.15163
Among populations within groups	1	0.643	0.01273	1.90	0.02240
Within populations	24	13.333	0.55556	82.94	0.17063

Appendix S3 Table o: Table showing the analysis of molecular variance (AMOVA) using mitochondrial sequence data for *Rhopalomesites proximus* populations grouped by host plant taxa.

Source of variation	d. f.	Sum of squares	Variance components	Percentage of variation	Φ statistics
Among groups (host plant)	1	1.679	0.04626	6.92	0.06921
Among populations within groups	2	2.024	0.06655	9.96	0.10698
Within populations	24	13.333	0.55556	83.12	0.16879



Appendix S3 Figure p: Median joining network obtained from mitochondrial sequences of *Rh. persimilis* (107 haplotypes; 1182 bp). Circle sizes and branch lengths are proportional to haplotype frequencies and to the number of nucleotide changes, respectively. Colours correspond to the localities shown in Appendix S1a. Red dot vertices are median vectors representing missing haplotypes. Ovals with discontinuous line indicate the optimum population grouping obtained by SAMOVA.

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Capítulo 4

Capítulo publicado:
Hernández-Teixidor, D., Lopez, H., Nogales, M., Emerson, B. C., Juan, C. & Oromi, P. (2014). Genetic, morphological, and dietary changes associated with novel habitat colonisation in the Canary Island endemic grasshopper *Acrostira bellamyi*. *Ecological Entomology*, 39(6), 703-715.

GENETIC, MORPHOLOGICAL AND DIETARY CHANGES ASSOCIATED WITH NOVEL HABITAT COLONISATION IN THE CANARY ISLAND ENDEMIC GRASSHOPPER *ACROSTIRA BELLAMYI*

ABSTRACT

The large flightless grasshopper *Acrostira bellamyi* Uvarov, endemic to the island of La Gomera (Canary Islands), inhabits two different environments: the xeric euphorb shrubland, as is typical for congeneric Canarian species, and the humid laurel forest, a novel habitat for the genus. We investigate genetic, morphological and ecological variation among individuals of *A. bellamyi* from the two habitats. DNA sequence data were used to evaluate whether grasshoppers from the two environments represent distinct lineages. Morphological and trophic analyses were performed to assess phenotypic differentiation between the two different habitats. Population genetic analyses support the hypothesis that the euphorb shrubland is the ancestral habitat for this species. Female laurel forest specimens are larger than those inhabiting the euphorb shrubland, and some external body parts exhibit significant morphometric differences between the two populations. Diet of shrubland individuals is completely different from that of laurel forest individuals. Although in each habitat they consume the most abundant plants, individuals are able to select food plants, which appears to be explained by their nutrient content. Our results suggest that *Acrostira bellamyi* has colonized the laurel forest from the shrubland, and that this habitat shift has resulted in genetic, morphological and ecological changes, perhaps as an adaptation to this new habitat.

INTRODUCTION

The evolutionary process by which new biological species arise in sexually reproducing eukaryotes (speciation) is one of the central topics in evolutionary biology (Butlin *et al.* 2012). Ecological speciation occurs when adaptation to divergent environments, such as different resources or habitats, leads to the evolution of reproductive isolation (Schluter 2000, Nosil 2012). However, for the completion of the speciation process, divergent (or disruptive) selection between environments or resources must cause the adaptive divergence of populations (Elias *et al.* 2012). Adaptation to environmental variability can be the outcome of phenotypic plasticity (the potentiality of a genotype to produce multiple phenotypes in response to variation in the environment) (Thompson 1991, Agrawal 2001, Pfennig *et al.* 2010). It has been proposed that phenotypic plasticity can induce differences enhancing assortative mating and restrict gene flow between populations, eventually leading to speciation (Whitman & Agrawal 2009). This has been suggested in particular for insect herbivore species, in which phenotypic plasticity can facilitate speciation prior to the development of reproductive isolation barriers (Görür 2005).

Oceanic islands are laboratories of evolution and their geographical, geological and ecological features make them ideal evolutionary and ecological case studies (Emerson 2002). The flora and fauna of oceanic islands have been extensively studied in the recent decades (e.g. Cronk 1997, Emerson 2002, Gillespie & Roderick 2002, Gillespie *et al.* 2008, Parent *et al*. 2008, Losos & Ricklefs 2009 and references therein). Many studies have focused on invertebrates, frequently with a phylogenetic interest (see Juan *et al.* 2000, Shaw 2002, Sequeira *et al.* 2008), however invertebrates may also be

used as models to investigate mechanisms of novel habitat colonization or resource exploitation (e.g. Nosil 2007, Funk 2010).

The Canary Islands is one of the most studied oceanic archipelagos concerning evolutionary processes due to their high variety of habitats, their outstanding biodiversity with high levels of endemicity, and a good knowledge of their geological history. Situated in the northeast Atlantic close to the Sahara coast, the archipelago comprises seven main volcanic islands with maximum ages ranging from 21 to 1 million years. The two eastern islands are older, lower and arid, having a limited number of habitats and poor biodiversity, whereas the remaining islands are younger, higher and more humid, with a variety of habitats and a rich biodiversity.

The Pamphagidae (Orthoptera) of the Canary Islands are a good model to study ecological processes because: (i) only six species occur in the Canaries, belonging the endemic genera *Purpuraria* (2 spp.) and *Acrostira* (4 spp.); (ii) all but one species occur on a single island (monoinsular); (iii) they are flightless and sedentary, slow moving and with low dispersal ability (López *et al.* 2007b); and (iv) there is a good understanding of their phylogenetic relationships and behavior (López *et al.* 2007a, 2008, 2013). All species are mainly arbusticolous, usually occurring in dry shrubland dominated by *Euphorbia* species, feeding on their leaves and shoots (López *et al.* 2005, 2007b). However, unlike other Canarian pamphagids, the only species inhabiting La Gomera (*Acrostira bellamyi*) also occurs in the humid laurel forest, a completely different habitat to the shrubland, within which euphorbs are extremely scarce. Almost all observations of this species in the laurel forest are of females that climb down from vegetation to lay their eggs in the soil. Males probably always remain in the canopy, given the extremely rare sightings of them. Despite the difficulty of finding

specimens in the forest vegetation due their cryptic behaviour, it does not appear to be a sink population, although ecological adaptations to the laurel forest habitat have not been reported. The forest population of *A. bellamyi* is unusual, since of the approximately 580 species of Pamphagidae described in the world (Otte 1994), there are no references or unpublished data (B. Massa, pers. comm.) about species inhabiting humid forests.

The aim of this study was to (i) assess genetic, trophic, and phenotypic differences between two populations of grasshopper surviving in adjacent divergent habitats, and (ii) test the hypothesis that the laurel forest population of *A. bellamyi* was derived from the shrub population. Given that other Canarian Pamphagidae species occur exclusively in shrub formations, we hypothesize that the laurel forest population of *A. bellamyi* is derived from a colonization event from the euphorb shrubland followed by an ecological niche-shift. We also hypothesize that the laurel forest population is genetically and morphologically distinct due to the limited dispersal capacity of the species and the large ecological differences between the dry shrub and wet forest habitats.

MATERIAL AND METHODS

STUDY AREA

The island of La Gomera is estimated to have emerged above sea level some 12 Ma (Llanes *et al.* 2009), it has a surface of 369.76 km^2 and an altitude of 1487 m. It is considered a mature island in which many types of environments are present, but *Acrostira bellamyi* has been only found between 400 and 1300 m a.s.l. in two different habitats: the dry euphorb shrubland (from sea level to 600 m on the northern and to

1300 m on the southern slopes) and the humid laurel forest (from 1000 m to the top of the island, in the altitudinal zone influenced by trade winds). The two habitats were formerly separated by a thermophilous transition forest that has almost disappeared due to alterations by human activities. Thus, the two habitats are now closer than they formerly were, because the shrubland has invaded the areas cleared of thermophilous forest, reaching the limits of the laurel forest.

The euphorb shrubland vegetation has an ancient tropical arid origin (Rand-Flora) and is mainly formed by species of the genus *Euphorbia* accompanied by other genera such as *Cistus*, *Chamaecytisus*, *Echium*, *Retama*, *Artemisia* and *Kleinia* among others (del Arco *et al.* 2010). It is an open xeric habitat, mainly dominated by shrubs of approximately 1-3 meters in height. The mean annual temperature is 16 ºC, although there are great differences between the southern and northern slopes of the island (mean above 23 °C in the summer on the southern slope) and the mean annual rainfall is 500 mm (650 mm/year in the north of island and 350 in the south) (Marzol & Sánchez-Megía 2009).

The laurel forest is considered a relict of the subtropical North Tethian forest from the end of the Tertiary, and it is dominated by perennial broadleaf laurifolious trees, although ericoid species like *Erica arborea* can dominate on ridge tops. It is the most plant diverse forest ecosystem of the island, being more widespread on the north slopes but also present on the south (Fig. 4.1). Its tree stratum is plurispecific (del Arco *et al.* 2010), being formed by a fairly continuous canopy that can reach heights between 5-30 meters. The mean annual temperature in the laurel forest ranges from 13 to 15 ºC (below 10 ºC in the winter), and the mean annual rainfall varies from 550 to 900 mm (Marzol & Sánchez-Megía 2009). The laurel forest usually has an additional

mist precipitation that can provide as much water as the rainfall itself, and it occurs mainly in the summer, especially in areas directly affected by the north-eastern humid trade winds (Gómez-González & Fernández-López 2009).

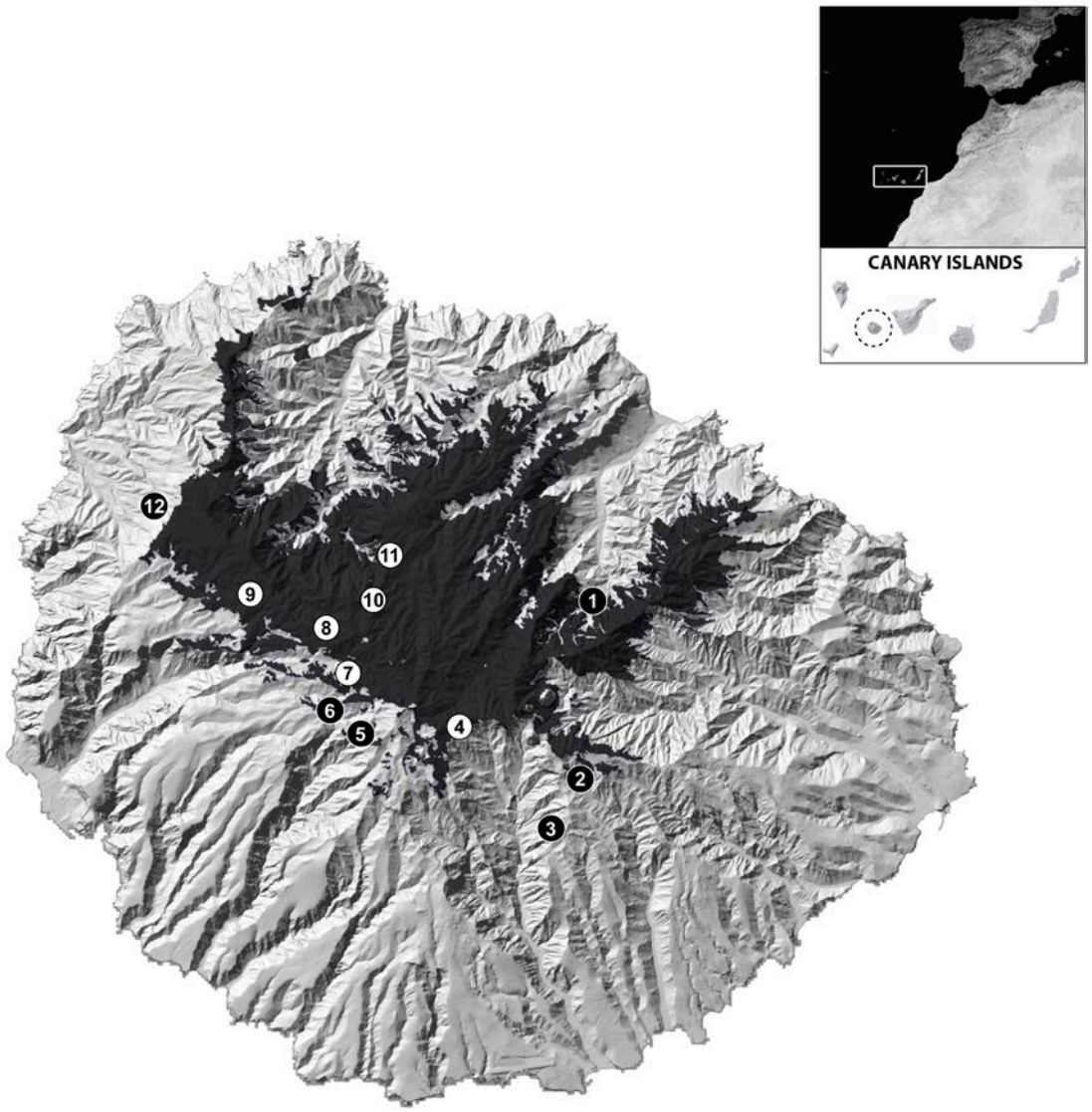

Figure 4.1. Sampling locations for *Acrostira bellamyi* used in this study. Laurel forest location codes in white circles; euphorb shrubland location codes in black circles. Dark area corresponds to the laurel forest (*sensu lato*).

SAMPLING

Canarian pamphagids in the euphorb shrubland are cryptic due to their homochromy with host plant stems, their passive behaviour and their extremely slow movement, usually remaining still during daytime. When they detect a potential threat they move around the stem to conceal themselves. These characteristics and behaviors make the species very difficult to find, including *A. bellamyi*, a species with a marked sexual size dimorphism (females 5.7-8.3 cm and approximate 2.5-4.5 gr; males 2.1-2.7 cm and appr. 1 gr). However, individuals of both sexes can be detected after careful inspection of host plants. This difficulty increases when they inhabit the laurel forest, in particular for males which are in the canopy, a challenging habitat for standard collection techniques. Males very rarely descend to the ground and only two have been collected in this particular habitat in the last 30 years (A. Hochkirch pers. comm., own data). In contrast, females are more conspicuous in the laurel forest when they descend from shrubs and trees to lay egg-pods in the soil. This has obliged our study to be performed with individuals of both sexes from the shrubland, but only with females from the forest. An intensive beating of branches was performed both in the shrub and the laurel forest, with some success in the former but not at all in the forest.

Specimens from the shrubland and laurel forest of La Gomera were collected between 2003 and 2012 (Table 4.1). After their capture, they were kept individually in containers for 24 h to collect droppings. The tibia of one foreleg of some specimens was cut off and preserved for molecular analysis, and individuals were subsequently released in their original locations and habitats. López *et al.* (2007a) found that this amputation procedure does not have a significant impact on survival rates.

Table 4.1. Number of female individuals of *Acrostira bellamyi* analysed per locality for the diet, morphological, and genetic analyses, and the corresponding haplotypes obtained. L.C.: Locality code

			Diet study	Morphological study	Genetic study	
	Locality	L.C.	Individuals	Individuals	MtDNA Haplotypes	Individuals
Euphorb shrubland						
	Vegaipala	2	13	1	be2, be3	ABE120, 121, 122, 123, 124, 125
	Barranco de Benchijigua	3	19	2	be3	ABE39
	Degollada Blanca	5	40	2	be5, be6, be7, be12,	ABE61, 64, 65, 66, 113, 115
	Casa forestal de Chipude	6	3	-	be12	ABE21
	Taguluche	12	19	8	be8, be9, be11, be13	ABE132, 133, 134, 135, 136, 137
	Banda de los Lairones	13	1	1	-	-
	Cerca Degollada de Pereza	14	2	2	-	-
	El Cercado	15	-	1	-	-
	El Rejo	1	1	-	be1	A29
Laurel forest						
	Caseta de los Noruegos	4	2	-	be4	ABE129
	Laguna Grande- Las Tajoras	7	42	18	be12, be7	ABE18, 19, 24, 25, 31, 47, 48, 50, 51, 95, 105
	Laguna Grande-Las Hayas	8	5	1	be12	ABE84
	Jardín de las Creces	9	15	-	be12	ABE58, 60, 72, 76, 98, 117
	Pista Mora Gaspar	10	3	1	be12, be7	ABE17, 41, 54, 55
	Mirador de Vallehermoso	11	3	1	be10	ABE78
	Laguna Grande	16	1	-	-	-

POPULATION GENETIC ANALYSES

Forty-six specimens of *Acrostira bellamyi* were used for molecular analysis, 21 were collected in euphorb shrubland localities and 24 were from laurel forest (Table 4.1). We used as an outgroup the related species *A. tamarani* Báez, which is the sister species of the group formed by the other three closely related species including *A. bellamyi* (López *et al*. 2007a). We amplified and sequenced a fragment of the mitochondrial gene cytochrome oxidase I (cox1) and the nuclear ribosomal internal transcribed spacer 2 (*ITS-2*), following López *et al*. (2007a) with primers and PCR conditions indicated therein. DNA sequences were assembled and edited using CodonCode Aligner v. 4.2.5 (CodonCode Corp., Denham, MA, USA) (www.codoncode.com), and then aligned with the online version of the MAFFT v. 7 automatic alignment software (Katoh & Standley, 2013) (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/).

Phylogenetic analyses based on maximum likelihood were performed using the software RaxML v. 7.2.8 (Stamatakis 2006). Haplotype networks were constructed using the statistical parsimony method of Templeton *et al*. (1992), using the software TCS v. 1.21 (Clement *et al.* 2000). This method links haplotypes with the smallest number of differences under a 95% confidence criterion. Ambiguities within networks were resolved following the criteria of Crandall & Templeton (1993). Estimates of nucleotide and haplotype diversity values were obtained with the program DnaSP v. 5.10.1 (Librado & Rozas 2009).

Coalescent theory predicts that recently colonised regions should have a lower genetic diversity and higher frequency of derived haplotypes compared to more ancestral areas (Wakeley 2008). Therefore, the average number of mutations of a

haplotype from the most recent common ancestor (MRCA) is expected to be significantly higher than would be expected by chance in recently colonised areas (Miraldo *et al.* 2011). The average mutational distance of DNA sequences from the MRCA sequence was estimated for both habitat types (euphorb shrubland and laurel forest), using the phylogenetic placement of *A. tamarani* to identify the MRCA sequence of *A. bellamyi*. To test whether empirical values were significantly higher or lower than chance expectations, data were randomized to generate a null distribution of mean haplotype distance from the MRCA.

Demographic history was investigated with the analysis of mismatch distributions (Rogers & Harpending 1992) within both habitat types. The shape of the mismatch distribution of pairwise differences is typically multimodal within samples drawn from populations at demographic equilibrium, whereas a unimodal distribution is generally found in populations having passed through recent demographic growth (Rogers & Harpending 1992, Harpending *et al.* 1998). In order to test whether both habitat populations conform to expectations of neutrality, Tajima's D (Tajima 1989), Fu's *Fs* (Fu 1997) and *R2* (Ramos-Onsins & Rozas 2002) tests were performed using DnaSP v. 5.10.1 (Librado & Rozas 2009), and the significance of results from each of three tests were evaluated using 5000 coalescent simulations.

MORPHOLOGICAL ANALYSES

A morphometric comparison of specimens from the two habitats was performed only with females, due to the difficulty of obtaining males in the laurel forest. Twelve linear morphological measurements were taken from 17 females from euphorb shrubland and 21 females from laurel forest, using a stereoscopic microscope with a micrometer.

Measurements were made on dried specimens from the DZUL collection (Department of Zoology, University of La Laguna), selecting body parts that do not change in shape or size either due to (i) variation in the fullness of the digestive tract, or (ii) as a consequence of the process of drying specimens. The morphological variables were: dorsal length (LP), maximum width (WP) and lateral height (HP) of pronotum; length of protibia (TL); length (LHF) and maximum height (HHF) of hind femur; maximum length of eye (LE) and dorsal distance between eyes (DBE); maximum height (HA3) and maximum length (LA3) of the 3rd antennomere; lateral height (HH) of head, as the distance from the base of clypeus to the vertex; and lateral length of head (LH), as the distance between the posterior margin of the eyes and the apex of fastigium.

Morphometric differences between populations of *A. bellamyi* from the two habitats were tested using multivariate analysis of covariance (MANCOVA). The "principal component size correction" (see Berner 2011) was used to amend body size effect in the variables. For this, the original variables were first divided in two sets: one matrix with variables related to the head, and another consisting of the remaining variables. Variables were mean-centered according to each habitat, and the PC1 of each set of variables was extracted in the resulting data matrices, reducing the number of variables to those that explained more than 80% of the variance. The PC1 scores obtained with each set of variables was used as a covariate to correct the body size effect in the matrices of original Ln-transformed variables of the other set. Given a significant MANCOVA, we also conducted univariate analyses of covariance (ANCOVA) independently for each morphological variable to determine which ones differed between the two habitats. All statistical analyses were conducted using the software Statistica (version 6.1, StatSoft Inc.).

DIET STUDY AND PLANT AVAILABILITY

The diet composition of *A. bellamyi* was determined by microhistological analyses of faeces using an optical microscope. Ninety-seven faecal pellets from the euphorb shrubland and 72 pellets from the laurel forest were analysed. The method has been successfully applied to determine the diet of different herbivores, including other Orthoptera (Isely & Alexander 1949, Gangwere 1962, Gangwere *et al.* 1998, Capello *et al.* 2012). One pellet from each specimen was diluted in water before placement on a slide. Thirty optical fields were randomly examined at 10X magnification for each faecal sample. Plant tissues observed in the faeces were identified on the basis of cellular characteristics (epidermal cells, stomata, trichomes, hairs, etc.), using a reference collection of leaf, flower and fruit tissues, and an identification key for the epidermis of Canary Islands plants (Marrero & Nogales 2005).

Two variables were used for diet characterisation: the frequency of occurrence (%FO) and the percentage of optical fields within which each plant component appeared (%OF). The Spearman correlation (r_s) was used to relate these two dietary variables. A likelihood ratio test (G) was applied to compare the number of plant species in each faecal sample (ie. dietary diversity in different habitats), the most important components of the diet, and diet according to sex and age. Diet similarity or overlap among the two habitats was evaluated using the Morisita index for percentage of optical fields, in which values close to 0 indicate low similarity, and values close to 1 a high similarity (Krebs 1999). Niche-breadth was assessed using the standardized Levin's niche-breadth index, where values close to 0 indicate a specialized diet, and values close to 1 a generalized diet (Krebs 1999). Diet selection was quantified using

the Savage index (Savage 1931) and the significance of selection evaluated by Chi-square test (Manley *et al.* 1993). A relative frequency of 0.01% was arbitrarily assigned to all species consumed but not recorded in the estimation of vegetation cover (Padilla *et al.*, 2005). Statistical analyses were performed using the IBM SPSS Statistics software (version 19.0, SPSS Inc.).

To evaluate the nutritional value of the components identified in the diet, main food items were collected in the field and their chemical composition determined. The proximate analysis of foods was performed by CANAGROSA Laboratories (http://www.canagrosa.com/), and focused on the main groups of chemical compounds: Ash or mineral matter (*MM*), crude protein (*CP*), crude fat or ether extract (*EE*), acid-detergent fibre (*ADF*), which represents the lignin and cellulose fractions of plant material, neutral-detergent fibre (*NDF*), which contains the fibres in *ADF* plus hemicellulose, and non-fibrous carbohydrates (*NFC*), which provides an estimate of digestible carbohydrates. The percentage of dry mass corresponding to each compound was estimated by the following methodologies: Dumas method for proteins, Soxhlet extraction with hexane for lipids, gravimetric plus digestion with acid-detergent solution for *ADF* and neutral-detergent digestion for *NDF.* Ash was measured by drying samples at 550ºC for three hours. *NFC* was calculated based on the remaining chemical groups following the equation by Mertens (1997):

$NFC = 100 - MM - CP - EE - NDF$

Plant composition of the two habitats was examined to explore the relationship between diet composition and plant availability. Plant cover was obtained following the line-intercept method, measuring the projection on the substrate of a line covering

either plants, bare ground or rock (Kent & Coker 1992). Five linear parallel transects of 25 m were selected in four different localities for each habitat.

RESULTS

POPULATION ANALYSES

A total of 773 bp of the mitochondrial *cox1* was obtained from 45 specimens of *A. bellamyi*, yielding 13 different haplotypes, nine exclusive to shrubland localities, two exclusive to laurel forest ones, and two present in both habitats (Table 4.1, Fig. 4.1). No sequence variation was found for ITS among localities or between habitats. The sequences obtained in the present study have been deposited in the EMBL nucleotide sequence database under accession numbers LM643814-LM643858 (*cox1*).

The most frequent *A. bellamyi* mitochondrial haplotype (be12) was shared among 23 individuals, 20 were from the laurel forest and three from the shrubland. The shrubland individuals showed much higher overall nucleotide and haplotype diversities (0.00172 and 0.65789, respectively) than those from the laurel forest (0.00035 and 0.08333, respectively). Both the total number of haplotypes and the number of haplotypes deduced to be ancestral were also higher for the shrubland population. The statistical parsimony analysis of the *cox1* gene sequences yielded a single network at the 95% confidence limit made of two haplotype groups separated by a total of 8 mutational steps corresponding to the eastern and western regions (see Fig. 4.2). The topology obtained in the phylogenetic analyses was similar to that from haplotype networks but with low bootstrap values, something expected given the low genetic variation at the population level.

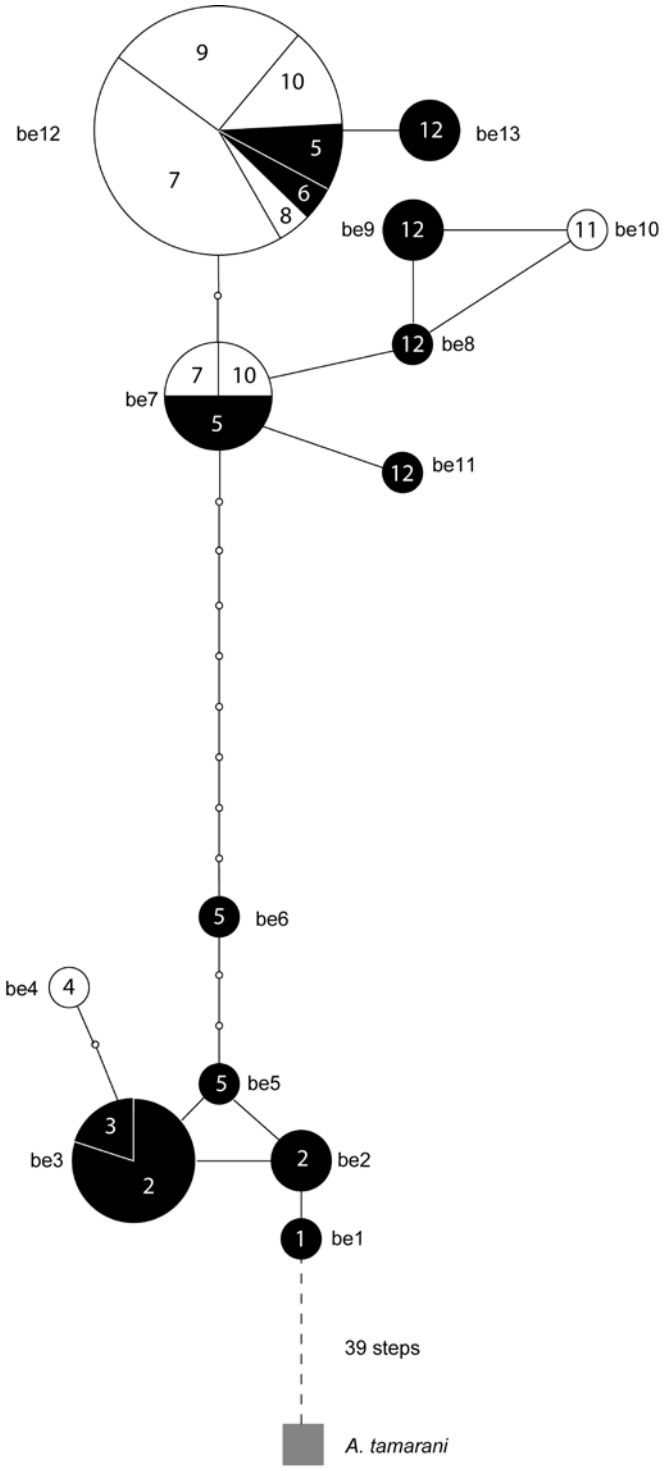

Figure 4.2. Statistical parsimony mtDNA haplotype network for *Acrostira bellamyi.* Haplotype codes as in Table 4.1. The size of each circle/square is proportional to the haplotype frequency. Small white circles indicate missing or extinct haplotypes. Colours in the network according to the type of habitat [white: laurel forest; black: euphorb shrubland; grey: outgroup (*A. tamarani*)]. Subsectors numbers correspond to sampling locality. Haplotype be1 is deduced to be the MRCA sequence within *A. bellamyi*, based on its connection to *A. tamarani*.

The network strongly suggests that the most likely ancestral habitat was the euphorb shrubland, and that more than one mitochondrial lineage was involved in the colonisation of the laurel forest. The average number of mutations of haplotypes from the MRCA sequence (*be1* in Fig. 4.2) in shrubland was significantly lower than that expected by chance (9.55 *vs.* 12.60). Conversely, laurel forest haplotypes exhibit a higher average number of mutations from the MRCA than is expected by chance (15.33 *vs.* 12.79) (Fig. 4.3). Mismatch distribution analyses revealed a unimodal distribution within the laurel forest population, together with a negative value of Tajima's D (D = -2.26, p <0.001), both genetic signatures suggestive of a recent population expansion (Fig. 4.4). In contrast, the shrubland population showed a multimodal distribution and positive value of Tajima's D = 2.48, p = 0.99. Other neutrality test statistics, however, did not yield significant deviations from neutrality for both the laurel forest (Fs = 2.5, p = 0.88; R2 = 0.18, p = 0.73) and shrubland (Fs = 0.74, p = 0.69; R2 = 0.24, p = 0.76) populations.

MORPHOMETRIC COMPARISON OF *ACROSTIRA BELLAMYI* POPULATIONS

Females from the laurel forest tended to be larger than those from the shrubland, as indicated by the mean values of most of the measured variables (see Table 4.2), and by the significant morphological differences found between females from the shrubland and the laurel forest (MANCOVA: Wilks' λ = 0.359, P < 0.001 for variables from the head; Wilks' λ = 0.315, P < 0.001 for variables from the body). The posterior univariate ANCOVAS showed differences among specimens from the two habitats in variables related with prothorax, protibia, maximum height of 3rd antennomere, and distance between eyes (Table 4.2). Covariates had significant differences in all analyses,

indicating their suitability for body size correction. In addition, heterogeneity of slopes was rejected in all analyses (MANCOVA and ANCOVAS), suggesting that there is no variation in the relationship between morphological variables and body size between the two habitats.

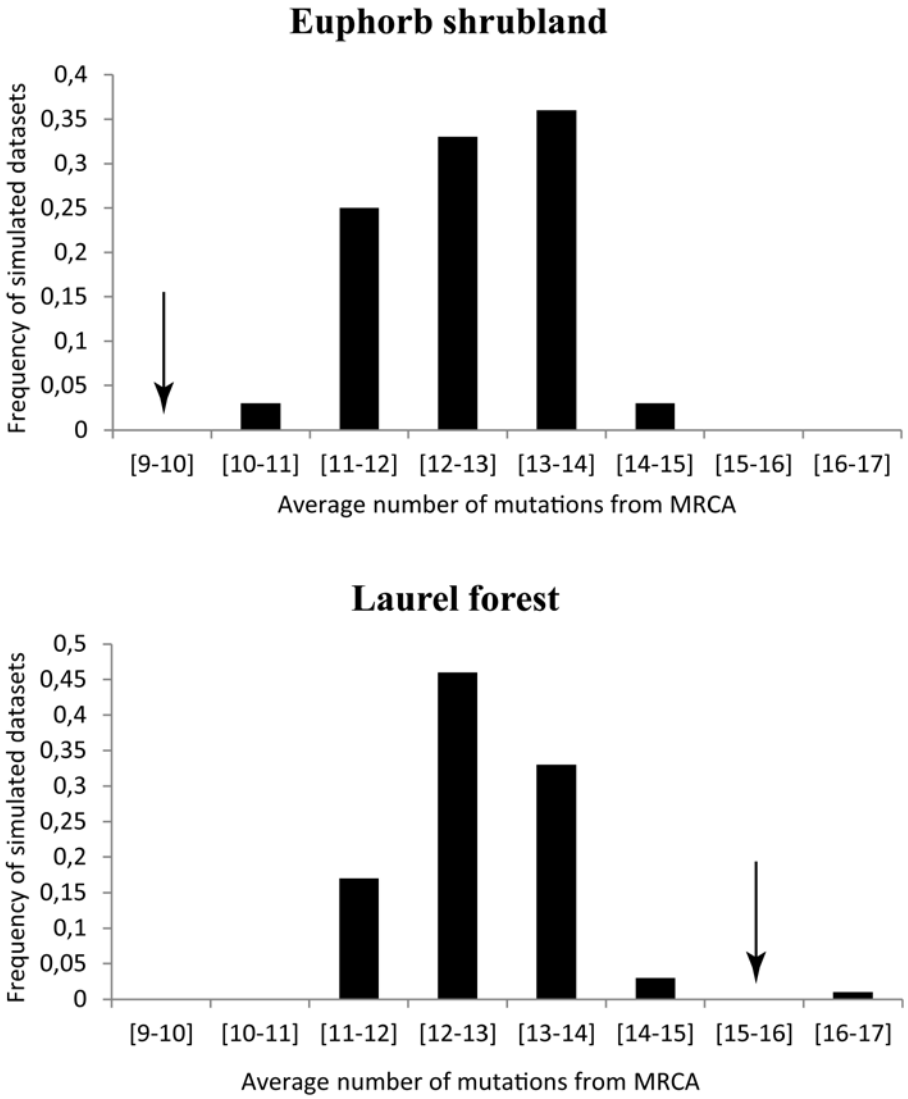

Figure 4.3. Observed and null model expected distances of haplotypes from the MRCA in euphorb shrubland and laurel forest. Arrows indicate observed distances.

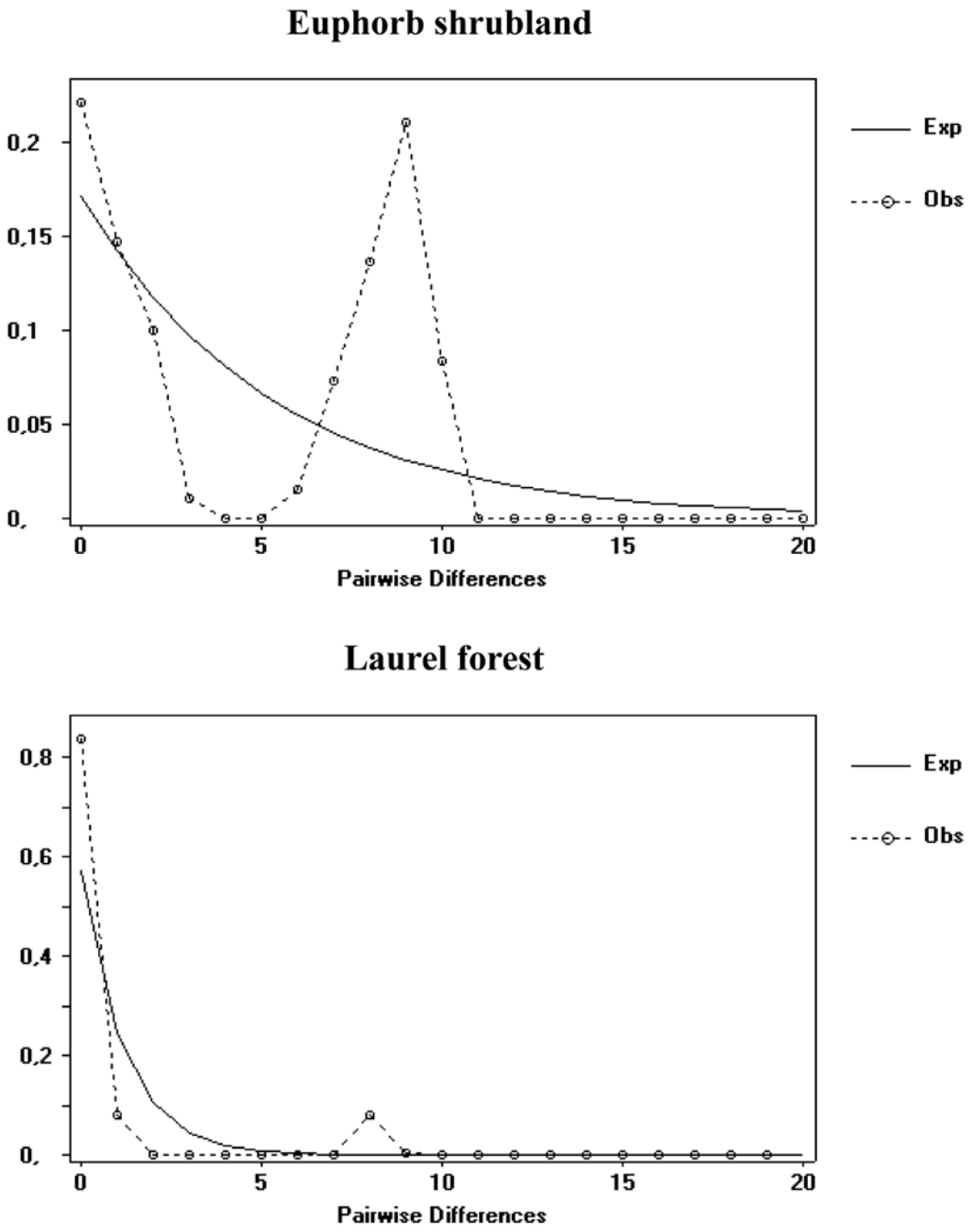

Figure 4.4. Mismatch distribution of mtDNA haplotypes for the two habitats. The expected frequency (Exp) is based on a population growth-decline model, and is represented by a continuous line. The observed frequency (Obs) is represented by a dotted line.

DIET COMPOSITION

A total of eight plant species were identified in faecal pellets from the euphorb shrubland, and 10 plant species in pellets from the laurel forest (Table 4.3). Due to the

high correlation found between the variables %FO (Frequency of occurrence) and %OF (Optical field) (r_s = 0.98 P < 0.001 for 8 types of food from shrubland, and r_s = 0.94 P < 0.001 for 10 types of food from laurel forest), we chose to use the variable %FO for diet description. The main component of the diet in the shrubland was *Euphorbia* spp. (66% of FO), but *Chamaecytisus proliferus* (Fam. Fabaceae) (24%) and *Cistus monspeliensis* (Fam. Cistaceae) (5%) were also identified. In the laurel forest the most consumed species were *E. arborea* (76%) and *Morella faya* (26%), while *C. monspeliensis, Laurus azorica* (Fam. Lauraceae) and *Rubus ulmifolius* (Fam. Rosaceae) among others appeared in much lower proportion.

Table 4.2. Results of ANCOVAS testing for differences in 13 morphological variables between populations of *A. bellamyi* from euphorb shrubland and laurel forest. All variables are $\log_{10}$-transformed (see Materials and Methods for variable abbreviations). Values in all interaction term and covariates are significant. In the column "Comparison" an interpretation of the results is made (E= euphorb shrubland; L= laurel forest). Significant variables are in bold.

	Euphorb shrubland (*n* = 18)	**Laurel forest** (*n* = 20)	**ANCOVAS**				
Variable	**Mean ± DS**	**Mean ± DS**	**d.f.**	**MS**	***F***	**P**	**Comparison**
HP	9.24 ± 0.48	10.23 ± 0.50	1	0.019	56.8	**0.000**	E<L
LP	8.82 ± 0.46	9.44 ± 0.64	1	0.008	19.23	**0.000**	E<L
WP	10.40 ± 0.48	10.78 ± 0.54	1	0.002	8.8	**0.005**	E<L
TL	7.47 ± 0.34	7.81 ± 0.48	1	0.003	7.91	**0.008**	E<L
LHF	23.62 ± 1.20	24.10 ± 1.36	1	0.001	1.8	0.190	E=L
HHF	4.07 ± 0.22	4.06 ± 0.24	1	0.000	0.08	0.782	E=L
LA3	5.35 ± 0.53	5.53 ± 0.56	1	0.001	1.82	0.186	E=L
HA3	1.18 ± 0.09	1.29 ± 0.11	1	0.013	16.63	**0.000**	E<L
LE	3.43 ± 0.17	3.44 ± 0.18	1	0.000	0.06	0.806	E=L
DBE	3.43 ± 0.13	3.67 ± 0.24	1	0.007	27.77	**0.000**	E<L
HH	6.46 ± 0.31	6.60 ± 0.40	1	0.000	2	0.166	E=L
LH	4.76 ± 0.33	4.76 ± 0.33	1	0.000	0.00	0.991	E=L

With regard to the most important diet components in each habitat, *E. arborea* was markedly more consumed in the forest than in the shrubland (G_1 = 95.05, P < 0.001), *C. monspeliensis* was similarly consumed in both habitats (G_1= 0.18, P = 0.66) and *Ch. proliferus* was consumed in greater amounts in the shrubland (G_1 = 27.51, P < 0.001). Comparisons for *M. faya* and *Euphorbia* spp. could not be analysed because they were only found in the diet of individuals from one of the two habitats.

Table 4.3. Plant species present in *A. bellamyi* diet from two habitats (euphorb shrubland and laurel forest). % FO: frequency of occurrence; % OF: optical fields. Main components are in bold.

	Euphorb shrubland (*n* = 97)		**Laurel forest** (*n* = 72)	
Plant species	**% FO**	**% OF**	**% FO**	**% OF**
Euphorbia spp.	**66.0**	**65.7**	-	-
Chamaecytisus proliferus	**24.7**	**23.5**	1.4	0.5
Cistus monspeliensis	5.2	4.3	**8.3**	**6.5**
Bituminaria bituminosa	3.1	2.3	-	-
Retama monosperma	1.0	1.0	-	-
Pinus canariensis	1.0	1.0	-	-
Didymodon cf. *vinealis*	2.1	2.1	-	-
Erica arborea	1.0	0.3	**76.4**	**68.2**
Morella faya	-	-	**26.4**	**20.9**
Laurus azorica	-	-	8.3	4.4
Rubus ulmifolius	-	-	4.2	4.2
Ilex canariensis	-	-	1.4	0.5
gen. indet. 1	-	-	1.4	1.3
gen. indet. 2	-	-	1.4	0.1
gen. Pteridophyta indet.	-	-	1.4	0.5

The analysis of diet by each sex was only possible for the shrubland samples (35 males and 59 females), as data for males from the laurel forest were not available. In the shrubland, *Euphorbia* spp. (57% in males and 72% in females) and *Ch. proliferus* (34% in males and 20% in females) were the most important components of the diet in

both sexes (Table 4.4). However, while *C. monspeliensis* and *Didymodon* cf. *vinealis* (Bryophyta: Dicranaceae) also appeared in lower proportions in the diet of males, four different species - but mainly *Bituminaria bituminosa* (Fam. Fabaceae), complemented female diet. The differences between the sexes were not significant (G_2 = 3.099, P = 0.212).

With respect to the relationship of diet and age in euphorb shrubland (no available data from forest males or nymphs), a total of 22 faeces of nymphs and 77 of adults were analyzed (Table 4.4). *Euphorbia* spp. and *Ch. proliferus* were the principal components in the two age groups, and no statistical differences were found in their consumption (G_2 = 3.8, P = 0.150).

Table 4.4. Plant species present in *A. bellamyi* droppings from the euphorb shrubland by sex (males *vs.* females) and age (adults *vs* nymphs). % FO: frequency of occurrence; % OF: optical fields. Main components are in bold.

	Males (n = 35)		**Females** (n = 59)		**Adults** (n = 76)		**Nymphs** (n = 21)	
Plant species	**% FO**	**% OF**	**% FO**	**% OF**	**% FO**	**% OF**	**% FO**	**% OF**
Euphorbia spp.	**57.1**	**57.1**	**72.9**	**72.4**	**71.0**	**70.7**	**47.6**	**47.5**
Chamaecytisus proliferus	**34.3**	**34.2**	**20.3**	**18.5**	**21.0**	**20.8**	**38.1**	**33.5**
Cistus monspeliensis	8.6	6.1	-	-	3.9	2.8	9.5	9.5
Pinus canariensis	-	-	1.7	1.7	1.3	1.3	-	-
Bituminaria bituminosa	-	-	5.1	3.8	3.9	2.9	-	-
Didymodon cf. *vinealis*	2.9	2.89	-	-	1.3	1.3	4.8	4.8
Retama monosperma	-	-	1.7	1.7	-	-	4.8	4.8
Erica arborea	-	-	1.7	0.6	1.3	0.4	-	-

DIET INDEX ANALYSIS AND TROPHIC ECOLOGY

Levin's niche breadth index was low in both habitats; *A. bellamyi* was slightly more specialized in the laurel forest (0.10) than in shrubland (0.15). The Morisita index indicated a very low trophic overlap between shrubland and laurel forest (0.008),

reflecting a remarkably significant diet shift in the laurel forest population. Dietary diversity (number of species present in each pellet) was more diverse in the forest (G_2 = 19.25, $P < 0.001$).

Acrostira bellamyi individuals were clearly more likely to consume the abundant plants in the habitats they occupied. However, *Euphorbia* spp. and *Ch. proliferus* were more frequently selected in the shrubland than expected from their respective availability in this habitat (1.86 and 2.57 of Savage index, respectively) (Fig. 4.5). In contrast, the main components of the diet in the laurel forest (*E. arborea* and *M. faya*) were not positively selected in this respect. However, there was a positive selection for species with less cover in this habitat, such as *C. monspeliensis* and *R. ulmifolius* (10.44 and 3.19 of Savage index, respectively) (Fig. 4.6).

Chemical analyses showed that the principal component of the diet in the shrubland (*Euphorbia lamarckii*) had the best balanced nutritional values, with high fat, fibre (ADF) and carbohydrate contents. However, the protein content was higher in plants eaten secondarily, except *C. monspeliensis* that had slightly lower values (Table 4.5). In comparison, the main plant consumed in the laurel forest (*E. arborea*) showed the lowest protein content, low fat and carbohydrates but the highest fibre levels.

The principal food for *A. bellamyi* in the shrubland had more than twice the fat content than the main components of diet in the forest (*E. arborea* and *M. faya*). Protein content was higher in plants from the shrubland, especially with respect to *E. arborea.* The principal secondary food items had a higher amount of protein in both habitats. Fibre content was higher in the most consumed plants from the forest, but carbohydrate composition was lower.

Table 4.5. Nutritional composition of the main food plants of *Acrostira bellamyi* in each habitat. All values correspond to the percentage of dry mass (see Materials and Methods for variable abbreviations).

	Ash	Protein	Fat	Fibre		Carbohydrates
Habitat	***MM***	***CP***	***EE***	***ADF***	***NDF***	***NFC***
Euphorb shrubland						
Euphorbia lamarckii	4.8	10.3	11.4	27.2	14.8	58.7
Euphorbia berthelotii	9.3	7.9	6.6	11.3	8.4	67.8
Euphorbia lambii	11.8	17.3	8.1	15.1	13.9	48.9
Chamaecytisus proliferus	3.3	17.3	2.5	22.3	26.4	50.5
Cistus monspeliensis	9.0	8.8	10.8	11.3	31.2	40.1
Pinus canariensis	5.6	14.7	5.5	50.9	44.7	29.2
Laurel forest						
Erica arborea	2.6	5.7	4.6	51.6	45.3	41.8
Morella faya	7.4	8.6	2.5	40.2	37.9	43.7
Cistus monspeliensis	9.0	8.8	10.8	11.3	31.2	40.1
Laurus azorica	4.9	11.8	5.9	42.9	36.4	41.1
Rubus umifolius	4.9	8.5	3.6	25.5	30.9	52.1
Ilex canariensis	4.5	9.2	2.4	19.4	14.0	69.9

DISCUSSION

GENETIC ORIGIN AND ENVIRONMENTAL VARIATION

Population genetic analyses support the idea that the ancestral habitat for *Acrostira bellamyi* is the euphorb shrubland. Across all 13 mtDNA haplotypes, the proportion of habitat specific mitochondrial haplotypes was higher in the shrubland (62%) compared to the laurel forest (15%). Additionally, both nucleotide and haplotype diversity values were higher in the shrubland compared to the laurel forest. These data are further supported by analyses of relative haplotype age which indicate that haplotypes sampled from the laurel forest were significantly younger (derived) than those sampled from shrubland. Neutrality tests also suggest that the laurel forest population

had experienced a recent expansion. All these results demonstrate that the laurel forest has been colonised secondarily from shrubland ancestors.

MORPHOLOGICAL VARIATION

Female laurel forest specimens of *A. bellamyi* were larger than those living in the euphorb shrubland, and some structures and parts of their bodies had slightly, but significantly larger morphometric differences between the two populations. Size is determined by genes, environmental factors, and their interaction, with many environmental factors implicated for Orthoptera (nutrition, elements and minerals, toughness of food, temperature, solar radiation, disease, predator threat, moisture, toxins, etc, see Whitman 2008 and references therein). The degree to which genes or environment control these differences is unknown, but temperature and nutrition have been found to be the most habitual environmental factors that induce insect size variation (Chown & Gaston 2010 and references therein). In ectothermic animals, body size generally increases when developmental temperature decreases, a phenomenon known as the temperature–size rule (Atkinson 1994). Thus, some Orthoptera follow Bergmann's rule, with larger individuals or species at higher latitudes and altitudes (Whitman 2008). In La Gomera, the mean yearlong temperature in the laurel forest is lower than in the euphorb shrubland, and that forest is located at higher altitude than the shrubland. Diet, temperature and altitude differences between populations of *A. bellamyi* in these two habitats could be important environmental factors promoting the body size differences found among females of this species.

The abundance, quality and diversity of food resources positively affect growth rate, which in turn affects instar size (Diamond & Kingsolver 2010, Ho *et al.* 2010).

Conversely, conditions and environments constraining growth rate (e.g. food shortage) normally produce smaller body sizes (Blanckenhorn & Demont 2004). Weather conditions in the laurel forest facilitate the availability of fresh food year-round. In comparison, in the shrubland there is significant water stress during the summer and part of the autumn, producing leaf shortage and dryness of stems. Thus, nymphs of *A. bellamyi* from the laurel forest probably experience a relatively constant food supply, which may positively affect their adult body size.

Morphometric differences among specimens of the two habitats occur mainly in the prothorax (dorsal length, maximum width and lateral height of pronotum, and length of protibia), a part of the body often associated with population divergence in grasshoppers (Tregenza 2002 and references therein). Furthermore, the vegetation of the laurel forest is more complex and dense than the euphorb shrubland, not only in the horizontal plane but also in the vertical plane, accompanied by a higher heterogeneity of branches and leaves. In the forest, specimens of *A. bellamyi* are probably faced with more obstacles or difficulties for moving in the vegetation, and a longer protibia and a higher pronotum may be related with more efficient locomotion in this habitat. The greater height of the 3rd antennomere recorded in the forest specimens implies a larger surface area of the antenna, which potentially increases the amount of sensilla. Grasshoppers use olfaction and gustation when finding and identifying food plants (Helms *et al.* 2003), with their antennal sensilla involved in plant odour sensitivity (Blaney & Simmonds 1990). Furthemore, it is well known that volatile and non-volatile compounds have a positive effect on the development of olfactory sensilla (Rogers & Simpson 1997, Bernays & Chapman 1998). Polyphagous grasshoppers that feed on a great diversity of plants probably have a higher number of

chemoreceptors to recognize the secondary metabolites associated with the consumed leaves (Dumas *et al.* 2010). In comparison to the shrubland, the laurel forest contains a large number of plant species belonging to different families, which could have selected for an increase in sensilla.

TROPHIC ECOLOGY

Acrostira bellamyi exhibits disjunct oligophagy (*sensu* Chapman 1990), because they feed on a limited number of unrelated plants belonging to different families. In the euphorb shrubland, independently of sex or age, *A. bellamyi* fed mainly on *Euphorbia* spp., supplemented with other species, such as *Ch. proliferus*. The colonisation of the laurel forest and exploitation of a different niche required a change to another plant spectrum, where practically no shrubland species are present. Our results indicate that *A. bellamyi* mainly consume two species in the laurel forest (*E. arborea* and *M. faya*) from two families (Ericaceae and Myricaceae, respectively) that are phylogenetically very distant from the Euphorbiaceae primarily consumed in the shrubland. Diet is also more diverse in the laurel forest, probably due to the higher vegetation richness and the persistence of leaves and tender stems throughout the year. Other Canarian pamphagids have been observed feeding on *Euphorbia lamarckii* or *E. regis-jubae*, but there are no detailed studies on their diet composition. Nevertheless, sightings of individuals resting on other potential food plants have been recorded, so their diets may be richer than previously recognized: *Acrostira tamarani* has been located on *Pinus canariensis* (Fam. Pinaceae), and has even been fed in captivity with their needles (Oromí *et al.* 2001); *A. euphorbiae* García & Oromí was found on *Kleinia neriifolia* (Fam. Asteraceae) (0.34% observations) and *Retama rhodorhizoides* (Fam.

Fabaceae) (1.7%) (López *et al.* 2007b); *A. tenerifae* Pérez & López has been observed on *R. rhodorhizoides* (27.5%) (López *et al.* 2005); *Purpuraria erna* Enderlein and *P. magna* López & Oromí on *Euphorbia balsamifera*, *K. neriifolia* and annual dry grass (López *et al.* 2013, pers. obs.). As a general pattern, almost 60% of grasshoppers that have been specifically investigated are polyphagous, a further 25% are oligophagous and monophagy is rare (Bernays & Chapman 1994). Polyphagy is the predominant feature of grasshopper feeding pattern and is presumed to be the ancestral state (Bernays & Chapman 2000).

With regard to food selection, *A. bellamyi* usually consumed mostly shrubby or arboreal plant species that constitute much of the plant biomass of each habitat. This probably reflects the necessity of feeding on plant species that are abundantly present throughout the year, and the need to obtain adequate refuge. *Acrostira bellamyi* spends most of the time on its food plant, both in the shrubland and (likely) in the forest. Most grasshopper species exhibit some degree of preference for the food they eat, and in general their diet is partially influenced by the relative abundance of potential host plants (Chapman 1990). In the case of the *Acrostira* shrubland population, the two principal plant species consumed were positively selected with respect to their own availability. However, in the laurel forest there was no positive selection of principal species consumed, possibly due to an incomplete adaptation to feeding on forest species. Furthermore, *A. bellamyi* positively selected other species to complete their food requirements too (e.g. *C. monspeliensis*), although these species were less abundant.

Food selection patterns might also be influenced by the macronutrient content of plants and by the presence of defensive plant-produced allelochemicals

(Raubenheimer & Simpson 2003). There is great variation (both seasonally and ontogenically) in the nutrient contents of plants, and insects may adapt their diet composition according to this variation. Many studies have demonstrated that insects can regulate the intake of different nutrients by eating a mix of available foods rather than a single type (Chambers *et al.* 1997). This could be the reason why *A. bellamyi* does not feed exclusively on *Euphorbia* spp., supplementing its diet with other plants. Nitrogen is often considered the key nutrient required by herbivorous arthropods and generally limits insect growth and fecundity (Scriber & Slansky 1981). This would explain why herbivorous insects tend to feed on high quality host plants in terms of nitrogen content (Price *et al.* 2011). In the case of *A. bellamyi,* it secondarily consumed plant species with high nitrogen content, such as *Ch. proliferus* in the shrubland and *M. faya* in the forest, to compensate for lower nitrogen content in the principal food plant species in each habitat. Carbohydrates are the main energy source for insects and they influence growth, life duration and fecundity (Parra 2012). However, the high carbohydrate content of all principal plants consumed by *A. bellamyi* renders this nutrient as non-essential for plant selection. The deficit of some lipids has been associated with lower survival and fecundity rates in insect species (Awmack & Leather 2002, Parra 2012). In grasshoppers lipids can be mild olfactory attractants and strong phagostimulants (Latchininsky *et al*. 2007), and also can be an important component of their eggs (Allais *et al*. 1964). *Euphorbia lamarckii* had the higher lipid content in the shrubland, thus *A. bellamyi* probably gains enough of this component in this habitat. However, in the forest the main food plant species had low lipid contents and the grasshoppers possibly consume other plant species such as *C. monspeliensis* to compensate for this. Fibre content is often considered a negative index of nutritional

quality - mainly ADF - because most phytophagous insects do not digest a significant portion of the fibre they consume (Hochuli 1996). The main food plants for *A. bellamyi* in the forest had twice the fibre (ADF) of the main diet components in the shrubland, so the digestibility of forest plants is probably lower. Therefore, the lower digestibility and the protein and fat scarcity of the principal forest plant species with respect to the shrubland may lead to an increase in grasshopper consumption in order to balance these nutrients; a common mechanism for balancing diets (see Slansky & Scriber 1985). Such compensatory feeding enables grasshoppers to reach or approach nutrient targets when exposed to highly unbalanced natural food (Berner *et al*. 2005).

Presence of toxic substances could be another factor for plant selection by a phytophagous insect. Price (1997) proposed that feeding on potentially toxic plants presents some advantages, such as reduction of competition with other herbivores, and protection against predators. However, the physiological adaptations to exploit this highly specialized diet are costly and require metabolic shifts (Karban and Agrawal 2002). In the shrubland *A. bellamyi,* like the other Canary Island species of Pamphagidae, has a diet based on *Euphorbia*, which contains toxic substances in its latex (Marco *et al*. 1999). They may use the active compounds of *Euphorbia* as predator repellents, as occurs with *Hyles tithymali* caterpillars in the Canary Islands (Hundsdoerfer *et al.* 2005). In the laurel forest there are some highly to moderately toxic plant species (see Delgado 1998). At least one such species, *Laurus azorica* (which is consumed by *A. bellamyi*), has been demonstrated to produce antifeedants that have proven lethal for some insect species (González-Coloma *et al.* 1994). The invasion of the laurel forest by *A. bellamyi* has enlarged its plant food spectrum with respect to

the other Canarian pamphagids, including some new toxic species not present in their ancestral habitat.

CONCLUSION

Acrostira bellamyi appears to have been able to colonize laurel forest from shrubland due to a dietary shift, as the two habitats differ considerably in ecological terms. The forest population displayed several morphological and ecological differences that could be interpreted as incipient adaptational responses to this new habitat. Alternatively, the morphological changes could have been due to environmental effects on development and growth, i.e. the product of phenotypic plasticity. The two populations are also differentiated in terms of neutral genetic markers, with the forest population exhibiting a genetic signature of colonization of this habitat from the shrubland. Our results suggest that adaptation to a new environment and food plants may represent the incipient stages of speciation. Divergent natural selection produced by adaptation to different environments can produce reproductive isolation (e.g. ecological speciation), as has been shown for phytophagous insects adapted to different host species (reviewed in Matsubayashi *et al.* 2010). It is unknown whether there is reproductive isolation between *A. bellamyi* populations from the two different habitats, although cases in which speciation is clearly incomplete are not uncommon, as observed in some situations previously described (Nosil 2009 and references therein).

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Capítulo 5

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A TAXONOMIC REVISION AND SPECIES DELIMITATION OF THE GENUS *PURPURARIA* ENDERLEIN, 1929 (ORTHOPTERA: PAMPHAGIDAE) USING AN INTEGRATIVE APPROACH

ABSTRACT

Recent studies on the endemic Canarian genus *Purpuraria* have shown that the taxonomy of its only recognized species (*P. erna*) is probably erroneous. In the present study an integrative revision of the genus is performed, based on a large number of specimens and geographical sampling. As a result, i) the diagnostic characters at the genus level are re-described, ii) *P. magna* n. sp. based on morphological, morphometric and genetic data is described, and iii) the taxonomic status of a formerly described subspecies is clarified. Intraspecific and interspecific morphometric differences have been found, indicating that the genus is undergoing a process of morphological diversification. Nevertheless, the possibility of interspecific mating between individuals of the two species is suggested, since no significant differences have been found between their respective calling songs. Genetic analyses using mitochondrial and nuclear DNA sequences suggest that *P. erna* and *P. magna* are recent species with evidences of secondary contact episodes in the past.

INTRODUCTION

The Canary archipelago originated from an atypical volcanic hot-spot (Geldmacher *et al.* 2001, 2005) and its islands have been isolated throughout their existence, except the easternmost Lanzarote, Fuerteventura and their outlying islets, which formed a single landmass during Pleistocene sea-regression episodes (Carracedo *et al.* 2003). These sea-level minima would have caused the emergence of submarine banks,

subsequently reducing the distance among islands and between those and the continent, thus facilitating dispersal processes (Fernández-Palacios 2011). Isolation between the other islands has promoted the formation of species, which are often endemic to a single island. Thus, the always isolated islands show the highest proportion of within-island endemics, while Lanzarote and Fuerteventura display the lowest single-island endemicity and the largest number of shared endemics due to their proximity and past land connections (see Arechavaleta *et al.* 2010). In addition, the eastern Canary islands and islets, in spite of their older age and proximity to the continent, show a depauperate biodiversity when compared to the others. This has been explained by their senescent geological stage and consequent long periods of erosion, which have led to an ecological uniformity (Fernández-Palacios 2011). However, Macías-Hernández *et al.* (2008, 2010) have demonstrated for *Dysdera* spiders that recent processes of speciation are still operating on Lanzarote and Fuerteventura.

In spite of the closeness of the Canary Islands to the mainland, its orthopteran fauna is relatively poor, including 95 species recorded so far (Arechavaleta *et al.* 2010). As much as 36 species are endemic, 23 of which being flightless as it is common on oceanic islands. New discoveries in these islands are not rare (4 new species in the last decade), but in a number far lower than in other groups like beetles or spiders. The grasshopper family Pamphagidae in the Canary Islands is composed of two endemic related genera: *Acrostira* Enderlein, 1929 with four monoinsular endemics of the central and western islands, and *Purpuraria* Enderlein, 1929 with the single species *P. erna* Enderlein, 1929, occurring on Lanzarote, Montaña Clara, Lobos and Fuerteventura. Later on, Bland (2001) described the subspecies *P. erna lanzarotensis*

based on a single female from Lanzarote, comparing it with a few female specimens of the nominal subspecies *P. erna erna* from Fuerteventura.

Based on molecular analysis, López *et al.* (2007a) suggested the existence of one *Purpuraria* species previously unrecognized under the phylogenetic species concept. However, the morphological characters then observed were not enough to support the description of a new species. Moreover, the genetic validation of the subspecies *P. e. lanzarotensis* from Lanzarote could not be verified, given that no individuals of this population were found, and its taxonomic status was also questioned because populations belonging to *P. e. erna* were discovered on this island too. The genetic, morphological and chorological results of López *et al.* (2007a) showed that the taxonomy of *Purpuraria* is probably incomplete and imprecise.

Description of species presenting taxonomic uncertainties should be based on multiple lines of evidence such as morphology, molecular, ecological, behavioural and geographical information (Stockman & Bond 2007, Bond & Stockman 2008). In this respect, it has been shown that recognition of song elements can be also a valuable tool for species discrimination both in invertebrates (see Ragge 1986, 1990, García *et al.* 1996b, 2005, Hernández *et al.* 1997, Clemente *et al.* 1999, Shaw 2000,Heller *et al.* 2006) and vertebrates (see Vaurie 1959, Payne 1986, McCracken & Sheldon 1997, Raposo *et al.* 1998, Tubaro 1999). In Pamphagidae grasshoppers songs are not very conspicuous, but it can be a useful character for taxonomic discrimination (Llorente *et al.* 1995, García *et al.* 1996a, Presa *et al.* 2000, López *et al.* 2008a). Sound production announcing receptivity to males with particular bioacoustical elements has been described in females of *Acrostira* and *Purpuraria* (López *et al.* 2008b).

In the present study we integrate morphological, morphometric, bioacoustic and genetic data in order to revise the taxonomy of *Purpuraria,* describing a new species and clarifying the taxonomic status of the formerly described subspecies. Additional morphological characters are also used to perform a re-description of the genus *Purpuraria* and its type species. Furthermore, we have expanded the geographical sampling of the two *Purpuraria* morphospecies and the number of individuals analysed to define better their distribution and phylogeography.

MATERIAL AND METHODS

DEPOSITORIES

MCNT: Museum of Natural Sciences of Tenerife (Canary Islands, Spain). MNCN: National Museum of Natural Sciences (Madrid, Spain). UMCZ: Zoology Collection, Faculty of Biology, University of Murcia (Spain). ZMA: Zoologisch Museum Amsterdam (The Netherlands). TAUI: National Collection of Insects, Department of Zoology, Tel Aviv University (Israel). CMUP: Bruno Massa Collection, University of Palermo (Italy). CHL: personal collection of H. López. CPO: personal collection of P. Oromí. All other material is in the collection of the Department of Animal Biology (DZUL), University of La Laguna (Canary Islands, Spain).

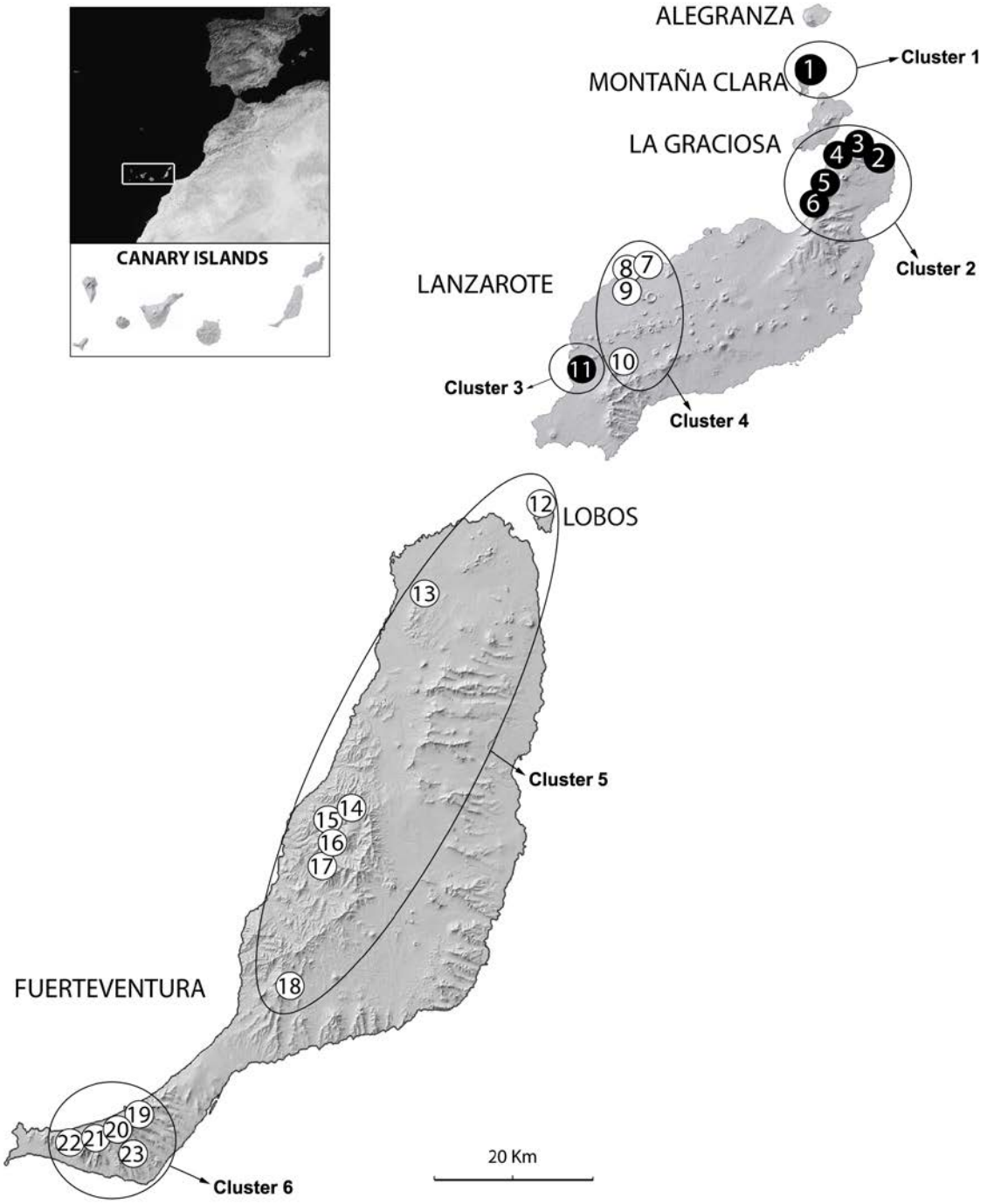

Figure 5.1. *Purpuraria* sampling sites in the eastern Canary Islands. Locality numbers as indicated in Table 5.1. Clusters referring to groups of *Purpuraria* populations for the morphometric and bioacoustic comparisons. Black circles: *Purpuraria magna* populations. White circles: *Purpuraria erna* populations.

Table 5.1. List of specimens of *Purpuraria* examined in the morphological, bioacoustic and genetic studies, and their geographical localities. Mitochondrial and nuclear haplotypes are indicated in the genetic study section. L.C.: Locality code, as in the map (Fig. 5.1). m: males and f: females.

			Bioacoustic study	Morphological study	Genetic study			
Taxa					**MtDNA**		**ITS2**	
Island	**Locality**	**L.C.**	**Individuals**	**Individuals**	**Haplotypes**	**Individuals**	**Haplotypes**	**Individuals**
Purpuraria magna								
Montaña Clara	La Caldera	1	11 m, 7 f	10 m, 12 f	ma4m, ma5m	A13, A127, A14, A73	ma1n	A13, A14
Lanzarote	Vega Grande	2	-	4 m, 2 f	ma6m, ma7m, ma10m	A63, A74, A17	ma1n	A124, A63, A74, A17
	Mirador de Las Rositas	3	8 m, 8 f	8 m, 8 f	ma1m	A117, A19, A18	ma1n	A19
	Bajo El Risco	4	4 m, 3 f	7 m, 3 f	ma1m	A120, A16, A121, A15	ma1n	A15, A16
	Bco. Elvira Sánchez	5	-	1 m, 1 f	ma2m	A27, A203, A218	ma1n	A203, A218
	Valle de Temisa	6	-	1 f	ma2m	A280	ma1n	A280
	El Mojón	11	3 m, 5 f	3 m, 8 f	er/ma1m[b], ma3m, ma8m, ma9m	A118, A23, A28, A22, A119	ma1n	A118, A119, A23, A417, A419, A28
Purpuraria erna								
Lanzarote	Islote Tabaibas	7	10 m, 17 f	10 m, 22 f	er1m, er6m	A139, A21, A138, A84, A123, A20, A26	er1n	A139, A20, A26, A21, A123
	Islote Camellos	8	-	8 m, 4 f	-	-	-	-
	Islote Betancores	9	-	2 f	er1m	A209	-	-

	Yaiza[a]	10	8 m, 7 f	8 m, 8 f	er15m	A282, A273	er1n, er2n	A283, A281, A273, A282
Isla de Lobos	Llanos	12	2 m, 3 f	3 m, 13 f	er2m	A9, A10, A136, A11, A137	er2n	A10, A9, A137, A11
Fuerteventura	Rosa Negrines	13	9 m, 6 f	12 m, 16 f	er3m, er8m	A85, A8	er2n	A130, A250, A252, A131, A85, A8
	Morro Velosa	14	-	1 m, 1 f	er4m, er9m	A77, A72	er1n	A77, A72
	Betancuria	15	-	1 f	-	-	-	-
	Mña. de La Cruz	16	-	2 f	er4m	A5	er1n	A5
	Degollada Honda	17	3 m, 8 f	12 m, 22 f	er3m	A6	er1n, er2n	A6, A132
	Mña. Cardones	18	-	2 f	er12m, er13m, er14m	A277, A286, A279, A278	er2n	A277, A278, A285, A286, A279
	Pico de La Zarza	19	-	3 f	er5m, er/ma1m[b]	A275, A274, A276	er3n	A274, A275
	Bco. de Vinamar	20	4 m, 3 f	5 m, 8 f	er5m	A135, A134, A2, A1, A133	er4n	A1, A2, A133, A299
	Bco. del Ciervo	21	13 m, 9 f	24 m, 20 f	er5m, er/ma1m[b], er11m, er16m	A4, A3, A262, A82	er2n, er3n, er4n	A3, A4, A82, A129, A255
	Bco. de Las Damas	22	-	1 m	-	-	-	-
	Jandía	23	-	1 f	-	-	-	-

[a] The holotype of *P. erna lanzarotensis* was included in this locality, according to its collecting data.

[b] Haplotype of specimens with discordance between mtDNA and nuclear DNA markers.

SAMPLING

Sampling was carried out from 2002 to 2009 in the eastern Canary islands and islets (Fuerteventura, Lobos, Lanzarote, La Graciosa, Montaña Clara and Alegranza). All the previously known localities for *Purpuraria* and many others were visited, to confirm and expand the knowledge on distribution and habitat use (Fig. 5.1, Table 5.1).

MORPHOLOGICAL STUDIES

A total of 124 females and 84 males of *Purpuraria erna* and 35 females and 34 males of the new species were used for the morphological analysis, including the holotype of *P. erna lanzarotensis,* lent by the Zoologisch Museum Amsterdam (The Netherlands). A redescription of the genus *Purpuraria* was necessary to fit the variation found in the new species, and to include diagnostic characters not considered previously, such as male genitalia and body colour of living specimens. Terminology follows Dirsh (1965) and Llorente & Presa (1997).

For the morphometric study, 26 males and 24 females of *P. erna*, and 31 males and 24 females of *Purpuraria magna* n. sp. were analysed, using individuals from all known localities. Ten linear morphological measurements were recorded for individuals of both sexes plus another three of only females, using a stereoscopic microscope with a micrometer. Body parts that do not change in size in dried specimens were selected for these measurements: dorsal length, maximum width and lateral height of pronotum; lateral height of pronotal crest; eye length; length and maximum height of hind femur; dorsal maximum width between external margin of eyes; 3rd antennomere maximum width; and mesosternal space maximum height. The

length of external and internal lobes of subgenital plate, and the difference of the alignment of these lobes at the base level were also measured in females.

Due to the marked sexual dimorphism of *Purpuraria*, the morphometric differences between the two species were analysed for each sex separately. Besides using *species* as a factor in the statistical analysis, the factor *cluster* was also considered to refer to six groups of populations (Fig. 5.1) in which species were never mixed. These clusters were assembled according to the geographical distribution and phylogenetic relationships (the latter according to López *et al.* 2007a) of the populations within them. Thus, inter- and intraspecific differences in *Purpuraria* could be tested with the morphometric analysis. A "principal component size correction" (see Berner 2011) was used in order to correct the body size effect. For this, the original variables were first divided into two sets (hereinafter SV- for set of variables): one matrix composed of variables related to the head, and a complementary one including the remaining body variables. According to each sex and factor (*species* and *clusters*), variables were mean-centred. Then, for each factor, the data matrices resulting from each sex and SV were joined to extract the pooled PC1 using the software STATISTICA (version 6.1, StatSoft Inc.). In the latter procedure, variables in each SV were reduced to those that explained more than 80% of the size variance. For each sex, the PC1 scores obtained for each SV were used as covariate to correct the body-size effect in the matrices of original Ln-transformed variables of their complementary SV. The morphometric comparisons within each factor, each sex and each SV were made using a distance-based permutational multivariate analysis of covariance (PERMANCOVA) (Anderson 2001, Anderson *et al.* 2008), with Euclidean distances calculated among variables. Pairwise comparisons between clusters were

executed (Anderson 2004), and relative dissimilarities per factor within each sex were visualized using a principal coordinate analysis (PCO). The software Primer v6 and PERMANOVA+ was used in these last analyses.

Because of its alterability, the body length was used only to describe the species and to evaluate their sexual size dimorphism (SSD). For the latter the ratio female/male body length was used as an SSD index (Hochkirch & Gröning 2008).

MOLECULAR ANALYSIS

A total of 48 specimens of *P. erna* and 26 of *Purpuraria magna* n. sp. were used for the molecular analysis, including specimens from all known localities (Fig. 5.1, Table 5.1).

MtDNA and nuclear internal transcribed spacer 2 (*ITS-2*) sequences of *Purpuraria* were obtained from GenBank after a previous study (López *et al.* 2007a). New sequences of the mitochondrial cytochrome oxidase I (*cox1*) gene fragment and the nuclear ribosomal *ITS-2* were amplified and sequenced following López *et al.* (2007a). The sequences from the present study were deposited in EMBL under accession numbers HF678518-HF678524 (*cox1*). DNA sequences were assembled and edited using CodonCode Aligner v. 3.5. (CodonCode Corp., Denham, MA, USA) (www.codoncode.com). Unlike protein coding genes, the ribosomal gene sequences showed length polymorphism and were aligned with the online version of the software MAFFT v. 6 (Katoh *et al.* 2002, 2005), using the 'auto-strategy' option. This procedure outperforms other approaches for ribosomal alignments (Wilm *et al.* 2006).

In order to reconstruct the phylogeographic pattern of both species, the haplotype networks of the *cox1* and *ITS-2* genes were constructed using the statistical parsimony method of Templeton *et al.* (1992). The analysis was performed using the

software TCS v. 1.21 (Clement *et al.* 2000), which can also identify the most ancient haplotypes in the sample using predictions from coalescence theory. Comparisons of uncorrected genetic distances (*P*-values) of *cox1* and *ITS-2* genes between the two species of *Purpuraria* and all species of *Acrostira* were calculated with the software MEGA v.5.0 (Tamura *et al.* 2011), using nucleotide p-distances. Given that López *et al.* (2007a) suggested a possible introgressive hybridization in three individuals of *Purpuraria,* these were removed from the data set for the calculations of the pairwise genetic distances between the two species.

BIOACOUSTIC STUDY

In order to study the sound emissions, 75 males and 76 females (Table 5.1) were individually kept in plastic containers to proceed as in López *et al.* (2008a, 2008b). Males and females were isolated to avoid mating, and to promote the emission of calling songs of females to communicate their availability for reproduction. As in previous studies (López *et al.* 2008a, 2008b), all songs were produced by females, confirming that males do not emit any sound. Recordings were made with an Optimus AVL-600 unidirectional supercardioid microphone and a Marantz PMD671 digital recorder. The software Avisoft® SAS Lab Pro version 5.1 was used to review and analyse all recordings, but finally only 25 females representing all known populations of *Purpuraria* were selected for inter- and intraspecific comparisons.

Ragge and Reynolds (1998) defined “syllable” as the sound produced by a complete movement of the stridulating mechanism. Songs of *Purpuraria* and *Acrostira* are made up of simple and/or double syllables containing one and two hemisyllables respectively (López *et al.* 2008a, 2008b). The hemisyllable *d* is present in both types of

syllables, being produced by a thorax dorsoventral contraction, whereas the hemisyllable *a* is present in the double syllables and is produced by a thorax lateral contraction. The oscillograms of a typical song are made up of the sequence: opening hemisyllable - silence - assemblage of syllables - silence - closing hemisyllable. In *P. erna* there are no significant differences between the coincident parameters of the two types of syllables, so they can be analysed together (López *et al.* 2008b). As hemisyllables *a* are present only in double-syllable songs, the comparisons between the *Purpuraria* species using parameters of hemisyllables may only be performed with the hemisyllables *d*.

Selected songs of each of these 25 females were used for measuring eight variables in the domains of time and frequencies, which provided independent information for statistical analyses. The following parameters were measured in the oscillograms for the domain of time: the effective duration of song or time elapsed only in the assemblage of syllables of one emission, the total number of syllables (double and simple) of the song, the number of syllables per second or effective emission rate, and the interval between consecutive hemisyllables *d*. In the domain of frequencies, the variables measured in the spectrograms of several hemisyllables *d* selected in each song were: frequency peak, lowest frequency, highest frequency, and band-width.

For the inter- and intraspecific bioacoustical comparisons the factors *species* and *clusters* were also considered. A distance-based permutational multivariate analysis of variance (PERMANOVA) was performed with Euclidean distances calculated among standardized and Ln-transformed variables. *Species* was considered as a fixed factor and *cluster* as a nested factor within species, since the two species do not

coexist in any cluster. The software Primer v6 and PERMANOVA+ was used for this analysis. A univariate analysis was performed afterwards to evaluate the differences of each variable between species and clusters, using the software SPSS Statistics vs. 19.

The sounds recorded are kept in the sound library at the Department of Animal Biology (DZUL), University of La Laguna, and some sample songs are available at Orthoptera Species File Online web (http://Orthoptera.SpeciesFile.org).

RESULTS

TAXONOMY

Purpuraria Enderlein, 1929

Purpuraria Enderlein 1929: 95, Uvarov 1943: 21, Johnston 1956: 101, Johnston 1968: 66, Herrera 1982: 64, Otte 1994: 194.

Type species. *Purpuraria erna* Enderlein, 1929.

Redescription

Medium to long-sized insects with a strong sexual dimorphism. Body subcylindrical, with tubercles covering it to different extents, crowning especially the most conspicuous carinae all over the body, being variable in size, number and colour (white, black and with tints of red, orange and ochre), and with separate, fine, white and upright hairs, especially dense in ventral parts. Live specimens present a general pale colouration in which either ochre, brown or grey can prevail; often more striking colours in the hidden part of the occiput, base of mandibles and antennal scape (bluish), and on the hind knees (yellow, orange, red or white). These colours are darker and/or less striking in dead specimens.

Head. Subconical with the vertex markedly protruding but strongly depressed at the level of the medain ocellus; fastigium arrowhead shaped in dorsal view and rather horizontal in lateral view; with two parallel fastigial carinae crowned with tubercles and not extending behind the eyes. Margins of fastigium and fastigial carinae marked and slightly prominent in both sexes (well-marked and prominent in males of *P. erna*). Longitudinal furrow of vertex short and shallow dorsally, but narrow and deep in the forehead. Facial carinae well developed and crowned with abundant tubercles. Antennae long, usually exceeding the posterior margin of the 1st abdominal segment; filiform, with 10 well-articulated segments, but frequently with 11-12 when the 3rd and/or 5th are divided into two unarticulated segments: 1-2 short and subcylindrical, 3-5 with marked lateral flat expansions that confer a triangular section and a general ensiform shape to the antenna; 6 and 7 antennomeres frequently with much lesser lateral expansions, and rest of antennomeres with circular section. Eyes strongly oval and prominent, 1.5 x higher than wide.

Thorax. Pronotum almost as long as wide and high, tectiform with a prominent crest (smaller in males of *P. erna*) extended along the dorsal line, generally including the typical suture and the metazona, and with the highest point in the middle of the pronotum. This crest is highly variable in shape even in the progeny of the same pod, its apex rounded or with either an acute or obtuse angle in lateral view; sides of the crest either convex or straight in dorsal view, with large prominent tubercles mainly on the median carina, variable in number and colour (white, orangish, ochraceous or black). Pronotal suture well defined and extending to almost the basal margin of the

paranotum, dividing the pronotum into a prozona 4x longer than the metazona. In dorsal view, the anterior margin of pronotum with a convex curvature at the midline, and the posterior margin straight or slightly concave. Anterior and posterior margins of the pronotum with 5-6 transverse, narrow dark spots on both sides, crowned with black or brown tubercles; these spots missing or obsolete in males. Mesonotum short and smooth, metanotum larger and with abundant tubercles, both with a median low crest lifted mainly at the posterior margin, being more prominent on the metanotum. Tegminae reduced and wings absent in both sexes: females with tegmina like a vestigial fold which can exceed the anterior margin of the mesonotum, and males squamipterous with narrow, subparallel and delicate tegminae totally or partially covering the tympanic cavity, but rarely exceeding the posterior margin of the 1st abdominal segment. Tegminae of males with a somewhat marked vestigial venation, without a clear colour pattern.

Basisternum and spinasternum of prosternum well defined. Anterior margin and disc of the basisternum lifted, the former like a collar and the latter like a broad and flat prosternal process with some tubercles. Spinasternum not lifted, broad, flat and divided by a narrow longitudinal pit. Sternum elongated, 8-shaped, frequently with not well defined sutures; mesosternal lobes with posterior margin rounded, mesosternal and metasternal interspaces narrow.

Legs with fine, abundant, white upright hairs, especially dense in ventral and internal parts. Hind femur with carinae crowned with tubercles, the dorsal and ventral carinae appearing slightly serrated, 4.7x longer than higher in males and 5.3x in females. Internal and external spines of the hind tibiae more developed and abundant

in the anterior half; males with 9-13 internal and 8-10 external spines, and females with 10-11 and 9-10 respectively. Brunner's organ present, well developed and sharp.

Abdomen. Elongated and subcylindrical; tergites 1-9 with a dorsal median crest more prominent in posterior half, but sometimes missing in tergites 2-4 in males, always more developed in the 1st abdominal tergite, where it is crowned by large tubercles. Posterior margin of tergites 1-8 with a transversal narrow, light coloured band with 5-6 dark spots along both sides, though missing or less notable in males. Tympanic opening rounded in females and wider, elongated and transversal in males. Krauss' organ present but very small and barely distinguishable.

Female subgenital plate pentalobulated with lobes well separated and apex rounded, except the central which is narrow and acuminated (Fig. 5.2B, E); ventral valves of ovipositor with variable shape and soft margins, ventrally slightly concave and with the posterior 1/3-1/2 strongly narrowed (the latter less appreciable in some old females). Male subgenital plate long, narrowing in the posterior half and acuminate at the tip. Cerci large and incurved down-inwards in males and short, conical and straight in females.

Phallic complex with basal and apical valves forming a very obtuse angle (Fig. 5.3A, F); valves of cingulum arrowhead shaped and densely covered by tiny teeth; in the rest of cingulum, sides of the rami and inferior fore border of apodemes with scattered tiny teeth (Fig. 53D, I). Large epiphallus, in lateral view with saddle shape (Fig. 5.3C, H), longer than high, and quadrangular in dorsal view (Fig. 5.3B, G); lophi prominent with large teeth, higher on top and smaller at the sides; sulcus wide with

parallel sides; ancorae incurved downwards (laterally) and with rounded apex (dorsally).

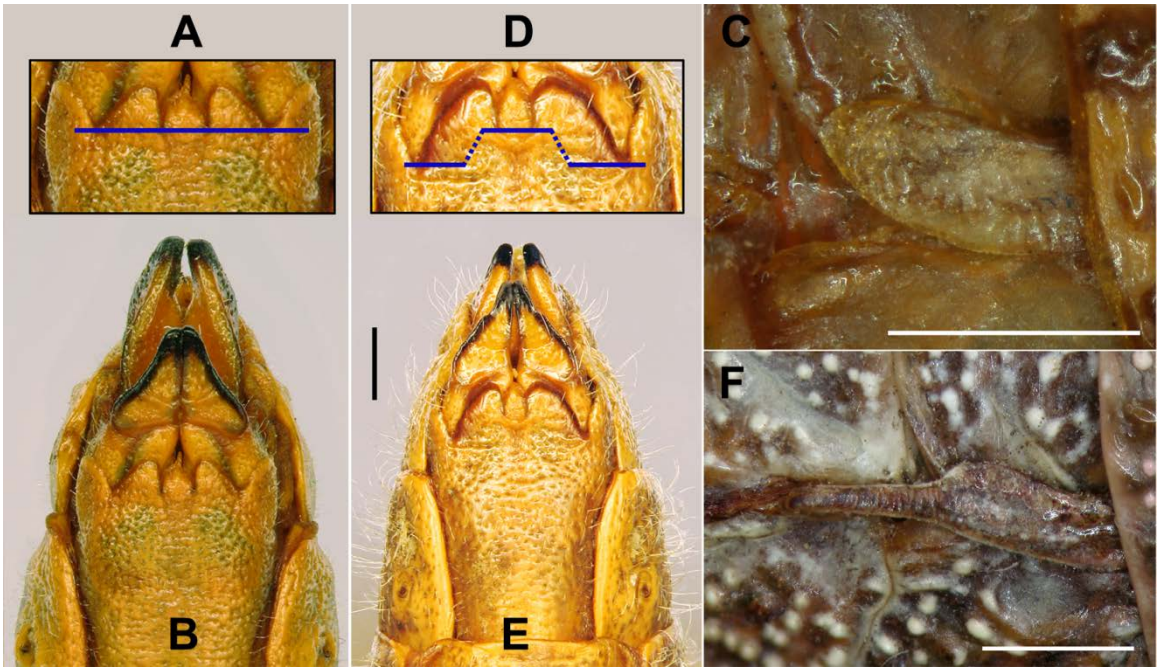

Figure 5.2. *Purpuraria erna*: A-C; *Purpuraria magna*: D-F. A, D: Detail of the female subgenital plate showing the different alignment of their lobes at the base level. B, E: ventral view of the female subgenital plate. C, F: detail of the female tegmina in the two species. Scales 1 mm.

Diagnosis of the genus. The mainland ancestor or sister genus of the Canary pamphagids has not yet been found (López *et al.* 2007a), but *Purpuraria* has the greater morphological similarity with *Acinipe* Rambur, 1938, *Paracinipe* Descamps and Mounassif, 1972 and *Orchamus* Stål, 1876, especially for the subcylindrical shape of the body and the appearance of the head and thorax. However, in *Purpuraria* the hind femora are longer, narrower and slender, and tegminae shorter and narrower. A genetic study has confirmed that *Purpuraria* is the sister group of *Acrostira*, the other Canary pamphagid genus (López *et al.* 2007a).

Purpuraria is smaller than *Acrostira* (*Purpuraria*: ♂♂ 12-27.9 mm, ♀♀ 31.25-53.12 mm; *Acrostira*: ♂♂ 25-33, ♀♀ 53-73), with a more prominent fastigium and

fewer antennomeres (*Purpuraria*: 10-12; *Acrostira*: 13-15), and the males have squamigerous tegminae while *Acrostira* are practically apterous. Laterally, the epiphallus is longer than wide in *Purpuraria* and wider than long in *Acrostira*; the lobes of the penis form an obtuse angle in *Purpuraria* but they are almost straight in *Acrostira*; these lobes are proportionally longer in *Purpuraria* than in *Acrostira*. Ventrally, the valves of the cingulum are arrowhead-shaped in *Purpuraria* and campaniform in *Acrostira*. Two species can be differentiated in *Purpuraria*, being their morphological descriptions detailed next.

***Purpuraria erna* Enderlein, 1929.**

Purpuraria erna Enderlein 1929: 97, Chopard 1954:1, Johnston 1956: 101, Dirsh 1965: 100, Johnston 1968: 66, Johnsen 1974, Herrera 1982: 64, Báez 1984: 40–41; García & Oromí 1992: 127–128, Gangwere *et al.* 1998: 1–21, Bland 2001: 113–119, Oromí *et al.* 2001: 92, Pérez *et al.* 2003: 244, López *et al.* 2005: 419, 430, Contreras-Díaz *et al.* 2006: 767–771, López *et al.* 2007a: 587–598, Hochkirch & Gröning 2008: 195, López *et al.* 2008a: 29–42, López *et al.* 2008b: 1–8.

Material examined

Fuerteventura. Jandía: 10/12/1971, 1♀ (E. Barquín leg.). Barranco de Las Damas (28.086494/-14.386014): 01/04/2007, 1♂, leg. E. Morales. Barranco del Ciervo (28.087967/-14.372539): 03/12/2002, 1♂, 1♀, leg. A.J. Pérez; 03/12/2002, 1♀, leg. H. López; 03/12/2002, 1♀, leg. B. Rodríguez; 04/04/2004, 7♂♂, 11♀♀, leg H. López and N. Macías; 21/06/2004 (eclosion after egg-pod P9), 16♂♂, 6♀♀, leg. H. López. Pico de la Zarza (28.098457/-14.351396): 18/01/2007, 3♀♀, leg. GIET. Barranco de Vinamar

(28.096132/-14.357884): 04/04/2004, 5♂♂, 8♀♀, leg. H. López. Montaña Cardones (28.263997/-14.147270): 17/01/2007, 2♀♀, leg. GIET. Degollada Honda (28.380382/-14.095515): 18/04/2003, 1♂, 1♀, leg. H. López; 18/04/2003, 1♀, leg. B. Rodríguez; 14/01/2005, 1♀, leg. A. Machado; 21/06/2004 (eclosion after egg-pod P8), 11♂♂, 10♀♀, leg. H. López. Montaña de la Cruz (28.441618/-14.057354): 01/04/2004, 2♀♀, leg. H. López. Morro Velosa (28.441190/-14.056775): 04/12/2002, 1♀, leg. B. Rodríguez; 04/12/2002, 1♂, leg. H. López. Betancuria: 12/05/1974, 1♀, leg. A. Machado. Rosa de los Negrines (28.643998/-13.944848): 06/12/2002, 2♂♂, 2♀♀, leg. H. López; 31/03/2004, 5♂♂, 11♀♀, leg. H. López and N. Macías; 03/06/2004 (eclosion after egg-pod P6), 5♂♂, 3♀♀, leg. H. López.

Isla de Lobos. Llanos (28.749320/-13.823835): 30/03/2004, 3♂♂, 13♀♀, leg. H. López and N. Macías.

Lanzarote. Yaiza (28.952033/-13.759432): 03-08/03/1977, 1♀ (holotype of *P. erna lanzarotensis*), leg. M. C. and G. Kruseman; 14/01/2007, 1♂♂, 2♀♀, leg. H. López and E. Morales; 11/07/2009, 7♂♂, 5♀♀, leg. H. López and E. Morales. Islote Tabaibas (29.062062/-13.765040): 22/04/2003, 5♀♀, leg. H. López; 28/11/2004, 5♀♀, leg. GIET; 01/07/2005, 10♂♂, 12♀♀, leg. H. López and E. Morales. Islote de Los Camellos (29.056143/-13.762317): 02/07/2005, 8♂♂, 4♀♀, leg. H. López and E. Morales. Islote de Los Betancores (29.053071/-13.767797): 12/07/2009, 2♀♀, leg. H. López and E. Morales.

Redescription

Male: very small size (16.17 ± 1.7 mm), with a light grey-brown ground colour, fairly contrasting when they are on females (Fig. 5.4A). In general, abundant tubercles in the carinae but scarce in the rest of the integument.

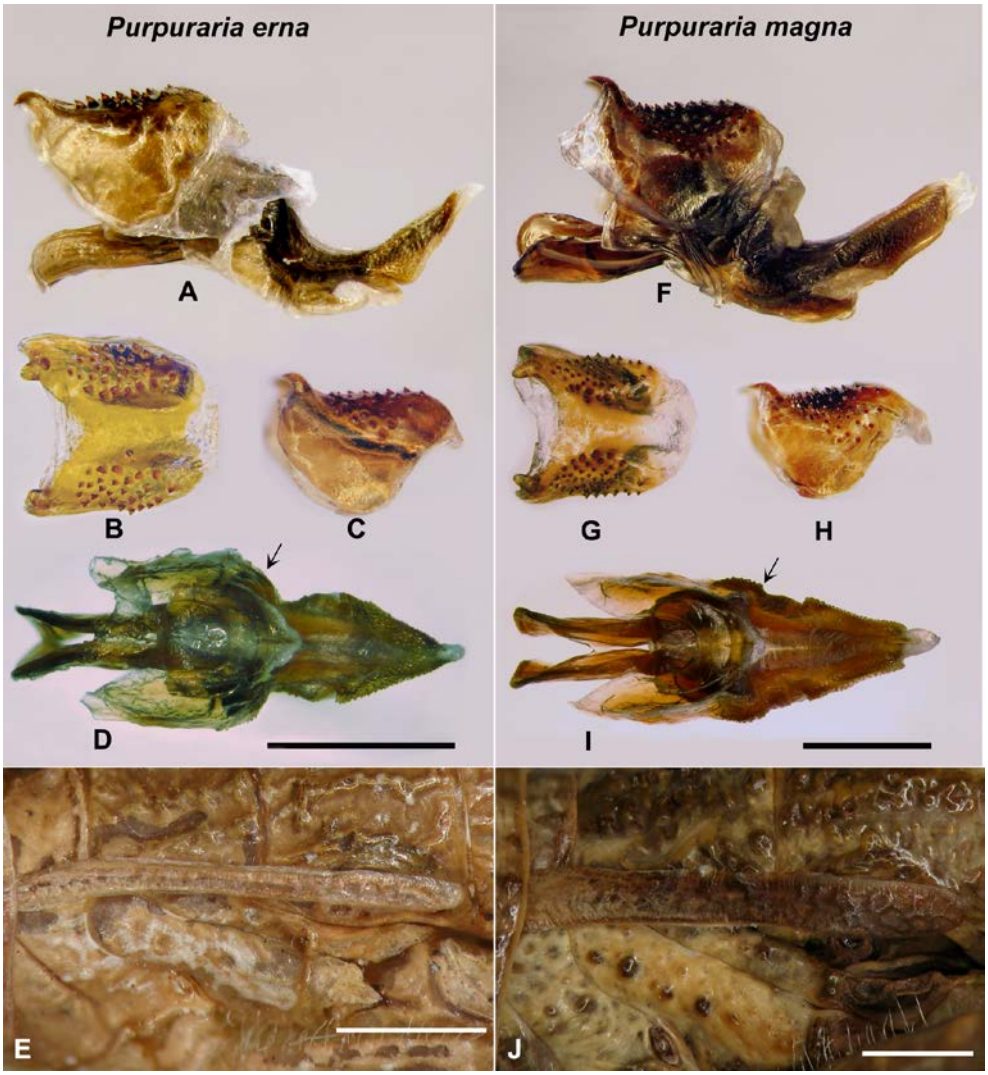

Figure 5.3. *Purpuraria erna*: A-E; *Purpuraria magna*: F-J. A, F: lateral view of the phallic complex. B, G: dorsal view of the ephiphallus. C, H: lateral view of the ephiphallus. D, I: dorsal view of the aedeagus, the arrows indicating the most important difference between the two species. E, J: detail of the male tegmina in the two species. Scales 1 mm.

In the occiput, presence of two short, oblique more or less marked carinae, each from posterior-dorsal margins of eyes and oriented backwards. Dorsally, interocular distance 0.22x (0.18-0.26) the maximum length of the eyes. Eyes with fine

longitudinal stripes more or less visible in the anterior half, resulting from postembrionic moults. Laterally, pronotum 1.09x higher than long (0.93-1.23), and dorsally 0.83x longer than wide (0.77-0.93). Pronotal crest small, short and bounded at the prozona, crowned by large tubercles, frequently dark. In general, crest of the first abdominal segment higher than pronotum. Tegminae very flexible (Fig. 5.3E).

Phallic complex in dorsal view with the lateral fore borders of apodemes very convex and the union of zygoma with the rami constrained (indicated with an arrow in Fig. 5.3D).

Female: Body length 43.38 ± 4.61 mm, with the general shape and colouration of the genus (Fig. 5.4A). Like in males, tubercles mainly on the carinae and scarce in the rest of the integument.

Occiput without the oblique carinae present in males. Dorsally, interocular distance 0.84x the maximum length of eyes (0.75-1). Longitudinal ocular stripes in some specimens replaced or mixed by scattered regular dark maculae. Laterally, pronotum 1.12x higher than long (1-1.26) and dorsally 0.97x longer than wide (0.89-1.06). Tegminae very short, concave-oblong and with a rounded posterior margin, very rarely exceeding the posterior margin of mesonotum, with a short posterior-dorsal prolongation (Fig. 5.2C). All lobes of the subgenital plate start with their bases aligned at the same level in most females (Fig. 5.2A, B) and rarely at two different levels like in *Purpuraria magna* n. sp. (see below).

Bland (2001) described the subspecies *P. erna lanzarotensis* using a single female from Yaiza (Lanzarote) and comparing it with females of *P. erna* from Fuerteventura. He found few differences in the shape of the pronotal crest, the fastigium and the subgenital plate. The holotype of *P. erna lanzarotensis* plus 91 males

(8 from Yaiza) and 123 females (7 from Yaiza) of *Purpuraria* have been analysed, thus we conclude that *P. e. lanzarotensis* is a synonym of *P. e. erna*. Diagnostic characters used by Bland are highly variable in *Purpuraria*, even among specimens of the same locality or pod, and they are not limited to an exclusive form in any population.

Some fixed differences have been observed in the isolated and closely geographically related populations from Islote Tabaibas, Islote Betancores and Islote Camellos (west Lanzarote): in females, the anterior margin of genital valves is not oblique but straight; in the phallic complex, both the sides of rami and the fore border of apodemes have fewer tiny teeth. However, we have not considered these morphological differences sufficient to establish a new taxon at any level.

Biology/ecology: individuals of *Purpuraria erna* were mostly found on bushes of *Euphorbia lamarckii* or *E. obtusifolia*, but in some localities on *Kleinia neriifolia* too. In Yaiza, some specimens were collected on annual dry grass and on nearby *E. lamarckii* and *K. neriifolia* shrubs. Nymphs have been found in different stages of development at the same time of the year in different parts of the islands. Like other Canary pamphagids, the phenology of *Purpuraria* is probably highly affected by rainfall (López *et al.* 2007b), since in Lanzarote and Fuerteventura precipitations are scarce, highly varying along the year and also depending on altitude and orientation of the localities.

Distribution: Fuerteventura, Lanzarote, Isla de Lobos (Canary Islands, Spain).

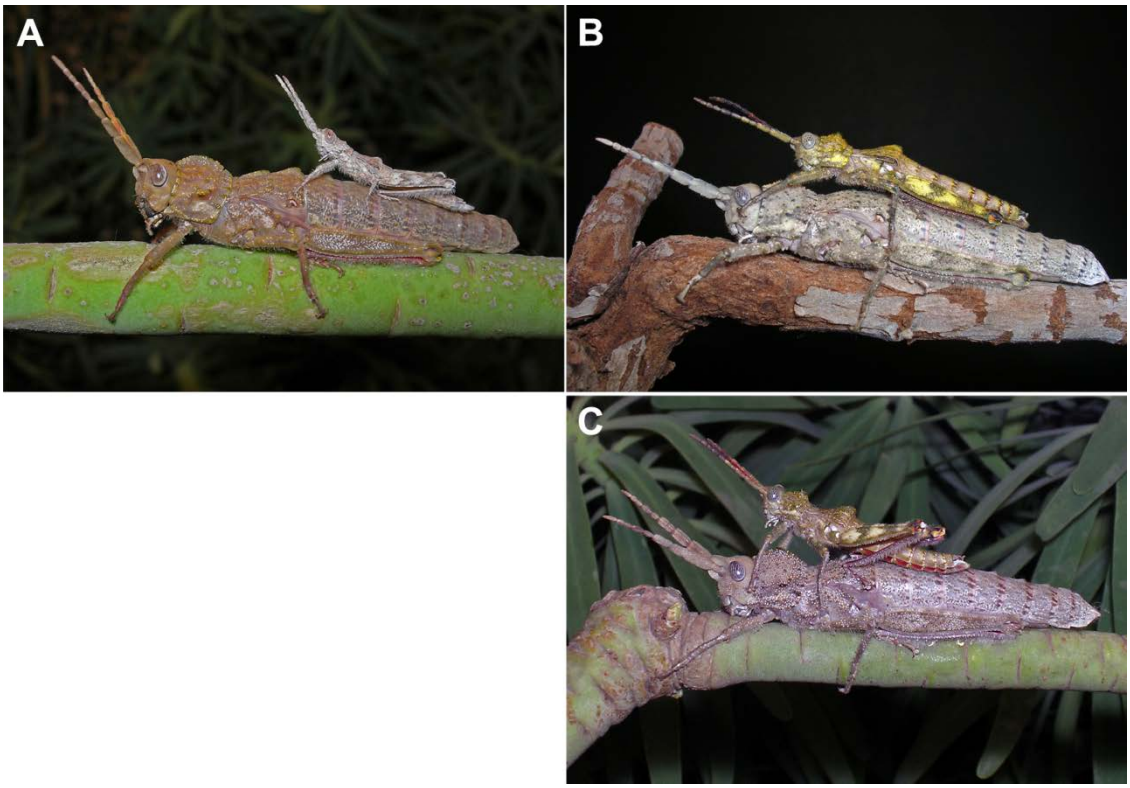

Figure 5.4. Aspect of *Purpuraria erna* (A), and *P. magna* from Lanzarote (B) and Montaña Clara islet (C).

***Purpuraria magna* n. sp. López and Oromí.**

Purpuraria n. sp. Pérez *et al.* 2003: 244, 430, López *et al.* 2007a: 587-598, Hochkirch & Gröning 2008: 195, López *et al.* 2008b: 1-8.

Material examined:

Holotype. Montaña Clara, La Caldera (29.298865/-13.535305): 25/04/2001, ♂ (DZUL 21134), leg. B. Rodríguez (depository DZUL).

Paratypes.

Montaña Clara. La Caldera (29.298865/-13.535305): 12/11/2000, 1♀ (DZUL 20966), leg B. Rodríguez; 24/03/2001, 2♀♀ (DZUL 20972, 20974), leg. B. Rodríguez; 25/01/2002, 1♀ (DZUL 20973), leg. H. López; 15/10/2002, 1♂ (DZUL 21133), leg. B. Rodríguez;

26/11/2002, 1♂ (DZUL 21128), leg. A.J. Pérez; 07/12/2004, 8♂♂ (DZUL 20998, 20999, 21126, 21127, 21129, 21130-21132), 8♀♀ (DZUL 20964, 20965, 20967-20971, 20978), leg. GIET (depositories MCNT 1♂ 2♀♀, TAUI 1♂ 1♀, CHL 1♂ 1♀, CPO 1♂ 1♀).

Lanzarote. Bajo el Risco (29.194412/-13.505188): 27/11/2004, 7♂♂ (DZUL 21142-21148), 3♀♀ (DZUL 20987-20989), leg. GIET (depositories MNCN 1♂ 1♀, MCNT 2♂♂). Barranco Elvira Sánchez (29.130723/-13.516902): 30/04/2003, 1♂ (DZUL 21156); 14/09/2005, 1♀ (DZUL 20801), leg. H. López. Mirador de Las Rositas (29.195648/-13.494680): 15/04/2001, 1♂ (DZUL 21157), 1♀ (DZUL 20997), leg. F. Acosta; 11/06/2003 (eclosion after egg-pod P1), 1♂ (DZUL 21137), 1♀ (DZUL 20994); 11/06/2003 (eclosion after egg-pod P2), 4♂♂ (DZUL 21138-21141), 3♀♀ (DZUL 20991, 20995, 20996); 11/06/2003 (eclosion after egg-pod P3), 1♂ (DZUL 21136), 1♀ (DZUL 20993); 12/06/2003 (eclosion after egg-pod P4), 1♂ (DZUL 21135), leg. H. López; 26/11/2004, 2♀♀ (DZUL 20990, 20992), leg. GIET (depositories MCNT 1♀, CPO 1♂, ZMA 1♂, UMCZ 1♂). Valle de Temisa (29.132990/-13.508904): 13/01/2007, 1♀ (DZUL 20979), leg. H. López. Vega Grande (29.202023/-13.462487): 28/11/2002, 3♂♂ (DZUL 21153-21155), 2♀♀ (DZUL 20985, 20986); 01/12/2004, 1♂ (DZUL 21152), leg. H. López leg. (depositories CHL 1♂ 1♀, CMUP 1♂ 1♀). El Mojón (28.995076/-13.824020): 30/11/2004, 3♂♂ (DZUL 21149-21151), 8♀♀ (DZUL 20975-20977, 20980-20984), leg. GIET (depositories CHL 1♂ 1♀, CPO 1♂ 2♀♀, ZMA 1♀, UMCZ 1♀).

Description

Male: small size (25.2 ± 1.51 mm), with an ochraceous, brown or greyish basal colour, but with geographical variations: specimens from Lanzarote have some striking colours while in Montaña Clara they are lighter (Fig. 5.4B, C). Internal side of antennomeres 1-

5 is red and external side brown; rest of antennomeres brown, becoming lighter towards the apical ones. Eyes greyish with scattered but very conspicuous regular dark maculae. Pronotum with scattered green tubercles in the upper half; crest crowned by large dark tubercles, but not on the anterior margin where they are yellow-greenish; paranotum with a broad yellow-greenish band in males from Lanzarote, and ivory-white in those from Montaña Clara. The first abdominal segment has abundant green tubercles, those on the crest larger and darker. First third of tegminae grey, and dark brown in the rest; tegminae completely grey in males from Montaña Clara. Posterior and ventral margins of abdominal tergites greenish, with some reddish spots (these not observed on Montaña Clara); margins of abdominal sternites reddish. First and second pairs of legs brown, with brown-yellowish spots on the knees. Epimeron, episternum and dorsal side of coxae of hind legs with white or ivory-white areas. Hind femur of Lanzarote males with alternated brown and yellow-greenish strips at both sides, and brown and ivory-white in males from Montaña Clara; in both cases some scattered green tubercles in the external brown strips. External and internal sides of hind femur knees with a variable range of reddish, yellowish, orange, black and brown shades. Hind tibiae reddish. Abundant tubercles on the carinae and rest of integument, usually ochraceous and white. In specimens from Montaña Clara, dense white tubercles covering most dorsal and lateral parts of the body (Fig. 5.4C).

Occiput in both sexes without the oblique carinae present in *P. erna* males. Dorsally, interocular distance 0.39x the maximum length of the eyes (0.32-0.45). Laterally, pronotum 1.16x higher than long (1.03-1.29) and dorsally 0.89x longer than wide (0.84-0.95). Crest of the pronotum very high, crowned by large and prominent tubercles, generally black, green or dark ochre. Apex of crest with a sharp angle,

though in some specimens more obtuse. First abdominal segment with a high crest (as high as or lower than the pronotal crest) crowned by large tubercles similar to those on the pronotal crest but less abundant, with rounded apex and posterior side straight or slightly inclined towards the second abdominal segment. Tegminae long and narrow but apparently not flexible or easy to break (Fig. 5.3J).

Phallic complex with the fore border of apodemes slightly curved or straight, and the union between zygoma and the rami not constrained (indicated with an arrow in Fig. 5.3I).

Female: body length 42.21 ± 2.88 mm, with the general shape and colouration of the genus. Tubercles like in males, showing the same pattern of white tubercles in most specimens from Montaña Clara (Fig. 5.4B, C).

Dorsally, interocular distance 0.80x the maximum length of the eyes (0.72-0.88). Eyes with scattered regular dark maculae, sometimes mixed with fine longitudinal strips in the anterior half. Laterally, pronotum 1.16x higher than long (1.09-1.24) and dorsally 0.91x longer than wide (0.85-0.97). Tegminae very short and always exceeding the posterior margin of the mesonotum with a posterior-ventral prolongation like a flagellum (0.65 mm; 0.23-1.25) (Fig. 5.2F).

Central and internal lobes of the female subgenital plate with their bases unaligned with the external ones, which start at a different level (Fig. 5.2D, E) and only rarely aligned at the same level. Genital valves with edges smooth except in females from Montaña Clara, where the valves have angular margins.

Despite some differences found in Montaña Clara specimens (colour, tubercles, female genital valves), we have considered these morphological features insufficient to establish a new taxon.

A comparative diagnosis of the two species is summarized in Table 5.2.

Table 5.2. Comparative morphological characters in the two species of *Purpuraria*.

	P. erna	***P. magna* n. sp.**
Males		
Body size	16.17 ± 1.7 mm	25.2 ± 1.51 mm
General colour	Light grey-brown	More contrasted and colourful, with some parts ivory-white, reddish, green or yellow-greenish.
Occiput	With two short carinae from posterior margins of eyes obliquely oriented backwards	Without such carinae
Interocular distance / maximum eye length	0.22x (0.18-0.26)	0.39x (0.32-0.45)
Pronotal crest	Small, short and bounded at the prozona	High and extended along the pronotum
Phallic complex	Lateral fore borders of apodemes very convex. Union of zygoma with the rami constrained	Lateral fore border of apodemes slightly curved or straight. Union of zygoma with the rami not constrained
Females		
Tegminae	Oblong, rarely with a very short dorsal prolongation	Subparallel, narrower and always with a distal, ventral flagellum-like prolongation exceeding the posterior margin of mesonotum
Alignment of the bases of the lobes in the subgenital plate	At the same level	At different levels

Biology/ecology: field observations suggest that *P. magna* and *P. erna* have no differences in habitat or food selection. A specific ecological study like that done with

Acrostira euphorbiae (López *et al.* 2007b) should be performed to confirm these observations.

Distribution: Lanzarote and Montaña Clara (Canary Islands, Spain).

MORPHOMETRIC ANALYSIS

Within each sex, statistical analysis showed significant morphometric differences among species (Table 5.3A, Fig. 5.5), and among specimens from the six clusters (Table 5.3B, Fig. 5.5), as well as in variables from head and body. The covariates also showed important differences in all comparisons, indicating that they were good to correct the body size effect. However, in males almost all interactions between covariates and factors had significant differences, indicating that differences in factors depend on the effect of covariates, probably due to the marked morphological disparity between males of the two species. The pairwise comparison showed a generalized morphometric segregation among specimens of the different clusters in both sexes, except between females from clusters 1 and 2, females from clusters 3 and 5, and males from clusters 2 and 3 (Table 5.4).

The PCOs yielded two components that explain more than 78% of the variance in all cases, with the higher variability grouped on the PCO1 axis, especially in males (more than 97% of the variance explained). In each sex, intragroup distances were lower than intergroup distances for all variables, especially in males which were clearly clustered into two separate groups according to species (Fig. 5.5). The PCO analyses supported the results of PERMANCOVA, indicating that *P. erna* and *P. magna* are different morphometric species. PCO showed that females of *P. erna* have a higher

variability than those of *P. magna*, whereas males of both species have a similar intraspecific variation. Some of the morphometric differences found in the pairwise analysis were not so evident in the PCO, probably because the variance explained by axis PCO1 and PCO2 was lower. In any case, the graphs show that some clusters have intragroup distances lower than the intergroup distances, for example females of cluster 6 and males of cluster 4 of *P. erna* (Fig. 5.5).

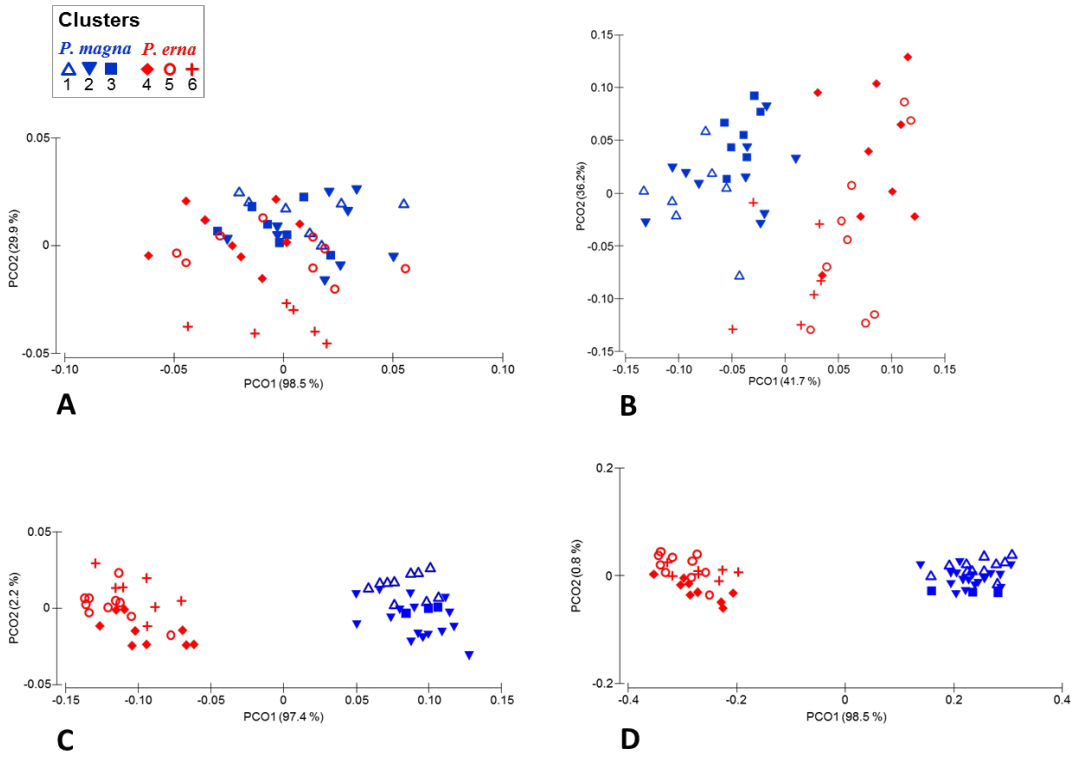

Figure 5.5. Principal coordinate analysis (PCO), based on Euclidean distances calculated among Ln-transformed data, of the relative dissimilarities per factor within each sex, in variables from the head (A, C) and body (B, D). Clusters are the groups of populations of *Purpuraria* formed for the morphometric and bioacoustic comparisons. Figures A and B show the comparison between females, and C and D between males. Red symbols are used for *P. erna* and blue ones for *P. magna*.

Table 5.3. Summary of PERMANCOVA on morphometric differences between sexes of both species of *Purpuraria* (A) and among clusters of populations (B) according to head or body variables, and covariates extracted from each of these two groups (see Material and Methods section). NS: not significant; *p < 0.05; **p < 0.01.

	Female head				Female body				Male head				Male body			
(A)																
Source	d		*F*	P	d		*F*	P	d		*F*	P	d		*F*	p
	1	0.	38.	*	1	0.	27.	*	1	0.	487	*	1	1.	688.	*
	1	0.	4.8	*	1	0.	34.	*	1	0.	372	*	1	2.	119	*
Cov. x	1	0.	0.3	N	1	0.	1.2	N	1	0.	1.9	N	1	0.	7.38	*
	4	0.			4	0.			5	0.			5	0.		
Total	4				4				5				5			
(B)																
	1	0.	66.	*	1	0.	24.	*	1	0.	58.	*	1	0.	55.3	*
Cluster	5	0.	11.	*	5	0.	14.	*	5	0.	424	*	5	0.	611.	*
Cov. x	5	0.	0.6	N	5	0.	0.9	N	5	0.	2.2	*	5	0.	1.89	*
	3	0.			3	0.			4	0.			4	0.		
Total	4				4				5				5			

Table 5.4. Morphometric pairwise comparisons among clusters of populations of *Purpuraria*. NS: not significant; *p < 0.05; **p < 0.01.

	Female head		Female body		Male head		Male body	
Clusters	*t*	P	*t*	P	*t*	P	*t*	P
1 vs. 2	1.44	NS	1.64	NS	3.34	**	2.04	*
1 vs. 3	1.96	*	2.99	**	2.20	**	2.40	**
1 vs. 4	4.06	**	5.67	**	31.60	**	34.31	**
1 vs. 5	2.51	**	4.74	**	37.80	**	37.88	**
1 vs. 6	5.77	**	3.65	**	28.74	**	26.72	**
2 vs. 3	2.31	**	1.75	*	0.83	NS	1.69	*
2 vs. 4	4.35	**	4.63	**	23.86	**	35.45	**
2 vs. 5	2.21	**	4.19	**	28.57	**	39.32	**
2 vs. 6	4.40	**	3.32	**	21.79	**	28.57	**
3 vs. 4	2.29	**	4.18	**	23.07	**	24.07	**
3 vs. 5	1.40	NS	4.61	**	28.10	**	26.73	**
3 vs. 6	5.01	**	4.57	**	20.97	**	16.43	**
4 vs. 5	2.52	**	2.81	**	5.19	**	3.73	**
4 vs. 6	4.80	**	4.11	**	3.72	**	2.05	**
5 vs. 6	3.42	**	2.20	**	3.05	**	2.40	**

A marked sexual size dimorphism (SSD) was found in the two species, being 1.6x higher in *P. erna* (2.61, n=52) than in *P. magna* (1.72, n=55).

MOLECULAR ANALYSIS

A total of 781 bp of the mitochondrial *cox1* and part of the tRNA leu gene were obtained from 37 specimens of *Purpuraria erna* and 23 of *P. magna,* yielding 15 and 10 haplotypes, respectively (Table 5.1). 18 haplotypes coincided with those found by López *et al.* (2007a) and the remaining seven were new. The average intraspecific pairwise difference between the *cox1* sequences of each species was 1 ± 0.2% for *P. erna* and 0.4 ± 0.1% for *P. magna.*

The nuclear *ITS-2* sequence (384 bp) was obtained from 40 individuals of *P. erna* and 18 of *P. magna,* yielding four and one haplotypes, respectively, which corresponded with those found by López *et al.* (2007a). Among the *ITS-2* sequences for each species, the average intraspecific pairwise difference was 0.43 ± 0.1% for *P. erna,* while *P. magna* showed no variation. The uncorrected genetic distance between the two species was 1.9 ± 0.4% for *cox1* and 3.6 ± 0.9% for *ITS-2* data.

The statistical parsimony analysis of the *cox1* gene yielded a single network at the 95% confidence limit with the two species separated by 11 mutational steps (see Fig. 5.6). The loop connections found were resolved following Crandall and Templeton's (1993) criteria. Thirteen of the 25 haplotypes were singletons and the rest were shared between specimens from close localities. Haplotypes from the same localities were often clustered together or separated by a few mutations, while many mutational steps separated haplotype clusters from different regions, indicating a

pattern of high genetic differentiation and moderate gene flow (see Fig. 5.6). Within *P. erna,* the groups of haplotypes clustered by geographical areas and separated by many mutational steps were mostly in accordance with the lineages reported in López *et al.* (2007a). Several cases of discordant patterns of mtDNA (er6m, er/ma1m and ma9m, see Fig. 5.6), that is *P. magna* individuals showing typical *Purpuraria erna* mtDNA haplotypes or conversely were found, one of them already detected by López *et al.* (2007a).

The statistical parsimony analysis of the nuclear *ITS-2* gene yielded a network of *P. erna,* separated by eighteen mutational steps from the single haplotype of *P. magna* (both groups beyond the 95% parsimony limit) (see Fig. 5.6). The most frequent haplotype of *P. erna* (er2n) was shared among 21 individuals from southern Fuerteventura to western Lanzarote. The second most frequent haplotype (er1n) was shared by 10 specimens from western Lanzarote and north-central Fuerteventura. The other two haplotypes are exclusive to nine specimens from southern Fuerteventura, showing a genetic differentiation between this area and the rest of Fuerteventura and Lanzarote. As indicated above, two individuals of *P. magna* had a *magna* nuclear background but an *erna* mtDNA, and one individual of *P. erna* had an *erna* nuclear background with a *magna* mtDNA.

All sequenced individuals of *P. magna* shared the same *ITS-2* haplotype, showing 18 fixed differences with respect to *P. erna* (12 corresponded to nucleotide substitutions and 6 to indels). In contrast, only five diagnostic nucleotide differences were observed among mtDNA sequences of the two taxa. The two species can therefore be diagnosed by fixed nucleotide differences in *cox1* as follows: positions 294 (C/T), 384 (A/T), 438 (T/C), 450 (T/C) and 666 (C/T) for *P. magna* / *P. erna*. The

diagnostic characters of *ITS-2* positions were as follows: 114 (C/A), 129 (G/-), 130 (G/-), 131 (C/-), 133 (T/C), 147 (T/G), 180 (T/C), 196 (T/C), 213 (C/T), 223 (T/A), 228 (C/G), 270 (A/-), 271 (C/-), 272 (A/G), 312 (A/G), 331 (A/G, C, T), 336 (A/T) and 338 (-/T, A).

The mitochondrial and nuclear markers showed no differences between close populations of the subspecies *P. erna lanzarotensis* and *P. erna erna*.

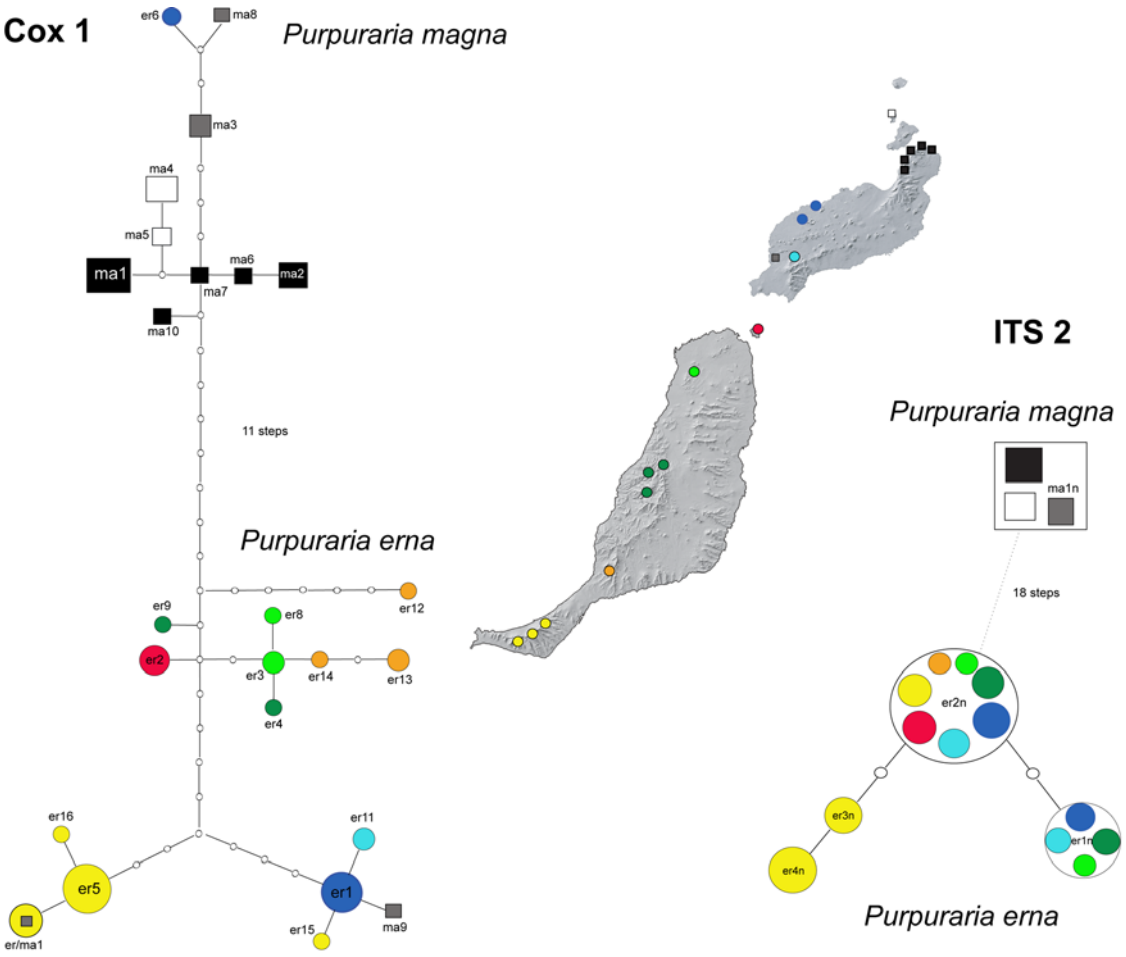

Figure 5.6. Statistical parsimony mtDNA and *ITS-2* haplotype network for *Purpuraria erna* (circles) and *P. magna* (squares). Haplotype codes as in Table 5.1. The size of each circle/square is proportional to the haplotype frequency. Small white circles indicate missing or extinct haplotypes. Colours in the network are according to those of localities.

BIOACOUSTIC COMPARISON

Results of the multivariate analysis (PERMANOVA) showed no significant differences between calling songs of females of both species, nor among females from the different population clusters. The same result was found with the posterior univariate analysis between species and clusters, except in the variable higher frequency of the domains of frequencies, which had significant differences between species (Mann-Whitney Test: U= 36; p = 0.04), the values in *P. erna* being higher than in *P. magna*.

DISCUSSION

The prospection of additional localities, examination of a larger number of individuals and the use of more taxonomic characters than in previous studies clearly shows that *P. magna* must be considered a new species, as it shows diagnostic morphological, morphometric and genetic differences to *P. erna*. The existence of two different species of pamphagids in Lanzarote was an unexpected result given that on the archipelago there is just one species per island, even on ecologically much richer islands. Maybe a similar situation to Lanzarote could be in Tenerife if a new species is finally discovered in the Anaga region, a part of the island with abundant local endemisms vicariant to those found in the geographically opposed Teno (Juan *et al.* 2000, Hochkirch & Gröning 2008), where *Acrostira tenerifae* is restricted. Furthermore, in order to increase the list of pamphagids from the Canary Islands, another possible species to be discovered would be on El Hierro island, from where no pamphagids are known so far in spite of repeated searching.

The former subspecies *P. e. lanzarotensis* is not a valid taxon, since its proposed morphological diagnostic characters have a range of variability displayed by all populations of *Purpuraria*. Moreover, the degree of mitochondrial and nuclear disparity between *P. erna lanzarotensis* and the geographically closest population of *P. erna erna* is the same as or even lower than that found among other populations of *P. erna erna*. These results highlight the drawbacks of making descriptions of new taxa based on a limited number of specimens, in which the differences found can be due to polymorphic variations of characters presumed to be diagnostic.

Despite females of both species being morphologically very similar, they showed significant morphometric differences, whereas in males such differences are in accordance with their noticeable dissimilarities. Within each sex, intraspecific morphometric differences have also been found among populations, suggesting a remarkable plasticity in the two species. Such differences among species are normally related to their different behavioural capacities and/or adaptations to the habitat (see Moran 1986, Richman & Price 1992, Larson & Losos 1996, Kohlsdorf *et al.* 2008). Although no studies on habitat use have been done in *Purpuraria*, apparently they do not perform a discriminant habitat selection. Normally, individuals select the predominant bush species of the habitat for feeding, this behaviour being common to both species throughout their distribution. Furthermore, no particular behavioural capacities have been detected in any species, but they could exist and be related with sensorial, mobile and/or reproductive functions, since morphometric differences have been detected for both head and body variables.

Although SSD values in *Purpuraria* are in agreement with the trend of other Pamphagidae, the female-biased SSD of *P. erna* is the highest so far known in

Orthoptera (see Hochkirch & Gröning 2008). The male-female SSD in *Purpuraria* may be related with the different number of nymphal stages between sexes, as we have observed in both species (unpublished data) and is also the case in other orthopterans (Berner & Blackenhorn 2006, Esperk *et al.* 2007, Hochkirch & Gröning 2008). In addition, *Purpuraria* use the reproductive strategy of protandry, in which males reach the adult stage and sexual maturity before females (Nylin *et al.* 1993, Candolin & Voigt 2003, Møller 2004), probably to gain early access to virgin females (Andersson 1994, Morbey & Ydenberg 2001). In protandry, females reach the adult stage when environmental conditions are optimal (Bidau & Marti 2008). Thus, in poor habitats where optimal conditions are concentrated into a short time period, males should be much smaller than females (Bidau & Marti 2005). Fuerteventura and Lanzarote are dry islands where high foliage density lasts for a short time, so the extreme SSD in *Purpuraria* is probably determined not only by the different number of nymphal stages but also by environmental conditions.

Many wing-reduced grasshoppers have developed alternative mechanisms to the femoro-elytral method for communication. In *Acrostira* and *Purpuraria*, the songs are produced by rubbing together the ventral edge of the metanotum and the reduced basalar sclerite, the only remaining piece of the wings (López *et al.* 2008b). This simple method produces isolated straightforward songs without modulation or temporal cadence, and without relation to environmental temperature (López *et al.* 2008a). These characteristics are probably hindering bioacoustic modifications among species and populations of *Purpuraria*. The bioacoustic isolation among populations of Orthoptera can cause specific sexual selection during the courtship, reducing gene flow and leading to speciation (Grace & Shaw 2012). However, the mechanisms that have

promoted speciation in *Purpuraria* may be of another nature, since differences in calling songs between species or populations are minimal and related to an almost irrelevant sound parameter.

The molecular analyses suggest that speciation within *Purpuraria* is a recent event. The limited bioacoustic differences found between the two species support this hypothesis of recent speciation and, possibly, an incomplete prezygotic isolation between them. A heterospecific attraction during courtships has been previously described in related species of orthopterans with limited variation in the calling songs (Doherty & Howard 1996, Gregory *et al.* 1998, Gray 2005, Vedenina *et al.* 2009).

The mitochondrial and nuclear gene differentiation also supports the species status of *P. magna*. However, contrary to general expectations in recent speciation processes, we have found a higher differentiation between *P. magna* and *P. erna* in nuclear than mitochondrial genes. This pattern was explained in López *et al.* (2007a) by the concerted evolution mechanisms of the rDNA multicopy gene family, to which *ITS-2* belongs. Basically, the time taken to reach complete lineage sorting within rDNA multicopy gene families may be shorter than in single-copy nuclear genes (Parkin & Butlin 2004).

In addition, several cases of discordance between mtDNA and nuclear DNA markers have been found between the two species of *Purpuraria.* Mito-nuclear discordance may be caused by several processes such as differential sorting of ancestral mtDNA polymorphisms, differential selection on mitochondrial and nuclear markers or sex-biased dispersal (see Toews & Brelsford 2012 for a review). Patterns of either mitochondrial introgression or incomplete lineage sorting have been documented in recently diverged species (or populations) sporadically co-occurring in

contact zones (e.g. Sota & Vogler 2001, Masta *et al.* 2002, Ribera *et al.* 2003, Weisrock *et al.* 2005). In *Purpuraria,* the geographic inconsistencies between mtDNA and nuclear markers suggest current or past secondary contact in at least one geographic region of Lanzarote.

Furthermore, the nuclear *ITS-2* network does not show geographical differentiation within the species (except in the south of Fuerteventura), unlike the mitochondrial network, in which high genetic differentiation between geographical areas and low gene flow between localities were observed. This pattern may be explained by the sex-biased dispersal abilities of the species (Hamilton 1967, Ortego *et al.* 2011): the results with the mitochondrial marker might be due to the low female dispersal abilities of *Purpuraria*, contrasting with high vagility in males, which tend to move actively to find females.

The phylogeographic pattern suggests that *P. erna* has been exchanging migrants between the two main islands, and *P. magna* between Lanzarote and Montaña Clara. The Pleistocene sea-level regressions may have facilitated inter-island movements. A similar pattern has also been observed in other organisms such as *Dysdera* spiders (Bidegaray-Batista *et al.* 2007, Macías-Hernández *et al.* 2013) and *Hegeter* beetles (Juan *et al.* 1998).

The widespread distribution of *P. erna* haplotypes over the older island Fuerteventura and several localities of Lanzarote, and its higher genetic diversity for both markers, both reflect an older evolutionary history for this species than for *P. magna*. The most recent lineage of *P. magna* appears in the north of Lanzarote and Montaña Clara, although it also has an isolated population in the south of Lanzarote. The recurrent volcanic activity during the last few millenia in central and southern

Lanzarote may have produced local extinctions of *P. erna* and *P. magna*, leaving only some small populations restricted to isolated scrubland spots. The volcanic activity on the eastern islands could have induced cycles of population expansion followed by fragmentation and local extinction by lava flows, as suggested in other local endemic species like the spiders *Dysdera lancerotensis* and *D. alegranzaensis* (Bidegaray-Batista *et al.* 2007, Macías-Hernández *et al.* in 2013) and the lizard *Gallotia atlantica* (Bloor *et al.* 2008).

The Jandía population of *P. erna* (SW Fuerteventura) has clear nuclear and mtDNA differences with respect to the remaining populations of the species. This was already pointed out by Juan *et al.* (1998) for the beetle *Hegeter politus* (presently *H. deyrollei*). Jandía peninsula has the highest mountains on the island (850 m a.s.l.) and is separated from the mainland by a low, sandy isthmus (< 100m) which may have acted as an effective ecological barrier. Besides the above mentioned cases, other animal species endemic to Jandía have their sister species living in other areas of Fuerteventura and even Lanzarote (Groh *et al.* 1993, Emerson *et al.* 2000, Macías-Hernández *et al.* 2008).

CONCLUSION

We have revised the taxonomy of the genus and described a new species of *Purpuraria* occurring in Lanzarote and Montaña Clara, based on morphological, morphometric and genetic diagnostic characters. The present study emphasizes the convenience of integrating multidisciplinary data to delimit and recognize species. This is particularly critical in recent or incipient taxa, whose geological and evolutionary histories have originated complex populations with imprecise limits between species.

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Capítulo 6

CONCLUSIONES GENERALES

Rhopalomesites

1. En la diversificación del género *Rhopalomesites* están implicados tanto factores ecológicos como geográficos. Las especies están agrupadas en función de factores ecológicos, fundamentalmente según su planta hospedadora. Sin embargo, en especies que cuentan con más de una planta hospedadora los factores geográficos han tenido un mayor peso en la diversificación de las mismas.

2. Se han encontrado evidencias de dos linajes monofiléticos en el género *Rhopalomesites*: uno de ellos especialista, estrictamente asociado a *Euphorbia*, con sendas especies vicariantes en Madeira y en Canarias; y otro generalista, compuesto por especies que se alimentan de gran variedad de árboles y muy raramente de *Euphorbia*. Este linaje polífago incluye especies de laurisilva endémicas de Madeira y de Canarias, además de otra especie presente en Azores y en parte de Europa.

3. La divergencia entre los dos linajes tuvo lugar durante la transición del Mioceno al Plioceno, hace alrededor de 5,3 Ma. Ambos linajes han diversificado independientemente de forma cuasi-paralela durante el Plioceno temprano, con diversificaciones dentro de las especies en el Pleistoceno tardío. Esto sugiere un aislamiento alopátrico en islas con la presencia de un hábitat adecuado, produciendo clados insulares sometidos posteriormente a diferenciaciones intrainsulares.

4. Las filogeografías intrainsulares sugieren que, en la estructuración genética de cada especie, los factores relacionados con la geografía, como la divergencia alopátrica, parecen haber sido más importantes que los cambios de hospedador o que el aumento en el rango de hospedadores.

5. Dentro del grupo vinculado a *Euphorbia* se ha detectado la existencia de una asociación ancestral con *Euphorbia mellifera* en Madeira y Canarias, y cambios posteriores a plantas hospedadoras del mismo género en zonas marginales.

6. Tras este estudio se realizan las correcciones siguientes: *Rh. palmi* se descarta como especie válida, pasando a sinonimia de *Rh. euphorbiae* de Madeira; *Rh. euphorbiae* es exclusivo de Madeira, siendo *Rh. proximus* la especie hermana presente en Canarias; *Rh. tardyii* es probablemente originario de Azores e introducido en Europa.

Acrostira

7. En *Acrostira bellamyi* la adaptación a entornos altamente divergentes, como recursos o hábitats diferentes, estaría produciendo una especiación incipiente.

8. Los análisis genéticos apoyan la hipótesis de que el tabaibal es el hábitat ancestral de *Acrostira bellamyi*. La posterior colonización desde este hábitat a uno tan diferente como la laurisilva se ha traducido en cambios genéticos,

morfológicos y ecológicos, que pueden interpretarse como una adaptación a este nuevo hábitat.

9. Las hembras de laurisilva son más grandes que las que habitan el tabaibal, mostrando además algunas partes del cuerpo diferencias morfométricas significativas entre las dos poblaciones. Éstas quizás pueden deberse a las diferencias de altitud, temperatura y complejidad de la vegetación entre los dos hábitats, así como a las dietas tan diferentes usadas por las poblaciones de *A. bellamyi* en el tabaibal con respecto a las de laurisilva.

10. La dieta en el tabaibal es completamente diferente a la de la laurisilva. Aunque en cada hábitat consumen plantas abundantes, los individuos son capaces de seleccionar las plantas de las que se alimentan, lo que parece explicarse por su contenido de nutrientes.

Purpuraria

11. Datos morfológicos, morfométricos y genéticos confirman la existencia de una nueva especie para el género, la cual se describe y denominamos *Purpuraria magna.*

12. Las dos especies del género *Purpuraria* aparentemente coexisten en el mismo hábitat e isla; sin embargo se desconoce el mecanismo de especiación que las originó.

13. Las hembras de las dos especies son próximas morfológicamente, aunque existen diferencias morfométricas que las diferencian. Los machos de ambas especies presentan diferencias morfológicas y morfométricas. Además, se han encontrado diferencias morfométricas intraespecíficas entre las poblaciones. Estas diferencias inter e intraespecíficas indican que el género está experimentando un proceso de diversificación morfológica.

14. A pesar de la diferenciación morfológica mencionada, los análisis genéticos sugieren que *P. erna* y *P. magna* son especies que han divergido recientemente, con evidencias de contactos secundarios en el pasado.

15. A pesar de las diferencias existentes entre las dos especies, parece que el aislamiento no se ha completado, ya que se han detectado casos de posible introgresión génica, que junto con la no diferenciación del canto entre las especies, sugiere la posibilidad de eventuales apareamientos interespecíficos, posibilitados por determinados eventos geológicos pasados.

16. Casos como los de *Purpuraria* y *Acrostira bellamyi* demuestran la importancia y conveniencia de la integración de datos multidisciplinares en la delimitación y reconocimiento de las especies, particularmente en taxones recientes o incipientes. Este tipo de estudios permite evaluar la divergencia entre poblaciones, y detectar posibles procesos de especiación.

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