UNIVERSIDAD AUTÓNOMA DE MADRID

TESIS DOCTORAL

Evolución en *Anacyclus* L. (Anthemideae, Asteraceae). Análisis de la zona de contacto entre *A. clavatus* (Desf.) Pers. y *A. valentinus* L.

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PhD THESIS

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REAL JARDÍN BOTÁNICO - CSIC

Alicia Agudo García

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La Dra. Inés Álvarez Fernández, Científica Titular del Departamento de Biodiversidad y Conservación, Real Jardín Botánico (RJB), Consejo Superior de Investigaciones Científicas (CSIC); y el Dr. Rubén Torices Blanco, Investigador Contratado del Departamento de Ecología Funcional y Evolutiva de la Estación Experimental de Zonas Áridas - CSIC y del Departamento de Ecología y Evolución de la Universidad de Lausana (Suiza); certifican:

Que la presente memoria titulada "Evolución en *Anacyclus* L. (Anthemideae, Asteraceae). Análisis de la zona de contacto entre *A. clavatus* (Desf.) Pers. y *A. valentinus* L." ha sido realizada bajo su dirección por Alicia Agudo García, que, a su juicio, reúne todas las condiciones para optar al Grado de Doctora.

Y para que conste a efectos legales, firman el siguiente documento en Madrid, a   de 2017.

Fdo. Inés Álvarez Fernández   Fdo. Rubén Torices Blanco

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De acuerdo con la mención de Doctorado Internacional

UNIVERSIDAD AUTÓNOMA DE MADRID   REAL JARDÍN BOTÁNICO - CSIC   CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS   GOBIERNO DE ESPAÑA MINISTERIO DE ECONOMÍA Y COMPETITIVIDAD

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Tabla de contenido

Abstract

Hybridization is one of the most relevant processes in evolutionary biology, and frequently is related to diversification by its effects on adaptation and colonization ability through the acquisition of novel genetic and morphological traits, and influencing reproductive isolation and therefore speciation. However, conceptual, methodological and technical limitations identifying and characterizing hybridization events in nature hamper the current understanding of hybridization. On the one hand, organisms of hybrid origin do not necessarily present intermediate morphologies, physiologies or genotypes with respect to the parental species, which could make their identification evident. On the other hand, it is possible that parental lineages may be confined to remote places or have become extinct and therefore totally unknown. Therefore, sympatric populations of inter-fertile species represent appropriate systems for the study for the study of hybridization process at small-scale.

This thesis presents the results of the four-year study on the contact zone between *Anacyclus clavatus* and *A. valentinus*, two annual plants of the Western Mediterranean. These species have distinct floral morphologies and adjacent distributions with overlapping ranges. In these overlapping areas sympatric populations can be found where there is a high frequency of intermediate floral morphologies, suggesting that they probably are hybrid zones. In addition, *A. valentinus* has been proposed as a hybrid origin species of *A. clavatus* and *A. homogamos*, which are inter-fertile and have contact zones in Morocco. The general objective of this work was to investigate the genetic, morphological and reproductive patterns that have occurred in the different species contact zones to infer the evolutionary processes and in particular to demonstrate if gene flow between these species is happening or has happened before. To this end, it has been collected data on reproductive biology, population structure and genealogical relationships in the species of the system. In particular, I have studied: (i) the germination rate and success, (ii) the pollination interactions, putting special attention to sympatric populations of *A. clavatus* and *A. valentinus*, (iii) the phenotype and fertility of individuals from both natural individuals and experimental hybrids, (iv) the genome size of individuals from both natural populations and experimental hybrids, (v) the diversity and genetic structure based on highly polymorphic loci genotyping (microsatellites) and their correlation to climatic variables, and (vi) the phylogenetic relationships based on sequences of several molecular markers of both chloroplast and nuclear DNA.

The three studied species, *A. clavatus*, *A. valentinus* and *A. homogamos* are heterocarpic and their different fruits follow a positional pattern within their capitula, which is probably related to the sequential seed release system and germination rate of their seeds. Observations of floral visitors showed that *A. clavatus* and *A. valentinus* share pollinators and in sympatric

populations rayed capitula were more attractive to pollinators than rayless ones. In addition, experimental crosses between species showed that all three species were inter-fertile, although they had reproductive barriers that reduce hybrid fitness. The studies on genome size, population genetics and phylogenetic signal were congruent with the existence of current gene flow between *A. clavatus* and *A. valentinus*. The phenotypic characterization of both the individuals obtained by crossing experiments and those from natural sympatric populations indicate that the morphology was unreliable for species identification in those populations distributed within *Anacyclus* contact zones. It was also observed that populations of *A. clavatus* from the SE of the Iberian Peninsula had a genome size lower than those from the inner areas of the Peninsula that also belong to a different genetic group with different climatic niches. This result raises questions about to biological identity of this species. Niche modelling in *A. clavatus*, *A. valentinus* and *A. homogamos* suggested different climatic optima, although there was some degree of overlap in any case. The area of highest overlapping, the region around the Strait of Gibraltar on both continents, was also the area with the greatest genetic diversity. Both the study of the experimental hybrids and their fertility and the species phylogenetic position were congruent with the hypothesis of a possible hybrid origin of *A. valentinus*, although in no case were conclusive. Finally, the inferred phylogenetic relationships supported the hypothesis that hybridization could have been a frequent phenomenon in *Anacyclus*, both present and past, and therefore the evolution of the species of this genus would fit better a model of reticular evolution.

In conclusion, all the genetic, morphological and reproductive results presented in this thesis offer all together a clear evidence to understand to understand evolutionary dynamics at small-scale in contact areas between the studied *Anacyclus* species. In this thesis I present different evidences supporting that homoploid hybridization is an essential process in the evolution of this genus, and highlight the necessity to develop analytical methods based on models of reticular evolution while taking into account different-sources evidences.

Resumen

La hibridación es uno de los procesos más relevantes en biología evolutiva, que en muchos casos está relacionado con la diversificación de los organismos, su capacidad adaptativa y de colonización a través de la adquisición de caracteres genéticos y morfológicos novedosos, el aislamiento reproductivo, e incluso la especiación. Sin embargo, los conocimientos sobre la hibridación son aún parciales, pues su estudio está sujeto a limitaciones conceptuales, metodológicas y técnicas que dificultan la identificación y caracterización de estos fenómenos en la naturaleza. Por un lado, los organismos de origen híbrido no presentan necesariamente morfologías, fisiologías o genotipos intermedios respecto de los parentales que hagan evidente su identificación. Por otro, es posible que los linajes parentales queden relegados a lugares remotos o haberse extinguido y por lo tanto ser totalmente desconocidos. Por lo tanto, los casos donde *a priori* se identifican poblaciones simpátricas de especies inter-fértiles representan sistemas idóneos para el estudio de los procesos de hibridación a pequeña escala.

Esta tesis recoge los resultados de cuatro años de estudio de la zona de contacto entre *Anacyclus clavatus* y *A. valentinus*, dos plantas anuales del Mediterráneo Occidental. Estas especies presentan morfologías florales diferenciadas y distribuciones adyacentes que solapan a lo largo de los límites de sus áreas de distribución. En estas áreas de solapamiento pueden encontrarse poblaciones simpátricas que presentan una elevada frecuencia de morfologías florales intermedias, lo que sugiere que probablemente se trate de zonas híbridas. Además, *A. valentinus* ha sido propuesta como especie de origen híbrido de parentales *A. clavatus* y *A. homogamos*, que son a su vez interfértiles y presentan zonas de contacto en Marruecos. El objetivo general de este trabajo consistió en investigar los patrones genéticos, morfológicos y reproductivos que tienen lugar en las zonas de contacto entre las diferentes especies para inferir los procesos evolutivos y en particular demostrar si existe o ha existido flujo génico entre ellas. Para ello se ha recogido información sobre la biología reproductiva, la estructura poblacional y las relaciones genealógicas en las especies del sistema. En particular se ha estudiado: (i) el éxito y ritmo de la germinación, (ii) las interacciones de polinización con especial atención a las poblaciones simpátricas de *A. clavatus* y *A. valentinus*, (iii) la fertilidad y el fenotipo de individuos de poblaciones naturales y de híbridos experimentales, (iv) el tamaño genómico de individuos de poblaciones naturales y de híbridos experimentales, (v) diversidad y estructura genética basadas en genotipado de loci altamente polimórficos (microsatélites) y su relación con variables climáticas, y (vi) las relaciones filogenéticas basadas en secuencias de varios marcadores moleculares tanto de ADN de cloroplasto como nuclear.

Las tres especies estudiadas, *A. clavatus*, *A. valentinus* y *A. homogamos* son heterocárpicas y sus frutos siguieron un patrón posicional en el capítulo probablemente

relacionado con el sistema de liberación secuencial y ritmo de germinación de sus semillas. Las observaciones de los visitantes florales mostraron que *A. clavatus* y *A. valentinus* comparten polinizadores y que en las poblaciones simpátricas los capítulos radiados resultaron más atractivos para los polinizadores que los discoideos. Además, los experimentos de cruces mostraron que las tres especies fueron inter-fértiles, aunque presentaron barreras reproductivas que producen una reducción de la eficacia biológica de las líneas híbridas. Los estudios sobre el tamaño genómico, la genética de poblaciones y la señal filogenética fueron congruentes con la existencia de flujo génico en la actualidad entre *A. clavatus* y *A. valentinus*. Los resultados de la caracterización fenotípica tanto de los individuos obtenidos mediante experimentos de cruces, como de los de las poblaciones simpátricas naturales, indican que la morfología es poco fiable para la asignación de especies en las poblaciones de las zonas de contacto entre especies de *Anacyclus*. Además se observó que las poblaciones de *A. clavatus* del SE de la Península Ibérica presentan un tamaño genómico menor que las del interior peninsular y que pertenecen a un grupo genético diferente con nichos climáticos diferentes, lo que nos abre una puerta para reconsiderar los límites de identidad biológica en esta especie. La modelización de nicho en *A. clavatus*, *A. valentinus* y *A. homogamos* sugirió óptimos climáticos diferentes, aunque hay cierto grado de solapamiento en cualquier caso. El área de mayor solapamiento, la región entorno al Estrecho de Gibraltar en ambos continentes, fue también la que contiene mayor diversidad genética. Tanto el estudio de los híbridos experimentales y de su fertilidad como la posición filogenética mostraron resultados congruentes con la hipótesis de un posible origen híbrido de *A. valentinus*, aunque en ningún caso fueron concluyentes. Por último, los patrones observados en el estudio filogenético apoyaron la hipótesis de que la hibridación podría haber sido un fenómeno frecuente en *Anacyclus*, tanto a tiempo presente como pasado, y por lo tanto la evolución de las especies de este género se ajustaría mejor a un modelo de evolución reticular.

En conclusión, los resultados de los análisis basados en datos genéticos, morfológicos y reproductivos presentados en esta tesis ofrecen en su conjunto evidencias claras para comprender la dinámica evolutiva a pequeña escala en las zonas de contacto entre las especies que se estudian de *Anacyclus*. En esta tesis se aportan diferentes evidencias coherentes con la idea de que la hibridación homoploide es un proceso esencial en la evolución de este género, subrayando la necesidad de desarrollar métodos de análisis en base a modelos de evolución reticular, considerando al mismo tiempo evidencias de fuentes diversas.

INTRODUCCIÓN

Antecedentes

Durante las últimas décadas, el papel de la hibridación en evolución ha tomado cada vez más relevancia desde ser entendido como un *dead-end* evolutivo o un evento sin interés (Mayr, 1963) hasta ser considerado el motor de procesos tan relevantes como la especiación y la adaptación, tanto en plantas como en animales (e.g. Arnold, 1997; Dowling, 1997; Seehausen, 2004). En la actualidad se desconoce con exactitud la frecuencia de los eventos de hibridación en la naturaleza, aunque los últimos estudios contradicen la idea de que es un fenómeno únicamente extendido en plantas (Stebbins, 1959; Raven, 1976; Whitham *& al.*, 1991) que raramente ocurre en animales (Dobzhansky, 1953; Mayr, 1942). Comparativas entre diferentes regiones señalan que la proporción de especies que hibridan con al menos otra especie varía entre un 1 y un 10% en animales (Schwenk *& al.*, 2008) y entre un ~3 y un 25% (Mayr, 1992; Mallet, 2005; Stace, 1975) en plantas. Según las últimas observaciones florísticas, la hibridación tiene una distribución taxonómicamente desigual (Ellstrand *& al.*, 1996), siendo particularmente habitual en plantas de cultivo (Ellstrand, 2003) y en especies invasoras (Ellstrand & Schierenbeck, 2000). Así mismo, aparece como un proceso ubicuo en la historia evolutiva de las angiospermas (Cui & al., 2006; Soltis & al., 2009; Van der Peer *& al.*, 2009, 2011), en la que varias familias (i.e. Brassicaceae, Poaceae y Solanaceae, Soltis *& al.*, 2009; Asteraceae, Cleomaceae y Fabaceae, Doyle, 2012; Schranz *& al.*, 2012) presentan a su vez un aumento de diversidad asociado a eventos de hibridación seguida de duplicación cromosómica (i.e. poliploidía). Igualmente, existen numerosos ejemplos de hibridación e introgresión relacionados con procesos de radiación y aislamiento (Barton, 2001; Joyce *& al.*, 2011; Mallet, 2005), así como de especiación en presencia de flujo génico (e.g. Feder *& al.*, 2012; Martin *& al.*, 2013; Nosil, 2008). De hecho, los estudios más recientes sugieren que la hibridación interviene, a excepción de los casos que contemplan especiación instantánea o alopatría completa, en casi todos los procesos de especiación propuestos hasta la fecha (Abbott & al., 2013).

Se define como hibridación el proceso reproductivo entre miembros de poblaciones genéticamente diferentes que resulta en la formación de individuos de ascendencia mixta (Barton & Hewitt, 1985). Así, los individuos de origen híbrido pueden derivar de un cruce de primera generación (F_1), de subsiguientes cruces entre híbridos, o de sucesivos retrocruces con alguno de los parentales (hibridación introgresiva). Esta heterogeneidad estructural se traduce igualmente en una gran variabilidad fisiológica y fenotípica, que comprende características propias de alguno de los parentales, así como intermedias, extremas o completamente nuevas (Rieseberg *& al.*, 1993). En cualquier caso, pese a ser una mezcla de materiales genéticos preexistentes, constituye una combinación novedosa, que de resultar ventajosa puede dar lugar a procesos adaptativos o de diferenciación respecto de las poblaciones de partida. En este sentido, los individuos híbridos suelen estar en desventaja, pues las incompatibilidades genéticas y

reproductivas asociadas a procesos meióticos como la recombinación (Gaeta & Pires, 2010) o las reorganizaciones cromosómicas (Navarro & Barton, 2003), adquieren mayor trascendencia cuanto mayores sean las diferencias entre los juegos cromosómicos combinados. Por otra parte, cuanto más divergentes sean los genomas que hibridan mayor será la probabilidad de que aparezcan caracteres novedosos (Stelkens & Seehausen, 2009) o de que se produzcan eventos de duplicación cromosómica (i.e. poliploidía, Ramsey & Schemske 1998). Las novedades morfológicas, genéticas o genómicas pueden en estos casos producir un refuerzo de las barreras pre y postcigóticas respecto de los parentales y facilitar procesos de especiación a través del aislamiento reproductivo (Rieseberg, 1997).

Otra de las características interesantes de la hibridación es la capacidad de crear "puentes" entre especies divergentes (Ellstrand, 2014). Por un lado, el flujo génico entre especies o poblaciones compatibles puede facilitar el intercambio y propagación de alelos ventajosos (Rieseberg & Burke, 2001; Morjan & Rieseberg, 2004). Por otro, la obtención de novedades funcionales permite en ocasiones una mejor adaptación, bien en las zonas híbridas o bien colonizando nuevos nichos o hábitats (Rieseberg & Wendel, 1993; Rieseberg & al., 2003). Así, los ejemplos de hibridación e introgresión son frecuentes tanto en especies invasoras (Currat & al., 2008) como en aquellas que muestran diversificaciones y radiaciones adaptativas rápidas (Price & Bouvier, 2002; Seehausen, 2004; Gourbière & Mallet, 2010; Schwatzer & al., 2012). Además, el flujo génico entre especies divergentes ofrece otras ventajas no meramente adaptativas, especialmente en poblaciones reducidas por efecto de la endogamia. Por ejemplo, las limitaciones (e.g. efectos Allee) a las que tienden algunas poblaciones fundadoras por la escasez de congéneres o polinizadores disponibles, puede contrarrestarse mediante el cruce con otra especie local (Mesgaran & al., 2014, Frankham, 2015). Igualmente, en poblaciones en riesgo de extinción por depresión endogámica, el flujo génico con poblaciones introducidas puede favorecer un aumento de la fecundidad y supervivencia de las primeras a través de lo que se ha llamado "rescate genético" (Frankham, 2015).

Estas razones han llevado a las zonas híbridas a ser propuestas como "laboratorios naturales" (Hewitt, 1988) y "ventanas" (Harrison, 1990) a través de las cuales entender los procesos evolutivos. Las zonas híbridas aparecen en áreas en las que existe flujo génico entre poblaciones genéticamente diferentes, dando lugar a individuos híbridos (Barton & Hewitt, 1985, 1989; Harrison, 1990). El análisis de estas áreas ha permitido desarrollar modelos que definen y explican sus procesos evolutivos (Moore, 1977; Barton & Hewitt, 1985, 1989; Harrison, 1990; Arnold, 1997). Las zonas híbridas ocurren en diferentes contextos espaciales, como, por ejemplo, en un mismo hábitat (sintopía) o zona geográfica (simpatría), así como áreas de distribución adyacentes (parapatría). Igualmente, existen contextos temporales diversos, según se trate de un encuentro secundario tras un periodo de alopatría o mantenido en el tiempo.

Otro contexto relevante es la dinámica poblacional, pues la evolución de la zona híbrida dependerá de si se encuentran poblaciones en contracción o en expansión, o de si se trata de especies locales, foráneas o invasoras (ver Abbott, 2013).

Si las barreras al intercambio genético entre las poblaciones de partida son débiles, puede producirse una reducción o pérdida de la diferenciación entre las mismas, dando lugar a una nueva población homogénea y de ascendencia mixta (Taylor & al., 2006). Por otro lado, si existe permeabilidad genética, pero predomina el éxito de los parentales sobre los individuos híbridos, el resultado sería el intercambio vía introgresión de sólo parte de los genomas de las poblaciones en contacto (Gay *& al*., 2008). Otra situación es que se produzca un fortalecimiento de las barreras al intercambio genético (i.e. refuerzo) y una tendencia a proteger extensas áreas del genoma de la introgresión (e.g. Via, 2009). Tanto la aparición de genotipos y fenotipos novedosos como el fortalecimiento de las barreras reproductivas pueden facilitar procesos de divergencia evolutiva. Así mismo, pequeñas fracciones de material genético introgredido pueden constituir una reserva genética que permita una mejor adaptación o supervivencia si las condiciones climáticas o ecológicas se vuelven adversas (Hewitt, 2011). En cualquier caso, los resultados posibles del contacto entre especies interfertiles constituyen escenarios propicios para que se produzcan eventos de especiación (Abbott *& al.*, 2013).

Por otro lado, las barreras pre- y postcigóticas que definen la viabilidad y la eficacia biológica de los individuos híbridos dependen tanto de factores endógenos como exógenos. Entre los factores endógenos se incluyen las organizaciones cromosómicas incompatibles o las alteraciones en la expresión de genes vitales o que afecten a la morfología de los órganos reproductores. En cambio, las preferencias de polinizadores, predadores o parásitos por individuos híbridos y/o parentales o la capacidad de adaptación a las condiciones ambientales de los híbridos se consideran factores exógenos (e.g., Campbell & Aldriage, 2006).

La simetría floral es uno de los caracteres florales más notables e interesantes desde el punto de vista evolutivo, por las implicaciones que tiene en la atracción de polinizadores (Donoghue *& al*., 1998; Harder & Barrett, 2006). En angiospermas, la transición hacia flores de simetría bilateral (flores zigomorfas) a partir de ancestros de simetría radial (flores actinomorfas; Stebbins, 1994; Donoghue, 1998; Citerne, 2010) ha favorecido la diversificación de estos organismos a través de la especialización de sus polinizadores (Sargent, 2004). En Asteráceas, la diferencia de simetría en las flores de un mismo capítulo es habitual, teniendo su máxima expresión en los capítulos radiados, que presentan flores liguladas marcadamente zigomorfas en el verticilo externo, mientras que las del resto de verticilos son flores en tubo comúnmente actinomorfas. Numerosos estudios sugieren que la evolución del capítulo radiado en Asteráceas está sujeto a un proceso funcionalmente equiparable al que determina la

morfología de las flores individuales (Kim *& al.*, 2008). Así, en esta familia, es la presencia de flores liguladas en el capítulo, así como su longitud, color y disposición, el carácter que mayor impacto tiene sobre la atracción de polinizadores (Leppik, 1977; Lack, 1982; Mani & Saravanan, 1999; Andersson, 2008; entre otros), lo que a su vez influye en las tasas de cruzamiento y de producción de frutos en las plantas que necesitan polinización mediada por insectos (Sun & Ganders, 1990; Marshall & Abbott, 1984).

Sin embargo, en ocasiones es habitual encontrar ambos tipos de capítulo entre especies cercanas e incluso entre poblaciones de la misma especie. Por ejemplo, en *Bidens pilosa* L. (Schultz Bipont, 1844), *Senecio vulgaris* L., y *S. sylvaticus* L. (Syme, 1875; y Záveský, 2004, respectivamente), se ha documentado la existencia de poblaciones con capítulos tanto discoideos, sin flores liguladas, como radiados. De todos ellos, el caso más estudiado desde el punto de vista de su origen (Stace, 1977; Abbott *& al.*, 1992; Lowe & Abbott, 2000), evolución, ecología, biología de la reproducción (Abbott & Schmitt, 1985; Theaker & Briggs, 1992; Abbott *& al*. 1998; Comes, 1998) y genética del desarrollo (Kim *& al.*, 2008) es el caso de *Senecio vulgaris*. En esta especie, la aparición de capítulos radiados en algunas poblaciones tiene su origen en la introgresión de *S. squalidus* (Kim & al., 2008), que es a su vez una especie de origen híbrido cuyos parentales (*S. aethnensis* y *S. chrysanthemifolius*) forman zonas híbridas en equilibrio (Brennan *& al.*, 2009) y que se ha convertido en planta invasora desde su introducción reciente en un nuevo entorno (Abbott *& al*., 2009).

El género *Anacyclus* (Anthemideae, Asteraceae) incluye tres especies con capítulos discoideos (*A. homogamos, A. monanthos* y *A. valentinus*) mientras el resto tiene capítulos radiados. Entre las de capítulos discoideos, *A. valentinus*, es la única que presenta flores femeninas en el verticilo externo, como ocurre habitualmente en las especies radiadas, con la peculiaridad de que se trata de flores sin lígula o con una lígula muy reducida. Esta morfología especial ha llevado a algunos autores a considerar la especie *A. valentinus* de origen híbrido (Humphries, 1979; Funk, 1985). Así mismo, algunas de estas especies (e.g. *A. clavatus* y *A. valentinus*) presentan hábitats y áreas de distribución solapantes, donde aparecen poblaciones simpátricas en las que, además de fenotipos claramente radiados y discoides, se observan otros intermedios tanto en la frecuencia y el tamaño de las flores del verticilo externo (i.e. flores liguladas o radiadas), lo que hace pensar en la existencia de híbridos entre ambas especies. Estos hechos, junto con la viabilidad entre cruces de diferentes especies, sugieren que la hibridación podría ser un fenómeno extendido y habitual en este género (Humphries, 1979, 1981), tanto en el pasado como en la actualidad. Por lo que *Anacyclus* resulta un sistema excepcional para explorar las dinámicas de las zonas híbridas y de los procesos evolutivos que han dado lugar a las especies que existen en la actualidad.

Sistema de estudio

Anacyclus es un pequeño género mediterráneo que comprende alrededor de 12 especies, mayormente hierbas anuales, que crecen en suelos arenosos o pedregosos y presentan una particular predilección por los hábitats ruderales (Humphries, 1979), como descampados y bordes de caminos, carreteras, cultivos, ramblas o lindes costeros (Figura 1).

Figura 1. Ejemplos de diferentes hábitats en los que se pueden encontrar las diferentes especies de *Anacyclus*.

La diversidad floral de este género incluye diferencias en los sistemas sexuales (i.e. ginomonoecia y hermafroditismo), diferentes tipos de capítulo (i.e. radiado y discoideo), y diferencias en el color, forma y longitud de las lígulas de las flores femeninas situadas en la periferia del capítulo. Todos estos caracteres florales (Figura 2) han sido clásicamente utilizados para distinguir las diferentes especies del género (Humphries, 1979).
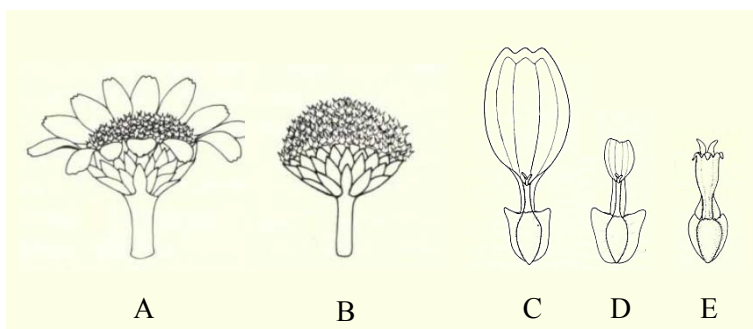
Figura 2. Caracteres florales en *Anacyclus*. A, capítulo radiado. B, capítulo discoide. C, flor femenina con lígula conspicua. D, flor femenina con lígula corta. E, flor tubular. Humphries in Bull. Brit. Mus. (Nat. Hist.), Bot 7(3): 126 fig. 19 (1979).

La mayor parte de las especies de *Anacyclus* son heterocárpicas (i.e. diferentes morfologías del fruto en un mismo individuo). Los aquenios de las posiciones más externas del capítulo presentan alas escariosas y conspicuas (Figura 3B) que se van reduciendo a medida que se avanza hacia el interior del capítulo donde están completamente ausentes (Figura 3C). En general, los aquenios son tardíamente caedizos (Figura 3A), y pueden permanecer fijados al capítulo entre las escamas del receptáculo durante varios meses. Así, es habitual observar en campo especímenes secos con aquenios persistentes junto a plántulas emergidas durante la nueva estación (Figura 3D).

Figura 3. Estrategias de dispersión. A, ejemplo de capítulo seco con aquenios persistentes. B, aquenio alado. C, aquenio no-alado. D, ejemplo de capítulos secos con aquenios persistentes de la estación previa junto con individuos de la nueva generación (en segundo plano en la imagen).
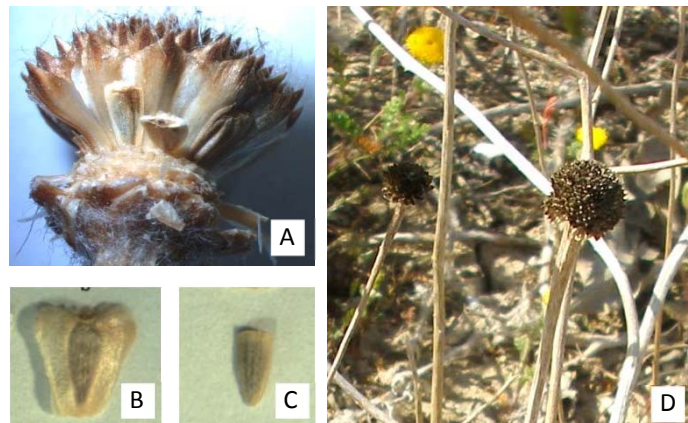
La presencia de heterocarpia ha sido interpretada como una estrategia mixta de dispersión (Imbert, 2002). En el caso de *Anacyclus* los aquenios se desprenden del capítulo empezando desde las posiciones más externas, quedando los del interior retenidos en el capítulo por periodos más largos (Bastida *& al.*, 2010). Estas especies presentan por tanto una forma secuencial de liberación de los frutos, permitiendo que las semillas de un mismo capítulo sean esparcidas y germinen durante varios y diferentes momentos favorables.

El género *Anacyclus* se incluye en la tribu Anthemideae, junto a un clado representado por varios géneros de entre las Anthemidiinae y las Matricariinae (Himmelreich *& al.*, 2008). La filogenia más completa del género hasta la fecha forma parte de un trabajo cuyo objetivo fue situar diferentes géneros monoespecíficos en la tribu Anthemideae (Oberprieler, 2004). Este análisis estuvo basado en un marcador nuclear (nrITS) y un solo individuo por especie, por lo que las relaciones filogenéticas obtenidas entre especies de *Anacyclus* en este trabajo no se pueden considerar concluyentes. Sin embargo, la localización del género *Anacyclus* en la tribu, junto con *Heliocauta*, *Achillea*, *Tanacetum* y *Matricaria* está apoyada por dos estudios independientes, con información de marcadores plastidiales y nucleares (Watson & al., 2000; Oberprieler, 2004, respectivamente).

Análisis previos sobre tamaño genómico en *Anacyclus* indicaron que existe una alta variabilidad entre especies, con valores entre 9.58 y 16.04 pg (Humphries, 1981). Por otro lado, todas las especies analizadas hasta la fecha (*A. clavatus*, *A. homogamos*, *A. valentinus*, *A. radiatus* and *A. pyrethrum*) presentan el mismo número de cromosomas 2n=18 (Schweizer & Ehrendorfer, 1976; Humphries, 1981), habitual en especies diploides especialmente de la tribu Anthemideae (Solbrig, 1977; Schweizer & Ehrendorfer, 1983; Vallés *& al.*, 2005), por lo que las diferencias entre tamaños del genoma no parecen deberse a eventos de duplicación cromosómica. Sin embargo, los individuos y localidades analizados en estos primeros trabajos fueron escasos (un individuo por población, de una a tres localidades por especie) y no representaban ni el área de distribución ni la zona de contacto entre estas especies, por lo que la caracterización de estos organismos en este sentido se considera aún preliminar.

El presente trabajo se centra en las especies *Anacyclus clavatus*, *A. homogamos* and *A. valentinus,* que por varios indicios morfológicos, reproductivos y ecológicos, pueden ser catalogadas como complejo de especies. Morfológicamente, estas especies difieren principalmente en el tipo de flores de la periferia del capítulo (Humphries, 1979; Bello *& al.*, 2013). Los capítulos en *A. clavatus* son radiados, pues presentan entre 8 y 15 flores periféricas femeninas que muestran lígulas blancas evidentes (de en torno a 0.5-1.5 cm de longitud), mientras que en *A. homogamos* son discoideos, debido a que no contienen flores femeninas. La especie *A. valentinus* contiene entre 2 y 10 flores femeninas en el verticilo externo que presentan lígulas cortas (~0.2 cm), a menudo escondidas bajo las brácteas del involucro, por lo que el capítulo presenta una morfología discoidea (Figura 4). Así, las especies *A. homogamos* y *A. valentinus* pueden ser fácilmente confundidas a menos que se haga una observación detallada de las flores periféricas.
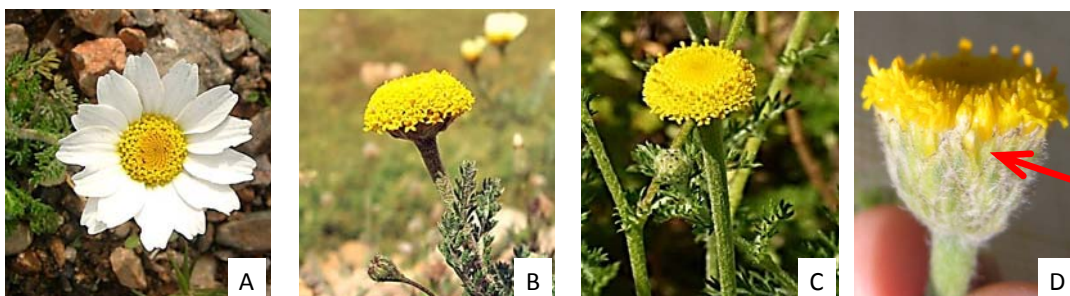

Figura 4. A, *A. clavatus*. B, *A.homogamos*. C y D, *A. valentinus*. La flecha roja señala una flor femenina protegida por una de las brácteas.

Anacyclus clavatus tiene una amplia distribución en toda la Cuenca Mediterránea, mientras que *A. valentinus* se encuentra en zonas costeras de la Península Ibérica, sur de

Francia, norte de Marruecos y Argelia, y *A. homogamos* se encuentra restringido a la región del Atlas Medio en el norte de Marruecos (Figura 5). La presencia de *A. homogamos* en algunos puntos de la costa mediterránea de la Península Ibérica ha sido documentada en base a cinco especímenes de herbario (Humphries, 1979; Álvarez I, Real Jardín Botánico – CSIC, España, 'pers. com.'). Sin embargo, pese a haberse llevado a cabo una exhaustiva búsqueda en estas localidades, esta especie no ha vuelto a encontrarse y por tanto su existencia en la Península Ibérica no ha sido confirmada.
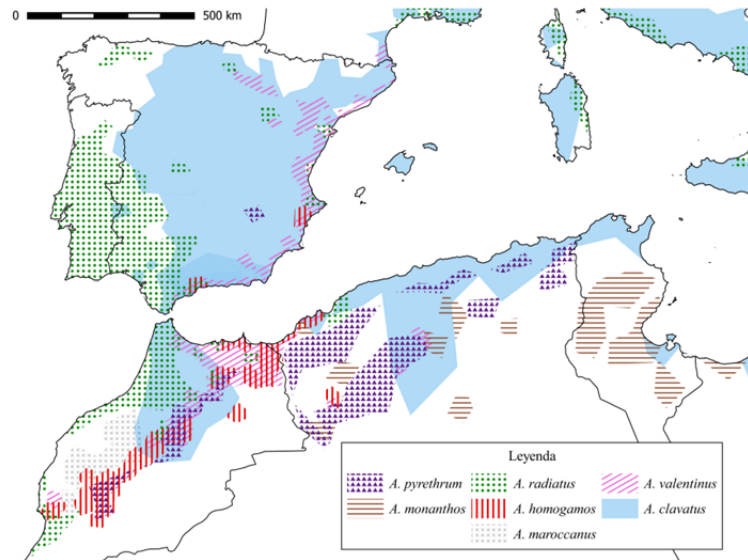
Figura 5. Distribución potencial de las especies de *Anacyclus* que entran en contacto en el Mediterraneo Occidental.

Las especies *A. clavatus* y *A. valentinus* presentan distribuciones solapantes en algunas áreas de la Península Ibérica y Marruecos, donde pueden encontrarse poblaciones en simpatría (i.e., poblaciones en las que al menos dos especies coexisten). Las poblaciones simpátricas habitualmente presentan individuos con caracteres intermedios y un alto grado de variación fenotípica. En estas poblaciones simpátricas, la identificación morfológica de estas especies se convierte en una tarea compleja (Figura 6).

Figura 6. Ejemplo de variación fenotípica en una población simpátrica de *A. clavatus* y *A. valentinus*.

Aunque no existen datos moleculares que apoyen la existencia de flujo génico entre las especies *A. clavatus*, *A. valentinus* y *A. homogamos*, existen varias evidencias que sustentan esta hipótesis. Primero, según los análisis de viabilidad de polen llevados a cabo por Humphries (1981), las tres especies están estrechamente emparentadas y son interfértiles. Los cruces entre *A. clavatus*, *A. homogamos*, y *A. valentinus* presentaron un 100% de éxito, mientras que se observó un significativo descenso (50%) en los experimentos de cualquiera de estas tres especies y *A. radiatus* Loisel., así como un fracaso absoluto en experimentos con la especie perenne *A. pyrethrum* (L.) Link. Segundo, la hipótesis del origen híbrido de *A. valentinus* propuesta por Humphries (1979) en base a los caracteres intermedios observados en esta especie. Finalmente, la observación de fenotipos florales intermedios en poblaciones simpátricas nos hace considerar que *A. clavatus*, *A. valentinus* y *A. homogamos* constituyen un complejo de especies en el que el flujo génico puede ocurrir de forma natural.

Adicionalmente, otras especies incluidas en este estudio son las plantas anuales *A. radiatus* (ambas subspecies *radiatus* y *coronatus*), que aparece en áreas de influencia atlántica de la Península Ibérica y Marruecos, así como en la costa mediterránea de Francia e Italia; *A. maroccanus*, que ocupa una reducida región en la meseta marroquí (Norte Atlas); y *A, monanthos*, que se encuentra en el norte de África, desde el NE de Argelia hasta el NE de Egipto. La única especie perenne del género, *A. pyrethrum*, se encuentra restringida a zonas montañosas del norte de África, aunque también puede encontrarse en la Sierra de Alcaraz en la Península Ibérica.

Objetivos

El principal objetivo de esta tesis es investigar los procesos evolutivos que acontecen en un sistema de especies presumiblemente cercanas en *Anacyclus*, que solapan sus áreas de distribución y que presentan actualmente poblaciones simpátricas. Para entender qué ocurre en las poblaciones simpátricas debemos previamente caracterizar tanto el fenotipo como el genotipo de las especies fuera y dentro de las áreas de contacto y demostrar que efectivamente existe flujo génico entre ellas. Los objetivos concretos son:

1. Caracterizar fenotípicamente las tres especies del foco de estudio: *A. clavatus*, *A. homogamos*, y *A. valentinus* (Capítulos 1.1, 1.3), tanto en campo como en condiciones controladas de cultivo en invernadero.
2. Explorar la existencia de diferencias en la preferencia de polinizadores y depredadores sobre *A. clavatus*, *A. valentinus*, y/o fenotipos florales intermedios entre estas especies tanto en poblaciones simpátricas como alopátricas (Capítulo 1.2).

3. Determinar el sistema reproductivo en *A. clavatus*, *A. homogamos*, y *A. valentinus*; estimar el éxito reproductivo de los cruces entre estas tres especies hasta una segunda generación híbrida; y finalmente caracterizar fenotípicamente dichas generaciones para explorar la herencia de los caracteres florales (Capítulo 1.3)

4. Caracterizar la diversidad y estructura genética en poblaciones de *A. clavatus*, *A. homogamos*, y *A. valentinus* a lo largo de sus rangos de distribución tanto en poblaciones simpátricas como alopátricas, y explorar los patrones en base a modelización de nicho de éstas y otras especies del Mediterráneo Occidental (Capítulo 2.2)

5. Caracterizar el tamaño de genoma en varias especies de *Anacyclus* y explorar su variación a lo largo de poblaciones simpátricas y alopátricas en *A. clavatus*, *A. homogamos*, y *A. valentinus* (Capítulo 2.1)

6. Establecer un marco filogenético de las especies de *Anacyclus* del Mediterráneo Occidental enfocado principalmente a recuperar una señal de la existencia de hibridación en la historia evolutiva de este género, y más en concreto entre el complejo de especies (Capítulo 3.1).

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CHAPTER ONE: REPRODUTIVE BIOLOGY

1.1.   Achene morphology affects time of seedling emergence in three heterocarpic species of *Anacyclus* L. (Anthemideae, Asteraceae).

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ABSTRACT

The production of two or more defined fruit types within an individual, i.e. heterocarpy, is considered to be related with a mixed dispersal strategy in which a proportion of the offspring is allocated to colonize new sites, whereas the rest stays near the maternal location. Here, we aimed to explore the effects of achene morphology (winged vs. unwinged achenes) and achene size on post-dispersal life history traits (probability and time of seedling emergence) in three heterocarpic *Anacyclus* species (Anthemideae, Asteraceae). Morphology, size and germination performance were studied in achenes from six populations in *Anacyclus clavatus* (Desf.) Pers*., A. homogamos* (Maire) Humphries*,* and *A. valentinus* L. Our results show that achene morphology and mass were related to its position within the head, so that outer achenes – winged-- were significantly heavier than inner ones –unwinged--. Additionally, winged achenes germinated faster than unwinged ones. This pattern may be related to the sequential achene release displayed by these species. Finally, our findings cast doubt on the role of wings as structures that favor dispersal by wind in *Anacyclus* achenes.

Keywords: bet-hedging, Compositae, heterocarpy, mixed strategy, position effects, weeds, winged fruits.

RESUMEN

La producción de dos o más tipos de frutos diferentes por un mismo individuo, i.e. heterocarpia, se considera relacionada con una estrategia mixta de dispersión en la que una parte de la descendencia está destinada a colonizar nuevos sitios, mientras que la otra permanece cerca de la planta madre. En este trabajo, nuestro objetivo fue explorar los efectos de la morfología del aquenio (aquenios alados *vs*. no alados) y de su tamaño en la etapa del ciclo de vida siguiente a la dispersión (probabilidad de germinación y tiempo de emergencia de las plántulas) de tres especies heterocárpicas del género *Anacyclus* (Anthemideae, Asteraceae). Se estudió la morfología, el tamaño y la germinación en aquenios de seis poblaciones de *Anacyclus clavatus* (Desf.) Pers*., A. homogamos* (Maire) Humphries*,* y *A. valentinus* L. Nuestros resultados indican que tanto la morfología del aquenio como su masa estaban relacionados con su posición en el capítulo, de manera que los aquenios más externos – alados – eran significativamente más pesados que los internos – sin alas –. Además, los aquenios alados germinaron más rápidamente que los no alados. Este patrón puede estar relacionado con la liberación secuencial de los aquenios que ocurre en estas especies. Por último, nuestros resultados ponen en duda la función de las alas como estructuras que favorecen la dispersión por viento de los aquenios en *Anacyclus*.

Palabras clave: bet-hedging, Compositae, heterocarpia, efectos de posición, estrategia mixta, malas hierbas, frutos alados.

INTRODUCTION

Seed dispersal is mainly determined by fruit characteristics, which usually vary continuously within an individual plant (Herrera, 2009). Besides, some plants produce two or more defined fruit types, i.e. heterocarpy, in which different fruit morphs may show different behavior on dispersal, germination recruitment or seedling survival (Imbert, 2002). Heterocarpy is usually interpreted as a bet-hedging or mixed strategy in which different seed subsets of one individual may successfully germinate under different conditions in time and space. This offers the chance to colonize new sites, free from sibling competition or other local sources of stress, whereas the rest of the offspring is staying in the same habitat (Gadgil, 1971; Levin & et al., 1984; Schoen & Lloyd, 1984; Venable & Brown, 1993; Imbert & Ronce, 2001). Bet-hedging strategy has been proposed to be favored in temporally variable environments because it increases geometric fitness, even if individual phenotypes might have relatively lower mean fitness (Gillespie, 1977; Venable, 1985; Venable & al., 1987; Venable, 2007; Simons, 2011; Tielbörger & al., 2012).

In Asteraceae, heterocarpy is relatively common (Mandák, 1997; Imbert, 2002). Heterocarpic species in this family usually produce different achene morphs within the same head (reviewed in Imbert, 2002), although exceptions in which achene variation occurs between aerial and subterranean heads are known in *Gymnarrhena micrantha* (Koller & Roth, 1964) and *Catananche lutea* (Ruiz de Clavijo, 1995; Ruiz de Clavijo & Jiménez, 1998), and in *Centaurea melitensis* (Porras & Muñoz, 2000) between cleistogamous and chasmogamous heads. Apart from these exceptions, most of achene variation is found within heads. For instance, beside variation in morphology, achenes may also extraordinarily vary in size (Venable & al. 1987; McGinley, 1989; Maxwell & al. 1994; Imbert & al. 1996; Van Mölken & al., 2005; Brändel, 2007). Both traits, achene size and morphology, usually covary, so that achenes without dispersal structures are usually the heaviest ones, whereas those adapted for longer dispersal are generally the lighter achenes (McEvoy, 1984; Tanowitz & al., 1987; Venable & al., 1987; Imbert & al., 1996; Imbert & Ronce, 2001; Brändel, 2004, 2007; Bastida & Menéndez, 2004, Bastida & al., 2010; among others). Regarding germination performance, recent research suggests that variation on achene size might be the main driver of divergent behavior of seeds from different achene morphs (Van Mölken & al., 2005; Torices & Méndez 2010). Achene size usually affects later performance on post-dispersal life history traits, particularly in competitive conditions (McEvoy, 1984; Andersson, 1996; Ruiz de Clavijo & Jimenez, 1998; Meyer & Carlson, 2001; Ruiz de Clavijo, 2005; Van Mölken & al., 2005; Benard & Toft, 2007). By contrast, seeds might have different behavior in germination inherent to its achene morphology, regardless of size, which would also affect later stages in plant development (Imbert, 2002). In the face of this controversy, it is needed to unravel whether the achene morphology within a

head has direct effects on post-dispersal life history traits, or if this effect is only mediated by achene size.

In spite of the high incidence of heterocarpy in Asteraceae and the knowledge of its influence on dispersal ability and germination performance, the proximate causes of achene variation remain unknown. As part of an ongoing project focused on reproductive biology and population genetics of *Anacyclus clavatus*, *A. homogamos* and *A. valentinus* species complex, we studied achene size variation and seedling emergence in these three species. *Anacyclus* is a Mediterranean genus of mostly annual weedy herbs that show an extraordinary variation in flower, achene morphology and sexual expression within capitula and among species (Bello & al., unpublished). Achenes in *Anacyclus* are dorsiventrally flattened and winged to unwinged from outermost to innermost positions respectively. Similar structures in achenes –wings-- are found in other Asteraceae (Anderberg & al., 2007; Manchester & O'Leary, 2010), mostly in the Calenduleae (*Garuleum*, *Norlindhia*, *Tripteris*, etc.) and Heliantheae (*Silphium*, *Verbesina*, *Wedelia*, etc.). As far as we know, no studies have been conducted on the role of wings in these achenes. It has been argued that the wings of the achenes in *Anacyclus radiatus* Loisel. might favor dispersion by wind (Bastida & al., 2010). These authors and Bastida & Menéndez (2004) found that in *A. radiatus* weight differences between central achenes –unwinged-- and peripheral ones –winged-- were not significant and that both morphs had non-dormant seeds.

We aimed to explore the effects of achene morphology (presence vs. absence of wings in achenes) and achene size on two important post-dispersal life history traits in three *Anacyclus* species: probability and time of seedling emergence. Finally, we discuss the implications that winged achenes may have on different dispersal mechanisms and germination performance regarding bet-hedging strategies in *Anacyclus* life history.

MATERIAL AND METHODS

Study species and plant material

Morphology, size and germination performance were studied in achenes from a total of 6 distant natural sites in *Anacyclus clavatus, A. homogamos,* and *A. valentinu*s (Table 1). A total of 9-10 complete and mature heads of several individuals in each site were collected and preserved in dry, dark, and cold place (-4ºC with desiccant material) until their use.

These three species occur in Western Mediterranean and their distribution areas partially overlap. *Anacyclus clavatus* is present throughout the Mediterranean both in coastal and inland areas, while *A. homogamos* is mainly restricted to inland areas of Morocco and

Argelia, and *A. valentinus* mostly occupies coastal areas in all Western Mediterranean (Humphries, 1979; pers. obs.). In areas where these species coexist, morphological variation of flowers increases remarkably (e.g., presence vs. absence of ray flowers within populations). In *A. homogamos* all flowers are bisexual and tubular (i.e. hermaphroditic heads) while *A. clavatus* and *A. valentinus* present female flowers in the periphery and hermaphroditic ones in the rest of the capitulum (i.e. gynomonoecious heads). Furthermore, while *A. clavatus* shows rayed female flowers, in *A. valentin*

Achene position and mass

Each head was manually disassembled to get all achenes, which were classified depending on their relative position within each head in 4-5 categories (from the outermost positions to the innermost): (1) produced by female flowers (peripheral and winged, only present in *A. valentinus* and *A. clavatus*), (2) outermost winged, (3) innermost winged, (4) outermost unwinged, and (5) innermost unwinged. Since achenes are too light (< 0.1 mg), groups of ten were weighed for each category in all heads using a Kern ABJ electronic precision balance (0.1 mg).

Achene morph and seed germination

Our aim was to study seed germination behavior in clearly distinct achene morphs: winged *vs.* unwinged achenes. Consequently, we selected only the outermost achenes (excluding those achenes from female flowers to have the same kind of achenes across all species since *A. homogamos* does not have female flowers), which displayed the largest wings, and the innermost achenes, which never showed wings (Fig. 1). Five fertile achenes (i.e. achenes slightly swollen and hard, resistant to tweezers pressure) of each category in each head were selected for the experiment adding up to 580 achenes. These were put on wet filter paper into Petri dishes with periods of 16 h white light and 8 h dark and temperature ranging 10º-27ºC in Real Jardín Botánico-CSIC greenhouse, maintaining a moist environment into the Petri dishes. Experiment was initiated on January 20th 2012 and seed germination was recorded daily until the experiment was terminated (120 days after). Once both cotyledons and radicle were developed, seedlings were transplanted to pots for further experiments.

Statistical Analyses

We evaluated how position, which was completely correlated with morphology, influenced achene mass, and afterwards, whether achene morphology and/or achene mass affected seed germination traits. Thus, firstly, to assess the effect of the achene

position/morphology on achene mass, we fitted Generalized Linear Mixed Models (GLMMs), via restricted maximum likelihood (Patterson & Thompson, 1971). GLMMs were employed because they provide a flexible way to model traits allowing the distinction between fixed and random factors in the model at the same time vs. a standard linear modeling. Satterthwaite's method was used to determine the approximate denominator degrees of freedom for these tests (Verbeke & Molenberghs, 1997). The explanatory variables included in the model were achene position, site and its interaction (achene position × site); whereas head was included as random factor. The response variable, achene mass, was modeled using a gamma distribution with a log link function.

Secondly, we also evaluated the effects of achene morphology and achene mass on probability of seed germination and time of germination by fitting GLMMs. In these two cases the explanatory variables were achene mass, achene morphology, site and its interaction (achene morphology × site). Again, head was the random factor. The probability of germination was modeled using a binomial distribution with a logit link function and time of germination was modeled using a Poisson distribution with a log link function. A different model for each species was fitted. All models were fitted using the GLIMMIX procedure of SAS (SAS Institute, Cary, NC) with the DIFF option in the LSMEANS statement.

RESULTS

Achene mass variation

Achene mass decreased from outermost –winged-- to innermost –unwinged-- positions in all studied sites (Table 2, Fig. 2). Furthermore, achene mass also differed within species in two out of three cases (Table 2). Achenes from Carchuna and Iznate were heavier than those from Miraflores de la Sierra and Castelló d'Ampuries in *A. clavatus* and *A. valentinu*s respectively (Fig. 2). Despite these differences, the general tendency to mass declining towards inner positions was observed for each respective pair of sites (Fig. 2). In *A. homogamos* this tendency was less marked since only the innermost achenes were statistically different from the remaining (Fig. 2).

Seed germination probability of winged vs. unwinged achenes

Mean percentage of total germination in all species analyzed was relatively high (74 %), while variation in observed germination within and among species was low. Seeds of winged *vs.* unwinged achenes did not show different germination probabilities for most of the sites (Table 3, Fig. 3a). Nevertheless, seeds of winged achenes from one site of *A. clavatus* (Miraflores)

germinated at significantly lower percentage than unwinged ones (Fig. 3a); although, seeds of winged achenes from this site also germinated at very high rates (Fig. 3a). Achene mass influenced positively the probability of seed germination in one species, *A. clavatus* (Table 3).

Additionally, our analyses pointed out that the probability of germination was significantly different between sites within species (i.e.: 54 % in Carchuna vs. 91 % in Miraflores de la Sierra for *A. clavatus*; 66 % in Asni vs. 94 % in Imouzzer for *A. homogamos*; 56 % in Iznate vs. 85 % in Castelló d'Ampuries for *A. valentinus*; Table 3).

Seedling emergence time of winged vs. unwinged achenes

Seeds showed an extraordinary variation in germination times, from one day after being sowed to more than 90 days. This variation was associated to achene morphology within each head, so that in general, seeds of winged achenes germinated much faster than those of unwinged ones (Table 4, Fig. 3b). Only achenes of *A. clavatus* from Miraflores did not show this pattern (Fig. 3b). Time of seedling emergence varied significantly among sites in *A. clavatus* and *A. homogamos*, but not in *A. valentinus* (Fig. 3b; Table 4). Thus, those sites having the fastest germinating seeds (Miraflores and Imouzzer) also showed smaller differences in germination time between seeds of winged *vs*. unwinged achenes (Fig. 3).

Achene morphology influenced the time of seedling emergence while achene mass has not a significant effect in *A. homogamos* (Table 4). However, in *A. valentinus* and *A. clavatus* both achene mass and morphology influenced significantly on seedling emergence times, although in *A. clavatus* the effect of achene morphology is also depending on the site. Beyond the significant effect of achene mass on emergence time in this two species, achene morphology, with the exception of Miraflores site, also affected seedling emergence times, suggesting that achene morphology could have a direct effect on germination timing independently of achene mass in these three species (Table 4).

DISCUSSION

Achene mass and morphology: their implications in dispersal

Our results show that in *Anacyclus* achene mass is related to its position on the capitulum, so that outer achenes are significantly heavier than inner ones, as commonly occurs in other heterocarpic Asteraceae (McEvoy, 1984; Venable & al., 1987; McGinley, 1989; Maxwell & al., 1994; Imbert & al., 1996; Imbert, 2002; Brändel, 2007; Sun & al., 2009; Filho & Takaki, 2011; but see Rocha, 1996). Usually, in these species, variation in achene mass and position is also correlated to a differential presence of dispersal structures within the same head.

In *Anacyclus clavatus*, *A. homogamos* and *A. valentinus*, outer achenes –the heaviest ones– showed lateral wings, while achenes in inner positions –the lightest ones– were unwinged. This result seems contrary to the general trend in heterocarpic species in which achenes presenting structures to favor dispersion by wind (i.e., pappus) are the lightest. Therefore, the role of achene wings in *Anacyclus* dispersal is unclear. Although in *A. radiatus* achene mass do not vary within a head, Bastida & al. (2010) suggest that winged achenes in this species might be secondarily dispersed by wind.

Winged fruits and/or seeds are considered wind dispersed because wings reduce the descent rate of diaspores (Harper 1977; Van der Pijl 1982; Cousens & al., 2008). Although morphology may help to understand how diaspores disperse, the conventional assignment of a plant species to a certain mode of dispersal based only on the morphology of its diaspore could result in misleading conclusions (Tackenberg & al., 2003). Several evidences indicate that winds might be only one of different vectors of dispersal in *Anacyclus* achenes. First, the very low height of *Anacyclus* species (almost always below 1 m) limits the effective dispersal by wind, since wind dispersal is strongly correlated with plant height (Thomson & al., 2011). Second, in *Anacyclus*, achenes are protected by the involucral bracts and dry heads become resistant structures that may remain on dead plant for long periods of time, resulting in an aerial seed bank (Bastida & al., 2010; pers. obs.). This type of head is present in other Asteraceae that grow in arid and semi-arid environments (Zohary, 1950), in which moisture seems the main factor for achene release (i.e., ombrohydrochory). Achenes are retained in the heads until the rainy season, in which are sequentially released during different periods of rain (Gutterman & Ginott, 1994 in *Asteriscus pygmaeus* (DC.) Coss. & Durieu; and Aguado & al., 2012 in *Anthemis chrysantha* Gay). This mechanism may be advantageous in fluctuating environments such as Mediterranean Climate zones because it increases the probability of establishment during different pulses of precipitation when the probability of establishment success is maximal (Gutterman, 1994; Peters & al., 2009). A similar mechanism was described in *A. radiatus* (Bastida & al., 2010) and observed in other *Anacyclus* species, in which achenes dampen by rain, are released centripetally, so that unwinged inner achenes can be set free several months after the outermost. Consequently, it is expected that heavy rains may be the main dispersal vector in all these ombrohydrochorous species, rather than winds. Furthermore, small diaspores –such as *Anacyclus* achenes-- usually show high floating abilities which may allow secondary dispersal by temporary watercourses (e.g. Telenius & Torstensson, 1989; Redbo-Torstensson & Telenius, 1995; Fumanal & al., 2007; Cousen & al., 2008; Lu & al., 2010). Finally, incomplete heads –the aerial seed bank-- are usually observed on dead plants even during the next blooming after the rainy season. These heads also might act as dispersal units dragged by animals (goats, sheep, etc.) or by human activity (transport by roads and/or railroad). Therefore, a wide range of agents may contribute to achene dispersal apart from

winds, and the effective dispersal of each achene morph will depend on what dispersal agent(s) is predominant.

The effects of achene morphology on seedling emergence

Achene morphology and mass influenced different stages on post-dispersal life history traits in *Anacyclus*, supporting previous findings about direct and indirect effects. The probability of seed germination was mainly related by achene mass instead of achene morphology in *A. clavatus*. Within an individual, seeds of larger achenes usually show higher probability of germination than those of smaller ones (Rai & Tripathi, 1982; Pandey & Dubey, 1988), regardless achene dispersal ability (McEvoy, 1984). These differences in germination have been mainly attributed to seed size instead of to the anatomical differences among the different achene morphs (Van Mölken & al., 2005).

In contrast, timing of seedling emergence in all the studied species was directly influenced by morphology. Thus, independently of achene mass, seeds of winged achenes germinated faster than those from unwinged ones. This pattern might have resulted from a coupling of seedling emergence to the sequential mechanism of head opening and achene releasing displayed by these species. Therefore, we expect a divergent selection on seedling emergence times of outer winged achenes *vs*. inner unwinged ones leading to a mixed strategy in germination. However, since morphology and position within heads are fully coupled, we cannot disentangle at present whether the observed effects should be solely attributed to achene morph or to its position within a head (i.e., positional effect). To answer this question it is required, for instance, manipulative experiments on wing size to assess the potential effects of winged morphology, achene position and achene mass on emergence times in this genus.

The contrasted pattern on the time of seedling emergence of winged *vs*. unwinged achenes may also influence the potential dispersal distance of each achene morph. If the main dispersal agent is a passive one such as water and/or wind, then, the time of exposure to dispersal vectors might influence the final ability of achieving longer distances. Hence, unwinged achenes have more time to be secondarily dispersed once they have been released from heads. By contrast, seed of winged achenes germinate fast –sometimes in 24 h--, reducing the probabilities of being long dispersed. This reduction in the probability of secondary dispersal occurs as well in other species with diaspores released by rains in harsh environments (Parolin, 2006). Thus, contrary to expectations, winged achenes might achieve shorter, or at least the same distances than unwinged and lighter ones. Whether achene release and achene variation on emergence times are adaptive strategies to cope with unpredictable rains and disturbed habitats we should expect that both traits (1) vary within a gradient of unpredictability in rains, and (2) were hereditable in some way.

On the other hand, the rapid germination of winged achenes might be a response for competing with neighbors. It is expected that accelerated germination is selected when competitive neighborhood is high (Orrock & Christopher, 2010). In *Crepis sancta,* early emergence of heavier achenes provides a competitive advantage which might have been selected to cope with sibling competition (Dubois & Cheptou, 2012). Therefore, winged achenes could be adapted to compete with siblings and not to be long dispersed since they are able to germinate rapidly. But then, have wings any adaptive significance beyond dispersal? Wings might contribute for the physiological mechanism by means the time of seedling emergence is controlled, because they are membranous expansions of the pericarp that increase the surface throughout water and gas can enter into the embryo leading to germination. Time of germination usually is influenced by pericarp anatomy of achenes (Imbert, 2002). For instance, thicker pericarps restrict gas exchange and water absorption by the embryo tissue (McEvoy, 1984; Tanowitz & al., 1987; Prinzie & Chmielewski, 1994).

Despite the lack of knowledge about the effective dispersal agent for each achene morph, several traits (i.e., high percentage of germination, non-dormancy, fast seed germination rate of the first subset of achenes released, lack of seed soil bank, and differences in seedling emergence coupled with achene release) are consistent with a bet-hedging strategy that ensures progeny establishment at different optimal germination frames.

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TABLES

Table 1. Sites and sampling for the three studied species.

Species	Origin/Voucher	Sampling
A. clavatus	Spain: Granada, Carchuna (36°41'49"N; 3°27'33"W), 13 m, *Agudo 1*	10 individuals/one head each (late summer 2011)
	Spain: Madrid, Miraflores de la Sierra (40°47'36.45"N; 3°43'46.97"W), 883 m, *Álvarez* 2173	10 individuals/one head each (early autumn 2011)
A. homogamos	Morocco: Imouzzer (31º19'55"N; 7°24'32"W), 2224 m, *Gonzalo 1275*	10 heads from an unknown number of individuals (summer 2009)
	Morocco: Asni (31°15'4"N; 7°58'40"W), 1160 m, *Álvarez 2115*	9 heads from an unknown number of individuals (late spring 2010)
A. valentinus	Spain: Girona, Castelló d'Empuries (42°15'47.2"N; 3°7'45.5"E), 0 m, *Álvarez 2059*	10 heads from 3 individuals (summer 2009)
	Spain: Málaga, Iznate (36°46'35"N; 4°10'45"W), 285 m, *Álvarez 2137*	9 individuals/one head each (late summer 2011)

Table 2. Effects of flower position and population on achene mass of three *Anacyclus* species. Data represent the Wald-type F-statistic with the degrees of freedom as subindex for fixed factors (the sign indicating the direction of the effects), and the estimate for covariance parameter and its standard error for the random factor.

	A. clavatus		*A. valentinus*		*A. homogamos*	
Fixed factors	*F*	*P*	*F*	*P*	*F*	*P*
Position	$70.15_{\ 4,\ 71}$	< 0.0001	$35.00_{\ 4,\ 62.04}$	< 0.0001	$20.37_{\ 3,\ 39}$	< 0.0001
Site	$4.07_{\ 1,\ 18.0}$	0.0589	$5.44_{\ 1,\ 16.92}$	0.0322	$0.88_{\ 1,\ 13}$	0.365
Position × Site	$1.26_{\ 4,\ 71}$	0.2953	$0.84_{\ 4,\ 62.04}$	0.5034	$1.38_{\ 3,\ 39}$	0.2618
Random factors	*Estimate*	*SE*	*Estimate*	*SE*	*Estimate*	*SE*
Head	0.047	0.017	0.050	0.019	0.035	0.015
Sample size	99		89		60	

41

Table 3. Effects of achene morphology, achene size and population on germination probability of three *Anacyclus* species. Data represent the Wald-type F-statistic with the degrees of freedom as subindex for fixed factors (the sign indicating the direction of the effects), and the estimate for covariance parameter and its standard error for the random factor.

	A. clavatus		*A. valentinus*		*A. homogamos*	
Fixed factors	F	P	F	P	F	P
Morphology	$0.73_{1, 45.8}$	0.3983	$0.23_{1, 68.6}$	0.6361	$2.54_{1, 149}$	0.1131
Site	$13.29_{1, 18.5}$	0.0018	$4.71_{1, 15.6}$	0.0458	$11.88_{1, 11.9}$	0.0653
Morphology × Site	$7.91_{1, 190}$	0.0054	$1.47_{1, 188}$	0.2267	$0.64_{1, 149}$	0.4239
Achene size	$+\ 6.27_{1, 24.1}$	0.0194	$0.25_{1, 23.51}$	0.6240	$0.00_{1, 26.3}$	0.9840
Random factors	*Estimate*	*SE*	*Estimate*	*SE*	*Estimate*	*SE*
Head	1.725	1.086	1.279	0.793	1.140	1.048
Sample size	195		193		154	

Table 4. Effects of achene morphology, achene size and population on time of seedling emergence of three *Anacyclus* species. Data represent the Wald-type F-statistic with the degrees of freedom as subindex for fixed factors (the sign indicating the direction of the effects), and the estimate for covariance parameter and its standard error for the random factor.

	A. clavatus		*A. valentinus*		*A. homogamos*	
Fixed factors	*F*	*P*	*F*	*P*	*F*	*P*
Morphology	$2.03_{1, 147}$	0.1563	$19.47_{1, 151}$	< 0.0001	$22.06_{1, 133}$	< 0.0001
Site	$15.92_{1, 17.4}$	0.0009	$0.12_{1, 18.9}$	0.7299	$10.06_{1, 13.2}$	0.0072
Morphology × Site	$47.79_{1, 147}$	< 0.0001	$3.30_{1, 151}$	0.0714	$1.20_{1, 133}$	0.2750
Achene size	$-42.30_{1, 147}$	< 0.0001	$-17.21_{1, 151}$	< 0.0001	$1.64_{1, 133}$	0.2022
Random factors	*Estimate*	*SE*	*Estimate*	*SE*	*Estimate*	*SE*
Head	0.799	0.304	1.226	0.047	0.150	0.062
Sample size	152		156		138	

FIGURES

Figure 1. Picture of winged and unwinged achenes of all species: a) *Anacyclus clavatus*, b) *A. valentinus*, c) *A. homogamos*. Scale = 2 mm
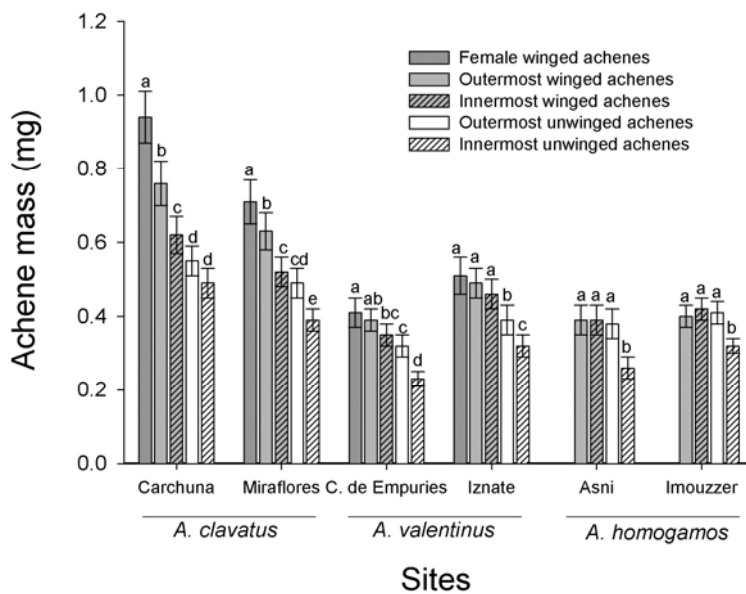
Figure 2. Least-square means (± SE) of achene mass of three species of *Anacyclus* from two sites each, and from different position within heads. Means sharing a superscript were not significantly different at the $P < 0.05$ level.
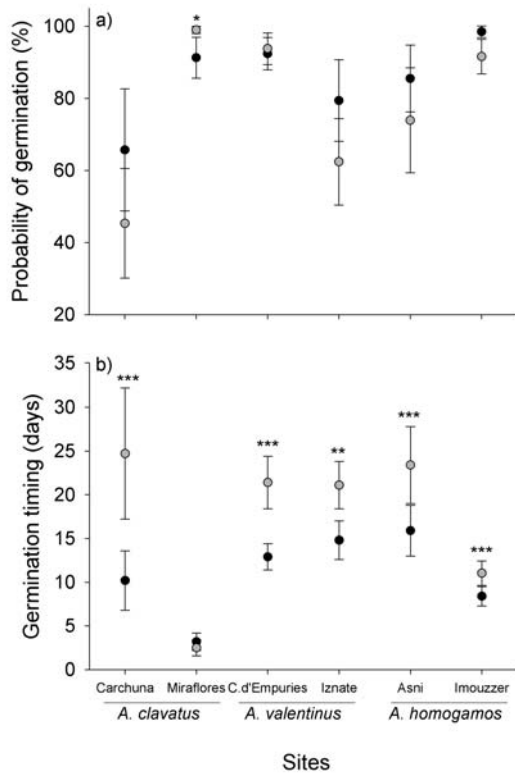
Figure 3. Least-square means (± SE) of a) probability of germination, and b) germination timing of winged (black) and unwinged (grey) achenes of three species of *Anacyclus* from two sites each. Significant differences among winged and unwinged achenes are shown above each site (* < 0.05, ** < 0.01, *** < 0.001).

1.2. Pollinator preference in a contact zone between *A. clavatus* (Desf.) Pers. and *A. valentinus* L.

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ABSTRACT

To attract pollinators, plants display conspicuous structures such as the rays surrounding the inflorescence in many species of the sunflower family. Rayed inflorescences usually attract a larger number of visits than rayless ones, nevertheless, these plants are commonly visited by a wide array of pollinator fauna and the preferences of different pollinator groups on the ray/rayless inflorescence remain largely unexplored. To fill this gap, we studied preferences of different pollinator groups for plant, inflorescence, and neighbourhood traits in a generalist system where the rayed *Anacyclus clavatus* and the rayless *A. valentinus* co-exist showing a wide range of phenotypic variation. Furthermore, we combined this observational study with two experiments manipulating the floral phenotype and the neighbourhood environment. We found that in the natural contact zone, the presence of rays influenced the probability of being visited by the overall pollinator assemblage and by Dipteran pollinators in particular. In contrast, bees showed no preference for a particular phenotype and its visitation pattern was mainly driven by the number of capitula simultaneously blooming in the plant or in the neighbourhood. Additionally, no differences were found between both phenotypes under solitary conditions; however, rayed plants benefited from having rayed neighbours, whereas rayless and intermediate phenotypes competed for pollinators when surrounded by neighbours. These results highlight differential pollinator preferences and the importance of the neighbouring composition in pollinator attraction for a focal plant and suggest a dynamic and complex framework affecting hybrid zone dynamics.

Keywords Asteraceae; Capitulum; Fly pollination; Head; Hybrid zone; Ray

INTRODUCTION

Pollinators mediate floral evolution by exerting selective pressures on plant traits (Wesselingh and Arnold 2000; Sánchez-Lafuente 2002; Gómez et al 2008a; Campbell et al 2008; Parachnowitsch and Kessler 2010; Penet et al 2012). The evolutionary process underlying floral evolution and pollinator attraction is often understood as a gradual co-adaptive mechanism in which the plant evolves in response to its most efficient pollinator, resulting in a unidirectional specialization for a specific pollinator or group of pollinators (Fenster et al 2004; van der Niet et al 2014). Consequently, generalist pollinated plant species have received less attention from an evolutionary perspective, under the light that pollinators may not be an efficient source of natural selection for these plants. Paradoxically, generalist plants seem to be 'the rule rather than the exception' (Waser et al 1996) and the above mentioned view was recently countered, being suggested that specific pollinator groups might effectively exert selective pressures on floral shapes in a generalist framework (Sánchez-Lafuente 2002; Gómez et al 2008b; Gómez et al 2014). If generalist species can be visited by hundreds of pollinator species and these effectively drive floral and plant evolution, which traits and conditions favour the attraction of generalist pollinators?

An exceptional model of generalist species is provided by the ray flower polymorphism within the sunflower family, the Asteraceae. The floral polymorphism of Asteraceae is one of the best documented polymorphisms in plant biology, having received considerable attention in genetics (Ingram and Noltie 1984; Marshall and Abbott 1984; Sun and Ganders 1990; Gibson 2001), ecology (Abbott and Schmitt 1985; Stuessy et al 1986; Gibson 2001; Nielsen et al 2002; Andersson 2008; Bertin et al 2010), and more recently, in developmental biology (Kim et al 2008; Bello et al 2013). The inflorescence of Asteraceae is usually composed by a large number of sessile florets on an axis extremely reduced in length but expanded (i.e. head or capitulum), representing the unit for pollinator attraction. In many Asteraceae species the outermost florets in the capitulum show a conspicuous petaloid structure (i.e. ray florets, rayed phenotypes; Fig. 1a), whereas in other species all florets of the capitulum are similar (i.e. disc florets, rayless phenotypes; Fig. 1b). Variation in ray display can occur between species within a genus (e.g. Nielsen et al 2002) or even between individuals of the same species (e.g. Andersson 1996). This diverse plant family is mostly composed by species visited by a broad assemblage of pollinators (Lane 1996) and the presence of rays seems to have significant consequences on pollination, mainly enhancing capitula attractiveness (Lack 1982; Celedón-Neghme et al 2007; Andersson 2008) and, consequently, influencing the levels of outcrossing (Marshall and Abbott 1984; Sun and Ganders 1990). Nevertheless, most of these studies in Asteraceae have been focused on a

small range of species visited by a limited number of pollinator groups assuming that the whole pollinator assemblage might equally affected by rays.

Hybrid zones between species that differ in floral phenotypes commonly display high levels of floral trait variation, providing the grounds to study pollinator preferences and pollinator-mediated selection under natural conditions (Barton and Hewitt 1985; Aldridge and Campbell 2007; Campbell and Waser 2007). Studies performed on these areas have already provided strong evidences on pollinator-mediated selection of floral traits (Hodges and Arnold 1994; Campbell et al 1997; Campbell 2003; Campbell 2008). Despite this, only a limited number of hybrid zones has been studied and these involved highly specialized plant species with contrasting pollination syndromes (Aldridge and Campbell 2007), where a strong reproductive isolation effect arises due to pollinator preferences (Schemske and Bradshaw 1999; Emms and Arnold 2000). In turn, generalist pollinated hybrid zones have received less attention. Considering the distinctive features of these areas, i.e. pollinator assemblage is shared among co-existing taxa, a generalist hybrid zone provides an ideal ground to disentangle pollinator preferences on generalist floral syndromes.

In this study, we explore the preferences of different pollinator groups on ray polymorphism, by studying a generalist-species contact zone and combining a set of manipulative experiments in single-species populations. We focus in the contact zone between two annual *Anacyclus* L. species (Asteraceae), which differ mainly in their floral phenotype: the rayed species *Anacyclus clavatus* (Desf.) Pers. (Fig. 1a), and a rayless one *Anacyclus valentinus* L. (Fig. 1b). Both species are inter-fertile (Humphries 1981) and show a large phenotypic diversity in the number and size of ray florets in sympatric areas (Bello et al 2013) . Preliminary results of experimental crosses between these species as well as genome size investigations in these areas (Agudo & al., unpubl.) support that hybridization might be occurring in contact zones between the two species. A preliminary survey revealed that both species and the intermediate phenotypes were visited by a large array of pollinator types including different groups of Diptera, Hymenoptera, Coleoptera and in a small extent Lepidoptera (R. Torices, field observations) ensuring an exceptional micro-evolutionary framework to quantitatively explore the preferences of pollinator groups relative to ray polymorphism. Also, because neighbouring conditions can affect the interplay between phenotypes (Williams 2007; Jones and Comita 2008), we explore pollinator preferences to ray phenotypes under different pollination contexts. We followed a complementary approach combining an observational study in the contact zone of both species, with two different experimental manipulations including: (i) floral phenotype manipulation, through removing rays in a site exclusively with rayed individuals and adding artificial rays on individuals in a site with exclusively rayless ones; and (ii) neighbouring plants removal to explore the effect of the floral phenotype on different social contexts: surrounded by

other conspecific plants or alone. Finally, we discuss how pollinator abundance and preferences might lead to reproductive isolation and drive hybrid zone dynamics.

MATERIALS AND METHODS

Study species

Anacyclus L. (Anthemideae, Asteraceae) is composed by around 12 species of mostly weedy annual herbs found in dry and disturbed habitats throughout the Mediterranean basin (Humphries 1979). This genus shows an extraordinary variation in reproductive traits and sexual expression within capitula and among species, which has been suggested to be derived from different evolutionary and hybridization events (Fig. 1 a-c; Humphries, 1981). One example is the species complex formed by *A. clavatus* (Desf.) Pers. and *A. valentinus* L. (Fig. 1a, b). Both species show notable differences in floral morphology and, in areas where both species coexist, morphological variation of the inflorescences is remarkably high (e.g. number, and size of the rays, Fig. 1a-d; Bello *et al.* 2013), including intermediate phenotypes that might be a product of hybridization between these two species.

Anacyclus clavatus is usually found in disturbed habitats, coastal beaches, fields and roadsides, within the Circum-Mediterranean Basin (Humphries 1979). This plant has gynomonoecious capitula (i.e. presenting female and bisexual flowers), with two types of flowers varying both in sex expression and morphology: ray female florets with white rays, displayed in the outermost position of the capitulum and yellow bisexual disc florets with a tubular-campanulate corolla displayed in the central part of the capitulum (Fig. 1a; Bello *et al.* 2013). *Anacyclus valentinus* is found mainly in coastal areas of Western Mediterranean, namely at Morocco, Spain, Algeria and Tunisia, occurring in disturbed grounds, sandy places, lowlands, river banks, fields, and roadsides (Humphries 1979). Likewise *A. clavatus*, this species bears gynomonoecious capitula but, in contrast, female florets present inconspicuous rays or no rays at all, resulting in a discoid-like or rayless capitulum (Fig. 1b, Humphries 1979; Bello et al 2013).

Both species are inter-fertile and self-incompatible (Humphries 1981; Agudo & al., unpubl.), and commonly bloom from February to July. Furthermore, both species display a mixed strategy in fruit dispersal presenting winged and wingless achenes with different germination phenology (Torices et al 2013; Afonso et al 2014).

Study sites

This study was conducted during the spring of 2013 within a contact zone between *A. clavatus* and *A. valentinus*, nearby Torre del Mar (southern Spain), at three different sites. The three sites showed similar sizes and ecological conditions, and were chosen because they presented a high number of individuals of *Anacyclus* species. The three selected sites included: i) an open field, 1 m a.s.l., 210 m distance from the sea, where both species grew and where an intermediate phenotype had previously been observed (hereafter *sympatric site*; 36° 43' 48.875" N, 4° 6' 8.154" W); ii) an abandoned area close to a road, 1 m a.s.l., 160 m away from the sea, only with *A. clavatus* (rayed species; hereafter *rayed site*; 36° 45' 4.186" N, 4° 5' 58.289" W); and iii) an open field area next to planted palm trees, 16 m a.s.l., 1 km distance from the sea, only with *A. valentinus* (rayless species; hereafter *rayless site*; 36° 43' 50.516" N , 4° 6' 4.697" W). The vegetation in these three sites was very similar, being characterized by several ruderal herbaceous species such as, *Leontodon longirostris* (Vill.) Mérat (Asteraceae), *Hirschfeldia incana* (L.) Lagr.-Foss. subsp. *incana* (Brassicaceae), *Chrysanthemum coronarium* L. (Asteraceae), and *Echium creticum* subsp. *granatense* (Coincy) Valdés (Boraginaceae).

Experimental design

Floral visitor preferences were assessed by two approaches. First, we performed an observational study at the sympatric area, where both species naturally inhabit; and second, a manipulative experiment controlling floral phenotypes and neighbouring conditions at both single-species sites (*rayed* and *rayless sites*) was conducted.

Sympatric site

To assess floral visitor's preferences under natural conditions, we randomly selected 107 plants and, to maximize the efficiency of field observations, we established 27 groups including 2 to 7 plants. Plant groups were monitored as described below in *Floral visitor observations* section. Plants were firstly characterized in three phenotypes: rayed, rayless and intermediate (Fig. 1 a-c); and were afterwards tagged and characterized focusing on general plant traits, capitulum traits, and neighbourhood traits. Specifically, plant traits included plant height (the distance from the ground to the tallest part of the plant), plant dimension (a circular area whose diameter was calculated through the mean value between the plant's largest diameter and its perpendicular axis), and floral display (total number of open capitula per individual at each observation day; quantified repeatedly through the field season). Inflorescence traits included capitulum size (total diameter of the capitula, from the tip of a ray to the tip of the opposite ray), disc size (diameter of the disc), and ray number. Ray length was

additionally estimated by the following formula: (Capitulum size – diameter of the disc)/2. Lastly, pollination context (the number of open *Anacyclus* capitula within a 0.5 m radius, assigned accordingly to each phenotypes) was surveyed at three different periods during the field season. Among the traits measured, the three phenotypes only differed significantly in those traits related to rays (Electronic Supplementary Material A).

Phenotypic manipulations at single-species sites (rayed and rayless sites)

To get further insights of pollinator's preferences, we performed two experiments of phenotypic manipulation, one involving the removal of rays in rayed individuals within the rayed site (Fig. 1e), and another involving the addition of artificial rays in rayless individuals present in the rayless site (Fig. 1f). We expected rayed plants to attract a higher number of insect visitors.

We selected 20 groups of three nearby plants, separated by around 1 to 2 m at the *rayed site*. Each triplet contained one un-manipulated individual (rayed individual; control phenotype), an individual, which had its rays carefully removed with the aid of tweezers (rayless individual; experimental phenotype; Fig. 1e) and a one individual equipped with artificial rays (artificially rayed phenotype; experimental control for the experiment described below; Fig. 1f). Plants within each triplet were carefully chosen to be similar in plant traits (height, dimension and floral display) and the plants belonging to the same group were maintained as homogeneous as possible by removing the floral buds produced after the beginning of the experiment.

At the *rayless site*, we selected 20 pairs of nearby plants, separated by around 1 to 2 m from each other. Within each pair, one individual was set as the rayless individual (rayless individual; control phenotype) while the other was equipped with artificial rays (artificially rayed phenotype; experimental phenotype; Fig. 1f). Artificial rays were made with synthetic paper and were added to the capitula to mimic the rayed phenotype (Fig. 1f) following a similar approach successfully employed by Nielsen and colleagues (Nielsen et al 2002). Plants from this population were kept homogeneous as described above for the *rayed site*.

Plant, inflorescence and neighbourhood traits were measured for each selected plant, following the same procedure as for the plants in the sympatric site. Floral visitors were monitored as described below in *Floral visitor observations* section.

Neighbourhood removal experiment at single-species sites

We experimentally manipulated the neighbours to reduce the abundance of capitula surrounding focal plants and assess how differences in the pollination context affect pollinator

preferences on different floral phenotypes. We expected that solitary plants would have a lower number of pollinator visits compared with accompanied plants and that this decrease would be stronger in rayless phenotypes.

To test this, we selected 10 additional pairs of plant in each single-species site (we excluded the rayed experimental control in the rayed site) and we manually removed all surrounding *Anacyclus* plants within a 1 m radius. Individuals belonging to the same pair were separated from each other by more than 2 m and plants were kept homogeneous as described above. Floral visitors were monitored as described in *Floral visitor observations* section.

Floral visitor observations

A reference collection of *Anacyclus* floral visitors was gathered in a preliminary survey of pollinator assemblage within the contact zone in the spring of 2012. In 2013, floral visitor observations were carried during the main flowering period of the study species, more specifically, during the central hours (from 10:30 to 18:00, GMT+1) of warm and sunny days from 30-Mar to 26-April, interchangeably in the three studied sites. Plant groups were observed during intervals of 5 minutes. Observers were positioned 1 to 2 m distance from the plant group and used small-range binoculars to avoid disturbing the foraging activity of floral visitors in tagged plants. A floral visit was only considered when there was a direct contact between the forager and the sexual organs of the capitulum (anthers or stigmas). When an insect visited various capitula within a single plant, we considered this as a single visit because both studied species are self-incompatible. After each observation, weather conditions, hour of the day and surrounding insect activity were recorded for data quality assessment. Only observations performed in sunny days with relevant insect activity were considered for the statistical analyses. A total of 7,885 minutes of observation time was performed in the *sympatric site* (mean: 90 minutes per individual; min – max: 75 – 95), 4,265 minutes in the rayed site (mean: 60 minutes per plant 55 - 65) and 3,760 minutes in the rayless site (mean: 70 minutes per plant; 50 - 80). Insect identification was based on the reference collection gathered in 2012; still, whenever a new taxon was observed, it was collected with a capture net or a vacuum container for subsequent identification. Smaller insects were conserved in ethanol 70%, while bigger ones were air dried (see Electronic Supplementary Material B for further information). All insects of reference collection are kept at the Centre for Functional Ecology (University of Coimbra).

Pollinator groups

Given the wide fauna of floral visitors on *Anacyclus* plants (Electronic Supplementary Material B), we assessed the preferences of particular pollinator groups on studied plant traits. 'Pollinator group' was defined as a group of pollinators which tends to interact with flowers in a

similar way and was established following the methodology employed in Gomez *et al.* (2008b), specifically considering similarities in size, proboscis length, foraging behaviour and feeding habits, rather than taxonomic relationships. Through this method we were able to obtain the following groups: ants, beeflies, beetles, big bees, big flies, bugs, butterflies, hoverflies, small bees, small flies and wasps. The relative abundance of some groups was very low, hindering the statistical analyses; therefore in order to bypass this, we merged some groups and excluded groups with lower visitation rates, ending up with the following four main and well represented groups: bees (including small and big bees), big flies, hoverflies and small flies. Bees included individuals from approximately 12 mm to 2 mm, with members from the Apidae family such as *Apis mellifera*, *Anthophora* sp. and *Eucera longicornis*, but also *Lasioglossum* sp. (Halictidae) and some unidentified species from Megachilidae and Sphecidae families. Hoverflies included individuals from approximately 15 mm to 9 mm, represented only by members of the Syrphidae family. Specifically, hoverflies included *Eristalis tenax*, *Eristalis arbustorum*, *Eupeodes* sp., *Episirphus* sp., *Sphaerophoria* sp., *Syritta pipiens*, *Chrysotoxum*, and a few non-identified hoverfly species. Finally, big and small flies included members of the Calliphoridae, Anthomyzidae, Tachinidae, Scathophagidae and few unidentified species. Big and small flies included individuals bigger or smaller than 2 mm, respectively. Finally, in the *rayed site* there was a very low visitation rate and no pollinator groups were established due to statistical constraints.

Statistical analyses

Broadly, we explored the effects of floral phenotype (the presence of rays), floral display, and neighbourhood context on pollinator attraction for the overall pollinator assemblage and for the defined pollinator groups. For this, we fitted several general linear mixed models (GLMM), using the lme4 package (Bates et al 2014) within the CRAN database (R 3.0.1 software; R Core Team 2013). Before fitting any model we carefully analysed and explored our data, searching for correlations and multicollinearity (Electronic Supplementary Material C) and, after fitting a model, we performed model validation routines, e.g. overdispersion was calculated using Pearson residuals (Zuur et al 2009) to assure that all models were within acceptable values (< 1.15). This analysis was partitioned into three separate sections: (a) assessing the effect of the floral phenotype on floral visitation considering natural floral display and pollinator context; (b) investigating which particular capitulum traits affect pollinator attraction; and (c) exploring how differential neighbouring conditions affect pollinator attraction.

Pollinator preferences

First, we assessed the effect of floral phenotype on floral visitation taking into account the effect of floral display and the pollination context. Thus, floral phenotype, floral display, and pollination context were included as explanatory variables. Plant identity was set as a random factor for the sympatric site while group identity was included as a random factor for both single species sites. Visitation rate was modelled with a Poisson distribution and a log link function. Visitation rate differences between floral phenotypes were tested using least square means function from the 'lsmeans' package (Lenth 2013). This analysis was performed in all three sites; however models for rayed and rayless sites only considered the 20 selected triplets and pairs, respectively, without manipulated neighbourhood conditions. We analysed the visits of all pollinator groups in one global model and, additionally we analysed specific pollinator groups independently for the sympatric and rayless sites. Again, we were unable to establish pollinator groups in the *rayed site*.

Pollinator preferences for particular capitulum traits

Secondly, we studied which capitulum components had an impact on floral visitor's attraction. In particular, we explored the specific role of ray length and disc size in floral visitor's attraction. For this purpose, we analysed only the sympatric site due to its natural high variability of floral traits (Electronic Supplementary Material A). We divided the data in two different groups based on the presence of rays: (i) a rayed group including rayed and intermediate individuals; and (ii) a rayless group including only rayless individuals. Both groups were analysed separately and the effect of disc size was analysed in both groups, while ray length effect was only analysed for the rayed group. Capitulum diameter was discharged in the rayed group because of its high correlation with ray length (Electronic Supplementary Material C). Beside disc size or ray length, each GLMM model included floral display and pollination context as explanatory variables and plant identity as a random factor. As above, we fitted a model with the visits of the overall pollinator assemblage and additional, independent models for each pollinator group. Visitation rate was modelled with a Poisson distribution and a log link function.

The effect of pollination context on visitor preferences

Thirdly, we further explored the effect of neighbouring traits on pollinator preferences by means of two complementary approaches: one based on the observational assessment in the sympatric site and another based on the experimental manipulation of the neighbourhood on both single species sites. On the sympatric site, the quantification of the number of open neighbouring capitula and assessment of their phenotypes (rayed, intermediate and rayless ones) around each focal plant in a radius of 0.5 m allowed to test the effect of particular neighbour types. In models above, pollination context was expressed as the total number of *Anacyclus*

capitula surrounding a focal plant, irrespective of phenotype. For this analysis, we divided pollination context in three independent explanatory variables: pollination context of rayed, intermediate and rayless capitula. Also, we were interested to see the effect of particular neighbours in each phenotype and, for this, we analysed rayed, intermediate and rayless focal plants independently. Visitation rate was modelled with a Poisson distribution and a log link function. Floral display and the specific pollination context (rayed, intermediate or rayless neighbouring capitula) were used as explanatory variables. Plant identity was set as a random factor. As in previous analyses, visits of all pollinators were fitted in a global model and additional models were performed for each pollinator group. For the single-species site, we explored the effects of pollination context by comparing solitary plants (whose neighbours were removed by us; see above) with plants which were surrounded by neighbours. Specifically, we compared ten solitary plant groups against two groups: (i) ten plant groups with an averaged pollination context, which had pollination context values around the median (median for the rayed site = 68; rayless site = 31); and (ii) ten plant groups with the highest pollination context (maximum of pollination context for the rayed site = 209; rayless site = 317). GLMM models included as explanatory variables the floral phenotype (rayed and rayless), pollination context (control vs. neighbourhood removed) and its interaction. Each pair of plants was included as a random factor. Visitation of the entire floral visitor's assemblage for both sites and independent pollinator group for the rayless site were modelled with a Poisson distribution and a log link function.

RESULTS

Pollinator preferences at the sympatric site

Floral phenotype significantly affected the total number of visits when the overall assemblage of floral visitors was considered, whereas floral display and pollination context only showed a significant effect for specific pollinator groups (Table 1). Rayed phenotypes were visited at a significantly higher rate than rayless phenotypes (Fig. 2a), with intermediate phenotypes receiving fewer visits than rayed phenotypes but more than rayless ones, but not differing statistically from them (Fig. 2a). Considering specific pollinator groups, Dipteran groups (big flies, hoverflies and small flies) were the main responsible for the differences considering the total visitation rate between rayed and rayless phenotypes (Table 1; Fig. 2a). Bees, on the other hand, visited rayed, intermediate and rayless plants at the same rate, preferentially visiting plants with larger floral displays (Table 1). Finally, hoverflies were impacted by the three analysed variables, showing preference for rayed plants with larger floral displays and with high number of *Anacyclus* capitula in the surrounding (Table 1; Fig. 2a).

Ray removal and addition experiments

On the *rayed site*, ray removal did not reduce the visitation rate (all floral visitors were analysed together due to the low number of interactions; floral phenotype: $\chi^2 = 2.69$, $P = 0.26$). Regardless of the floral phenotype, those plants with a higher floral display received a higher number of floral visitors ($\chi^2 = 26.01$, $P < 0.0001$). In contrast, the pollination context did not influence visitation rate ($\chi^2 = 0.72$, $P = 0.395$). Finally, the decrease on visitation rate observed in artificial rayed phenotype compared to control naturally-rayed plants was not significant (Fig. 2).

On the *rayless site*, the artificially-rayed phenotype did not attract significantly more floral visitors than rayless ones (Table 2, Fig. 2c). Floral display had no significant effect on the visitation rate of any pollinator group (Table 2). In addition, pollination context only had an effect on bee's visitation rates (Table 2). Bees visited more frequently plants with a higher pollination context, i.e., with a higher number of *Anacyclus* capitula in the surrounding (Table 2).

Pollinator preferences for particular capitulum traits

Disc size had no significant effect on floral visitor's attraction in both rayed and rayless plants (Table 3). Conversely, the length of the rays had a significant impact in pollinator attraction for rayed plants (Table 4). Longer rays significantly increased the visit of the total assemblage of pollinators, hoverflies and small flies (Table 4).

The effect of pollination context on visitor preferences

Natural variation in pollination context

The impact of the pollination context varied with the floral phenotypes of the focal plants (Table 5). Two distinct patterns were observed: a positive effect of neighbouring plants on focal plants with rayed phenotype, and a negative impact of neighbours on plants with intermediate and rayless phenotypes. Rayed plants were significantly more visited by big flies and hoverflies when surrounded by rayed individuals and also more visited by hoverflies when were surrounded by intermediate neighbours (Table 5). By contrast, intermediate and rayless

plants received significantly less visits from bee pollinators, when surrounded by neighbours of the same phenotype (Table 5).

Neighbourhood experimental removal

The removal of all *Anacyclus* neighbours did not produce a significant reduction in the visitation rate of the overall pollinator assemblage in both sites (Fig. 3a, b), with the exception of hoverflies within the rayless site. The removal of neighbours significantly decreased hoverflies visitation rates to rayless plants compared to those plants surrounded by an average number of capitula (Fig. 3c). By contrast, the artificially rayed plants whose neighbouring plants were removed did not suffer a reduction in hoverfly visitation (Fig. 3c).

DISCUSSION

The studied species were pollinated by a large assemblage of pollinators from an extensive variety of taxonomic groups, at least from 17 different families, belonging to several orders. The studied plant traits, namely floral phenotype, floral display and pollination context affected differently pollinator attraction and, interestingly, we found a contrasting foraging pattern between pollinator groups, specifically between Diptera and bees. Below we discuss the implications of different pollinator preferences in the evolution and maintenance of the rayed phenotype and in the degree of reproductive isolation in a generalist species contact zone.

The presence of rays was shown to have a general positive effect in pollinator attraction (Lack 1982; Marshall and Abbott 1984; Sun and Ganders 1990; Nielsen et al 2002; Celedón-Neghme et al 2007; Andersson 2008), however, rays might not affect all pollinator groups in the same way. In *Anacyclus*, rays triggered mainly the attraction of Dipteran functional groups. Although, it has been suggested that Hymenopteran are the main pollinators of Asteraceae (Lane 1996), Dipteran pollinators could also be important pollinators of rayed species. For instance, the rayed species *Achillea ptarmica* (Andersson 1991) and *Senecio vulgaris* are mainly visited by syrphids, having their rayless phenotypes a lower pollinator visitation. Moreover, a ray removal experiment in *Helianthus grosseserratus*, a rayed species visited mostly by Diptera and Hymenoptera pollinators, produced a reduction in Dipteran visitation but not in Hymenoptera (Stuessy et al 1986). These studies are in accordance with our observations and point to an effect of rays on Dipteran functional groups similar to the one observed in this study. Still, a comparative study aimed to test differences between species visited by Hymenoptera and Diptera would allow the further understanding of ray ecology and evolution.

Our ray removal/addition experiment unfortunately did not replicate the pattern observed in sympatric sites. Although we followed the same methodological approach of Nielsen et al. (2002) to create artificial rays, the ray addition seemed to fail since the artificial rayed plants attracted less pollinator than control rayed ones. This is most probably due to the complexity of this structure (Thomas et al 2009), rays can display UV reflection (Ron et al 1977) and show different micro-characters that could significantly influence pollinator attraction (Lane 1996). The microstructure of the used paper might thus not have effectively mimic natural rays. For instance, these artificial rays showed a residual reflection of UV light, while natural rays of *Anacyclus* did not. Future experiments should be careful enough not to ignore this.

Visitation rates of rayed and rayless plants were differently affected when surrounded by different flowering neighbourhood conditions. Several works have sought to understand how neighbouring conditions affect a focal plant, concluding that factors such as the scale (Bartkowska and Johnston 2014; Hegland 2014), the neighbouring density (Makino et al 2007; Hegland 2014), and the area and/or density under different population sizes (Williams 2007; Dauber et al 2010) affect pollination visitation rates. Our study adds a level of complexity to this framework, showing that the neighbours' floral phenotype might affect the visitation rate of a focal plant depending on its own phenotype. In particular, rayed plants in the sympatric site were visited more frequently by Dipterans when surrounded by other rayed individuals, whereas rayless plants did not benefit from having rayed neighbours on the surroundings and also receiving less visits by bees when surrounded by rayless neighbours. This asymmetrical relationship of rayed and rayless plants with their neighbours might deeply influence mating patterns and gene flow, and require further study. Therefore, the neighbours' phenotype might play an important role with potential consequences to pollinator selection on floral traits and should receive attention in further studies.

Understanding the foraging behaviour of pollinators is important as they can be an efficient source of prezygotic barrier and drive speciation (Lowry et al 2008). However, our results do not support the existence of reproductive isolation produced by pollinators, i.e. ethological isolation, in the generalist pollinated contact zone studied. Although Dipteran showed a preference for rayed plants, *A. clavatus*, compared to the rayless, *A. valentinus*, the same assemblage of pollinators visited both species. In addition, we observed movements of both bees and Dipteran pollinators between rayed and rayless phenotypes. Since no reduction in visitation rates is observed to intermediate phenotypes and since hybrids between the two plant species are able to produce viable seeds (Agudo & al., unpubl.), a non-discriminated visitation pattern may ultimately cause gene introgression from one species to the other.

In the absence of other forms of selection, the visitation patterns of the wide variety of pollinator groups observed in the studied hybrid zone might cause a contrasting phenotypic selection due to pollinators' differential foraging behaviour, with insect abundance being the main regulator of the direction of the selection. Generalist species have been given less attention in trait selection mediated by pollinators with only a few exceptions (Gómez et al 2009; Gómez and Perfectti 2010; Gómez et al 2014). The available studies with the generalist *Erysimum mediohispanicum* show that some pollinator groups selected for different corolla shapes whereas other groups visited flowers indiscriminately, attenuating the selection (Gómez et al 2008b). Similarly, we found that in this contact zone, Dipteran pollinators preferred rayed individuals and individuals with larger rays, whereas bees visited plants independently of the floral phenotype. We could thus expect ray selection if the preference produced by Dipteran is translated into a higher fitness on those rayed plants, while bees might diminish the pollinator-mediated effect of the Dipteran visitors (Schmid-Hempel and Speiser 1988; Thompson 2001).

Despite the observed advantages of rayed capitula in pollinator attraction, several independent reversals towards rayless capitula have occurred throughout the evolutionary history of Asteraceae, suggesting that rayless capitula could also be adaptive (Bremer and Humphries 1993; Torices et al 2011). The production of rays might entail a cost, reducing available resources for fruit and seed production (Andersson 1999; Andersson 2001; Celedón-Neghme et al 2007; Andersson 2008), and/or attracting more seed predators (Fenner et al 2002). In these *Anacyclus* species, the long flowering duration of capitula might reduce the advantage of the rayed phenotype of having more pollinator visits because stigmas within capitula could be displayed for periods longer than two weeks when there is no pollination and because a single pollinator visitor can interact and pollinate a high number of open flowers within the capitula (J. Cerca de Oliveira, R. Torices; field observation). Consequently, plants receiving fewer visits, such as rayless phenotypes, could still secure a sufficient amount of pollen, ultimately ensuring a high reproductive success and avoiding costs in ray production. However, despite apparently securing similar pollination services in terms of realised fitness, receiving a reduced number of pollinator visits will have a negative impact on male reproductive success (pollen dispersal), and indirect negative impact on female fitness, through effects of biparental inbreeding (Williams 2007; Jones and Comita 2008).

In conclusion, we found that the production of rays influenced the probability of being visited by pollinators, however, in a generalist plant hybrid zone, not all foraging groups showed a preference for the rayed phenotype, resulting in potential contrasting selection patterns, which might ultimately be regulated by insect diversity and abundance. Also, we found support for the importance of the neighbours' phenotype and conditions when assessing pollinator preference on a focal individual, with rayed plants benefiting from having other

conspicuous neighbours, whereas rayless and intermediate phenotypes did not. Still, to better understand the potential selective role of pollinators in a generalist context, studies focusing in plant distribution in a fine-scale pattern and studies of genetic patterns and parentage identification are needed to assess if differential behaviour between Dipteran groups and bees are driving different matting patterns.

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FIGURES

Fig. 1 Natural and experimentally manipulated phenotypes used in this study: a) rayed capitulum observed in *Anacyclus clavatus*; b) rayless capitulum common in *A. valentinus*; c-d) intermediate phenotypes observed in populations where *A. clavatus* and *A. valentinus* grow in sympatry; e) artificially rayless capitula (highlighted with black arrows) in a pure rayed site; f) artificially rayed capitula (highlighted with black arrows) in a pure rayless site.
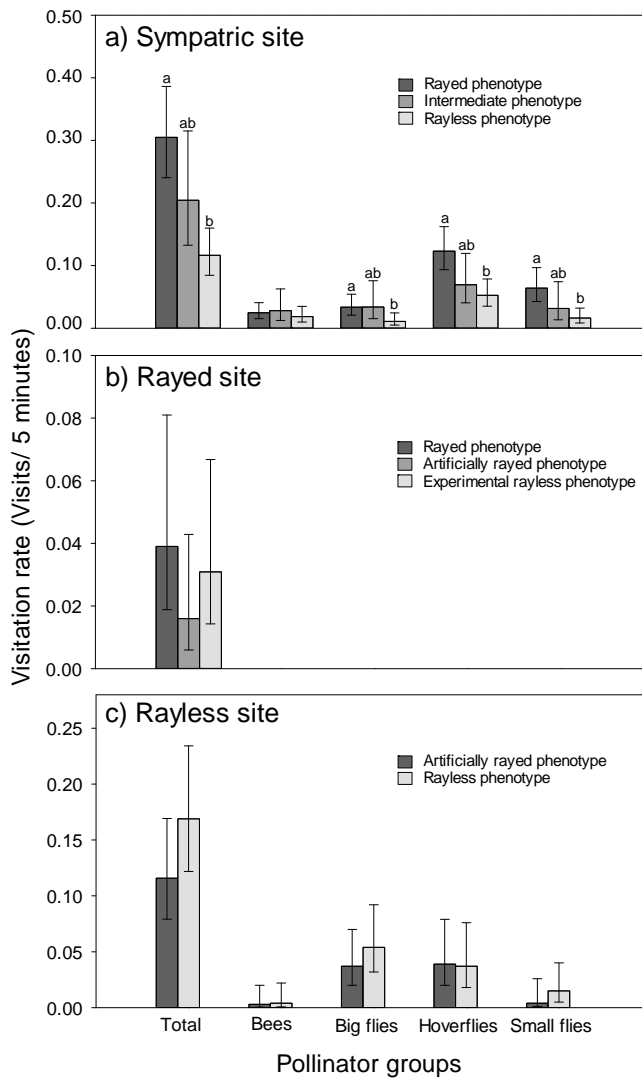
Fig. 2 Least square means (± 95% confidence intervals) of the visitation rate (number of visits per 5 minutes intervals) for the entire pollinator assemblage (Total) and for particular pollinator groups observed (bees, big flies, hoverflies and small flies) in the studied sites: a) sympatric site, b) the rayed site and c) the rayless site. Means sharing different letters were significantly different at $P < 0.05$. No letter is displayed in cases where no statistically differences were found.
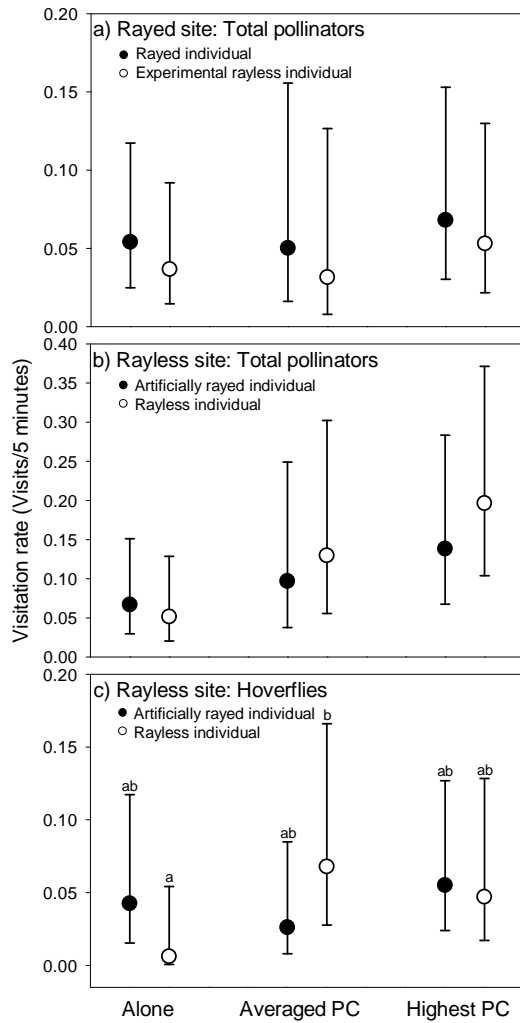
Fig. 3 Least square means (± 95% confidence intervals) of pollinator visitation rate (number of visits per 5 minutes intervals) in rayed (black dots) and rayless (white dots) plants under different neighbouring conditions, namely solitary plants, plants with averaged pollination context and plants with the largest pollination context. The entire pollinator assemblage is displayed for: a) the rayed site and b) the rayless site, while c) displays hoverfly in the rayless site. Means sharing different letters were significantly different at $P < 0.05$. No letter is displayed in cases where no statistically differences were found. PC = Pollination context.

TABLES

Table 1. Results of the generalized linear mixed model analysis (GLMM) on the effects of floral phenotype (rayed, intermediate and rayless phenotypes), floral display and pollination context on pollinator attraction for the entire pollinator assemblage (Total) and for particular pollinator groups (bees, big flies, hoverflies and small flies) in the sympatric site. Plant identity was used as a random variable. Statistical significances ($P < 0.05$) are shown in bold. The sign of significant slopes ($P < 0.05$) for continuous variables (floral display and pollination context) is indicated with (+) or (-)

Sympatric site		Total		Bees		Big flies		Hoverflies		Small flies	
Variables	*Df*	χ^2	***P***	χ^2	***P***	χ^2	***P***	χ^2	***P***	χ^2	***P***
Fixed											
Floral phenotype	2	20.76	**0.00003**	0.85	0.656	7.36	**0.025**	12.46	**0.002**	13.04	**0.002**
Floral display	1	1.07	0.302	(+) 9.09	**0.003**	0.0001	0.994	(+) 4.63	**0.032**	0.78	0.377
Pollination context	1	0.92	0.337	0.26	0.612	(+) 3.34	0.068	(+) 4.20	**0.040**	0.07	0.797
		Variance	**SD**	**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**
Random											
Plant		0.405	0.637	0.631	0.795	0.827	0.963	0.355	0.596	0.776	0.881

Table 2. Results of the generalized linear mixed model analysis (GLMM) on the effects of floral phenotype (rayless and artificially rayed phenotypes), floral display and pollination context on pollinator attraction for the entire pollinator assemblage (Total) and for particular pollinator groups (bees, big flies, hoverflies and small flies) in the rayless site. Plant identity was used as a random variable. Statistical significances ($P < 0.05$) are shown in bold. The sign of significant ($P < 0.05$) slopes for continuous variables (floral display and pollination context) is indicated with (+) or (-)

Rayless site		Total		Bees		Big flies		Hoverflies		Small flies	
Variables	*Df*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*
Fixed											
Floral phenotype	1	2.39	0.112	0.11	0.742	0.88	0.349	0.01	0.909	2.01	0.156
Floral display	1	0.40	0.526	0.11	0.737	0.98	0.322	0.59	0.443	0.37	0.543
Pollination context	1	0.11	0.745	(+) 4.03	**0.040**	0.55	0.458	1.95	0.163	0.04	0.838
		Variance	**SD**	**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**
Random											
Plant		0.031	0.117	3.395	1.843	0	0	0.757	0.870	0	0

70

Table 3. Results of the generalized linear mixed model analysis (GLMM) on the effects of disc size, floral display and pollination context on floral visitor attraction on pollinator attraction for the entire pollinator assemblage (Total) and for particular pollinator groups (bees, big flies, hoverflies and small flies) in the sympatric site. Rayed and intermediate individuals were analysed (*RAYED PHEN.*) independently from rayless individuals (*RAYLESS PHEN.*). Plant identity was used as a random variable. Statistical significances ($P < 0.05$) are shown in bold. The sign of slopes ($P < 0.10$) for continuous variables (disc size, floral display and pollination context) is indicated with (+) or (-)

Sympatric site		Total		Bees		Big flies		Hoverflies		Small flies	
Variables	*Df*										
RAYED PHEN.		χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*
Fixed											
Disc size	1	2.20	0.138	1.05	0.306	1.82	0.170	1.40	0.237	0.07	0.790
Floral display	1	0.31	0.575	(+) 7.38	**0.007**	0.04	0.836	(+) 2.94	0.086	0.62	0.432
Pollination context	1	1.04	0.307	0.01	0.943	(+) 4.34	**0.037**	(+) 3.03	0.082	0.10	0.756
Random		**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**
Plant		0.491	0.701	0.638	0.799	0.864	0.930	0.864	0.690	1.166	1.080
RAYLESS PHEN.		χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*
Fixed											
Disc size	1	1.58	0.209	1.73	0.188	0.16	0.691	0.47	0.494	0.00086	0.977
Floral display	1	1.65	0.199	(+) 3.02	0.082	0.59	0.444	0.85	0.356	2.13	0.145
Pollination context	1	0.64	0.422	2.49	0.115	0.07	0.792	0.10	0.749	2.21	0.137
Random		**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**
Plant		0.055	0.235	0	0	0	0	0	0	0	0

Table 4. Results of the generalized linear mixed model analysis (GLMM) on the effects of ray length, floral display and pollination context on pollinator attraction for the entire pollinator assemblage (Total) and for particular pollinator groups (bees, big flies, hoverflies, small flies) for rayed and intermediate individuals in the sympatric site. Plant identity was used as a random variable. Statistical significances ($P < 0.05$) are shown in bold. The sign of significant slopes ($P < 0.05$) for continuous variables (floral display and pollination context) is indicated with (+) or (-)

Sympatric site		Total		Bees		Big flies		Hoverflies		Small flies	
Variables	*Df*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*
Fixed											
Ray length	1	(+) 9.10	**0.0026**	0.001	0.976	1.30	0.255	(+) 7.95	**0.005**	(+) 7.58	**0.006**
Floral display	1	0.71	0.401	(+) 7.58	**0.006**	0.04	0.849	(+) 4.13	**0.042**	0.31	0.577
Pollination context	1	1.46	0.227	0.0075	0.936	(+) 4.33	**0.037**	(+) 3.22	0.072	0.19	0.663
		Variance	**SD**	**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**	**Variance**	**SD**
Random											
Plant		0.410	0.640	0.698	0.836	0.918	0.958	0.366	0.605	0.948	0.973

Table 5. Results of the generalized linear mixed model analysis (GLMM) on the effects of specific neighbourhoods (rayed, intermediate or rayless neighbourhood) on subsets regarding rayed, intermediate and rayless separately (focal individuals) for the entire pollinator assemblage (Total), and for different pollinator groups (bees, bees, big flies, hoverflies, small flies). Floral display and pollination context were also used for this analysis though results are not displayed. Plant identity was used as a random variable (results not shown). Statistical significances ($P < 0.05$) are shown in bold. Significant ($P < 0.05$) and marginally significant ($P < 0.10$) regression coefficients (b) slopes are also displayed.

Focal plant phenotype	Neigbourhoods	Total	Bees	Big flies	Hoverflies	Small flies
Rayed	Rayed	ns	ns	$b = 0.05, P = 0.045$	$b = 0.06, P = 0.023$	ns
	Intermediate	ns	ns	ns	$b = 0.12, P = 0.011$	ns
	Rayless	ns	ns	$b = 0.06, P = 0.090$	ns	ns
Intermediate	Rayed	ns	ns	ns	ns	ns
	Intermediate	ns	$b = -0.65, P = 0.023$	ns	ns	ns
	Rayless	ns	$b = -0.19, P = 0.055$	ns	ns	ns
Rayless	Rayed	ns	$b = -0.09, P = 0.071$	ns	ns	$b = -0.09, P = 0.090$
	Intermediate	ns	ns	ns	ns	ns
	Rayless	ns	$b = -0.58, P = 0.018$	ns	ns	ns

Supplementary information

Electronic Supplementary Material A. Descriptive characterization of plant, capitulum and surrounding traits of the *Anacyclus* individuals selected in the sympatric site. Description includes mean and SE of all the measured traits for all the plants selected and for each phenotype (Rayed, Intermediate and Rayless). Statistical significances ($P < 0.05$), obtained by means of a glm model, comparing trait values across the different phenotypes are shown in bold (degrees of freedom, *Df*, are also provided). Description and statistical *P* values from the number of rays and ray length variables include only plants with rays (rayed and intermediate phenotypes; n = 55). Studied traits include: Plant height - distance in mm from the ground to the tallest part of the plant; Plant dimension - circular area in cm^2 whose diameter was the mean value between the plants' largest diameter and its perpendicular axis; Floral display - number of open capitula per individual at each observation day; Capitulum diameter - diameter in mm from the tip of a ray to the tip of the opposite ray; Disc diameter - diameter in mm of the central yellow disc; Number of rays - number of rayed female florets per capitulum; Ray length – ray extension from the tip to the central yellow disc in mm; Pollination context – number of open capitula of *Anacyclus* neighbours within a 0.5 m radius. Sample size (n) is also provided.

	All phenotypes	Rayed	Intermediate	Rayless		
Sympatric site	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	*P*	*Df*
Plant traits						
Plant height (mm)	220.53 ± 10.07	220.17 ± 15.01	223.57 ± 29.57	219.71 ± 15.38	0.99	2
Plant dimension (cm^2)	516.41 ± 79.02	568.96 ± 135.06	506.26 ± 144.96	457.23 ± 115.34	0.81	2
Floral display	5.99 ± 0.58	6.41 ± 0.89	7.91 ± 2.04	4.70 ± 0.62	0.13	2
Capitulum traits						
Capitulum diameter (mm)	21.20 ± 0.87	28.89 ± 0.60	19.26 ± 1.27	12.72 ± 0.31	**2.2e-16**	2
Disc diameter (mm)	12.48 ± 0.15	12.33 ± 0.19	12.47 ± 0.36	12.68 ± 0.30	0.58	2
Number of rays	5.78 ± 0.52	9.40 ± 0.37	9.20 ± 0.63	-	0.79	1
Ray length (mm)	4.36 ± 0.44	8.28 ± 0.29	3.39 ± 0.60	-	**2.517e-11**	1
Neighbourhood traits						

Pollination context	7.44 ± 1.19	6.25 ± 1.62	13.39 ± 4.07	6.44 ± 1.70	0.09	2
n	89	41	14	34		

Electronic Supplementary Material B. Absolute and relative frequencies of the floral visitors of Anacyclus spp. capitula in each studied site. Data is taxonomically displayed, including the orders Coleoptera, Diptera, Hemiptera, Hymenoptera and Lepidoptera. On the sympatric site the rayed, intermediate and rayless phenotypes are respectively displayed. On the rayless site the artificially-rayed and the rayless phenotype are displayed respectively. Lastly, for the rayed site the rayed and the rayless phenotypes are displayed respectively. Insects where identification was complex were identified until family whenever possible and included in a category of not identified (non id.).

	Sympatric site										Rayless site								Rayed site						
		%		%		%	Total	%		%		%	Total	%		%		%	Total	%						
Coleoptera	10	**4**	2	**3.1**	2	**2.2**	14	**3.4**	6	**6.4**	1	**1.2**	7	**4**	2	**7.1**	0	**0**	2	**3.5**
Diptera	203	**80.9**	51	**79.7**	63	**67.7**	317	**77.7**	43	**45.7**	39	**48.2**	82	**46.9**	6	**21.4**	4	**13.8**	10	**17.6**
Hemiptera	1	**0.4**	0	**0**	1	**1**	2	**0.5**	0	**0**	1	**1.2**	1	**0.6**	2	**7.1**	4	**13.8**	6	**10.5**
Hymenoptera	36	**14.3**	11	**17.2**	22	**23.7**	69	**16.9**	42	**44.7**	37	**45.7**	79	**45.1**	11	**39.4**	10	**34.4**	21	**36.8**
Lepidoptera	1	**0.4**	0	**0**	5	**5.4**	6	**1.5**	3	**3.2**	3	**3.7**	6	**3.4**	7	**25**	11	**38**	18	**31.6**
Total	251	**100**	64	**100**	93	**100**	408	**100**	94	**100**	81	**100**	175	**100**	28	**100**	29	**100**	57	**100**
Coleoptera																				
Cetoniidae																				
Oxythyrea funesta	0	**0**	0	**0**	0	**0**	0	**0**	1	**14.3**	0	**0**	1	**14.3**	0	**0**	0	**0**	0	**0**
Cantharidae																				
Rhagonicha fulva	0	**0**	0	**0**	0	**0**	0	**0**	2	**28.6**	0	**0**	2	**28.6**	0	**0**	0	**0**	0	**0**
Malachiidae																				

Clanoptilus abdominalis	1	**7.1**	0	**0**	1	**7.1**	2	**14.2**	0	**0**	1	**14.3**	1	**14.3**	0	**0**	0	**0**	0	**0**
Oedemeridae																				
Oedema simplex	1	**7.1**	0	**0**	0	**0**	1	**7.1**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
Dermestidae																				
Attagenus sp.	0	**0**	1	**7.1**	0	**0**	1	**7.1**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
Others																				
non id.	8	**57.2**	1	**7.1**	1	**7.1**	10	**71.6**	3	**42.8**	0	**0**	3	**42.8**	2	**100**	0	**0**	2	**100**
Total	10	**71.6**	2	**14.2**	2	**14.2**	14	**100**	6	**85.7**	1	**14.3**	7	**100**	2	**100**	0	**0**	2	**100**
Diptera																				
Syrphidae																				
Eristalis tenax	23	**7.5**	4	**1.3**	8	**2.6**	35	**11.4**	3	**3.7**	0	**0**	3	**3.7**	2	**20**	2	**20**	4	**40**
Eristalis arbustorum	1	**0.3**	1	**0.3**	0	**0**	2	**0.6**	1	**1.2**	0	**0**	1	**1.2**	0	**0**	0	**0**	0	**0**
Eupeodes sp.	17	**5.6**	6	**1.8**	5	**1.6**	28	**9**	6	**7.2**	3	**3.7**	9	**11**	0	**0**	0	**0**	0	**0**
Episirphus sp.	0	**0**	0	**0**	5	**1.6**	5	**1.6**	1	**1.2**	1	**1.2**	2	**2.5**	0	**0**	0	**0**	0	**0**
Sphaerophoria sp.	51	**16.3**	15	**4.8**	19	**6**	85	**27.1**	6	**7.2**	7	**8.6**	13	**15.9**	1	**10**	0	**0**	1	**10**
Syritta pipiens	2	**0.6**	0	**0**	0	**0**	2	**0.6**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
Chrysotoxum sp.	1	**0.3**	0	**0**	0	**0**	1	**0.3**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
non id.	8	**2.6**	0	**0**	1	**0.3**	9	**2.9**	4	**5**	3	**3.7**	7	**8.7**	0	**0**	0	**0**	0	**0**
Bombyliidae																				
Conophurus sp.	0	**0**	0	**0**	0	**0**	0	**0**	6	**7.2**	3	**3.7**	9	**11**	0	**0**	0	**0**	0	**0**
non id.	0	**0**	0	**0**	0	**0**	0	**0**	4	**4.9**	1	**1.2**	5	**6.1**	0	**0**	0	**0**	0	**0**
Tachinidae																				
Tachina fera	0	**0**	1	**0.3**	1	**0.3**	2	**0.6**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
Scathophagidae																				
Scathophaga stercoraria	1	**0.3**	0	**0**	0	**0**	1	**0.3**	2	**2.4**	1	**1.2**	3	**3.6**	0	**0**	0	**0**	0	**0**

non id. Miltogramminae	0	**0**	1	**0.3**	0	**0**	1	**0.3**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
Calliphoridae																				
Calliphora vomitoria	3	**1**	2	**0.6**	0	**0**	5	**1.6**	1	**1.2**	2	**2.4**	3	**3.6**	0	**0**	0	**0**	0	**0**
Lucilia caesar	1	**0.3**	0	**0**	2	**0.6**	3	**0.9**	1	**1.2**	3	**3.7**	4	**4.9**	1	**10**	0	**0**	1	**10**
Anthomyzidae																				
non id.	12	**3.9**	2	**0.6**	3	**1**	17	**5.5**	0	**0**	1	**1.2**	1	**1.2**	0	**0**	0	**0**	0	**0**
Others																				
non id. Diptera	31	**8.7**	11	**3.4**	7	**2.2**	49	**14.3**	6	**7.2**	10	**12.4**	16	**19.2**	1	**10**	2	**20**	3	**30**
non id. small Diptera	52	**16.6**	8	**2.6**	12	**3.8**	72	**23**	2	**2.4**	4	**5**	6	**7.4**	1	**10**	0	**0**	1	**10**
Total	203	**64**	51	**16**	63	**20**	317	**100**	43	**52.4**	39	**47.6**	82	**100**	6	**60**	4	**40**	10	**100**
Hemiptera																				
Non id. Hemiptera	1	**50**	0	**0**	1	**50**	2	**100**	0	**0**	1	**100**	1	**100**	2	**33.3**	4	**66.7**	6	**100**
Total	1	**50**	0	**0**	1	**50**	2	**100**	0	**0**	1	**100**	1	**100**	2	**33.3**	4	**66.7**	6	**100**
Hymenoptera																				
Formicidae																				
non id.	1	**1.5**	0	**0**	1	**1.5**	2	**3**	30	**37.9**	22	**27.8**	52	**65.7**	0	**0**	1	**4.8**	1	**4.8**
Apidae																				
Apis mellifera	12	**17.4**	4	**5.8**	11	**15.8**	27	**39**	1	**1.3**	5	**6.3**	6	**7.6**	6	**28.6**	7	**33.2**	13	**61.8**
Anthophora sp.	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	2	**9.5**	0	**0**	2	**9.5**
Eucera longicornis	0	**0**	0	**0**	0	**0**	0	**0**	1	**1.3**	0	**0**	1	**1.3**	0	**0**	0	**0**	0	**0**
Ammobates sp.	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
non id.	0	**0**	0	**0**	2	**3**	2	**3**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
Megachilidae																				
non id.	1	**1.5**	0	**0**	0	**0**	1	**1.5**	0	**0**	0	**0**	0	**0**	0	**0**	1	**4.8**	1	**4.8**

Halictidae																				
Lasioglossum sp.	3	**4.3**	5	**7.1**	2	**3**	10	**14.4**	3	**3.8**	4	**5.2**	7	**9**	0	**0**	0	**0**	0	**0**
Sphecidae																				
non id.	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	1	**4.8**	1	**4.8**
Others																				
non id.	19	**27.5**	2	**3**	6	**8.6**	27	**39.1**	7	**8.9**	6	**7.5**	13	**16.4**	3	**14.3**	0	**0**	3	**14.3**
Total	36	**52.2**	11	**15.9**	22	**31.9**	69	**100**	42	**53.2**	37	**46.8**	79	**100**	11	**52.4**	10	**47.6**	21	**100**
Lepidoptera																				
Pieridae																				
Colias croceus	0	**0**	0	**0**	1	**17**	1	**17**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
Nymphalidae																				
Pararge aegeria	1	**17**	0	**0**	0	**0**	1	**17**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0	**0**
Others																				
non id.	0	**0**	0	**0**	4	**66**	4	**66**	3	**50**	3	**50**	6	**100**	7	**38.9**	11	**61.1**	18	**100**
Total	1	**17**	0	**0**	5	**83**	6	**100**	3	**50**	3	**50**	6	**100**	7	**38.9**	11	**61.1**	18	**100**

Electronic Supplementary Material C: Pearson correlation coefficient characterization of plant, capitulum and surrounding traits for *Anacyclus* individuals selected in the sympatric site. Characterization includes correlation values for each trait, with statistically significant Pearson correlation coefficients highlighted in bold. Statistical significances ($P < 0.05$) were adjusted for multiple tests. Correlation and statistical P values from the number of rays, disc diameter and ray length variables include only plants with rays (rayed and intermediate phenotype; n = 55). The remaining traits were performed with data from all phenotypes (n = 89).

Sympatric site	Plant			Capitulum			
Traits	Plant height	Plant Dimension	Floral display	Capitulum diameter	Disc diameter	Number of rays	Ray length
Plant							
Plant height							
Plant dimension	0.02						
Floral display	0.02	**0.79**					
Capitulum							
Capitulum diameter	0.08	0.10	0.14				
Disc diameter	0.11	0.01	0.04	0.18			
Number of rays	-0.04	0.09	0.06	0.15	-0.12		
Ray length	0.05	0.11	-0.02	**0.98**	-0.04	0.18	
Neighbourhood							
Pollination context	**0.70**	-0.08	- 0.04	-0.07	0.01	0.06	- 0.08

1.3. Relative reproductive isolation and inheritance of floral traits in *Anacyclus* L. (Anthemideae, Asteraceae) species complex

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Abstract

Reproductive isolation is one of the criteria for species delimitation and its assessment is crucial to understand hybrid speciation. *Anacyclus valentinus*, a gynomonoecious species with discoid capitula has been considered of hybrid origin between *A. clavatus*, a gynomonoecious species with radiate capitula, and *A. homogamos*, which is hermaphroditic with discoid capitula. In the present these three species partially overlap their areas of distribution where gene flow between them may occur. Evidence of current hybridization was already revealed by genetic data and genome size in natural populations and synthetic F_1 hybrids, in which intermediate floral phenotypes were reported. Here, we determine the mating system for these species and estimate the existence of reproductive barriers between them by experimental crosses (F_1, F_2 and BCs). The inheritance of the sexual system and floral traits such as the number of female flowers and its ray length was also explored. Despite these three species were inter-fertile, a significant decrease in fertility of the F_1 hybrids between all the species pairs indicate the existence of post-zygotic barriers among these species. Ratios of sexual systems observed in the F_1s, F_2s, and BCs indicated that two genes interact by duplicate recessive epistasis for the gynomonoecy expression. This model implies fixed heterozygosis in one of these loci in *A. valentinus*, whereas in *A. homogamos* heterozygosis is not necessarily fixed, and homozygosis for the two loci is required in *A. clavatus*. This model is congruent with the hybrid origin of both *A. homogamos* and *A. valentinus*.

Introduction

The reproductive biology is a keystone in understanding species biogeographic and genetic patterns, and important processes such as hybridization and speciation (Darwin 1876; Richards 1986; Morgan & Schoen 1997; Holsinger 2000; Barrett 2002; Wright *et al*. 2008). In plants, the huge diversity observed in sexual systems across lineages (Barrett 2010) makes necessary its study in each system at both specific (Case *et al.* 2008; Blambert *et al.* 2016) and population levels (Krawczyk *et al.* 2016; Broz *et al.* 2017). Sexual selection and inbreeding have relevant implications for the evolution of the different sexual and mating systems (Shuster 2009; Losdat *et al.* 2014; Lankinen and Green 2015).

Among the sexual systems in plants, hermaphroditism is the most common, whereas others such as dioecy, monoecy or gynomonoecy are much less frequent (Richards 1997). However, in Asteraceae there is a disproportionate representation of the gynomonoecy compared to any other plant families (Yampolsky and Yampolsky 1922; Torices *et al.* 2011). Gynomonoecy in Asteraceae is usually linked to the presence of ray peripheral female flowers (i.e., the radiate capitulum) (Torices and Anderberg 2009; Torices *et al.* 2011). This association between ray and female flowers could indicate a functional link between both structures. Thus, it has been proposed that selection for this showy inflorescence might have led to subsequent reduction of stamens in these flowers to pay off the cost of the ligule production (Bawa and Beach 1981).

The presence of radiate and non-radiate capitula is frequent in some Asteraceae tribes (e.g., Anthemideae, Inuleae, Senecioneae, etc.), within the same genus (e.g., *Layia*, *Matricaria*, *Senecio*, *Tanacetum*, among others), and even within the same species (e.g., in *Bidens pilosa* L., *Senecio vulgaris* L.), motivating investigations on the evolution of this floral trait. In *Senecio*, hybridization was identified as the process involved in the origin of some

radiate morphs and species (Abbott *et al.* 1992, 2009, Lowe and Abbott 2000, James and Abbott 2005). This was a relevant fact in determining the participation of two major loci in the ray flower expression (Andersson 2001) in detriment of the previous ideas in which one dominant locus would control the presence of ray flowers (Trow 1912, Richards 1975, Ingram & Taylor 1982). The hypothesis of the existence of at least two loci was suggested in other genera (i.e. in *Layia* by Ford and Gottlieb 1990, and in *Dubautia*, *Madia* and *Raillardiopsis* by Carr *et al.* 1996). In addition, it is known that the *cycloidea* family genes (CYC genes) that control floral symmetry are involved in the expression of Asteraceae ray flowers (Gillies *et al.* 2002, Broholm *et al.* 2008, Kim *et al.* 2008, Chapman and Abbott 2010). However, despite all this knowledge on the expression of this trait, the genic interactions among loci are still uncertain, and gynomonoecy have been obviated in all these studies due to its link to the radiate capitula. This association does not exist in different species across the family, including some genera of Anthemideae (*Anthemis*, *Cotula*, *Soliva*, and *Anacyclus*) in which some species with non-radiate capitula present female peripheral flowers.

Anacyclus is a Mediterranean genus represented by around ten species of mostly annual herbs (Humphries 1979). Two of these species (*A. homogamos* and *A. monanthos*) are hermaphroditic with non-radiate capitula, and the remaining are all gynomonoecious with radiate capitula except in *A. valentinus* which capitula are non-radiate. This morphology in a gynomonoecious species was interpreted by Humphries (1979) as a consequence of hybridization between one hermaphroditic and another gynomonoecious species, based also on the frequent overlapping areas of distribution among species. Additionally, current gene flow between *A. valentinus* and *A. clavatus* was documented to occur in sympatric populations in which radiate, non-radiate capitula and intermediate phenotypes (i.e., shortly radiate capitula) were found (Agudo et al. in prep), suggesting no reproductive barriers

between them. The extremely rare presence of *A. homogamos* in scattered places in the Iberian Mediterranean coast, a species mainly distributed in the Mid-Atlas region in Morocco (Humphries 1979), adds complexity to this system in which the knowledge of its reproductive biology seems crucial to understand its evolution. Here, we experimentally crossed the three species of the complex: *A. clavatus*, *A. homogamos* and *A. valentinus* to assess reproductive isolation and investigate the segregation of floral traits and gynomonoecy. Our specific aims were: 1) to determine the mating system for the three studied species; 2) to estimate the magnitude of the reproductive barriers among these three species; and 3) to determine the type of inheritance of the sexual system and radiate capitula to understand the frequency of phenotypes observed in natural sympatric populations.

Materials and methods

Study system

The three studied species, *Anacyclus clavatus*, *A. homogamos*, and *A. valentinus* are all annual herbs growing in anthropogenic habitats in Western Mediterranean that partially overlap their areas of distribution (Humphries, 1979; Álvarez, in rev.). *Anacyclus clavatus* occupies the largest area, from coastal to inland regions; *A. homogamos* is mainly restricted to Middle and High Atlas in Morocco, although a few and scattered observations in coastal regions in Algeria, Morocco, and Spain were made in the recent past; and *A. valentinus* is widely distributed in coastal regions, although may also be observed in Morocco inland areas. Morphologically, they only differ by their sexual systems (i.e., hermaphroditic in *A. homogamos* vs. gynomonoecious in *A. clavatus* and *A. valentinus*), and by the ray length (i.e., ligule length hereafter) and width in the corolla of the female flowers (i.e., absent to

inconspicuous ligules in *A. valentinus* resulting in discoid capitula vs. showy white ligules in *A. clavatus* that form radiate capitula). Capitula in *A. homogamos* are discoid, and all flowers are tubular. Phylogenetic analysis based on sequences of several nuclear and chloroplast markers indicate a close relationship between *A. clavatus* and *A. valentinus*, whereas *A. homogamos* belongs to a different clade (Oberprieler, 2004; Agudo *et al.* "unpubl."). Although all species in this genus present the same chromosome number (2n = 18), differences in genome size between them were observed (Nagl and Ehrendorfer, 1974; Humphries, 1981). A recent study focused on genome size populations in our study system (Agudo *et al.*, "in rev.") revealed a significant different genome size in *A. valentinus* (8.39 pg/2C) compared to those in *A. clavatus* and *A. homogamos* (~ 10 pg/2C), which are not distinct to each other.

Plant material

Seeds from six natural populations of *A. clavatus*, *A. homogamos*, and *A. valentinus* (two populations per species), were collected for sowing and cultivation in the Research Greenhouse of the Royal Botanic Garden-CSIC in Madrid (Table S1). The two populations selected of each species are located far enough (at least 50 km apart) to ensure geographic isolation between them. Sampling and sowing was previously described in Torices *et al.* (2013). After germination, seedlings were grown individually in a mix of COMPO SANA® Universal Potting Soil and siliceous sand (3:1) until the first 4-6 leaves were developed. After that, around 20-30 plants per population were planted out in a similar soil mix for the experimental crosses and phenotypic characterization. Out of these, 4-7 plants per population were selected as ovule donors (Table S2) and the remaining were designated as pollen donors.

Floral traits of female flowers were also characterized in plants collected in the field (Table S1).

Mating system and interspecific crosses

The mating system of the studied species was determined based on the effective number of mature seeds per capitulum (seed set) produced by the plants selected as ovule donors after each treatment. Eight different treatments (one treatment per capitulum) were performed on each mother plant: 1) no pollen addition to test autogamy in the absence of pollen vectors; 2) pollen addition of the same individual to test self-fertilization in the presence of pollen vectors, hereafter the self-compatibility test; 3) pollen addition of individuals from the same population of the mother plant to test outcrossing, used as reference for the intra-population cross; 4) pollen addition of individuals from different population of the same species, used as reference for the inter-population cross; 5-8) pollen addition of each of the four remaining populations of different species to test viability of inter-specific crosses. All treated capitula were bagged before anthesis until fruits were collected. A mix of pollen from different individuals was used for each treatment to ensure viability and to favour pollen saturation. Pollen was collected from the tip of the style with the aid of a tweezers and was kept and mixed in 1.5 ml Eppendorf tubes for its immediate use. Pollen addition was made using a paintbrush for each capitulum treated. Each treatment started when the first stigmatic branches are developed in the capitulum of the mother plant and it finished at least a week after the last stigmatic branches were developed in each case (i.e., 2-4 weeks depending on the size of the capitulum). All treatments were performed in 2012. Fruits were collected at least 4 weeks after the treatment was finished. Since in the Asteraceae each flower may

produce only one seed, the total number of mature seeds and flowers were counted in each capitulum to calculate the seed set rate for each treatment (i.e., number of mature seeds / number of flowers). All these seeds constituted the F_1s used in subsequent generations. Additionally, germination success following Torices *et al*. (2013) was also analysed in each case.

The second hybrid generation

To explore the existence of post-zygotic barriers, a second generation of hybrids (F_2s) and back-crosses (BCs) were obtained using the methods described above. In this case one population per species and 3-8 ovule donor plants were selected from each type of cross (Table S3). In order to couple phenology, winged achenes, whose seeds have faster germination times (Torices *et al*., 2013), were selected for sowing in all cases. Three treatments (one per capitulum) were performed on each hybrid individual: 1) pollen addition of individuals of the same cross (F_2); 2) pollen addition of individuals of one of the parents (BC); 3) pollen addition of individuals of the other parent (BC). Additionally, pollen from F_1 hybrids was added to individuals from the parents' populations to test pollen viability of the hybrids. Due to room limitation in the greenhouse, the crosses to obtain the F_2s and BCs corresponding to the species pair *A. clavatus* and *A. homogamos* were performed in 2013, whereas those corresponding to *A. clavatus* and *A. valentinus*, as well as those between *A. homogamos* and *A. valentinus* were done in 2014.

Phenotypic characterization

All cultivated plants including parental lines and hybrid generations (F_1s, F_2s, and BCs) were used for phenotypic characterization. One capitulum per individual was haphazardly selected for floral traits observations. Four traits of female flowers were studied: number of flowers, corolla length (i.e., ligule and tube length), ligule length, and ligule maximum width. Additionally, other morphological characters studied were: maximum length of the aerial part of the individual (i.e., length from the base of the stem to the tip of the longest branch), maximum diameter of the stem, and number of capitula. Floral traits observations were made with the aid of Olympus SZX7 binoculars.

Statistical analyses

Experimental crosses were assessed by Generalized Linear Mixed models (GLMMs). The effect of different crosses on the probabilities of setting a viable seed and on variation of morphological traits were investigated by fitting GLMMs via restricted maximum likelihood (Patterson & Thompson, 1971) with the SAS 9.4 software (SAS Institute, Cary, NC) using the GLIMMIX procedure with the DIFF option in the LSMEANS statement. Satterthwaite's method was used to determine the approximate denominator degrees of freedom. The probability of producing a viable seed was modelled using a binary distribution with a logit function; whereas the number of female flowers was modelled using a negative binomial or Poisson distribution with a log function. Finally, corolla length of female flower, ligule length, and ligule width, were modelled using a Gaussian distribution with a log function. All models included one fixed factor: the pollination treatment (i.e., fertilization with the different types of pollen), and one random factor: the ovule donor plants.

In addition, we assessed whether the observed segregation of F_1s, F_2s and BCs phenotypes fitted to the expected values under a double recessive epistasis model for the gynomonoecy genetic control. *p*-values were computed by Monte Carlo simulation with 5000 replicates. All these tests were performed in R 3.3.2 (R Core Team, 2016). Finally, to explore the existence of vegetative characters that could distinguish among species, a discriminant analysis was performed with the SPSS software (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.).

Results

Mating system

The three studied species showed very low ability of producing fruits by self-fertilization. Both autogamy and self-pollination treatments led to very low number of seeds being always significantly lower than the outcrossing intra-population control (Fig. 1, Table 1).

Intra- and inter-specific crosses, F_1s, F_2s, and BCs

In *A. clavatus* the seed set yielded by both intra- (min-max: 86-92%, n = 694) and inter-specific crosses (66-95%, n = 2665) were not significantly different to those of the corresponding intra-population controls (Table 1), although specifically in two treatments (pollen from population "At" of *A. homogamos* and pollen from population "W" of *A. valentinus*) a significant decrease on seed set was observed (Fig. 1). In contrast, the *A. homogamos* inter-specific crosses produced significant higher seed sets than the intra-population controls (Table 1), with the exceptions of pollen addition from populations "V" of

A. clavatus and "W" of *A. valentinus* that showed similar and significantly lower values, respectively (Fig. 1). In the case of *A. valentinus*, a high variation on seed set produced by all treatments was observed, including the intra-population control. The seed set was significantly lower for the intra-specific crosses and when pollen from populations "At" of *A. homogamos* and "V" of *A. clavatus* was added (Fig. 1).

Results of germination tests, including those seeds obtained by autogamy and self-pollination, showed high success in all cases (>75%, n = 691) and were similar to those obtained by natural populations in the field (data not shown). Individuals from autogamy survived in 76% (n = 45) of cases, of which 62% (n = 34) developed flowers, although in 24% (n = 21) of flowering individuals the pollen production failed. In contrast, after all interspecific crosses, the 98% of germinated individuals (n = 610) survived, of which 98.5% (n = 601) developed flowers, and only 0.7% did not produced pollen.

Seed set in all F_1 hybrid lines decreased significantly compared to their corresponding parental lines (Fig. 2). This decline on fertility was observed on both F_2s and BCs for each line (Fig. 2, Table 2). A remarkable higher variation in fertility was observed in those crosses in which *A. valentinus* was involved (Fig. 2).

In addition, fertilization treatments with pollen from hybrid individuals (F_1s) on non-hybrid lines yielded lower seed set compared with the intra-specific controls and were statistically significant in *A. clavatus* and *A. homogamos* but not in *A. valentinus* (Fig. 3). However, germination in the second generations for all crosses in both hybrid and non-hybrid lines was successful at least in 75% (n = 800) of cases, similar to the corresponding F_1s. Out of these, on average, the 88% (n = 738) developed flowers.

Phenotypic characterization on the species complex

Floral traits differed between the three species. All these traits resulted significantly different between *A. clavatus* and *A. valentinus* ($F_{1, 43} = 48.43$, $p \leq 0.0001$ for number of female flowers; $F_{1, 43} = 65.45$, $p \leq 0.0001$ for corolla length; $F_{1, 43} = 15.84$, $p = 0.0003$ for ligule length; and $F = 19.86_{1, 38}$, $p \leq 0.0001$ for ligule width), whereas in *A. homogamos* the absence of female flowers allowed to unambiguously distinguish this species from *A. clavatus* and *A. valentinus*.

However, other non-floral morphological characters did not show clear differences between species. A discriminant analysis was not able to clearly distinguish among species, although a trend of larger individuals (longer and thicker stems) were observed in *A. clavatus* and *A. valentinus* vs. *A. homogamos*, and a higher number of capitula were observed in *A. homogamos* and *A. valentinus* vs. *A. clavatus* (Fig. S1). Nevertheless, these traits were highly variable within each species and significant differences were observed between populations for each species, except in *A. valentinus* for the maximum diameter of the stem and the number of capitula (Fig. 4C-D). Taking into account that vegetative characters did not allowed distinguishing among species, only differences in sexual systems and female floral traits were analysed in the subsequent generations.

Sexual systems and female floral traits on hybrid lines

All F_1 individuals from *A. clavatus* × *A. homogamos* and of *A. homogamos* × *A. clavatus* crosses were gynomonoecious (100%, n = 158; Table 3); and showed intermediate values comparing to their parental species in all female floral characters, although the level of significance in these differences depended on the lines crossed (Fig. 5A-C). In the F_1s of both

A. homogamos × *A. valentinus* and *A. valentinus* × *A. homogamos,* the number of female flowers was in most of the cases significantly lower than in *A. valentinus* (Fig. 5G). In all these hybrid lines hermaphroditic individuals, without any female flower, were present. On average, the 76% of the F_1s (n = 136) was gynomonoecious, although this value ranged between 63-95% (n = 13-19) depending on the cross (Table 3). Ligule size of female flowers in the F_1s was significantly higher than those for *A. valentinus* in all cases (Fig. 5H, I).

In the case of *A. clavatus* × *A. valentinus* and *A. valentinus* × *A. clavatus* all F_1s were gynomonoecious as their both parental lines (n = 130, Table 3). Intermediate values were obtained for the number of female flowers (Fig. 5D), but remarkable differences in the length and width of the ligules were observed depending on the populations crossed (Fig. 5E, F).

As in their corresponding F_1s, the BCs to *A. clavatus* in the hybrid lines of *A. clavatus* × *A. homogamos* and of *A. homogamos* × *A. clavatus* produced only gynomonoecious individuals (n = 42; Table 4), whereas in the BCs to *A. homogamos* and F_2s, the gynomonoecy was present in 34.5% (n = 58) and 68.9% (n = 45) of the individuals, respectively (Table 4). Female floral characters showed a pattern of decreasing values from the highest, represented by the BCs to *A. clavatus,* to the lowest, represented by the BCs to *A. homogamos*; whereas the F_2s usually presented intermediate values (Fig. 6A-C).

All individuals of F_2s and BCs in lines of *A. clavatus* × *A. valentinus* and *A. valentinus* × *A. clavatus* were gynomonoecious, as both parental species are (n = 88; Table 4). There was no a clear pattern for the female floral characters, although a trend to show higher number of flowers and larger ligules than in *A. valentinus* was observed, except for the BCs to *A. valentinus* whose values were similar to this species (Fig. 6D-F).

In *A. homogamos* × *A. valentinus* and *A. valentinus* × *A. homogamos* the gynomonoecy was present on 59.2 % (n = 37) of the F_2s, on 81.5% (n = 26) of the BCs to *A. valentinus*, and on 26.5% (n = 22) of the BCs to *A. homogamos* (Table 4). As in other type of crosses no clear pattern was observed for floral traits. Nevertheless the observed variation increased and F_2s showed a trend to fewer female flowers but with larger ligules (Fig. 6G-I).

Sexual system and floral traits on non-hybrid maternal lines

Floral traits of individuals from non-hybrid maternal lines that were treated with pollen from different F_1 hybrids were also characterized. In these cases, all *A. clavatus* maternal lines produced 100% (n = 82) of gynomonoecious individuals (Table 5). The number of female flowers was similar to *A. clavatus*, but ligules were smaller, except when pollen from the F_1s of *A. valentinus* × *A. clavatus* was added which ligule size was similar to those of *A. clavatus* (Fig. 7A-C).

In the case of *A. homogamos* treated with pollen of hybrid origin, gynomonoecy was present in around 40.5 % (n = 84; Table 5). In such cases, the number of female flowers was similar to those of *A. valentinus* in all cases, although ligule size tended to be larger and highly variable (Fig. 7D-F).

In *A. valentinus* the crosses with pollen of hybrid origin produced 100 % (n = 47) of gynomonoecious individuals, except when pollen from *A. valentinus* × *A. homogamos* was added, in which case gynomonoecy was present in 94.4 % (n = 18) of individuals (Table 5). All female flower characters obtained were similar to those of *A. valentinus* in all cases except when pollen from *A. clavatus* × *A. valentinus* was added, in which case the ligules were

significantly longer and wider and number of female flowers were similar to *A. clavatus* (Fig. 7G-I).

Discussion

Our common garden study of experimental crosses between *Anacyclus clavatus*, *A. homogamos* and *A. valentinus* has allowed to quantitatively assess the reproductive isolation between these three species. Our results pointed out general patterns at specific and population levels that help to understand the evolution of hybrid zones among these species. In addition, we provide new insights on the inheritance model of gynomonoecy based on the segregation of this sexual system between the three species.

Mating system

Our results clearly support that the three species under study were mostly self-incompatible. Although in specific individuals autogamy occurred at very low frequencies, it might be significantly increased with the aid of vectors (pollinators). Despite this increment, differences with outcrossing treatments were remarkable enough to consider the success of self-pollination irrelevant. Additionally, the decrease on survival and flowering in individuals generated by autogamy reduced to extremely low the probability of reproductive success of selfing progeny. In these cases, high rates of failure in pollen production also prevents self-pollination in a second generation. However, the existence of rare self-compatibility in *Anacyclus* might give an opportunity for reproductive assurance in range borders or colonizing events where mate availability could be highly restricted.

Reproductive isolation between the species of the complex

The three species under study were inter-fertile and were able to produce F_1s in all the possible combinations, although slight differences were found between populations. This kind of differences could be due to an inbreeding effect at population level (i.e., populations genetically poor, low fertility, small effective size), or a maternal effect that cannot be masked by small sample size (i.e., some ovule donor individuals have low fertility). Additionally, the differences in flowering phenology between some population pairs could have led to mismatches in the optimal maturity of reproductive organs and pollen quality, decreasing the reproductive success. In both *A. clavatus* and *A. homogamos*, maternal lines showed moderate to high success and low variation within each type of cross in all cases. However, a different pattern was showed by *A. valentinus*, in which a high variation affected to all type of crosses including the intra-population controls. Additionally, this variation was accompanied by a notable lower fertility in all cases compared to *A. clavatus* and *A. homogamos*. In *A. valentinus*, the high variation seemed to be due to maternal effects, since within the two populations sampled there were individuals with very low fertility while others presented moderate values. Therefore, our results indicate that the sampled populations of *A. valentinus* presented relatively lower fertility than any other population sampled in *A. clavatus* and *A. homogamos*, which would be in accordance with the hypothesis of its hybrid origin, in which variation in reproductive success would still remain.

However, the fertility of F_1s fell down drastically for all the inter-specific hybrid lines (Fig. 2), suggesting the existence of post-zygotic barriers whose effects were observed in both F_2s and BCs, indicating a relative reproductive isolation among the three species. The slightly

higher seed set values observed in most of the F_2s and BCs involving *A. valentinus* might suggest weaker isolation in these cases. In contrast, the fertility of hybrid lines between both *A. clavatus* and *A. homogamos* was always lower, which could be the result of a higher evolutionary divergence between these two species than between any of these two and *A. valentinus*.

Phenotypic variation and inheritance of sexual systems and female floral traits

The most relevant result in sexual system inheritance was the difference observed in the frequency of gynomonoecy depending on the type of cross. The inheritance of gynomonoecy in Asteraceae has always been associated to the expression of radiate capitula (Ford & Gottlieb, 1990; Carr & al., 1996; Comes, 1998; Andersson, 2001). However, given this association (i.e., species with radiate capitula are almost always gynomonoecious whereas species with discoid capitula used to be hermaphroditic), it is unclear the type of gene interactions controlling the inheritance of both radiate capitula and unisexual female flowers leading to gynomonoecy (e.g. Trow, 1912; Richards, 1975; Ingram & Taylor, 1982; Ford & Gottlieb, 1990; Comes *et al.* 1998; Andersson, 2001).

In *Anacyclus* was possible to dissociate the sexual system and the type of capitula since *A. valentinus* is a gynomonoecious species with discoid capitula, which allows analysing segregation of both traits independently. Ratios of sexual systems observed in F_1, F_2, and BCs indicated that two genes interact by duplicate recessive epistasis for gynomonoecy expression. First, our results obtained in the crosses between *A. clavatus* and *A. homogamos*, in which all F_1 individuals were gynomonoecious, and a ratio 3:1 in favour of gynomonoecy was observed in the F_2, would be in accordance with a monogenetic control by dominance. In this case, *A.*

clavatus would be homozygous dominant (*AA*), whereas *A. homogamos* would be homozygous recessive for that locus (*aa*). The absolute frequency of gynomonoecy in all hybrid generations (F_1, F_2, and both BCs) between *A. clavatus* and *A. valentinus* lend us to infer the homozygous dominance for this locus (*AA*) in both species. Second, the variation in the ratios observed in the F_1s (3 gynomonoecy: 1 hermaphroditism) between *A. homogamos* and *A. valentinus* (Table 3) implies the existence of a complementary different locus that must be heterozygous (*Bb*) in both *A. homogamos* and *A. valentinus*. Moreover, the approximate ratio average in the F_2 (9 gynomonoecy: 7 hermaphroditism) suggests a double recessive epistasis between the two allelic pairs (Table 4). Therefore, the fixed allelic combination in the three species should be: (*AA BB*) in *A. clavatus*, (*AA Bb*) in *A. valentinus* and (*aa Bb*) in *A. homogamos*.

The deviated values from the expected ratios under this hypothesis were not significant for any hybrid line generation (Tables 3, 4). However, when non-hybrid lines were fertilized with pollen of F_1s hybrids, significant deviations were observed (Table 5). Although the most probable explanation could be the unbalanced representation of the different possible gametes in the pollen mixture (e.g.,. an overrepresentation of gametes *ab* in the pollen mix of "ZZ2772", and of gametes *AB* in the pollen mix fertilizing "FF3077" maternal lines), we cannot discard the occurrence of incomplete fixed heterozygosis for the alleles *Bb* in *A. homogamos*. This hypothesis was tested as well, and it could be only partially rejected in the case of maternal line "Z420" (Table 3), and it would alternatively explain the deviant values observed in the maternal line "ZZ2772" for a balanced pollen mixture. Moreover, the marginally significant values on the F_1 hybrids of the maternal line "W575" when pollinated with population "Z" of *A. homogamos* (Table 3) may suggest a biased representation to *aB* gametes. This unbalance may be explained by the presence of homozygote individuals *aa BB*

in population "Z" (i.e., incomplete fixed heterozygosis) or alternatively by the existence of gamete unbalanced incompatibilities in the F_1 hybrids following the Bateson-Dobzhansky-Muller (BDM) model (Bateson, 1909; Dobzhansky, 1936; Muller, 1942) driving to a variable reproductive isolation (VRI) among species (Cutter, 2012).

A model of allelic evolution compatible with all these alternative hypotheses, fitting with a double recessive epistasis for the control of gynomonoecy in *Anacyclus*, is presented here (Figure 8). Although further experiments are needed to understand the evolution of these lineages, this model is also congruent with the hybrid origin of both *A. homogamos* and *A. valentinus*, and the variation of the relative reproductive isolation observed between the three species. This is in accordance with the low and heterogeneous reproductive success observed at inter- and intra-population levels in *A. valentinus*, and the lower reproductive barriers with the other two species, its putative parental lineages (Fig. 8). This scenario would be also in accordance with the observation that *Anacyclus homogamos* is reproductively the most isolated species respecting the other two.

The imperfect fixed heterozygosity for alleles *Bb* in *A. homogamos*, in which no floral phenotypic change would be produced (i.e., all individuals are homozygous recessive *aa*, and therefore, hermaphroditic), fits well in this model. However, in *A.valentinus* (*Aa*) the presence of homozygotes (*bb*) could allow the existence of hermaphroditic individuals in its populations. In fact, the rare and scattered field observations of hermaphroditic individuals of *Anacyclus* (identified as *A. homogamos*) in the Iberian Peninsula and other coastal sites in North Africa could correspond actually to individuals of *A. valentinus* with the rare allelic combination (*bb*) that would reflect a hermaphroditic phenotype. A positive selection pressure to heterozygotes *Bb* in *A. valentinus* might also explain the variation on fertility observed in

the populations studied, in which the incompatible allelic combinations *BB* and/or *bb* in this species would prevent from fertility.

The analyses of segregation for quantitative floral traits are more complex. For example, considering that the number of female flowers expands from 0 (hermaphroditic) to several (gynomonoecious), it might be expected that at least one of the locus involved in gynomonoecy also controls the number of female flowers, probably in codominance. According to our results, this locus might be presumably the allelic pair *B*, which would explain the higher number of female flowers in *A. clavatus* (*BB*) compared to *A. valentinus* (*AA Bb*), and the similar patterns of segregation for this trait observed in the BCs to both *A. homogamos* (*aa Bb*) and to *A. valentinus* (*AA Bb*) in the crosses with *A. clavatus* (*AA BB*). However, other alternative hypothesis such the existence of a third locus linked to these two (a duplicate locus of one of these) or several loci interacting in codominance cannot be discarded for the control of the number of female flowers. In the same way, the variation observed for the ligule length and width in female flowers in the F_1s suggest the involvement of several linked loci with different heterozygous levels at least in *A. clavatus*. Thus, the different floral trait segregation in the F_1s in the crosses between population "V" of *A. clavatus* and any population of *A. valentinus* (Figure 5) could be explained by the presence of one to several recessive loci in this population of *A. clavatus* that is not present in population "B" of the same species. It is important to note that these loci should be different to those involved in the sexual system, which may explain the homogeneous phenotype for ligule length observed in all F_1s in crosses between *A. valentinus* and *A. homogamos*, and the general trend to show larger ligules than in *A. valentinus*, more similar to a "*clavatus*" phenotype.

Concluding Remarks

In only few homoploid hybrid lineages the evidence of their origin is clearly supported, mainly due to the complexity of the patterns observed when reproductive isolation is not absolute and sympatry with recent relatives is frequent. In *Anacyclus*, the existence of current hybridization between related species was previously suggested by different sources of evidence (Humphries, 1979, 1981; Agudo et al. in rev, "unpubl."). However, there was no molecular data supporting the hybrid origin of *A. valentinus* (Oberprieler, 2004; Agudo et al. unpubl.), a species that was classically consider a hybrid based on its floral morphology and distribution range (Humphries 1979; Funk, 1985). Our results indicated a relative reproductive isolation between the three species, although one of these, *A. valentinus* showed a weaker isolation with both *A. clavatus* and *A. homogamos* than these two to each other.

Sexual systems and female floral traits were the only morphological characters to distinguish between the three studied species. The analysis of character segregation in F_1, F_2, and BCs between these three species allowed us to infer a double recessive epistasis between two complementary loci controlling the gynomonoecy; and the existence of mostly fixed heterozygosis for the same locus in *A. homogamos* and *A. valentinus*, whereas *A. clavatus* needs to be homozygous for the two loci. This hypothesis, jointly with the variation in reproductive isolation and incompatibilities in hybrids (BDM model) is congruent with the model presented for the evolution of these three species of *Anacyclus*.

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TABLES

Table 1. Effects of different treatments on the probability of setting a viable seed in the three studied species. Control represents the intra-population crosses in each case. No viable seeds were observed in any autogamy treatment for *A. valentinus*. Data represent the Wald-type F-statistic with the degrees of freedom as sub-index for fixed factors, and the estimate for covariance parameter and its standard error for the random factor: Plant. Significant p-values are in bold.

	A. clavatus		*A. valentinus*		*A. homogamos*	
Autogamy vs. control	*F*	*P*	*F*	*P*	*F*	*P*
Treatment	$165.7_{\,1,\,1588}$	**<0.0001**	-	-	$169.34_{\,1,\,966}$	**<0.0001**
Plant (Estimate ± SE)	0.108 ± 0.122		-		0.395 ± 0.457	
Sample size	1590		1919		968	
Self-compatibility vs. control						
Treatment	$451.79_{\,1,\,1493}$	**<0.0001**	$184.39_{\,1,\,1773}$	**<0.0001**	$411.51_{\,1,\,1026}$	**<0.0001**
Plant (Estimate ± SE)	0.292 ± 0.281		1.422 ± 1.193		0	
Sample size	1495		1775		1028	
Autogamy vs. self-compatibility						
Treatment	$9.01_{\,1,\,1495}$	**0.0027**	-	-	$9.29_{\,1,\,1050}$	**0.0024**
Plant (Estimate ± SE)	1.620 ± 1.751		-		5.522 ± 6.6	
Sample size	1497		1886		1052	
Intra-specific vs. control						
Treatment	$0.03_{\,1,\,675.6}$	0.855	$18.4_{\,1,\,1579}$	**<0.0001**	$8.98_{\,1,\,894}$	**0.0028**
Plant (Estimate ± SE)	0.025 ± 0.045		0.771 ± 0.642		0.482 ± 0.536	
Sample size	1488		1581		896	
Interspecific crosses vs. control						
Treatment	$2.55_{\,1,\,3447}$	0.1104	$9.9_{\,1,\,4199}$	**0.0017**	$8.34_{\,1,\,1846}$	**0.0039**
Plant (Estimate ± SE)	0.138 ± 0.122		1.42 ± 1.167		0.236 ± 0.256	
Sample size	3449		4201		1848	

Table 2. Effects of different treatments on the probability of setting a viable seed in the six hybrid lines generated. Control represents the F_1 in each case. Data represent the Wald-type Chi square test for the fixed factor, and the estimate for covariance parameter and its standard deviation for the random factor: Plant. The order of the species in the type of cross indicates the ovule (first) and the pollen donor (second).

Type of cross	Pollination treatment			Plant
	n	χ^2	P	Estimate ± SD
A. clavatus × A. homogamos	5108	86.1	<0.0001	0.44 ± 0.66
A. homogamos × A. clavatus	3879	184.6	<0.0001	0.09 ± 0.31
A. clavatus × A. valentinus	2325	15.0	0.0005	1.28 ± 1.13
A. valentinus × A. clavatus	2347	6.63	0.0364	1.48 ± 1.21
A. homogamos × A. valentinus	1949	48.2	<0.0001	0.09 ± 0.30
A. valentinus × A. homogamos	2752	8.14	0.0171	1.52 ± 1.23

Table 3. Observed gynomonoecy in the F_1s for the different type of crosses. Gyn = number of gynomonoecious individuals observed; n = total number of individuals studied. The χ^2 tested the hypothesis of double recessive epistasis for all possible types of ovule donors assuming a balanced representation of gametes in pollen mixture. Significant p-values are in bold. ([ms]) indicates marginally significant p-values (p < 0.10).

(**Table 3** on page 110)

Table 4. Observed gynomonoecy in the F_2s and BCs for the different hybrid lines. Gyn = number of gynomonoecious individuals observed; n = total number of individuals studied. The χ^2 tested the hypothesis of double recessive epistasis for all possible types of ovule donors assuming a balanced representation of gametes in pollen mixture. Significant p-values are in bold. ([ms]) indicates marginally significant p-values (p < 0.10).

Table 4 on page 111)

Type of cross	Gyn	n	Possible types of ovule donors					
A. clavatus × A. homogamos			*AA BB*					
B23 x At	20	20						
B177 x Z	12	12						
B186 x Z	16	16						
V50 x At	18	18						
V50 x Z	19	19						
A. clavatus × A. valentinus			*AA BB*					
B23 x F	15	15						
B23 x W	19	19						
V50 x F	19	19						
V50 x W	14	14						
A. homogamos × A. clavatus			*aa BB, aa Bb, aa bb*					
At492 x B	19	19						
At492 x V	6	6						
Z420 x B	15	15						
Z420 x V	18	18						
Z747 x B	15	15						
A. homogamos × A. valentinus			*aa BB*		*aa Bb*		*aa bb*	
			χ^2	p-value	χ^2	p-value	χ^2	p-value
At492 x F	12	17	∞	n/a	0.17647	0.7784	2.8824	0.1359
At492 x W	13	18	∞	n/a	0.074074	1	3.5556	0.09499[ms]
Z420 x F	16	18	∞	n/a	1.8519	0.2773	10.889	**0.0015**
Z420 x W	10	14	∞	n/a	0.09524	1	2.5714	0.1761
A. valentinus × A. clavatus			*AA Bb*					
F151 x B	17	17						
F151 x V	15	15						
W575 x B	19	19						
W575 x V	12	12						
			AA Bb					
A. valentinus × A. homogamos			χ^2	p-value				
F151 x At	14	18	0.074074	1				
F151 x Z	12	19	1.4211	0.2935				
W575 x At	9	13	0.23077	0.7431				
W575 x Z	18	19	3.9474	0.06409[ms]				

Table 3. Observed gynomonoecy in the F_1s for the different type of crosses

Hybrid lines	Gyn	n	Possible types of ovule donors					
A. clavatus × A. homogamos			*Aa BB*		*Aa Bb*			
			χ^2	p-value	χ^2	p-value		
BZ1119 (F$_2$)	9	12	0	1	0.8	0.5367		
BZ1358 (F$_2$)	5	10	3.3333	0.1354	0.66667	0.5172		
BZ1119 (BC to *A. clavatus*)	10	10						
BZ1358 (BC to *A. clavatus*)	10	10						
BZ1119 (BC to *A. homogamos*)	7	13	0.076923	1	1.5158	0.2567		
BZ1358 (BC to *A. homogamos*)	4	13	1.9231	0.2706	0.23919	0.7792		
A. clavatus × A. valentinus			*AA BB, AA Bb*					
BF2767 (F$_2$)	15	15						
BF2767 (BC to *A. clavatus*)	10	10						
BF2767 (BC to *A. valentinus*)	11	11						
A. homogamos × A. clavatus			*Aa BB*		*Aa Bb*			
			χ^2	p-value	χ^2	p-value		
ZB1249 (F$_2$)	8	12	0.44444	0.7373	0.088889	1		
ZB1250 (F$_2$)	9	11	0.27273	0.7422	1.7515	0.2302		
ZB1249 (BC to *A. clavatus*)	10	10						
ZB1250 (BC to *A. clavatus*)	12	12						
ZB1249 (BC to *A. homogamos*)	4	14	2.5714	0.1088	0.45907	0.5918		
ZB1250 (BC to *A. homogamos*)	5	18	3.5556	0.0951^{ms}	0.70204	0.4705		
A. homogamos × A. valentinus			*Aa BB*		*Aa Bb*		*Aa bb*	
			χ^2	p-value	χ^2	p-value	χ^2	p-value
ZF2780 (F$_2$)	9	18	6	**0.0247**	0.81724	0.4637	0.51386	0.6242
ZF2780 (BC to *A. homogamos*)	4	13	1.9231	0.2705	0.23919	0.7771	0.23077	0.7491
ZF2780 (BC to *A. valentinus*)	11	12	∞	n/a	1.7778	0.3047	8.3333	**0.0059**
A. valentinus × A. homogamos			*Aa BB*		*Aa Bb*		*Aa bb*	
			χ^2	p-value	χ^2	p-value	χ^2	p-value
FZ2675 (F$_2$)	13	19	0.4386	0.5893	0.50862	0.4917	5.594	**0.0193**
FZ2675 (BC to *A. homogamos*)	2	9	2.7778	0.1778	0.87806	0.4892	0.037037	1
FZ2675 (BC to *A. valentinus*)	10	14	∞	n/a	0.095238	1	2.5714	0.1775
A. valentinus × A. clavatus			*AA BB, AA Bb*					
FB2733 (F$_2$)	14	14						
FB2733 (BC to *A. clavatus*)	17	17						
FB2733 (BC to *A. valentinus*)	21	21						

Table 4. Observed gynomonoecy in the F$_2$s and BCs for the different hybrid lines

Table 5. Observed gynomonoecy in non-hybrid lines after treated with hybrid pollen. Gyn = number of gynomonoecious individuals observed; n = total number of individuals studied. The χ^2 tested the hypothesis of double recessive epistasis for all possible types of ovule donors assuming a balanced representation of gametes in pollen mixture. Significant p-values are in bold. (^{ms}) indicates marginally significant p-values (p < 0.10).

| Non-hybrid lines	Type of pollen	Gyn	n	Possible types of ovule donors					
A. clavatus				*AA BB*					
BB1115	*A. clavatus x A. homogamos*	15	15						
BB1115	*A. homogamos x A. clavatus*	12	12						
BB1292	*A. clavatus x A. homogamos*	10	10						
BB1292	*A. homogamos x A. clavatus*	13	13						
BB2799	*A. clavatus x A. valentinus*	16	16						
BB2799	*A. valentinus x A. clavatus*	16	16						
A. homogamos				*aa BB*		*aa Bb*		*aa bb*	
				χ^2	p-value	χ^2	p-value	χ^2	p-value
ZZ1690	*A. clavatus x A. homogamos*	10	18	0.22222	0.8138	1.0644	0.3521	2.5037	0.1452
ZZ1690	*A. homogamos x A. clavatus*	6	14	0.28571	0.7919	0.00235	1	0.17143	0.7872
ZZ1691	*A. clavatus x A. homogamos*	5	13	0.69231	0.5792	0.13428	0.7835	0.00513	1
ZZ1691	*A. homogamos x A. clavatus*	6	11	0.09091	1	0.54604	0.5674	1.3636	0.3455
ZZ2772	*A. valentinus x A. homogamos*	1	13	9.3077	**0.0035**	4.8811	**0.0413**	2.0769	0.2079
ZZ2772	*A. homogamos x A. valentinus*	6	15	0.6	0.5984	0.045708	1	1.8	0.228
A. valentinus				*AA Bb*					
				χ^2	p-value				
FF3077	*A. clavatus x A. valentinus*	15	15						
FF3077	*A. valentinus x A. clavatus*	13	13						
FF3077	*A. valentinus x A. homogamos*	17	18	3.6296	0.097^{ms}				
FF3077	*A. homogamos x A. valentinus*	19	19	6.3333	**0.0124**				

FIGURES
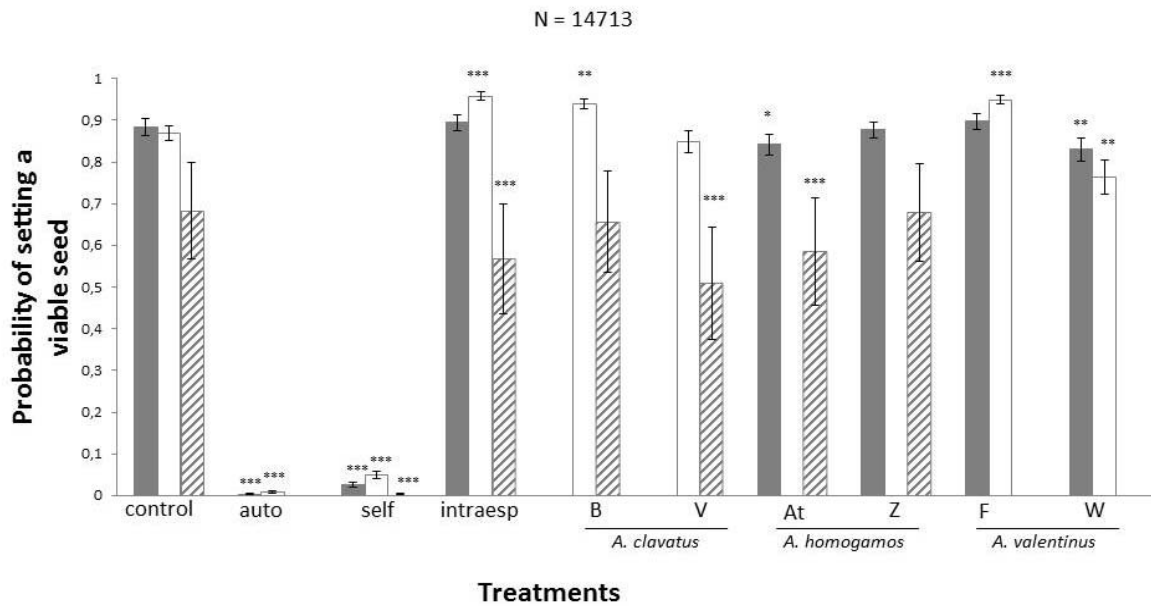
Figure 1. Least-square means (± 95% CI) of the probability of setting a viable seed by *Anacyclus clavatus* (grey bars), *A. homogamos* (white bars) and *A. valentinus* (rayed bars) mother plants. Treatments are pollen addition from different sources: pollen from individuals of the same population which the mother plant is from (control, outcrossing test); no pollen addition (autogamy test); self-pollen addition (self-compatibility test); pollen from individuals of the same species but different population which the mother plant is from (intra-specific crosses); and pollen from individuals of populations B, V, At, Z, F, and W, which are from different *Anacyclus* species in each case. Only significant differences with the corresponding intra-population controls treatment are indicated (*P < 0.05, ** P < 0.01, *** P < 0.001).
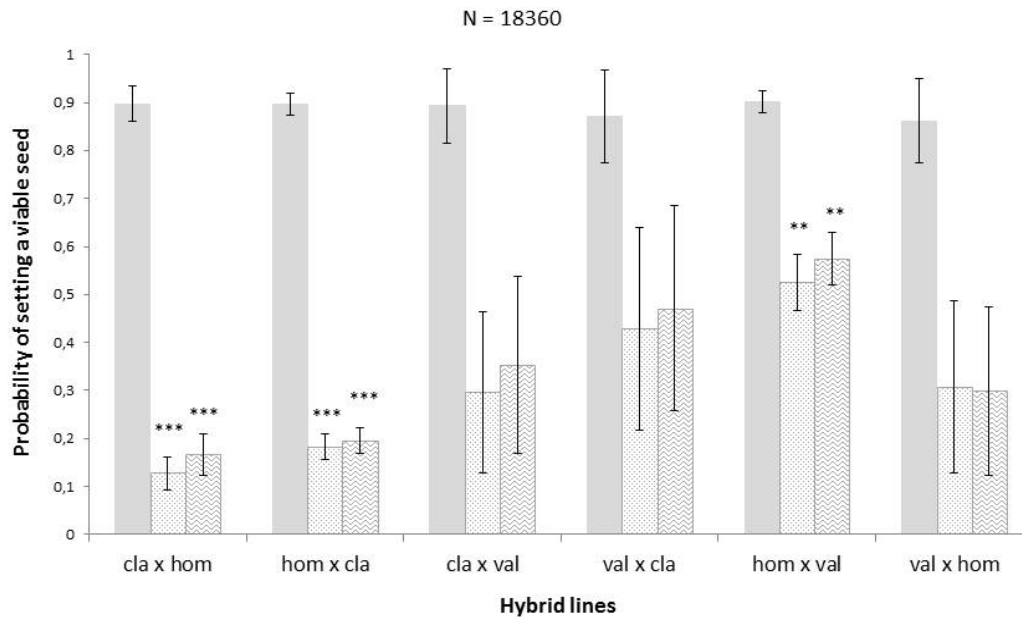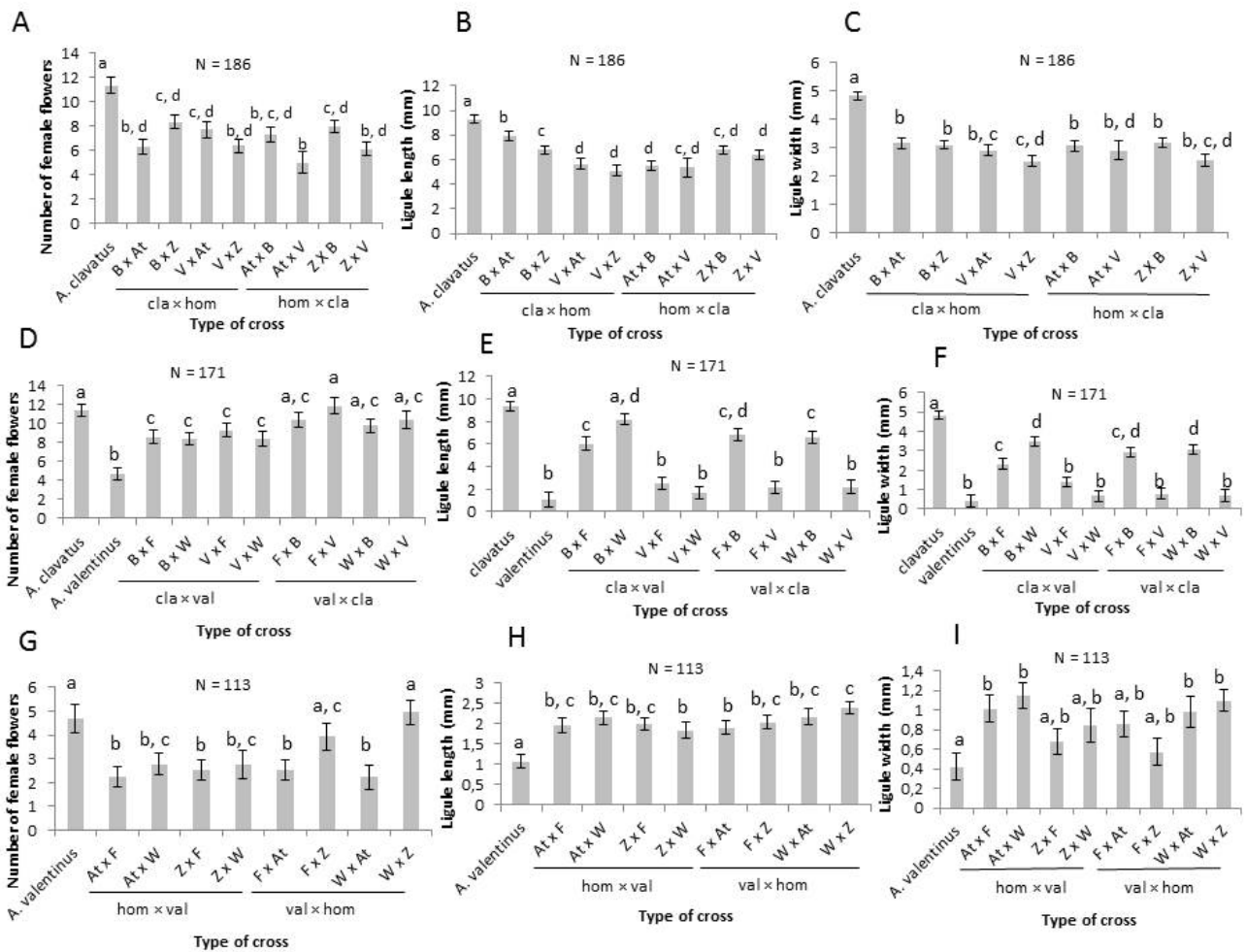
Figure 2. Least-square means (± 95% CI) of the probability of setting a viable seed by the F_1 hybrids when fertilized with pollen from the same line of F_1s hybrids (F_2: grey bars) and from non-hybrid lines (BCs**:** white bars) produced by the different type of crosses: *Anacyclus clavatus* × *A. homogamos* (cla × hom); *A. homogamos* × *A. clavatus* (hom × cla); *A. clavatus* × *A. valentinus* (cla × val); *A. valentinus* × *A. clavatus* (val × cla); *A. homogamos* × *A. valentinus* (hom × val); and *A. valentinus* × *A. homogamos* (val × hom). Data observed on the intra-specific crosses were included as the corresponding control in each case (black bars). Different letters above each bar indicate means statistically different ($P < 0.05$).
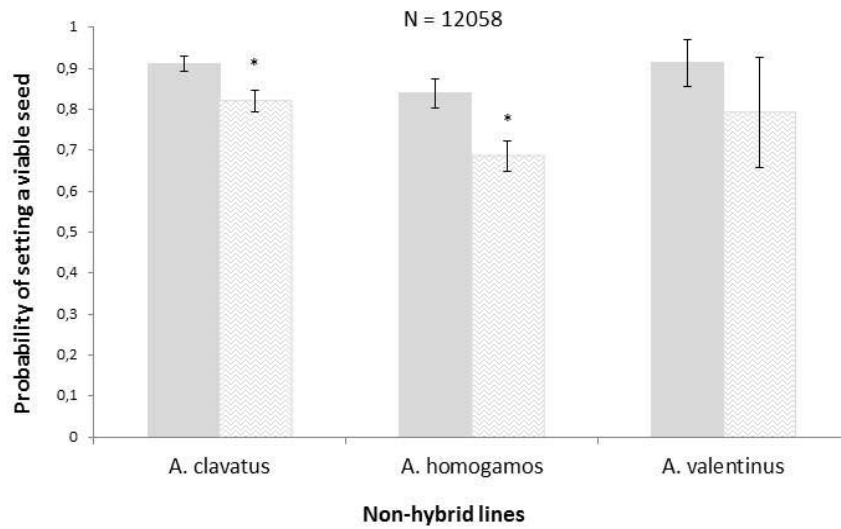
Figure 3. Least-square means (± 95% CI) of the probability of setting a viable seed by non-hybrid lines of *Anacyclus clavatus*, *A. homogamos* and *A. valentinus* treated with pollen from a F_1 hybrid in which one progenitor represents the corresponding non-hybrid line. Data for each corresponding non-hybrid intra-specific crosses were included as control (black bars). Treatments with pollen of hybrid origin were represented by waved motif bars. Only significant differences with the corresponding control are indicated (*$P < 0.05$).
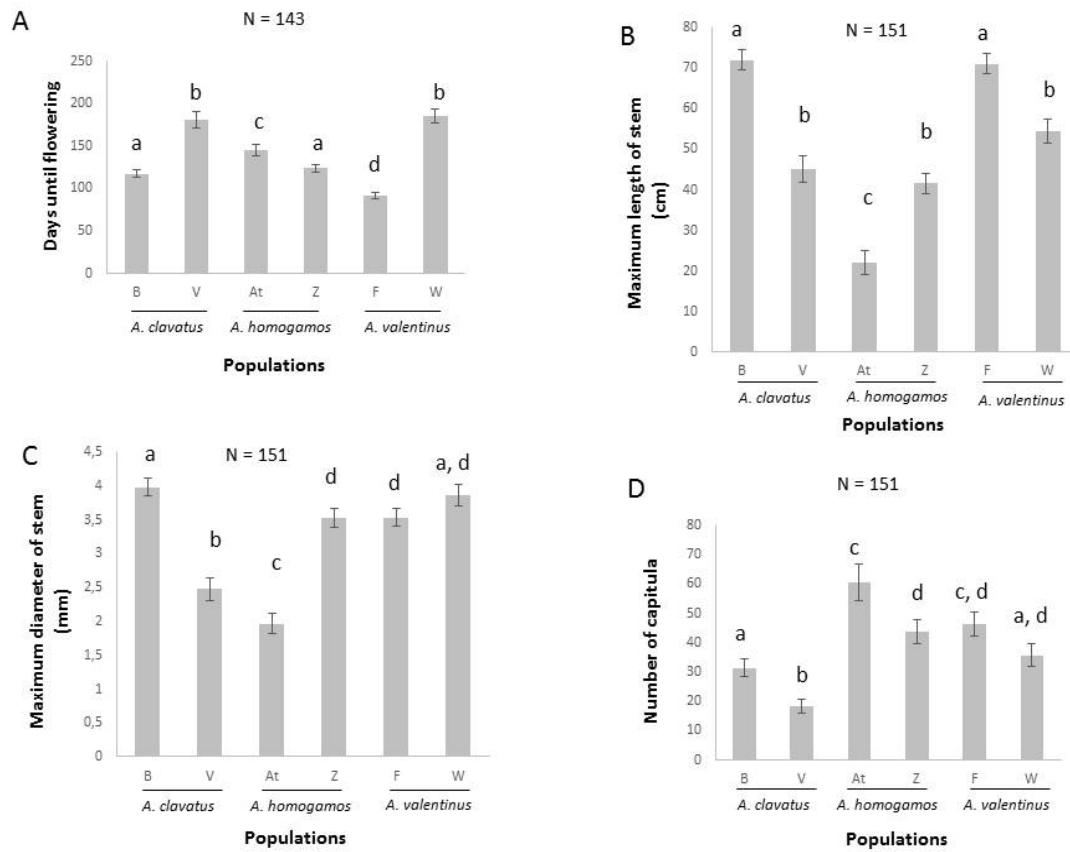
Figure 4. Least-square means (± 95% CI) of maximum length of stem (A), maximum diameter of stem (B), and number of capitula (C) in populations of *Anacyclus clavatus*, *A. homogamos*, and *A. valentinus*. Different low case letters indicate significant differences between populations (P < 0.05).

Figure 5. Least-square means (± 95% CI) of the number of female flowers (A, D, G), ligule length (B, E, H), ligule width (C, F, I), in F_1s of different types of crosses and populations (upper case letters in each type of cross): *Anacyclus clavatus × A. homogamos* (cla × hom), *A. homogamos × A. clavatus* (hom × cla), *A. clavatus × A. valentinus* (cla × val), *A. valentinus × A. clavatus* (val × cla), *A. homogamos × A. valentinus* (hom × val), and *A. valentinus × A. homogamos* (val × hom). When involved in a cross, values for *A. clavatus* and *A. valentinus* were also included, whereas values of *A. homogamos* were omitted since they do not produce female flowers. Different low case letters indicate significant differences between populations (P < 0.05).
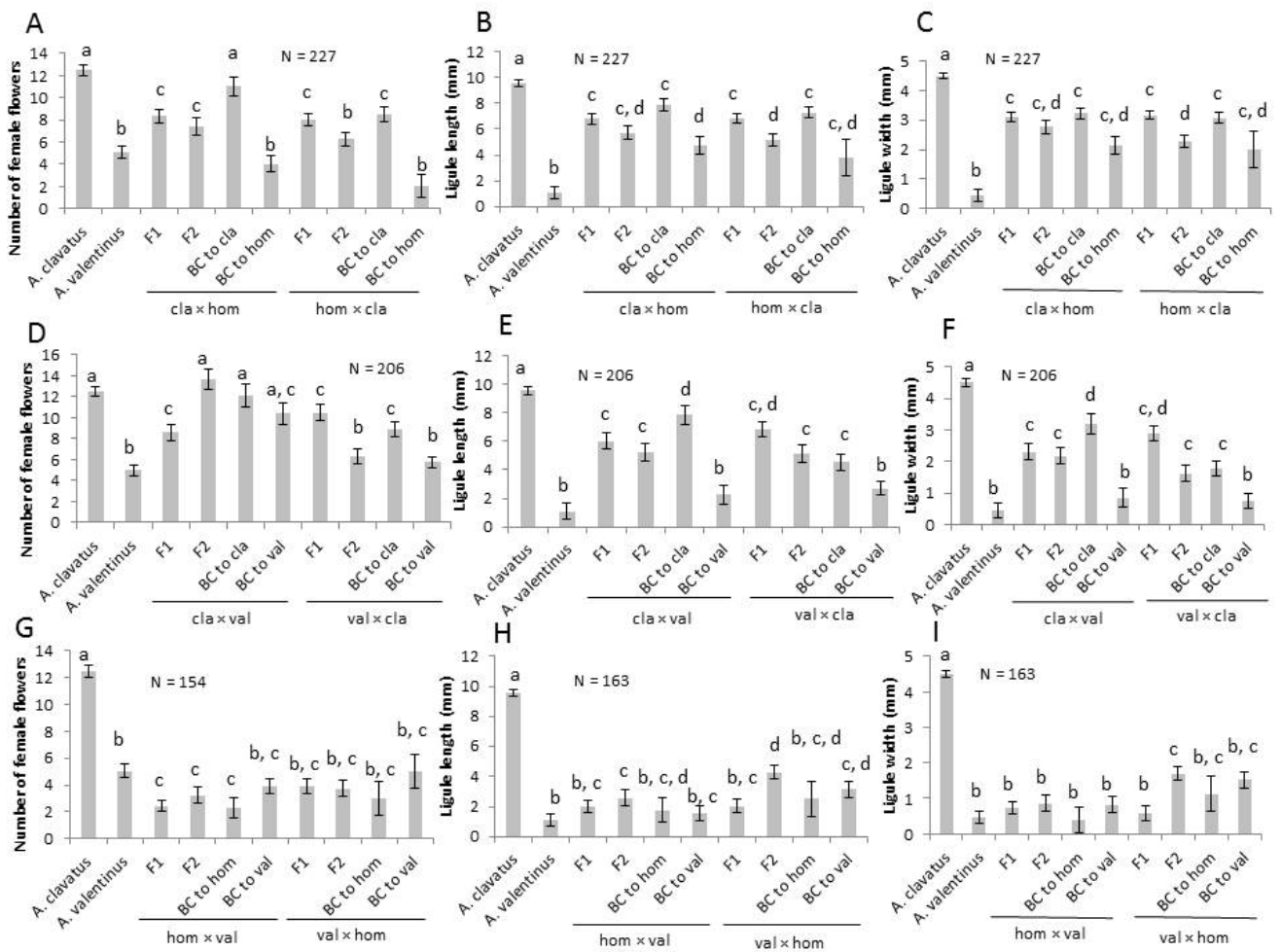
Figure 6. Least-square means (± 95% CI) of the number of female flowers (A, D, G), ligule length (B, E, H), ligule width (C, F, I), in F_2s and BCs of different types of crosses: *Anacyclus clavatus × A. homogamos* (cla × hom), *A. homogamos × A. clavatus* (hom × cla), *A. clavatus × A. valentinus* (cla × val), *A. valentinus × A. clavatus* (val × cla), *A. homogamos × A. valentinus* (hom × val), and *A. valentinus × A. homogamos* (val × hom). Values for *A. clavatus* and *A. valentinus* were also included, whereas values of *A. homogamos* were omitted since they do not produce female flowers. Different low case letters indicate significant differences between populations ($P < 0.05$).
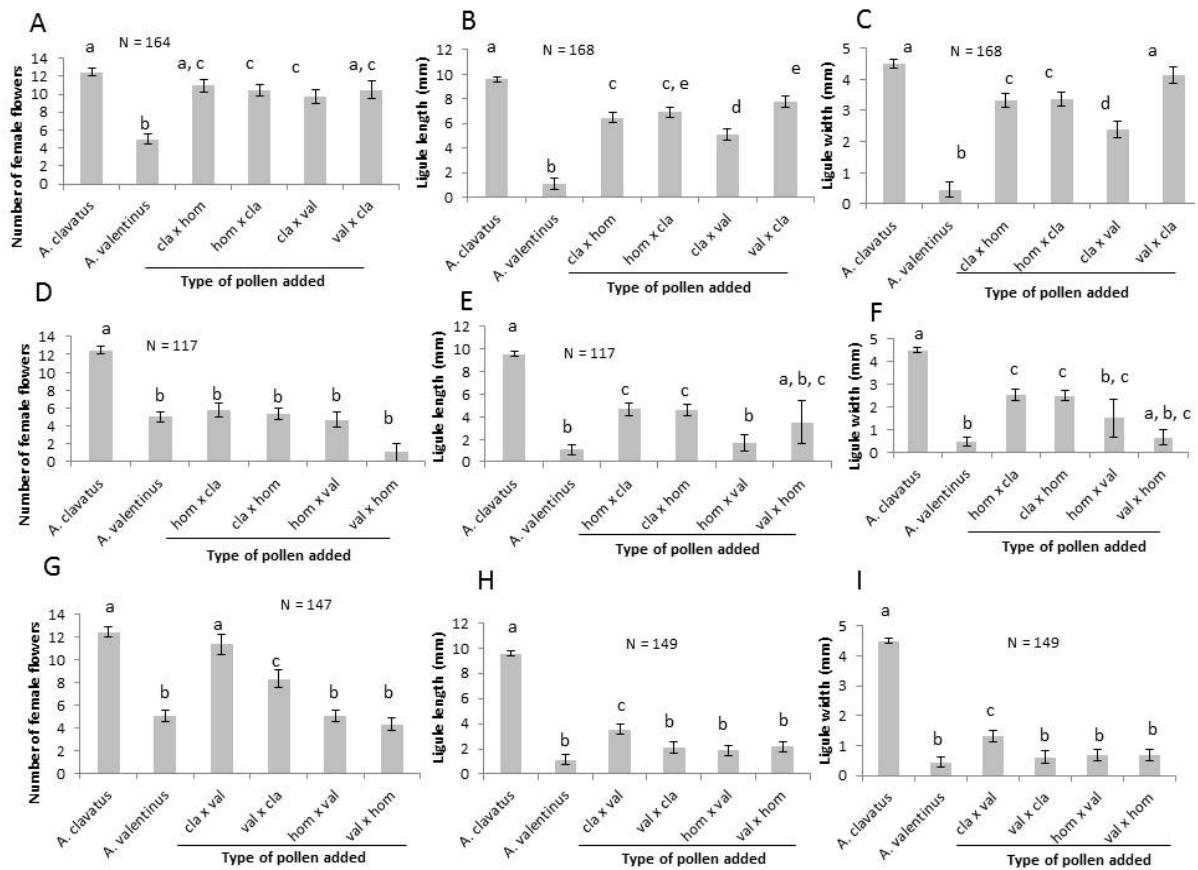
Figure 7. Least-square means (± 95% CI) of the number of female flowers (A, D, G), ligule length (B, E, H), ligule width (C, F, I), in F$_1$s of *A. clavatus* (A-C), *A. homogamos* (D-F), and *A. valentinus* (G-I) treated with pollen of hybrid origin: *Anacyclus clavatus* × *A. homogamos* (cla × hom), *A. homogamos* × *A. clavatus* (hom × cla), *A. clavatus* × *A. valentinus* (cla × val), *A. valentinus* × *A. clavatus* (val × cla), *A. homogamos* × *A. valentinus* (hom × val), and *A. valentinus* × *A. homogamos* (val × hom). Values for *A. clavatus* and *A. valentinus* were also included. Different low case letters indicate significant differences between populations (P < 0.05).
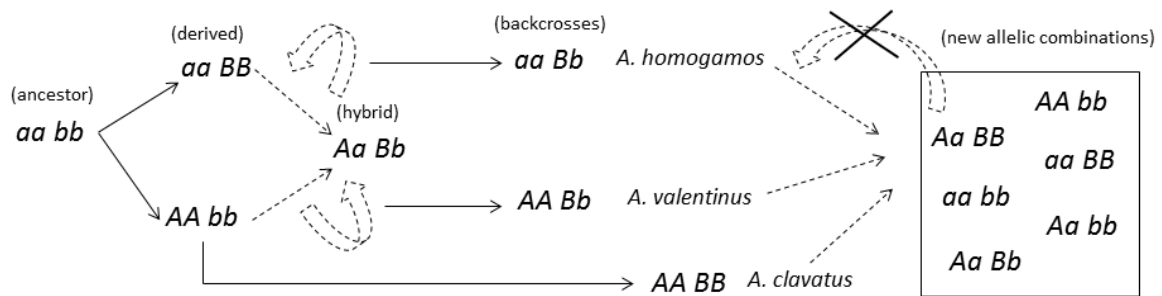
Figure 8. Allelic model evolution in *Anacyclus clavatus* (*AA BB*), *A. homogamos* (*aa Bb*) and *A. valentinus* (*AA Bb*) according to the hypothesis of the double recessive epistasis of gynomonoecy. From a common ancestor (*aa bb*) two divergent lineages evolved and hybridized. The hybrid might have backcrossed to each parental to give rise the *A. homogamos* and *A. valentinus* lineages, whereas *A. clavatus* would have evolve from one of the original divergent lineages. In the present, might be that some of the new allelic combinations obtained by crosses among these three lineages are incompatible to backcross.

SUPPLEMENTARY MATERIAL

Table S1. Plant material used in this study indicating the species, the code of the populations selected for the experiments, the origin and voucher information that includes country, locality, latitude and longitude, altitude (meters above sea level), date of collection, collector's number (in italics), and the herbarium where the voucher is deposited.

[1]Data not available in the original label

Species	Population code	Origin and voucher information
A. clavatus		Algeria: Constantine, 36º 21' N 6º 38' E[1], 660 m[1], 04.05.1840, *de Maisonneure s.n.*, LISU
		Italy: Sicily, Palermo, 38º 2' 32" N 13º 30' 51" E, 116 m, 24.04.2012, *Tomasello 421*, MA
		Morocco: Idriss dam, 34º 7' 39.2" N 4º 30' 50.8" W, 226 m, 14.04.2012, *Álvarez 2228*, MA
		Morocco: Azrou, 33º 29' 27.7" N 5º 15' 44.5" W, 1310 m, 14.04.2012, *Álvarez 2235*, MA
	B	Spain: Carchuna, 36º 41' 49" N 3º 27' 33" W, 13 m, 27.04.2011, *Agudo 1*, MA
		Spain: Pruna, 37º 2' 2.87" N 5º 16' 51.52" W, 362 m, 04.05.2010, *Álvarez 2091*, MA
		Spain: Antequera, 37º 2' 34" N 4º 30' 54.3" W, 471 m, 28.03.2011, *Álvarez 2122*, MA
		Spain: Málaga, 36º 43' 8.5" N 4º 28' 8.9" W, 53 m, 29.03.2011, *Álvarez 2130*, MA

Spain: Almuradiel, 38º 30' 26.7" N 3º 29' 48.2" W, 808 m, 01.04.2011, *Álvarez 2146*, MA

Spain: Mojácar, 37º 7' 45" N 1º 49' 50.8" W, 11 m, 17.04.2011, *Álvarez 2148*, MA

Spain: Dehesa de Campoamor, 37º 54' 3.3" N 0º 44' 49.8" W, 0 m, 18.04.2011, *Álvarez 2154*, MA

Spain: El Pozuelo, 36º 44' 51.8" N 3º 9' 55" W, 16 m, 19.04.2011, *Álvarez 2158*, MA

Spain: Argüeso, 43º 1' 20" N 4º 11' 58.78" W, 962 m, 23.07.2011, *Álvarez 2172*, MA

V   Spain: Miraflores de la Sierra, 40º 47' 36.45" N 3º 43' 46.97" W, 883 m, 22.10.2011, *Álvarez 2173*, MA

Spain: Madrid, 40º 24' 28.49" N 3º 41' 18.33" W, 632 m, 04.04.2012, *Álvarez 2176*, MA

Spain: Ataquines, 41º 10' 58.11" N 4º 48' 9.28" W, 795 m, 11.05.2012, *Álvarez 2273*, MA

Spain: Tarancón, 40º 1' 54" N 3º 2' 33.7" W, 763 m, 22.05.2012, *Álvarez 2275*, MA

Spain: Valdeganga, 39º 8' 36.1" N 1º 45' 43.3" W, 621 m, 22.05.2012, *Álvarez 2281*, MA

Spain: El Pulpillo, 38º 40' 12.6" N 1º 12' 47" W, 651 m, 22.05.2012, *Álvarez 2284*, MA

Spain: Aras de los Olmos, 39º 55' 17.1" N 1º 7' 38.5" W, 918 m, 23.05.2012, *Álvarez 2292*, MA

Spain: Els Rossildos, 40º 17' 21.4" N 0º 2' 42.9" W, 453 m, 24.05.2012, *Álvarez 2296*, MA

Spain: Calaceite, 41º 0' 58.6" N 0º 11' 36.3" E, 489 m, 24.05.2012, *Álvarez 2300*, MA

Spain: Gandesa, 41º 3' 30.1" N 0º 26' 30.6" E, 353 m, 24.05.2012, *Álvarez 2301*, MA

Spain: Chalamera, 41º 39' 43" N 0º 9' 58.9" E, 171 m, 24.05.2012, *Álvarez 2304*, MA

Spain: Bujaraloz, 41º 29' 58.7" N 0º 9' 26.7" W, 331 m, 24.05.2012, *Álvarez 2306*, MA

Spain: Alcolea del Pinar, 41º 2' 25.7" N 2º 27' 0.9" W, 1205 m, 25.05.2012, *Álvarez 2313*, MA

Spain: Talavera de la Reina, 39º 58' 57.1" N 4º 43' 49.5" W, 378 m, 31.05.2012, *Álvarez 2318*, MA

Spain: Belvís de la Jara, 39º 45' 33.4" N 4º 57' 38.1" W, 491 m, 31.05.2012, *Álvarez 2325*, MA

Spain: Cambil, 37º 40' 35.92" N 3º 33' 52.50" W, 771 m, 01.06.2012, *Álvarez 2326*, MA

Spain: Villena, 38º 38' 50.9" N 0º 52' 0.2" W, 525 m, 03.06.2013, *Álvarez 2332*, MA

A. homogamos	Morocco: *Agudo 34*, MA
	Morocco: Ighrem N'ougdal, 1918 m, *Agudo 67*, MA
	Morocco: Ait Ben Ammar, 1534 m, *Agudo 73*, MA
	Morocco: *Agudo 82*, MA
	Morocco: *Agudo 86*, MA
	Morocco: Tnine-des-Oudaya, 31º 37' 55.66" N 8º 15' 10.67" W, 380 m, 21.05.2010, *Álvarez 2097*, MA
	Morocco: Chafarni, 30º 50' 11.92" N 8º 23' 36.81" W, 1390 m, 24.05.2010, *Álvarez 2111*, MA
	Morocco: Tizi-n-Test, 30º 52' 6.44" N 8º 22' 45.37" W, 2100 m, 24.05.2010, *Álvarez 2113*, MA

		Morocco: between Tizi-n-Test and Asni, 30º 58' 51.51" N 8º 13' 26.48" W, 1262 m, 24.05.2010, *Álvarez 2114*, MA
	Z	Morocco: Asni, 31º 15' 4.5" N 7º 58' 40.18" W, 1160 m, 24.05.2010, *Álvarez 2115*, MA
	At	Morocco: Imouzzer, 31º 19' 55" N 7º 24' 32" W, 2224 m, 13.06.2009, *Gonzalo 1275*, MA
A. valentinus		Spain: Vilanova i la Geltrú, 41º 13' 15.6" N 1º 40' 33.8" E, 43 m, 28.06.2009, *Álvarez 2041*, MA
	W	Spain: Castelló d'Empuries, 42º 15' 47.2" N 3º 7' 45.5" E, 0 m, 29.06.2009, *Álvarez 2059*, MA
		Spain: La Concepción, 36º 46' 43.2" N 4º 25' 39.6" W, 121 m, 28.03.2011, *Álvarez 2124*, MA
		Spain: Benajarafe, 36º 42' 58.1" N 4º 11' 7.8" W, 0 m, 29.03.2011, *Álvarez 2132*, MA
	F	Spain: Iznate, 36º 46' 35" N 4º 10' 45.2" W, 285 m, 30.03.2011, *Álvarez 2137*, MA
		Spain: La Garrucha, 37º 10' 2.9" N 1º 49' 26.4" W, 4 m, 17.04.2011, *Álvarez 2149*, MA
		Spain: Dehesa de Campoamor, 37º 54' 48.5" N 0º 44' 13.3" W, 31 m, 18.04.2011, *Álvarez 2155*, MA
		Spain: Santa Pola, 38º 11' 29.5" N 0º 31' 29.4" W, 0 m, 18.04.2011, *Álvarez 2156*, MA
		Spain: L'Alcudia, 34º 7' 39.2" N 4º 30' 50.8" W, 226 m, 23.05.2012, *Álvarez 2288*, MA
		Spain: Torís, 39º 21' 54.1" N 0º 39' 23" W, 199 m, 23.05.2012, *Álvarez 2289*, MA
		Spain: Villamarxant, 39º 35' 1.4" N 0º 37' 1.1" W, 102 m, 23.05.2012, *Álvarez 2290*, MA

Spain: Losa del Obispo, 39º 42' 11.6" N 0º 48' 34.4" W, 401 m, 23.05.2012, *Álvarez 2291*, MA

Spain: Novelda, 38º 23' 8.1" N 0º 44' 34.3" W, 222 m, 03.06.2013, *Álvarez 2329*, MA

Spain: San Vicent de Raspeig, 38º 26' 41.3" N 0º 33' 46.7" W, 260 m, 03.06.2013, *Álvarez 2331*, MA

Table S2. Species, populations and individuals selected as ovule donors for experimental crosses, phenotypic characterization of the synthetic F_1s, and the treatments achieved on each individual. The treatments were: no pollen addition (0); pollen from the own individual (selfing); pollen from other individuals indicating by letters (At, B, F, V, W, and Z) the population which the pollen added was from.

[1] Treatment used for phenotypic characterization.

[2] Treatment not included in seed set analysis.

Species	Population	Individual	Treatments
A. clavatus	B	B23	0, selfing, At[1], B[1], F[1], V, W[1], Z
		B37	B, F, Z
		B177	0, selfing, At, B[1], F, V, W, Z[1]
		B184	F[1,2]
		B186	B[1], Z[1]
		B517	F[1,2]
		B751	selfing, B
	V	V50	0, selfing, At[1], B, F[1], V, W[1], Z[1]
		V55	selfing, B, F, V, Z
		V226	0, selfing, At, B, F, V, W, Z
		V250	At
A. homogamos	At	At321	At, F
		At412	0, selfing, At, B, F, V, W, Z
		At450	At, B
		At492	0, selfing, At, B[1], F[1], V[1], W[1], Z
	Z	Z416	At, B, F, Z
		Z420	0, selfing, At, B[1], F[1], V[1], W[1], Z[1]
		Z747	0, selfing, At, B[1], F, W, Z[1]
		Z983	0, selfing, V, Z[1]
A. valentinus	F	F151	0, selfing, At[1], B[1], F, V[1], W, Z[1]
		F156	B[1,2], Z[1,2]
		F349	At, B, F, Z
		F469	F, W, Z
		F470	B[1,2], Z[1,2]
		F617	0, selfing, At, B, F, V, W, Z
	W	W291	selfing, W
		W527	0, selfing, At, B, F, V, W, Z
		W529	F, W, Z
		W575	0, selfing, At[1], B[1], F, V[1], W, Z[1]

Table S3. Individuals selected as ovule donors for the synthetic F_2s, BCs, and phenotypic characterization indicating the type of crossing of the individuals' origin, the mother plant of the individuals, and the treatments achieved on each individual. Single letters in the treatments (B, F, and Z) indicate the code of the population which the pollen added was from.

(Table S3 continuation) Two letters indicate pollen from a hybrid (F_1) in which the first letter means the maternal origin and the second one the paternal origin.

[1] Treatment used for phenotypic characterization.

Cross of origin	Maternal ID	Individual	Treatments
A. clavatus × A. clavatus	B37	BB2799	B, BF, FB
(B × B)		BB3098	B, BF, FB
	B177	BB1115	BZ[1], ZB[1]
		BB1114	BZ, ZB
		BB1298	BZ, ZB
	B186	BB1110	BZ, ZB
		BB1292	BZ[1], ZB[1]
	B751	BB2794	B, BF, FB
A. clavatus × A. homogamos	B177	BZ1118	B, BZ, Z
(B × Z)		BZ1119	B[1], BZ[1], Z[1]
		BZ1309	B, BZ, Z
		BZ1310	B, BZ, Z
	B186	BZ1358	B[1], BZ[1], Z[1]
A. clavatus × A. valentinus	B184	BF3013	B, BF, F
(B × F)		BF2767	B[1], BF[1], F[1]
	B517	BF2668	B, BF, F
A. homogamos × A. clavatus	Z420	ZB1249	B[1], Z[1], ZB[1]
(Z × B)		ZB1250	B[1], Z[1], ZB[1]
		ZB1689	B, Z, ZB
		ZB2274	B, Z, ZB
	Z747	ZB2049	B, Z, ZB
A. homogamos × A. homogamos	Z420	ZZ1257	BZ, ZB
(Z × Z)		ZZ1260	BZ, ZB
	Z747	ZZ1252	BZ, ZB
		ZZ1690	BZ[1], ZB[1]
		ZZ1691	BZ[1], ZB[1]
	Z983	ZZ2771	FZ, ZF, Z
		ZZ2772	FZ[1], ZF[1], Z
		ZZ3027	FZ, ZF, Z
A. homogamos × A. valentinus	Z420	ZF2780	F[1], Z[1], ZF[1]
(Z × F)		ZF2781	F, Z, ZF
	Z747	ZF3056	F, Z, ZF
A. valentinus × A. clavatus	F156	FB2733	B[1], F[1], FB[1]
(F × B)		FB2734	B, F, FB
		FB3006	B, F, FB
A. valentinus × A. homogamos	F156	FZ2723	F, FZ, Z
(F × Z)	F470	FZ2675	F[1], FZ[1], Z[1]
		FZ2676	F, FZ, Z
A. valentinus × A. valentinus	F349	FF2783	BF, F, FB, FZ, ZF
(F × F)	F469	FF2792	BF, F, FB, FZ, ZF
		FF3077	BF[1], F, FB[1], FZ[1], ZF[1]

Supplementary figure

Figure S1 shows a scatter plot with "Función discriminante 2" on the y-axis (ranging from -4 to 4) and "Función discriminante 1" on the x-axis (ranging from -4 to 4). Legend: A. clavatus (light blue circle), A. valentinus (green triangle), A. homogamos (red square), + Centroide de grupo.

Structure matrix

Discriminant function

	1	2
HEIGHT	,838*	,539
NCAP	-,476	,814*
DIAM	,336	,507*
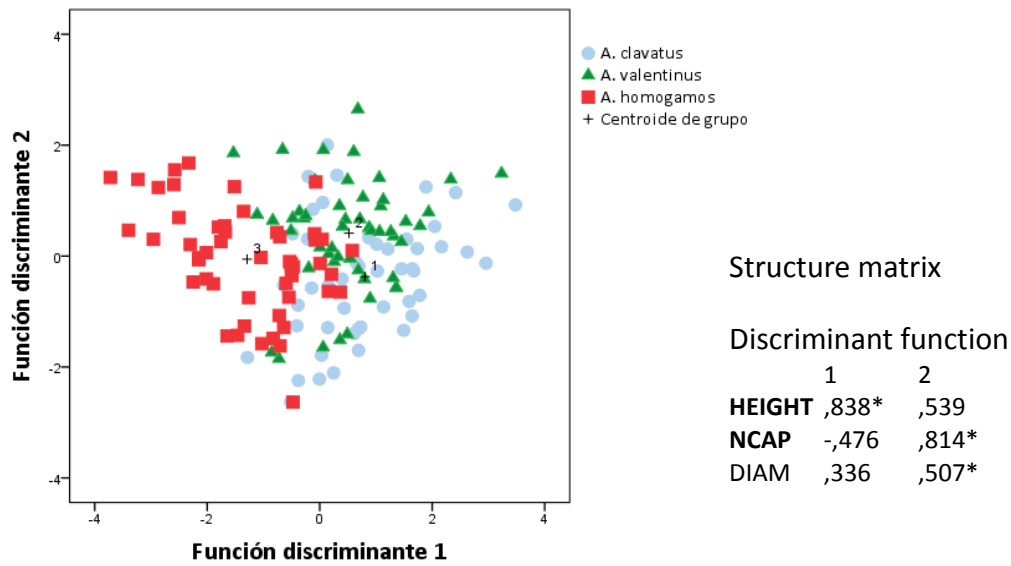
Figure S1. Representation of the two first discriminant functions using vegetative characters for individuals of *A. clavatus*, *A. homogamos* and *A. valentinus*. In the structure matrix, the two characters that most contribute in each discriminant function are in bold. Maximum length of stem (HIGH), number of capitula (NCAP), and maximum diameter of stem (DIAM). The main function for each character is also indicated (*).

CHAPTER TWO: POPULATION STRUCTURE

2.1 Genome size variation in a hybridising homoploid species complex in *Anacyclus* L. (Anthemideae, Asteraceae)

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Original Article

Genome size variation in a hybridising homoploid species complex in *Anacyclus* L. (Anthemideae, Asteraceae)

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Running Title: Genome size variation in *Anacyclus* homoploid species complex

- **Background and Aims** The genus *Anacyclus* comprises several diploid species that may hybridize in their overlapping distribution areas. In this study our main goal was to investigate genome size variation in natural populations of three *Anacyclus* species with special emphasis in their contact areas to evidence the existence of natural hybridization. Additionally, genome size variation in F_1 synthetic hybrids was also studied and compared with the estimates obtained in sympatric sites.

- **Methods** Flow cytometry was used to estimate the genome size of 564 individuals of the species complex of *A. clavatus*, *A. homogamos*, and *A. valentinus* from 30 sites, as well as of 173 individuals obtained from artificial crosses between these three species.

- **Key Results** Differences in genome size between *A. clavatus* and *A. valentinus* were significant in non-overlapping areas of species distribution, whereas in overlapping areas the variation in genome size increased, preventing a clear differentiation. In sympatric sites of *A. clavatus* and *A. valentinus*, individuals with intermediate genome size values between these two species were detected; these values were significantly similar to those obtained from the F_1 experimental hybrids between these species. Genome sizes between *A. clavatus* and *A. homogamos* were not different enough to allow a clear discrimination between them.

- **Conclusions** The different patterns of genome size variation observed in both sympatric populations and overlapping areas of distribution of *A. clavatus* and *A. valentinus* and in their non-overlapping areas, support the existence of gene flow between these species and suggest the occurrence of hybrid zones between them.

Key words: *Anacyclus*, Asteraceae, genome size variation, homoploid, hybridization.

INTRODUCTION

Reproductive isolation and ecological differentiation between hybrids and parental lines leading to homoploid hybrid speciation (i.e., speciation via hybridization without a change in chromosome number) has been considered rare in plant evolution (Rieseberg, 1997; Abbott *et al*., 2013; Yakimowski and Rieseberg, 2014). However, the existence of gene flow and homoploid hybrid swarms between closely related species seems to be relatively common. In overlapping distribution areas between species that may hybridize, the existence of large phenotypic variation together with genetic diversification may precede adaptive radiation and speciation (Abbott *et al*., 2013; Seehausen, 2013; Yakimowski and Rieseberg, 2014).

Although homoploid hybrid speciation may be evidenced by genetic markers (Arnold *et al*., 1991; Rieseberg, 1991; James and Abbott, 2005; Pan *et al*., 2007; Sherman and Burke, 2009; Brennan *et al*., 2012), studies of cytogenetics, reproductive biology and ecology of the involved species are fundamental to understand the mechanisms behind the speciation process. For instance, Lai *et al*. (2005) revealed that different chromosomal rearrangements are involved in the partial reproductive isolation of homoploid hybrids in *Helianthus*. Contrarily, in the homoploid hybrid *Iris nelsonii*, karyotype differences did not contribute substantially to the isolation between this species and its progenitors, and ecological barriers were suggested to be the main determinant for the absence of gene flow (Taylor *et al*., 2013).

As the amount of nuclear DNA is characteristic of a particular species, this character has been considered increasingly useful in the fields of systematics, ecology, and plant evolution (García *et al*., 2004; Kron *et al*., 2007; Loureiro *et al*., 2010; Greilhuber and Leitch, 2013; Suda *et al*., 2015). The advent of more robust and high-throughput techniques as flow cytometry (FCM) has allowed, not only the study of genome size at the population level with the screening of a large number of individuals, but also a more accurate evaluation and interpretation of genome size differences (either absolute or relative) among the analysed individuals. Cases of homoploid hybridization may particularly benefit from a population level survey of genome size when differences are sufficient to be detected by current methods (Loureiro *et al*., 2010).

The evolution of genome size is a highly dynamic process. The major mechanisms responsible for genome changes in homoploid hybrid plants include changes in chromosomal structure and in the number of copies of transposable elements (Bennetzen, 2002; Leitch and Bennett, 2004). The detection of homoploid hybrids using genome size is challenging and, technically, it requires that the parental taxa differ sufficiently in genome size (by at least 7%; Loureiro *et al.,* 2010). Genome size studies focusing on homoploid hybrid speciation or on homoploid hybrids contact zones are still scarce. Some of those studies report intermediate, but non overlapping genome sizes between the hybridizing species (Jeschke *et al*., 2003; Trucco *et al*., 2005, 2006 in *Amaranthus*), whereas others

show intermediate overlapping genome size values (Šiško *et al.*, 2003 in *Cucurbita*; Mahelka *et al.*, 2005 in *Elytrigia*; Bennert *et al.*, 2011 in *Diphasiastrum*; for a review see Loureiro *et al.*, 2010). Still, in other cases, the genome size of the hybrids is closer to the progenitor species with smaller genome (Bureš *et al.*, 2004 in *Cirsium*), whereas in others the established homoploid hybrid species present even more nuclear DNA content than the parents, possibly indicating a positive selection for this trait in the habitats where they occurred (Baack *et al.*, 2005 in *Helianthus*). Indeed, correlations between intraspecific genome size variation and type of habitat were already observed in both monoploid (e.g., *Knautia arvensis*, Kolář *et al.*, 2009) and polyploid hybrids (e.g., *Claytonia perfoliata*, McIntyre, 2012), suggesting a potential adaptive role of genome size in these populations.

Anacyclus is a genus composed by around 12 species of mostly annual herbs, predominantly distributed in western Mediterranean with a few species reaching the Middle East (Humphries, 1979). In this monograph the existence of individuals with intermediate floral characters (mainly related with the length of ray florets) in areas where species ranges overlap was attributed to hybridization. Several studies focused on the cytogenetics and reproductive biology of *Anacyclus* species (Nagl and Ehrendorfer, 1974; Schweizer and Ehrendorfer, 1976; Humphries, 1981) reported remarkable differences in nuclear DNA content among some species (from 9.58 pg in *A. homogamos* to 16.04 pg in *A. radiatus*) and homogeneity in the somatic chromosome number ($2n = 2x = 18$). Humphries (1981) also concluded that the only perennial species, *A. pyrethrum*, is reproductively isolated from all the annuals, whereas crosses between all annual species were successful. Experimental crosses among individuals of *A. clavatus*, *A. homogamos* and *A. valentinus* revealed self-incompatibility and some degree of postzygotic reproductive isolation among them (Álvarez I, Real Jardín Botánico – CSIC, Spain, 'unpubl. res.'). Apart from this, little is known about the relationships among these species. Phylogenetic analyses confirm the inclusion of *Anacyclus* in the tribe Anthemideae within a clade integrated by several genera of the subtribes Anthemidinae and Matricariinae (Agudo A., Real Jardín Botánico – CSIC, Spain, 'unpubl. res.'). However, incongruences between nrDNA and cpDNA based phylogenies, and the scarce representation of the genus in the only partial phylogeny available (Oberprieler, 2004) preclude having a conclusive frame for the evolution of the genus as well as detailed evidence of hybridization.

In the present study, our goal was to investigate genome size variation in natural populations of *Anacyclus clavatus*, *A. homogamos*, and *A. valentinus* to unveil the nature, extent and spatial structure of hybridization in their contact zones. To achieve this, genome size was estimated inside and outside overlapping areas, including sympatric sites, and compared with synthetic F_1 hybrids.

MATERIALS AND METHODS

Study system

This study is focused in the annual species *Anacyclus clavatus*, *A. homogamos* and *A. valentinus*, which differ mainly in the type of peripheral florets in the capitulum (Humphries, 1979; Bello *et al.*, 2013). The taxonomic treatment for species delimitation follows Humphries (1979) with few modifications based in the revision of the genus for the Iberian flora (Álvarez I, Real Jardín Botánico – CSIC, Spain, 'unpubl. res.'). Following this criterion, in *A. clavatus*, around 8-15 peripheral female flowers displaying a white 0.5-1.5 cm ligule form a radiate capitulum, whereas in *A. valentinus*, peripheral female flowers are fewer and display up to 0.3 cm white or yellow ligules, usually hidden behind the involucral bracts. By contrast, in *A. homogamos* all the flowers are tubular and bisexual, and therefore the capitula are discoid. The discoid appearance of the capitula in *A. valentinus* can lead to confusion with *A. homogamos*, unless detailed observations on peripheral florets are made. These three species grow in similar anthropogenic disturbed habitats. Following this taxonomic criterion and based on our own field observations of 290 populations and 586 herbarium specimens, we determined the areas of distribution for these species in western Mediterranean (Fig. 1). *Anacyclus clavatus* occurs all over the Mediterranean Basin; *A. valentinus* is known from coastal areas of the Iberian Peninsula, southern France, northern Morocco and Algeria; and *A. homogamos* is mainly restricted to the Middle Atlas region in northern Morocco. *Anacyclus homogamos* was also reported in different sites along the Iberian Mediterranean coast based on five herbarium specimens (Humphries, 1979; Álvarez I, Real Jardín Botánico – CSIC, Spain, 'unpubl. res.'). However, an exhaustive search for this species across all these Iberian sites did not enable us to find it, and therefore we could not confirm its existence in the Iberian coast. The areas of overlapping distribution between the species were confirmed by the presence of sympatric populations (i.e., populations in which at least two species coexist), where it was also common to find individuals with intermediate floral phenotypes between those of the coexisting species (i.e., hybrids between those coexisting species). Besides sympatric populations, more abundantly in these areas, populations of different species separated by few kilometres were be found, in which in some occasions intermediate or new floral phenotypes (Bello *et al.*, 2013) were also observed. Although the limits of the overlapping zones are not easy to be clearly defined, we arbitrarily considered to be outside an overlapping area when only individuals of a unique species (i.e., unique floral phenotype, excluding the intermediate ones) were observed within a 30 km radius. Considering this, the whole area of distribution of *A. valentinus* is mostly overlapped with part of the distribution areas of *A. clavatus* and *A. homogamos* (Fig. 1).

Plant material

A total of 564 individuals from 30 sites were included to assess genome size variation in natural sites (Fig. 1, Table S1 [**Supplementary Information**]). Generally, 10-19 individuals per site were analysed, except for most of the sympatric sites and for sites selected for experimental crosses in which a more extensive sampling were performed. *Anacyclus clavatus* was represented in a total of 12 sites (i.e., four sites outside overlapping areas, and eight inside overlapping ones, of which four were sympatric sites with *A. valentinus*). In *A. homogamos*, seven out of 8 sites were outside overlapping areas with the other two species; while *A. valentinus* was present in 10 sites plus the four in sympatry with *A. clavatus*. Along our previous field survey we identified the southern and eastern Iberian coast as hotspots for floral phenotype variation in *Anacyclus* (i.e., high variation in ligule length, number and colour, and presence of semi-tubular to tubular ligules), and therefore these areas were sampled in more detail (Fig. 1).

Six individuals cultivated from seeds collected in sites 2, 14 and 29 were selected as mother plants for the experimental crosses. At flowering, each mother plant was hand pollinated to receive two treatments, one per capitulum: 1) self-incompatibility test (i.e., pollen from the same individual); 2) inter-specific pollination (i.e., pollen from one of the other species, one capitulum per species). All treated capitula were bagged before anthesis until seeds were collected. Viable seeds obtained from these treatments were germinated and cultivated in the greenhouse. Finally, a total of 173 individuals (i.e., 21-38 per treatment, except for the self-incompatibility test in which no viable seeds were obtained) were included for FCM estimation of genome size.

Fresh leaf tissue of all individuals was collected either directly in the field and maintained at 4ºC until FCM analysis (usually within 2-3 days) or from greenhouse cultivated plants obtained from seeds collected in natural populations or after experimental crosses. Field sampling was carried out haphazardly with a minimum distance of 5 m between individuals. For plant cultivation a minimum set of 30 seeds per population or type of cross were sown. The outermost winged achenes were preferentially used since they show higher and faster germination rates (Torices *et al.*, 2013).

Genome size estimations using flow cytometry

Genome size was estimated by flow cytometry according to Galbraith *et al.* (1983) procedure for nuclear isolation. In brief, nuclei were released by chopping 0.5 cm^2 of fresh leaf material from each individual of *Anacyclus* together with 0.5 cm^2 of leaf tissue of the internal standard, *Vicia faba* "Inovec" (2C = 26.9 pg, Doležel *et al.*, 1998), with a razor blade in a Petri dish containing 1 mL of WPB (0.2 M Tris.HCl, 4 mM MgCl$_2$.6H$_2$O, 1% Triton X-100, 2 mM EDTA Na$_2$.2H$_2$O, 86 mM NaCl, 10 mM metabisulfite, 1% PVP-10, pH adjusted to 7.5 and stored at 4 ºC; Loureiro *et al.*, 2007). The

nuclear suspension was filtered through a 30 µm nylon filter. Afterwards, nuclei were stained with 50 µg mL^{-1} of propidium iodide (PI, Fluka, Buchs, Switzerland) and 50 µg mL^{-1} of RNAse (Fluka, Buchs, Switzerland) were added to avoid staining of double stranded RNA. After a 3-5 min incubation period, samples were analysed in a Partec CyFlow Space flow cytometer (Partec GmbH., Görlitz, Germany) equipped with a 532 nm green solid-state laser, operating at 30 mW. After the initial analyses, the amplifier system was set to a constant voltage and gain. Each day, prior to analysis, the instrument stability and linearity was checked with fluorescent beads. Results were acquired using Partec FloMax software v2.4d (Partec GmbH, Münster, Germany) in the form of four graphics: fluorescence pulse integral in linear scale (FL); forward light scatter (FS) vs. side light scatter (SS), both in logarithmic (log) scale; FL vs. time; and FL vs. SS in log scale. To analyse only intact nuclei, the FL histogram was gated using a polygonal region defined in the FL vs. SS cytogram. The stability of each sample was controlled by the analysis of the results of the FL vs. time; also, the possible presence of secondary metabolites was evaluated in the FL vs. SS cytogram. At least 1,300 particles were analysed per *Anacyclus*' G$_1$ peak. A coefficient of variation (CV) < 5% was set as quality criterion; whenever higher CV values were obtained, the sample was discarded and repeated until this threshold was achieved. In all cases, two G$_1$ peaks were obtained, one from the *Anacyclus* sp. nuclei and the other from standard nuclei, with minor to negligible G$_2$ peaks, and the measurements obtained were reproducible. Additionally, in order to discard that genome size variation was due to differences in the amount of secondary compounds among species, populations, and individuals, several combined flow histograms were prepared.

The genome size in mass units (2C in pg; *sensu* Greilhuber *et al.*, 2005) was assessed using the formula: sample 2C nuclear DNA content (pg) = (sample G$_1$ peak mean / *Vicia faba* G$_1$ peak mean) * genome size of *Vicia faba*.

Statistical analyses

We explored our results following Zuur *et al.* (2010) recommendations to assure that our data met the assumptions of linear modelling. Differences among species, sites, phenotypes within sympatric sites, and experimental crosses were assessed using analysis of variance (ANOVA). Additionally, we evaluated whether the genome size varied in those sites that were located inside or outside overlapping areas. All models were fitted in the R software (R Core Team, 2015). Genome size differences between different levels of each factor were tested using least-square mean comparison tests from the 'lsmeans' package by applying the Tukey's HSD adjustment (Lenth and Hervé, 2015).

RESULTS

Genome size in natural sites

A whole analysis of genome size by species, excluding sympatric sites, showed high variation and complex patterns. First, *Anacyclus valentinus* significantly differed of both *A. clavatus* and *A. homogamos* (Table 1, n = 310, $F_{2,22}$ = 33.95, P < 0.0001). Second, intra-specific variation in genome size was observed in the three species (Fig. 2). In *A. valentinus* the values ranged from 7.70 to 9.26 pg/2C, with a coefficient of variation (CV) of 3.58%, whereas in *A. homogamos* values from 9.57 to 10.74 pg/2C were observed, with a CV of 2.16% (Table 1). However, in this latter species the CV decreased to 1.47% when only sites outside overlapping areas were considered. The values obtained outside and inside overlapping areas of distribution in *A. homogamos* were significantly different (n = 71, $F_{1,69}$ = 16.09, P = 0.0001). *Anacyclus clavatus* showed the highest variation of the three species, ranging from 8.52 to 11.09 pg/2C (CV of 6.31%), i.e., almost two to three-fold the variation observed in the remaining ones. In this species, when only the sites outside overlapping areas were considered (i.e., sites 19-22), the variation decreases dramatically to a CV of 1.71% with values ranging from 10.16 to 11.09 pg/2C, which are significantly higher than those observed inside overlapping areas (Table 1, n = 115, $F_{1,6.9}$ = 75.62, P < 0.0001).

The variation observed in sympatric sites of *A. clavatus* and *A. valentinus* was the highest overall (CV of 6.87%), with values ranging from 8.03 to 10.72 pg/2C (Table 1). Moreover, these extreme values where obtained from the single site 12 (Fig. 2; Table S2 [**Supplementary Information**]). A detailed analysis in this site also revealed a very high variation in the phenotype (i.e., individuals with intermediate phenotype showed a CV of 6.87%, those with a '*valentinus*' phenotype a CV of 6.38%, and those with a '*clavatus*' phenotype a CV of 4.4%), with an overlap in the genome size values being observed among phenotypes (Table S2 [**Supplementary Information**]). The same was observed in the remaining sympatric sites although lower CV values (2.07-3.33%) were obtained (Table S2 [**Supplementary Information**]).

Genome size variation in synthetic hybrids and intraspecific crosses

All F_1 synthetic hybrids between *A. clavatus*, *A. valentinus* and *A. homogamos* showed intermediate genome sizes to those of their respective parental intraspecific crosses (Fig. 3), and were significantly different in each case (Fig. 3A, *A. valentinus* × *A. clavatus*, $F_{3,128}$ =168, P < 0.0001, n = 132; Fig. 3B, *A. valentinus* × *A. homogamos*, $F_{3,128}$ = 64.4, P < 0.0001, n = 132; Fig. 3C, *A. clavatus* × *A. homogamos*, $F_{3,105}$ = 359.7, P < 0.0001, n = 109). Moreover, the mean values of the synthetic hybrids were very similar to the arithmetical average of the mean values of the intraspecific offspring for each corresponding species pair. Mean 2C-values of synthetic hybrids were not significantly

affected by the direction of the crosses (Fig. 3), although some differences in minimum and maximum values and in the ranges of variation were found (Table S3 [**Supplementary Information**]). Finally, all intraspecific crosses presented genomes sizes similar to the corresponding natural populations for each species (Fig. 2).

DISCUSSION

The genome size values estimated were within the range of variation found in other Asteraceae and in other diploid members of the tribe Anthemideae, using similar techniques (Garnatje *et al.*, 2011; Bennett and Leitch, 2012). The few estimates on genome size for *Anacyclus clavatus*, *A. homogamos* and *A. valentinus* found in the literature fall mostly within the range of our values (i.e., 10.48 pg/2C for *A. clavatus* in Nagl and Ehrendorfer (1974); 11.55 pg/2C for *A. clavatus*, 9.58 pg/2C for *A. homogamos* and 10.54 p/2Cg for *A. valentinus* in Humphries 1981) or are very similar (i.e., 7.41 pg/2C for *A. valentinus* in García *et al.*, 2013). The exceptions found were the reported values of 12.71 pg/2C for one cultivated individual of *A. clavatus* (Humphries 1981), and 11.40 pg/2C for one individual of *A. valentinus* from Liège, Belgium (Nagl and Ehrendorfer 1974). The differences in the methods and sampling used in these previous works, including the methodology and standards used (i.e., Feulgen photometry with *Allium cepa* as reference standard), and the samples origin (i.e., cultivated or escaped from cultivation), prevent a valid comparison with our data. In these two cases both authors reported a chromosome number of 2n = 18 in all samples, and therefore trisomy or presence of B-chromosomes may be discarded as responsible for the differences observed.

Our results revealed continuous and overlapping patterns of variation in genome size among species and populations (Fig. 2) that do not seem to be explained by differences in the number of chromosomes. Supporting the hypothesis of a mostly invariant chromosome numbers within the genus, are the results of a recent work on *Anacyclus* ribosomal loci variation using FISH (Rosato *et al.*, in rev.). In this work we did observations on 196 individuals from 47 populations of all *Anacyclus* species, including several populations for which genome size was presented here (Fig. 2; Table S1 [**Supplementary Information**], i.e., populations 14 "Carchuna", 17 "Ouaoumana", 15 "Salobreña", and 16 "Tighassaline" of *A. clavatus*; population 29 "Asni" of *A. homogamos*; and populations 2 "Castelló d'Empúries" and 9 "Iznate" of *A. valentinus*) and two sympatric populations of *A. clavatus* and *A. valentinus*. Only two individuals of *A. valentinus* from L'Ametlla de Mar presented 2n = 19 chromosomes, in both cases due to the presence of a B-chromosome; the remaining individuals showed the expected number of 2n = 18 chromosomes (Rosato *et al.*, in rev.). Therefore, the occurrence of individuals with deviant chromosome numbers is considered of minor relevance and cannot explain the patterns of genome size variation observed in the present study. This is also

supported by previous chromosome number reports in *Anacyclus* species. No exception to the chromosome number of 2n = 18 was observed in a survey of 33 published works (Rice *et al.*, 2015) that reported chromosome counts for 113 individuals of several *Anacyclus* species, comprising 16 individuals of *A. clavatus*, 6 of *A. homogamos*, and 14 of *A. valentinus* representing their whole areas of distribution. Other factors such as the presence of varying amounts of secondary metabolites in each sample that might affect the fluorescence signal were also discarded based on the results of combined FCM histograms (Fig. 4). In these histograms, prepared with individuals of *A. valentinus* and *A. clavatus* (Fig. 4A), with individuals of *A. clavatus* from populations inside *vs.* outside overlapping areas of distribution with *A. valentinus* (Fig. 4B), and with parentals and F_1 hybrids (Fig, 4C), multiple peaks were observed. Therefore, other causes, such as the number of copies of transposable elements (Bennetzen, 2002; Leitch and Bennett, 2004), rather than chromosome number and technicalities should be the responsible for the variation observed.

The range of genome size variation in sites where *A. valentinus* and *A. clavatus* distribution areas overlap was up to threefold higher than the ranges recorded outside these areas. Within this ample range of variation, intermediate values for both species were also found, which were significant in sympatric sites (Fig. 2). Intermediate genome size values between the putative hybridizing species in sympatric sites were also observed in other hybrids (Trucco *et al.*, 2005, Bennert *et al.*, 2011). Additionally, the values recorded in sympatric sites of *A. clavatus* and *A. valentinus* were similar to those obtained in the experimental hybrids (F_1s) from crosses between those two species, similarly to what has been observed as well in other genera (*Amaranthus*, Jeschke *et al.*, 2003 and Trucco *et al.*, 2006; *Cucurbita*, Šiško *et al.*, 2003; *Elytrigia*, Mahelka *et al.*, 2005).

Despite the significant decrease of seed viability in the F_2s and BCs observed on experimental crosses between *A. clavatus* and *A. valentinus*, reproductive isolation is not complete and gene flow between these species may occur (Álvarez I, Real Jardín Botánico – CSIC, Spain, 'unpubl. res.'). The variation in the mean genome size values found within sympatric sites, coupled with differences in phenotype, might be partially explained by an unequal representation of parental species and hybrids (F_1s, F_2s and BCs) in the contact zones or by an erroneous assignation to an entity based on its phenotype. The phenotypic characterization of synthetic hybrids, revealed an extreme variation in the frequency of phenotypes, which in some cases was completely biased to one parent (Álvarez I, Real Jardín Botánico – CSIC, Spain, 'unpubl. res.'). This might explain that individuals assigned to *A. clavatus* based only on their phenotypes actually may correspond to hybrids between *A. clavatus* and *A. valentinus*, and presented genome size values that are beyond the variation observed in *A. clavatus*.

As a consequence of hybridization between *A. clavatus* and *A. valentinus*, different floral phenotypes are produced that might be adaptive and thus influence plant fitness. Detecting hybrid variants involved in ecological adaptation has been considered a challenging task (Yakimowski and

Rieseberg 2014). In the specific case of *A. clavatus* and *A. valentinus* crosses, floral traits showed a large variation, and have been commonly used for species delimitation (Humphries, 1981; Bello *et al.*, 2013). Our research on pollinator's behaviour in sympatric sites of these two species has showed that the presence of rays can be determinant for plant fitness. Rayed plants attracted more pollinators, and therefore are expected to be less pollen-limited than rayless plants, although the latter plants may compensate the lack of rays by producing a higher number of flowering heads (Cerca de Oliveira J, Natural History Museum, Norway, "unpubl. res."). Along this line, in a similar system, two CYC-like genes, involved in the development of ray flowers in the sunflower family, were expressed in the self-compatible species *Senecio vulgaris* via natural introgression from the rayed *S. squalidus* (Kim *et al.*, 2008). The resulting new hybrid variant was adaptive because rayed *S. vulgaris* became more attractive to pollinators and, thus, presented a higher outcrossing rate (Abbott and Irwin, 1988). In *Anacyclus*, the rayed phenotype is controlled by a similar set of genes (Bello M.A., Real Jardín Botánico – CSIC, Spain, 'unpubl. res.'), but unlike *Senecio vulgaris*, both *A. valentinus* and *A. clavatus* are self-incompatible species. Thus, the study of the adaptive significance of ray expression in the contact zones between these two species provides an opportunity to study the effect of self-incompatibility in the evolution of floral traits in homoploid hybrids.

Our results also suggest that gene flow and hybridization might be occurring across overlapping areas of other *Anacyclus* species in Morocco. Although no sympatric sites between *A. homogamos* and *A. clavatus* or between *A. homogamos* and *A. valentinus* were found during our field work, significant differences across *A. homogamos* sites together with similarities observed in genome size values between some natural populations and the F_1s offspring from crosses between *A. clavatus* and *A. homogamos*, suggest that hybridization between these species has taken place. However, in this case, identification of contact areas based solely in genome size differences is much more challenging since genome size of the putative parents is quite similar (Fig. 2).

In conclusion, the characterization of genome size of *A. clavatus*, *A. valentinus*, and *A. homogamos* from populations outside and inside their overlapping areas confirms that the variability observed in floral phenotypes inside these areas is also reflected in terms of genome size, and the pattern of variation is congruent with the existence of hybridization in these areas. Although *Anacyclus* species are all diploid with identical chromosome number and their hybrids are thus homoploid, the different ranges of genome size variation detected in two of these species allowed to document intermediate genome sizes in contact zones where hybridization is occurring. Our investigations also confirmed the potential usefulness of flow cytometry for a rapid assessment of genome size at the population level in homoploid organisms.

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TABLES

Table 1. Genome size (2C-values in picograms, 2C/pg) variation observed in the species and areas studied. (Description on next page)

Species/phenotype	Areas of distribution [1]	Genome size variation (2C/pg)				N	Sites
		Mean ± SD	Min	Max	CV (%)		
A. clavatus	outside	10.53 ± 0.18 a	10.16	11.09	1.71	61	4
	overlapping	9.40 ± 0.23 b	8.52	10.18	3.34	79	4
	whole	9.97 ± 0.63 A	8.52	11.09	6.31	140	8
	sympatric sites	9.46 ± 0.31	8.36	10.72	4.50	54	4
A. homogamos	outside	10.22 ± 0.15 a	9.86	10.74	1.47	82	7
	overlapping	9.93 ± 0.20 b	9.57	10.21	2.01	10	1
	whole	10.10 ± 0.22 A	9.57	10.74	2.16	92	8
A. valentinus	overlapping	8.36 ± 0.30 B	7.70	9.26	3.58	160	10
	sympatric sites	9.00 ± 0.35	8.14	9.90	6.38	46	3
intermediate	sympatric sites	9.03 ± 0.36	8.03	10.20	6.87	72	3

Table 1. Genome size (2C-values in picograms, 2C/pg) variation observed in the species and areas studied. SD, standard deviation; Min., minimum value; Max., maximum value; CV, coefficient of variation (%); N, number of individuals analysed; Sites, number of sampled sites. [1] *outside* - sites distributed outside the overlapping area with other *Anacyclus* species; *overlapping* – sites distributed in the overlapping area excluding sympatric sites. Different letters denote significant differences (*P* < 0.05) according to the least-squares means comparison test. Lower-case letters were used to show within-species differences between outside and overlapping areas and upper-case letters to show statistically different mean GS values between the whole distributions of the three species. Please note that only phenotypes intermediate between *A. clavatus* and *A. valentinus* were analysed.

FIGURES
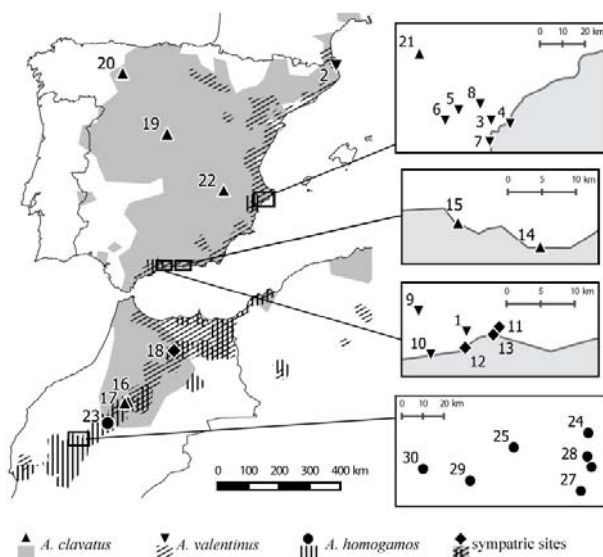
Figure 1. Potential distribution areas of *Anacyclus valentinus, A. clavatus* and *A. homogamos* in western Mediterranean based on previous taxonomic revisions (Humphries, 1979) and in our own field observations.
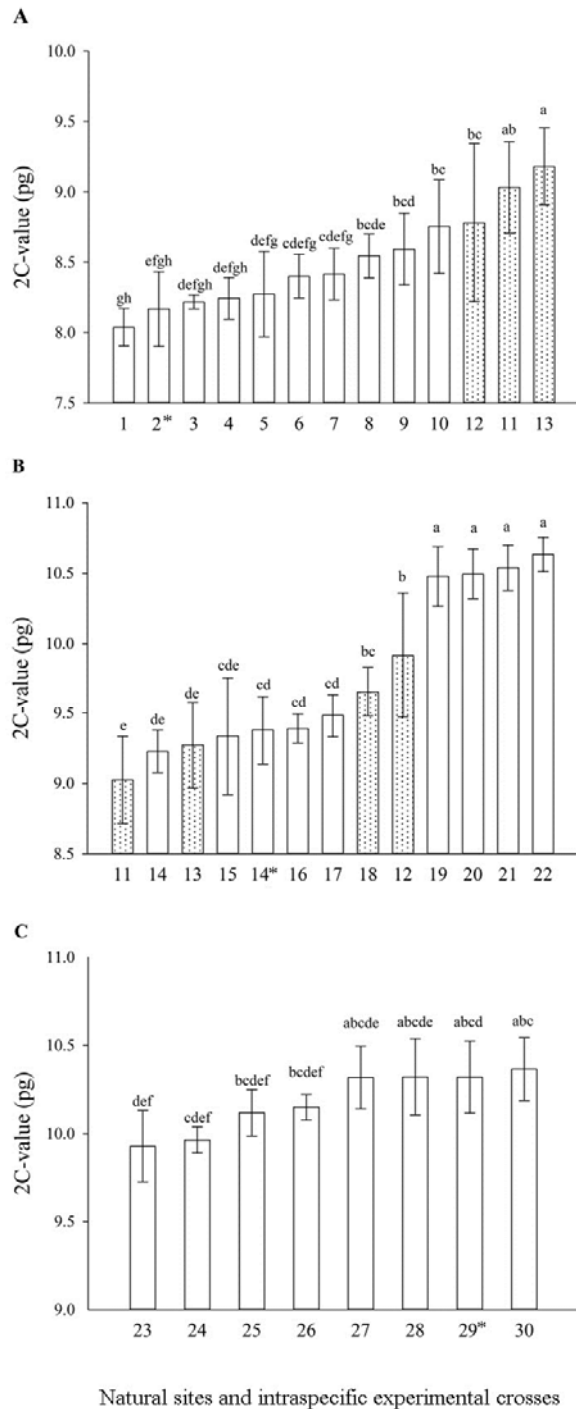
Figure 2. Mean 2C-values (pg) and standard deviation from natural sites and intraspecific experimental crosses (F_1) of: (A) *Anacyclus valentinus*; (B) *A. clavatus*; and (C) *A. homogamos*. Dotted motif indicates sympatric sites. An asterisk alludes to the values obtained from intraspecific experimental crosses (F_1). Different letters denote significant differences ($P < 0.05$) according to the least-squares means comparison test.
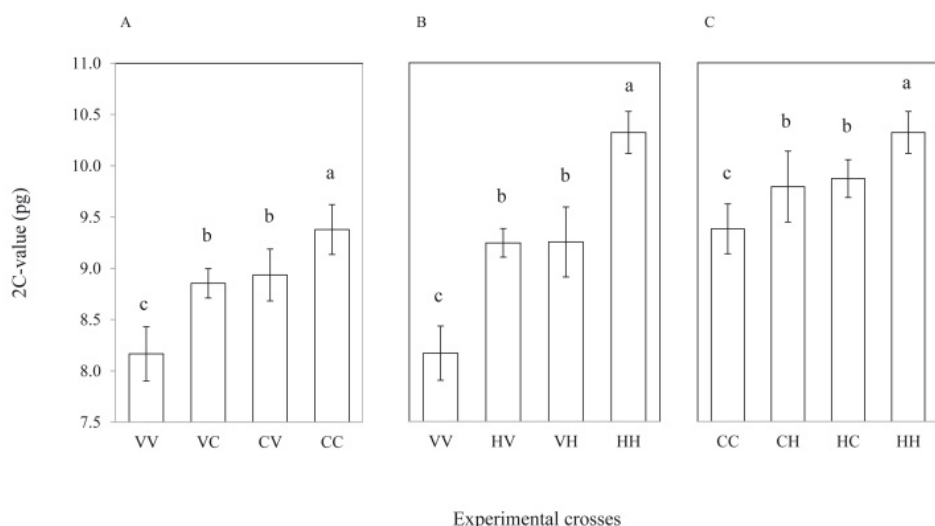
Figure 3. Mean of 2C-values (pg) and standard deviation of F_1s obtained by experimental crosses. (A) Crosses between *Anacyclus clavatus* and *A. valentinus*. (B) Crosses between *A. valentinus* and *A. homogamos*. (C) Crosses between *A. clavatus* and *A. homogamos*. In all cases, the respective intraspecific crosses are included for comparison. Note that the first letter of the cross corresponds to the ovule donor and the second one to the pollen donor. V, *A. valentinus*; C, *A. clavatus*; H, *A. homogamos*. Different letters above bars denote significant differences ($P < 0.05$) according to the least-squares means comparison test.
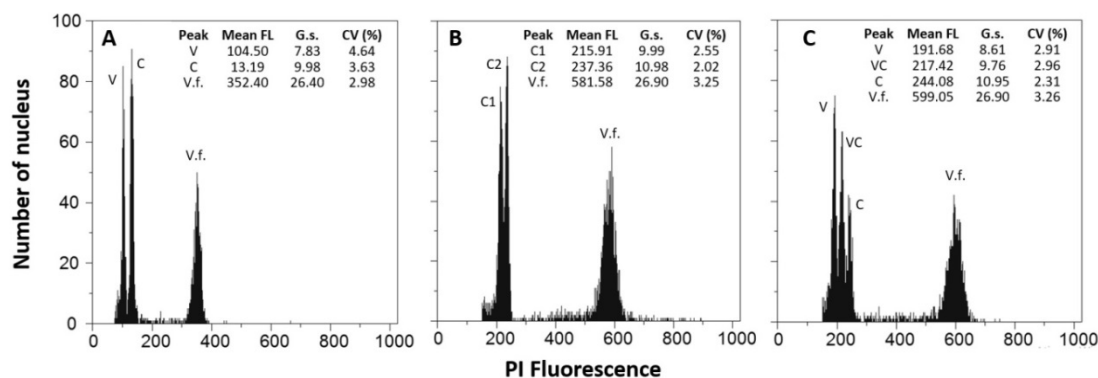
Figure 4. Relative fluorescence histograms of propidium iodide stained nuclei isolated from fresh leaf tissues of the reference standard *Vicia faba* "Inovec" (V.f.) and *Anacyclus* species: (A) *A. valentinus*, V (individual from site 3), and *A. clavatus*, C (individual from site 20), (B) *A. clavatus* from different populations, C1 (individual from site 14) and C2 (individual from site 22), and (C) *A. valentinus*, V, hybrid between *A. valentinus* and *A. clavatus*, VC, and *A. clavatus*, C.

SUPPLEMENTARY DATA

TABLES

Table S1. List of the sites included in the study, the species and/or phenotypes presented in each site, locality, latitude and longitude expressed in decimal degrees, altitude in meters above sea level, Collector ID, and type of sample collected. Vouchers of species collected in each site were deposited at MA Herbarium. *, sites sampled for experimental crosses.

Site	Species/phenotype	Locality	Latitude	Longitude	Altitude (m a.s.l.)	Collector ID	Sample type
1	*A. valentinus*	Torre del Mar, Spain	36.7510	-4.0998	514	RT72	leaves
2*	*A. valentinus*	Castelló d'Empuries, Spain	42.2631	3.1293	0	IA2059	achenes
3	*A. valentinus*	Alicante, Spain	38.3813	-0.5091	92	IA2020	achenes
4	*A. valentinus*	Cabo de Huertas, Spain	38.3658	-0.4135	33	IA2021	achenes
5	*A. valentinus*	Agost, Spain	38.4248	-0.6732	313	IA2330	leaves
6	*A. valentinus*	Novelda, Spain	38.3856	-0.7429	222	IA2329	leaves
7	*A. valentinus*	Urbanova beach, Spain	38.2993	-0.5198	1	IA2018	achenes
8	*A. valentinus*	San Vicent de Raspeig, Spain	38.4448	-0.5630	260	IA2331	leaves

9	*A. valentinus*	Iznate, Spain	36.7764	-4.1792	285	IA2137	leaves
10	*A. valentinus*	Benajarafe, Spain	36.7210	-4.1581	8	IA2133	leaves
11	*A. valentinus*, *A. clavatus*, and intermediate phenotypes	Algarrobo, Spain	36.7282	-3.9566	10	IA2134a	leaves
12	*A. valentinus*, *A. clavatus*, and intermediate phenotypes	Vélez-Málaga, Spain	36.7305	-4.1017	1	IA2144	leaves
13	*A. valentinus*, *A. clavatus*, and intermediate phenotypes	Algarrobo Costa, Spain	36.7478	-4.0557	5	IA2134b	leaves
14*	*A. clavatus*	Carchuna, Spain	36.6968	-3.4591	13	AA1	achenes
15	*A. clavatus*	Salobreña, Spain	36.7250	-3.5807	0	IA2135	achenes
16	*A. clavatus*	Tighassaline, Morocco	32.7565	-5.6753	871	AA49	achenes
17	*A. clavatus*	Ouaoumana, Morocco	32.7172	-5.7990	762	AA47	achenes
18	*A. valentinus*, *A. clavatus*, and intermediate phenotypes	Taza, Morocco	34.2231	-3.9703	486	IA2224	achenes
19	*A. clavatus*	Soto del Real, Spain	40.7531	-3.7814	934	IA2327	leaves
20	*A. clavatus*	León, Spain	42.6377	-5.4239	860	RT2	achenes
21	*A. clavatus*	Villena, Spain	38.6475	-0.8667	525	IA2332	leaves
22	*A. clavatus*	Chinchilla, Spain	38.9377	-1.7578	761	IA2328	leaves
23	*A. homogamos*	Bin El Ouidane, Morocco	32.1128	-6.4207	845	AA38	achenes
24	*A. homogamos*	Toufliht, Morocco	31.4686	-7.3997	1448	AA74	achenes
25	*A. homogamos*	Douar Ouriki, Morocco	31.3952	-7.7679	867	AA78	achenes
26	*A. homogamos*	Ait Ben Ammar, Morocco	31.3719	-7.3998	1534	AA73	achenes
27	*A. homogamos*	Ighrem N'ougdal, Morocco	31.2289	-7.4257	1918	AA67	achenes
28	*A. homogamos*	Col du Tichka, Morocco	31.3293	-7.3773	1744	AA72	achenes
29*	*A. homogamos*	Asni, Morocco	31.2511	-7.9778	1160	IA2115	achenes
30	*A. homogamos*	Wawizelt, Morocco	31.2900	-8.2141	844	AA88	achenes

Table S2. Genome size estimated (2C-values in picograms, 2C/pg) for the individuals sampled in each site. SD, standard deviation; Min., minimum value; Max., maximum value; CV, coefficient of variation; N, number of individuals analysed. In sympatric sites individuals were analysed also by phenotype, except for site 18, in which only seeds of individuals of *A. clavatus* were available.

Site	Species/phenotype	Genome size (2C/pg)				N
		Mean ± SD	Min.	Max.	CV (%)	
1	*A. valentinus*	8.04 ± 0.13	7.73	8.28	1.62	19
2	*A. valentinus*	8.17 ± 0.26	7.70	9.04	3.18	36
3	*A. valentinus*	8.22 ± 0.05	8.13	8.28	0.61	10
4	*A. valentinus*	8.24 ± 0.15	8.04	8.46	1.82	10
5	*A. valentinus*	8.27 ± 0.31	7.92	8.99	3.75	15
6	*A. valentinus*	8.40 ± 0.16	8.17	8.76	1.90	15
7	*A. valentinus*	8.42 ± 0.18	8.14	8.70	2.14	10
8	*A. valentinus*	8.54 ± 0.16	8.22	8.76	1.87	15
9	*A. valentinus*	8.59 ± 0.26	8.05	9.07	3.03	15
10	*A. valentinus*	8.75 ± 0.33	8.14	9.26	3.77	15
11	all species and phenotypes	9.02 ± 0.30	8.36	9.89	3.33	42
	A. valentinus	9.03 ± 0.32	8.36	9.68	3.54	14
	A. clavatus	9.02 ± 0.31	8.36	9.54	3.44	14
	intermediate phenotypes	9.00 ± 0.29	8.63	9.89	3.22	14
12	all species and phenotypes	9.07 ± 0.71	8.03	10.72	7.83	86
	A. valentinus	8.78 ± 0.56	8.14	9.90	6.38	18
	A. clavatus	9.91 ± 0.44	8.92	10.72	4.44	17
	intermediate phenotypes	8.88 ± 0.61	8.03	10.20	6.87	51
13	all species and phenotypes	9.22 ± 0.27	8.80	9.81	2.93	34
	A. valentinus	9.18 ± 0.27	8.80	9.81	2.94	14
	A. clavatus	9.27 ± 0.31	8.81	9.72	3.34	13
	intermediate phenotypes	9.20 ± 0.19	8.98	9.51	2.07	7
14	*A. clavatus*	9.38 ± 0.24	8.91	10.18	2.56	43
15	*A. clavatus*	9.34 ± 0.42	8.52	10.15	4.50	15
16	*A. clavatus*	9.39 ± 0.10	9.24	9.54	1.06	10
17	*A. clavatus*	9.48 ± 0.15	9.31	9.85	1.58	11
18	*A. clavatus*	9.65 ± 0.18	9.49	9.97	1.87	10
19	*A. clavatus*	10.48 ± 0.21	10.22	11.09	2.00	15
20	*A. clavatus*	10.49 ± 0.18	10.16	10.91	1.72	16
21	*A. clavatus*	10.54 ± 0.16	10.21	10.79	1.52	15
22	*A. clavatus*	10.63 ± 0.12	10.35	10.84	1.13	15
23	*A. homogamos*	9.93 ± 0.20	9.57	10.21	2.01	10
24	*A. homogamos*	9.96 ± 0.07	9.86	10.08	0.70	10

25	*A. homogamos*	10.12 ± 0.13	9.93	10.39	1.28	10
26	*A. homogamos*	10.15 ± 0.07	10.04	10.28	0.69	11
27	*A. homogamos*	10.32 ± 0.18	10.07	10.67	1.74	10
28	*A. homogamos*	10.32 ± 0.22	10.07	10.68	2.13	10
30	*A. homogamos*	10.37 ± 0.18	10.17	10.71	1.74	10

Table S3. Genome size estimated (2C-values in picograms, 2C/pg) for the F_1 individuals in each type of experimental cross. SD, standard deviation; Min., minimum value; Max., maximum value; CV, coefficient of variation; N, number of individuals analysed.

Type of cross	Genome size (2C/pg)				N
	Mean ± SD	Min.	Max.	CV (%)	
A.valentinus × A. clavatus	8.85 ± 0.14	8.54	9.08	1.58	21
A.clavatus × A. valentinus	8.94 ± 0.25	8.55	9.59	2.80	32
A.homogamos × A. valentinus	9.24 ± 0.14	8.93	9.53	1.52	31
A.valentinus × A. homogamos	9.25 ± 0.34	7.99	9.73	3.68	21
A.clavatus × A. homogamos	9.79 ± 0.35	8.99	10.42	3.58	38
A.homogamos × A. clavatus	9.87 ± 0.18	9.64	10.42	1.82	30

2.2. Geographic and environmental patterns in the genetic structure of the Western Mediterranean *Anacyclus* L. (Anthemideae, Asteraceae) species complex

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153

INTRODUCTION

As one of the world biodiversity hot-spots, the Mediterranean Basin harbours also high variation at population levels (Myers & al. 2000, Comes 2004). Gradual range expansion, radiations, changes in reproductive system, and hybridization were identified as some of the main processes that have contributed to generate such diversity (Nieto Feliner 2014). These processes have taken place in complex geological, climate, and ecogeographical scenarios (Blondel & Aronson 1999, Blondel & al. 2010, Hewitt 2011, Cowling & al. 2015) that increase the difficulty to interpret current distribution patterns of species and populations. In these cases, genetic population approaches are indicated to understand the biodiversity observed and the evolutionary history of lineages, specifically in cases of recent radiations (Guzmán & Vargas 2009, Balao & al. 2010, Mayol & al. 2012, López-Vinyallonga & al. 2015) and hybridization (Belaj & al. 2007, López de Heredia & al. 2007, Lo Presti & Oberprieler 2011, Ortego & al. 2016) where phylogenetic footprint is usually unclear.

Integrative approaches combining genomic phylogeography (e.g. Ren et al. 2017) and population genetic structure (e.g. Marcer et al. 2016) with species niche modeling (Guisan & Zimmermann 2000) have shown excellent results to understand current species patterns and the processes behind. The genetic structure of a species reflects its demographic history by the distribution patterns of its genetic diversity (i.e. clines, patches, and clusters) whereas the species niche modelling provides the ecological niche of such species. In addition, this framework allows the study of adaptive and neutral genetic variation at the population level and the factors that may influence it (i.e., natural selection, genetic drift, gene flow, etc.) over evolutionary time (Schoville & al. 2012). Thus, this integrated approach is essential to understand evolutionary and ecological processes in complex situations such as the previously

154

mentioned for the Mediterranean Basin (Jakob & al. 2007, Fernández-Mazuecos & Vargas, 2013, Temunovič & al. 2013).

The genus *Anacyclus* is mainly represented by annual herbs that grow on anthropogenic environments in Western Mediterranean (Humphries 1979). As human activities form an extended net connected by roadsides, which is one of the preferred habitats of *Anacyclus* sp. (authors' *pers. obs.*), a continuous occurrence of these species is observed in places with high urban density such as some places in the Mediterranean coast. This continuum is usually formed by a single species, but sometimes different ones cohabit and phenotypic variation increases preventing a clear identification of individuals that were considered as hybrids (Humphries 1979, 1981). The phenotypic variation observed occurs on floral traits and includes differences in sexual systems (i.e., gynomonoecy and hermaphroditism), types of capitula (i.e., rayed and discoid), and in the colour, shape, and length of the ligule in female peripheral florets. This type of variation is moderated by strong selection pressure due to their direct implications in plant fitness (Endler 1986, Donoghue & al. 1998, Fenster & al. 2004, Harder & Barrett 2006, Armbruster & al. 2009).

Giving the biological and geographical complexity of this study case, a landscape genetic framework seems appropriate to investigate the factors involved in the patterns of the variation observed. Based on two sampling strategies that jointly represent the inter- and intra-population variation observed at interspecific level, we aimed to analyse the genetic variation of this system and its distribution patterns to compare them with the species potential distribution by SDMs. As a novelty, the spatial distribution of the different genetic groups identified was modelled as well (see also Marcer & al. 2016, Ikeda & al. 2016). This approach allowed to understand the geographic patterns of the genetic and phenotypic variation observed, specifically in

sympatric populations and in trailing edges of species ranges, to document the genetic identity of the species, and the existence of current natural gene flow between them.

The objectives of this work were: (1) to test the genetic identity and explore the genetic structure of species complex *A. clavatus* (Desf.) Pers., *A. valentinus* L. and *A. homogamos* (Maire) Humphries and their closest relatives; (2) to test the hypothesis of current natural hybridization between the species of the complex; and (3) to explore in which degree environmental factors might explain species biogeography and genetic clusters distributions. To achieve these goals we genetically characterized a representative number of populations of the species complex with special emphasis on sympatric populations to draw the genetic landscape on their areas of distribution. Additionally, species and genetic distribution modelling based on environmental factors were performed to assess how the species and the genetic groups differed in their ecological niches.

Study system

This study is mostly focused in three species, *Anacyclus clavatus*, *A. homogamos* and *A. valentinus*, which are morphologically similar. They differ mainly in the type of peripheral florets in the capitulum (Humphries, 1979; Bello *et al.*, 2013; Álvarez, in rev.). *Anacyclus clavatus* presents heterogamous capitula, with 8-15 peripheral female flowers that display a white ligule of 0.5-1.5 cm length. *Anacyclus valentinus* presents heterogamous capitula as well, but in this case peripheral female flowers are scarce and they may be white or yellow with a ligule up to 0.3 cm in length, which are usually hidden by the involucral bracts (Álvarez, in rev.). In *A. homogamos* all flowers are tubular and bisexual. Therefore, both *A. homogamos* and *A. valentinus*

show discoid capitula, whereas *A. clavatus* displays rayed ones. The sympatric populations of *A. clavatus* and *A. valentinus* present a high variation of phenotypes, which comprises the typical morphology of these two species and an unclassifiable intermediate phenotypes according to ligule length and number.

Study area and sampling

The sampling of this study (Figure 1) covers the main distribution areas of the three species: *A. clavatus*, *A. valentinus*, and *A. homogamos*, which are in Western Mediterranean. Other *Anacyclus* species, *A. radiatus* subsp *radiatus*, *A. radiatus* subsp. *coronatus* and *A. monanthos* were also collected for comparative purposes. The sampling was carried between 2010 and 2013 following two strategies in order to screen the whole areas but also to survey the genetic structure at population level. First, one to three individuals were collected from each of 177 locations; and in the second strategy a total of 31 representative populations where sampled in detail (13 to 42 individuals per population). Within population, individuals were haphazardly collected separated at least by 10 m. Leaves of each individual were stored in silica gel. In total, the sampling included 798 individuals, of which 315 were from 89 populations of *A. clavatus*, 233 were from 40 populations of *A. valentinus*, 122 were from 17 populations of *A. homogamos*, and 110 were from 14 sympatric populations of *A. clavatus* and *A. valentinus*. The remaining samples were 1-2 individuals from each five, four, and eight populations of *A. radiatus* subsp *radiatus*, *A. radiatus* subsp. *coronatus* and *A. monanthos*, respectively (Figure 1 and see Table S1 in supporting information).

DNA extraction and amplification

Total genomic DNA was extracted from silica-dried leaves using the DNeasy Plant Minikit (QIAGEN, Hilden, Germany). For genotyping we used eight of the microsatellite markers previously developed for *A. clavatus* and *A. valentinus* that also amplified in the other three closely related species included in this study (Agudo *et al.* 2013). The selected markers were the loci 9, 15, 17, 19, 20, 21, 24 and D3. In order to discard homoplasy when comparing genotypes between different species (German-Aubrey *et al.*, 2016), sequences of at least two different alleles per locus and species were analyzed. The following wet-lab experiments were conducted by AllGenetics & Biology SL (A Coruña, Spain). Fragment analysis: PCRs were carried in a final reaction volume of 12.5 µL, containing 1 µL of DNA (10 ng/µL), 6.25 µL of the Type-it Microsatellite PCR Kit (Qiagen), 4 µL of PCR-grade water, and 1.25 µL of the primers mix, with forward primers labeled with PET, NED, VIC or 6-FAM dyes. The PCR protocol consisted in an initial denaturation step at 95 ºC for 5 min, followed by 35 cycles of 95 ºC for 30 s, 56 ºC for 90 s, 72 ºC for 30 s; and a final extension step at 68 ºC for 30 min. PCR products were pooled in two multiplexes (PCR 1: loci 17, 19, 20 and 24; PCR 2: loci 9, 15, 21 and D3) and analyzed on a 3130XL DNA Analyzer (Applied Biosystems). Sequencing: PCRs were carried out in a final volume of 25 µL, containing 12.50 µL of Supreme NZYTaq Green PCR Master Mix (NZYTech), 0.5 µM of each primer, 25 ng of template DNA, and PCR-grade water up to 25 µL. The thermal cycling conditions were as follows: an initial denaturation step at 95 ºC for 5 min, followed by 35 cycles of denaturation at 95 ºC for 1 min; annealing at 56 ºC for 1 min; extension at 72 ºC for 45 s; and a final extension step at 72 ºC for 5 min. A negative control was included in each PCR round to check for cross-contamination during the experiments. PCR products were run on 1 % agarose gels stained with GreenSafe

Premium (NZYTech), and imaged under UV light to verify the amplicon size. PCR products were sequenced using the PCR primers in a in a 3730XL DNA Analyzer (Applied Biosystems) from the short flanking region side primer or both sides when needed.

Microsatellite screening and analysis

The length of the fragments was automatically scored and checked by eye with the software Geneious v. 7.1.2 (Kearse *et al.,* 2012). The sequences obtained were aligned using as reference the known sequences of each loci (Agudo *et al.* 2013) and edited with Geneious. Only the samples with at least a 75 % of amplification across the eight loci were selected for succeeding analyses. Homozygote excess was tested with Micro-Checker v. 2.2.3 (Van Oosterhout et al. 2004) to evaluate the presence of null alleles per locus among species and populations.

Genetic diversity and structure

Number of haplotypes (Nh), sample size (N), percentage of polymorphic loci (PL), number of alleles (Na), number of private alleles (Np) and observed heterozygosity (Ho) were measured using GenAlex v. 6.501. Gene diversity per locus and population (Hs) and inbreeding factor (Fis) per population was obtained with FSTAT v. 2.9.3 (Goudet, 2001). Wright's Fst (Wright, 1950) and Nei's genetic distance (Nei, 1972) were calculated in Genalex and Weir and Cockerham Fst estimator (1984) in FSTAT with a 0.1% nominal level of significance for multiple tests after Bonferroni corrections. Additional analyses to calculate unbiased Fst values in the presence of null

alleles were also performed using the locus-by-locus analysis of molecular variance (AMOVA) with 10,000 permutations for significant tests with Arlequin 3.5 (Excoffier, *et al.*, 2005) and following the ENA method with the software FreNA (Chapuis & Estoup 2007), with 1000 replicates of bootstrap resampling over loci. Since all these different methods produced similar results, the Fst values obtained with FSTAT were hereinafter referred for standardization. As misleading inbreeding coefficient (*Fis*) values can be obtained in the presence of subpopulations within a population, the scores obtained were confronted with the percentages of individuals belonging to each genetic cluster per population. Isolation by distance (IBD) per species was tested through Mantel tests using Arlequin.

Genetic clusters assessment

To further understand the genetic structure of the species complex we performed two clustering analysis including all samples. In the first one, a Bayesian model-based method was used to estimate the number of genetic clusters (K) of the data with STRUCTURE version 2.3.4 (Pritchard *et al.*, 2000, Falush *et al.*, 2003). Both uncorrelated (Pritchard *et al.*, 2000) and correlated (Falush et al., 2003) allele frequencies models, were tested under admixture ancestry model. Burning period was set as 50,000 runs and MCMC repetitions after burning to 100,000. Per simulation, two to ten K (number of genetic populations) and 20 iterations per K were run. Analysis and visualization of the population structure was performed by the R package *pophelper* 1.1.9 (Francis, 2016). Best K was inferred using the ΔK method (Evanno *et al.*, 2005). The average matrix of multiple runs per K, which was used for subsequent calculations, as well as the highest value of the symmetric similarity coefficient (H') across runs per

K, were obtained with CLUMPP v 1.1.2 (Jakobsson & Rosenberg, 2007). Visualization of pie plots on a map was performed using the R package *mapplots* (Gerritsen, and Gerritsen, 2014).

The second method used was a Principal Component Analysis (PCA), which allows for visualization of the data across a coordinate system defined by a covariance matrix. This analysis was performed with the R package *adegenet* (Jombart, 2008), based on standardized allele frequencies. The three eigenvectors accounting for the maximum amounts of variance were selected for subsequent cluster analysis.

Genetic assignment

Previous studies (e.g. Cullingham *et al*., 2011; Hasselman *et al*., 2014, Ortego *et al.*, 2016) often used a posterior probability of Q≥0.90 as a threshold to assign complete membership to one genetic group, in contrast of a mixed ancestry, which is usually interpreted as a hybrid signal. In this study we followed this threshold limit and the analyses of genetic diversity and differentiation among genetic groups were preformed considering these samples of mixed ancestry apart.

Taxonomic assignment on sympatric populations

Individuals from three of the five sympatric populations were classified according to their floral phenotypes as *A. clavatus*, *A. valentinus*, and intermediates (Figure S1). In this way, we tested if floral phenotypes in these sympatric sites were correlated with any of the observed genetic groups. For testing this association between floral phenotype and genetic group, we fitted generalized linear models where the

probability of membership to each one of the genetic groups was considered the response variable using a binomial distribution. Floral phenotype was included as an explanatory variable and population as random factor. All models were fitted in R.

Ecological modelling

We modelled the potential distribution of the five species and the intraspecific genetic structure (the four genetic clusters obtained from previous analysis).

Our own field observations during sampling and data obtained from a survey of 1,559 *Anacyclus* specimens revised across 12 herbaria fieldwork were used as presences to calibrate the species distributions models (SDMs). To avoid sampling bias (Syfert et al., 2013), only points that were separated by at least 1 km from each other (i.e. matching the resolution of the climatic data) were retained, resulting in 1,141 occurrences (Figure S2).

The bioclimatic variables available in WorldClim 1.4 at 1km resolution (http://www.worldclim.org, Hijmans et al., 2005) were employed as predictors of the species distribution models. As background, we randomly selected 10,000 points over the entire study area. To avoid multicollinearity, we ran a correlation analysis on the background points and eliminated one of the variables in each pair with a Pearson correlation value >0.8. The variables finally included in the models were: bio01 (annual mean temperature), bio03 (isothermality), bio08 (mean temperature of the wettest quarter), bio09 (mean temperature of the driest quarter), bio15 (precipitation seasonality), and bio19 (precipitation of coldest quarter).

SDMs were carried out to determine suitable present potential macroclimate niche of *Anacyclus* species in the Western Mediterranean area. For this purpose, we produced an ensemble model (Araújo & New, 2007; Mateo et al., 2012) using two modelling techniques, i.e., generalized linear models (GLM; McCullagh & Nelder, 1989) and gradient boosting machine (GBM; Friedman, 2001). We used the BIOMOD 2.0 package in R (Thuiller et al., 2009, www.R-Forge.R-project.org) for the modelling, using the parameters set by default.

Models were calibrated with 70% of the data, and evaluated with the remaining 30%, area under the ROC curve (AUC) and true skill statistic (TSS). For each technique, presences and pseudo-absences used to calibrate the model were weighted such as to ensure neutral (0.5) prevalence. The procedure was replicated 10 times, with random training and evaluation datasets, such that we obtained 30 models (10 replicates x 3 techniques). To remove spurious models, the ensembles were generated using the models that had an AUC higher than 0.8 and a TSS higher than 0.7. The contribution of each model to the final ensemble model was proportional to their goodness-of-fit statistics. The potential distribution was considered as a consensus across statistical techniques and their contribution to the ensemble was proportional to their AUC values. The consensus model was converted in a binary model (presence/absence) applying three different threshold criteria: thresholds that allow a maximum of 5% or 10% of omission error (i.e. omission error is the percentage of the real presence predicted as absences in the model; Fielding & Bell, 1997), and the threshold maximizing AUC statistic.

We used Beta regression (package betareg v3.1-0, R computing environment), and Regression trees (package rpart v4.1-10, R computing environment) to model the potential distribution of genetic groups. Instead of relying on the widely used species

distribution modelling techniques, which force a binarization of the response variable with the consequent loss of data, we directly modelled the continuous probability of membership to each genetic cluster as given by STRUCTURE (see Genetic clusters assessment section above). We built our final model as a weighted average of both models based on their mean absolute error. The degree of niche overlap between species, between species and genetic groups, and between genetic groups was estimated according to the Pianka Index (Pianka, 1974). To calculate this index binary data are needed, and thus consensus models were performed based on species presence/absence and genetic group binarized data obtained by assigning the presence/absence according to a 0.90 membership threshold.

RESULTS

Microsatellite amplification and variation across species

The patterns of amplification and variation of the eight microsatellite markers for the five *Anacyclus* species are presented in Table S2. The low amplification and polymorphism observed in these loci could be due to polymorphisms across primer regions, as it has been commonly observed in other inter-species studies using SSR (Barbara *et al*., 2007). Sequences of a representative sampling of flanking regions in our markers found some polymorphisms of 1-2 bps within these regions in loci 17, 20, 21 and D3, whereas loci 15, 21 and 24 presented indels among different alleles (Table S3). However, in any case these polymorphisms and indels caused misinterpretation on the length of the scored fragments across species, and thus homoplasy was dismissed.

Genetic diversity and structure

A total of 517 different haplotypes were identified among the set of 31 populations, with no haplotype shared between different species (Tables 1 and S4). The genetic variation among the populations of the complex was high (39.5%). Among species, the complex presented a moderate differentiation, with a 21.6% of the total genetic variation, reaching a 29.6% when the sympatric populations were excluded from the analysis.

The Mantel tests of pairwise geographic and genetic distances measured as allele differences among populations (Fst), detected a significant correlation (r = 0.34, P < 0.001) accounting for up to 12% of the variance within the 31 populations dataset. However, no significant correlation (0.119 > *P* > 0.064) within each species was found.

On average, the number of polymorphic loci (PL), number of alleles (Na), effective number of alleles (Ne), number of private alleles (Np), gene diversity (Hs), as well as the difference between populations according to pairwise Fst tests (Table 1), presented minimum and maximum values in *A. valentinus* and *A. clavatus*, respectively. Among populations, similar results were obtained per species (Table S4), excepting on two populations of *A. homogamos* (5 and 18), which presented higher Np than *A. clavatus*.

The most variation found between populations of the same species was 36.8% in *A. clavatus*, following by 13.2% in *A. homogamos* and 8.7% in *A. valentinus*. The sympatric populations of *A. clavatus* and *A. valentinus* presented a 17.1% of variance. In line with this, not a single haplotype was shared among *A. clavatus* populations, whereas five haplotypes were present in two to five populations of *A. homogamos*, and 27 haplotypes were found in two to seven populations of *A. valentinus*. Additionally,

the most similar population of *A. valentinus* according to pairwise Fst tests (0.00) were 51, from North Morocco, and 75, from the SE Iberian Peninsula.

The inbreeding coefficients (*Fis*) per population were variable in every species (Table S4). This coefficient describes the allele frequencies distribution of a population according to Hardy Weinberg expectations (*Fis* = 0), ranging from -1 (excess of heterozygotes or exogamy) to 1 (excess of homozygotes or inbreeding). Across the populations analysed, only marginal negative values were observed in one population of *A. clavatus* (75). One population of *A. valentinus* (76) and two of *A. clavatus* (71 and 77) presented positive values below 0.10, whereas all populations of *A. homogamos* presented values above 0.16. Among the populations showing higher Fis values (> 0.30), four sympatric populations (30, 64, 65 and 74), two of *A. clavatus* (124 and 148) and one of *A. homogamos* (18) contained two genetic clusters, more than a 20% of individuals presenting genetic admixture or both (Figure S3), implying that *Fis* values may be misleading due to Wahlund effects.

According to the unbiased genetic identity matrix (Nei, 1978), *A. valentinus* presented similar values of genetic similarity (69%) with both *A. clavatus* and *A. homogamos*. In the same way, *A. homogamos* and *A. clavatus* were notably similar to each other (61%). The sympatric populations presented maximum values with *A. clavatus* (0.92) followed by *A. valentinus* (0.77) and *A. homogamos* (0.65).

The Bayesian analysis of genetic structure estimated four as the most probable number of genetic clusters (Figure 2). The average symmetric similarity coefficient (H') obtained in K = 4 was 0.99, revealing a high similarity of all ancestry membership matrices among runs. Independent analyses on the 31 populations of the species

complex dataset produced the same result. The genetic groups identified presented a clear geographic pattern, noticeable at all K values (Figure 2B).

These genetic clusters (hereafter named GC) consistently grouped the species of the complex. Amongst the samples with Q>=0.90, a 53% of *A. clavatus* individuals belonged to GC1 and 46% to GC2 whereas the 99% of *A. valentinus* clustered within GC3, and the 100% of *A. homogamos* were included in GC4. All individuals of the related species *A. radiatus* belonged to GC4, while *A. monanthos* presented a mixed membership (60% to GC4, 29% to GC3, 11% to GC2, and 1% to GC1).

The genetic groups GC1 and GC3 presented respectively the highest and lowest genetic diversity, in terms of number of polymorphic loci, number of alleles, number of private alleles, observed heterozygosity and gene diversity (Table S5). According to the unbiased genetic identity matrix (Nei, 1978), group GC3 is highly similar to all groups (0.6-0.64), whereas the less similar group pairs were GC4 with GC1 and GC2 (0.42 and 0.45 respectively). Intermediate values of similarity were obtained between GC1 and GC2 (0.53).

The PCA analysis resulted in three maximum rank eigenvectors that explained the 4.60, 3.71 and 2.74 % of the diversity. The four genetic groups obtained in STRUCTURE described likely distinct distributions among axes combinations (Figure S4). Roughly, the axis 1 clearly separates GC1 from GC2, the axis 2 the clusters GC1 and GC2 from GC3 and GC4 and the axis 3 the GC4 from the remaining clusters.

Genetic admixture

A total of 138 individuals presented a mixed ancestry, representing the 17% of the samples of *A. clavatus*, the 19% of *A. homogamos* and the 6% of *A. valentinus*, as well as a 35% of the individuals from sympatric populations. Samples of mixed ancestry were identified from all non-sympatric populations in all species (Figure 3), excepting in populations 122 (GC1) and 77 (GC2) of *A. clavatus*, 51, 56, 73 and 88 (GC3) of *A. valentinus*, and the sympatric population 69 (GC2).

Several populations presented percentages of individuals of mixed ancestry per population above 25%: populations 8 (28%) and 18 (53%) within *A. homogamos* and populations 72 (45%), 124 (31%) and 148 (59%) within *A. clavatus*. Moreover, a similar pattern was observed when only 1-3 individuals per populations were sampled (not shown).

Excepting population 69, the percentage of individuals of mixed ancestry in sympatric populations ranged between 25-63% of the total. Within the remaining samples two different genetic clusters were observed in each case: GC1 and GC3 occurred in population 65 (representing the 32% and 16% of the samples, respectively) and the GC2 and GC3 in populations 30 (32 and 5%), 64 (52 and 14%) and 74 (19 and 56%) (Figure 2A).

In sympatric populations, the floral phenotype was significantly associated with specific genetic groups (Figure S5). In particular, *A. clavatus* and *clavatus*-like phenotypes showed a significantly higher probability of belonging to GC2 group while *A. valentinus* phenotypes had a higher membership probability to the GC3 group (Likelihood Ratio Test = 15.97, *P* = 0.001). In addition, among these individuals belonging to GC3 (membership probability to GC3 > 0.9), no *clavatus*-like phenotypes

were assigned to this group (Figure S5B). The individuals with mixed ancestry in these sympatric populations did not showed significant correlation with any particular floral phenotype (Figure S5B), although intermediate floral phenotypes presented higher mean values.

Microclimatic niche according to genetic groups and species

The variables that most contributed to explain the potential distribution of the species were the isothermality and precipitation of the coldest quarter in *A. clavatus*, the precipitation seasonality in *A. homogamos*, the precipitation of the coldest quarter in *A. monanthos*, and both the precipitation of the coldest quarter and mean temperature of the driest quarter in *A. valentinus*. The distributions predicted by both the taxonomic assignment and the genetic cluster membership of the populations comprised the areas of distribution of the species complex in a high percentage, and the degree of overlapping is relatively high in some cases (Figure 4). Per genetic clusters, the potential distributions of CG1 and GC3 overlapped moderately (Figure 5A), whereas very low to no overlap was found between GC3 and GC4 and between GC1 and GC4, respectively (Figure 5B-C). The predicted area of GC2 was very small and overlapped with the predicted area of GC3 (Figure 6).

Niche overlap based on Pianka index (PI) was higher between the species *A. clavatus* and *A. valentinus* (0.77 PI), and *A. homogamos* and *A. valentinus* (0.62 PI), than between *A. clavatus* and *A. homogamos* (0.41 PI), whereas the lowest values were observed for all species pairs between *A. monanthos* (0.39 maximum PI, Figure S6). Among genetic clusters, GC1 presented similar degree of niche overlap respecting both GC3 (0.52 PI) and GC4 (0.56 IP) while these overlap was notably higher between GC3

and GC4 (0.83 PI) and maximum between CG2 all remaining clusters (GC1, 0.81 PI; GC3, 0.90 PI and GC4, 0.85 PI).

DISCUSSION

The combined analysis of the current genetic structure and climatic niche modeling of the *Anacyclus* species complex provided significant insights about the role of geographic and environmental factors on the evolutionary history of the group. The present genetic differentiation and the existence of current gene flow between these species were documented.

Species genetic identity

Our results revealed that in general, the genetic structure agreed with the taxonomical classification of the species of study, as found in other organisms (e.g., *Merluccius*, Henriques et al. 2016; *Robinsonia*, Takayama et al., 2014; *Ficus*, Wei et al., 2014). However, in the case of *A. clavatus*, the presence of two genetic clusters with a clear geographic pattern (i.e., GC2 at both sides of Strait of Gibraltar and its neighbor Mediterranean coastal areas, and GC1 extended along the remaining area of distribution of this species) was an unexpected result that revealed a great genetic variation with a remarkable geographic pattern for this species. The presence of clear divergent genetic groups with a geographic pattern has been also found in species of other genera (e.g., *Arabidopsis*, Brennan et al., 2014; *Alnus*, Mandák et al., 2015).

Other evidences, such the analysis of genome size variation in this species (Agudo *et al.* in rev.) showed a similar pattern (i.e., two types of populations within *A. clavatus*, one from coastal areas in SE Iberia, and another one from inland populations).

Despite this clear genetic and genomic differentiation within *A. clavatus*, no morphological characters were found so far to distinguish between these two groups. Neither former taxonomical revisions nor recent studies in morphological traits have observed phenotypic differences between *A. clavatus* from SE Spain and from other areas (Humphries 1979, Álvarez "unpubl."). In addition, each of the two genetic groups found within this species, GC1 and GC2, analyzed independently showed the highest genetic diversity within our system. This is expected in GC1, which area of distribution is the most extended, but it is quite remarkable for GC2 that is confined to a more reduced area, suggesting also the Strait of Gibraltar area as one of the hotspots for diversity (genetic and morphological) for *Anacyclus*.

A different genetic structure was observed in both *A. valentinus* and *A. homogamos*, in which one genetic group was found for each species. In the case of *A. valentinus*, its genetic group (GC3) was exclusive in this species or at least in the *valentinus*-like phenotype, whereas the genetic group present in *A. homogamos* (GC4) was also present in *A. radiatus* and *A. monanthos*. It is remarkable that in *A. valentinus* the genetic diversity is lower than in *A. clavatus* and *A. homogamos*.

Low genetic diversity was observed in cases of highly isolated populations (Johansson and Ehrlén, 2003), inbreeding, bottlenecks or recent speciation (Tarvin et al., 2017), but had shown to be advantageous among some highly adapted species, which presented no negative effects due to population fragmentation or the lack of genetic refreshment (Ge and Sun, 1999; Aguilar *et al.*, 2004; Vischer *et al.*, 2001). In the case of *A. valentinus*, the low diversity observed may be due to a recent origin or a bottle neck, in line with the hypothesis of a hybrid origin of the species suggested by Humphries (1979). The lower degree of genetic admixture observed among individuals and populations within *A. valentinus* suggests higher reproductive barriers between this

species and the species to which it occurred in sympatry (*A. clavatus*) preventing introgression, supporting the hypothesis of a low diversity due to inbreeding as well.

Gene flow, introgression, and phenotype variation

The analysis of sympatric sites (populations in which at least two different phenotypes were observed) clearly revealed the genetic admixture intra and inter-individually (Figure 3, Figure S5), which as in other systems (e.g., Emanuelli, et al., 2013; Ortego et al., 2017), indicated the existence of current gene flow between different genetic groups and species. However, there was an unbalanced representation of phenotypes vs. genotypes that could be related with different hybridization or introgression stages within populations (i.e., different frequencies of non-hybrid individuals, F_1, F_2, BCs, etc.). The fact that one phenotype may harbor different genotypes or and admixture of them (Figure S5) adds complexity to the system. It is important to note that the presence of GC2 in sympatric sites of *A. clavatus* and *A. valentinus* was frequent, and was the unique genetic group present in the sympatric population 69. Therefore, in sympatric sites there was not a clear correspondence between phenotype and genotype, which by extension it might occur also in species overlapping and limit areas of distribution.

Species and genetic groups distribution according to climate factors

In general, the model predictions fitted well for both species and genetic groups, although the predicted areas based on species distribution were more extended than expected. In contrast, the predicted areas based on genetic clusters were more restricted

and accurate with its corresponding species (i.e., the genetic groups present in each species). For example, all species were predicted for the Atlantic areas, in which actually only *A. radiatus* is present, but only GC4 was predicted in these areas, whereas the remaining genetic clusters were restricted to the Mediterranean climate zones, according to the species that they represent. This is very remarkable in the Strait of Gibraltar area, a place where the influences of both the Atlantic and Mediterranean climates are present. Models based on species (i.e., phenotypes) presented a higher degree of overlapping here, whereas models based on genetic clusters exclude the part with more Atlantic climate influence on this area (Figures. 4 and 5). This indicates a strong correlation between climatic variables, mostly related to differences in the precipitation regime, and genetic groups distribution. The highest overlapping between genetic groups (GC1, GC2, and GC3) was predicted in SE Iberia, one region in which precipitation is lower than in any other part of the Iberian coast.

The genetic landscape draws more extended contact zones where gene flow might occur than the presence of intermediate phenotypes showed. This would explain the scattered and few observations of sympatric populations and the predominance of a phenotypic mosaic pattern of populations (i.e., *clavatus*-like and *valentinus*-like phenotypes) along the Iberian Mediterranean coast. The accurate prediction of models indicates a clear correlation between climatic factors and genetic groups, although the extended overlapping in optimum distributions prevents isolation and a clear delimitation of the entities at least between *A. clavatus* and *A. valentinus*. Therefore, a gradation of aridity will determine the optimum distribution of species, of which *A. valentinus* (GC3) and *A. clavatus* (GC2) occupied the most arid environments and *A. radiatus* (GC4) the milder ones. Intermediate environmental conditions would be optimal for *A. clavatus* (GC1) and *A. homogamos* (GC4).

The Baetic (Iberian) and the Riffean (Morocco) regions, on both sides of the Strait, jointly conform one of the biodiversity hot spots in the world (Médail & Quézel, 1997). This is mainly attributed to a complex geological and climatic history that resulted in high mountain ranges separated by few kilometers of the Mediterranean sea, providing an extraordinarily diversity of environments geographically isolated in this area (Molina-Venegas et al., 2015). In *Anacyclus*, all genetic groups were present here (Figure 2B), and it may be considered the center of the genetic diversity for our system. The pattern of a continuous distribution of genetic groups and species at both sides of the strait suggests that the origin of this system might have preceded the splitting of the two continents after the Messinian Crisis about 5.5 Ma. This is congruent with the most probable short distance fruit dispersal strategy by ombrohydrochory in the annual species of *Anacyclus* studied (Bastida et al., 2010; Torices et al., 2013), although a secondary less probable second strategy of long-dispersal cannot be discarded (i.e., to explain the presence of *A. radiatus* subsp. *coronatus* in the Canary Islands and the rare presence of the genus in the Balearic Islands). From the Strait of Gibraltar area, each of the genetic group extended occupying areas according to their adaptation to different climatic factors. Hence, the geographic pattern observed in our system, in which no clear genetic barriers exist, seems to be ruled by environmental factors such as the rain pattern and aridity in which adaptation and selection plays an important role.

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TABLES

Table 1. Minimum and maximum genetic diversity parameters observed in the populations of *Anacyclus* species complex. N pop, number of populations. N, number of individuals per population. Nh, number of haplotypes. The following parameters represent values on average over all loci: PL, percentage of polymorphic loci; Na, number of alleles; Ne, effective number of alleles; Np, number of private alleles; Ho, observed heterozygosity; Hs, expected heterozygosity; Fis, inbreeding coefficient. Pairwise Fst refers to values among population pairs within each cluster.

	A. homogamos	*A. valentinus*	*A. clavatus*	sympatric
Npop	7	9	10	5
N	13-21	19-42	16-39	19-21
Nh	12-20	14-24	16-36	16-21
PL	0.50-0.75	0.25-0.75	0.50-1.00	0.63-1.00
Na	1.88-3.88	1.38-2.38	2.25-4.00	2.50-3.63
Ne	1.17-2.29	1.11-1.43	1.71-2.54	1.63-2.48
Np	0-0.38	0-0.13	0-0.13	0-0.38
Ho	0.126-0.306	0.097-0.182	0.221-0.476	0.163-0.393
Hs	0.351-0.533	0.144-0.292	0.271-0.583	0.321-0.559
Fis	0.168-0.616	0.041-0.482	-0.051-0.515	0.114-0.419
pairwise Fst range	0.025-0.145	0-0.301	0.118-0.604	0.071-0.322
Mean pairwise Fst	0.093	0.083	0.344	0.171

FIGURES
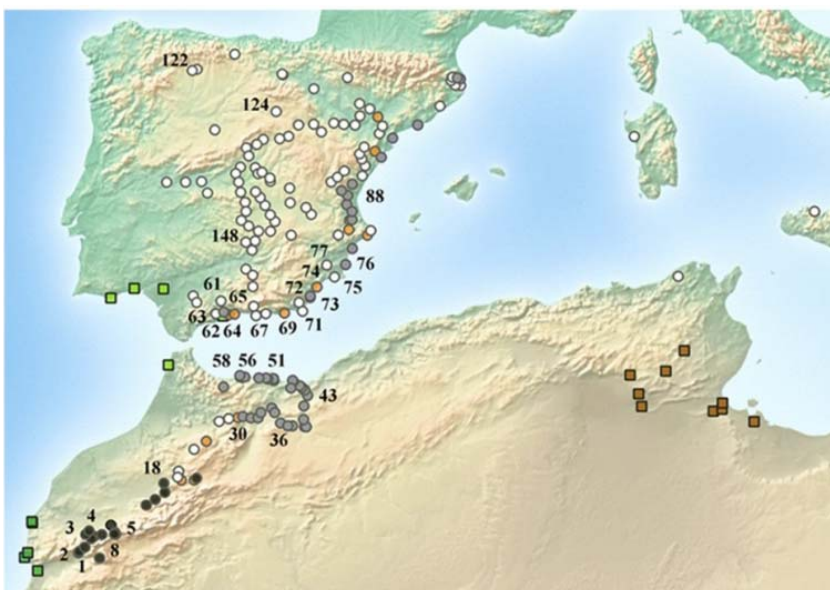
Figure 1. Map of the sampled populations (continuation on the following page).

Figure 1. (previous page) Map of the sampled populations. Circles represent populations of the complex of species: *A. clavatus* (white), *A. valentinus* (grey), *A. homogamos* (black) and sympatric populations of *A. clavatus* and *A. valentinus* (orange). Squares represent the related species *A. radiatus* (green) and *A. monanthos* (brown). Numbered labels refer to the 31 populations sampled in detail (13-42 individuals).
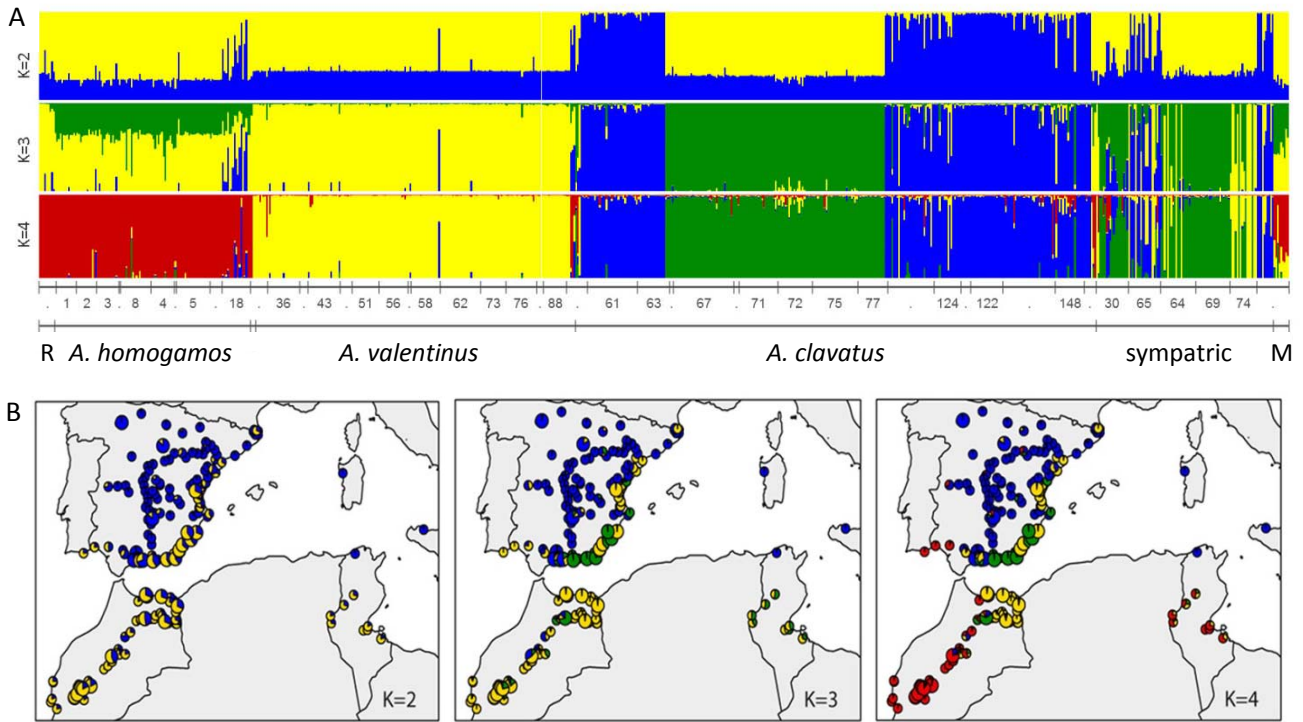
Figure 2. Results of genetic assignment based on the STRUCTURE analysis according to different K values. Each color represents a genetic cluster: blue, GC1; green, GC2; yellow, GC3; red, GC4. (**A**) Each line represents one individual. Numbered labels represent the 31 populations sampled in detail. R: *Anacyclus radiatus*, M: *Anacyclus monanthos*. (**B**) Maps representing the averaged memberships to the different genetic clusters per population. Larger pies represent the 31 populations sampled in detail, while the smaller ones represent values based on 1-3 individuals.
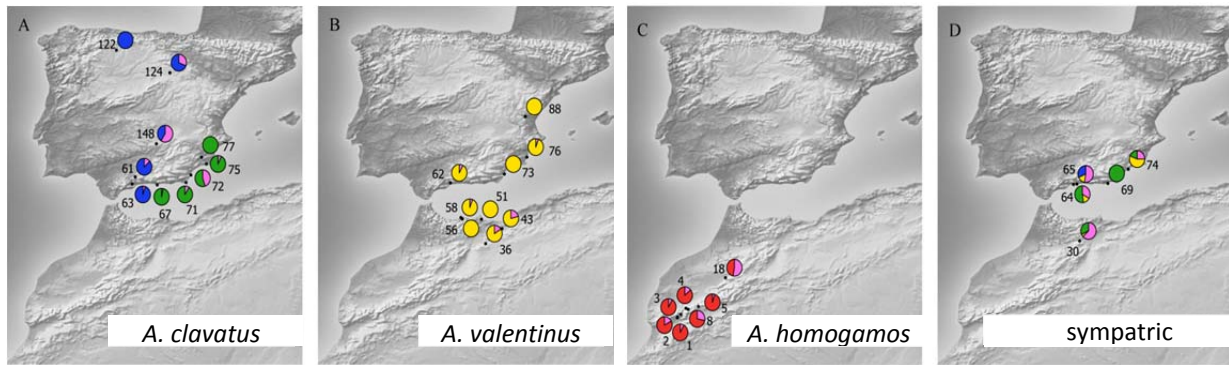
Figure 3. Mixed ancestry distributions. Pie charts on A, B, C, and D, represent the proportion of individuals presenting complete ancestry to one genetic cluster (colored according to Figure 3) or admixed ancestry (pink) per population and species. An adjacent black point represents the location of the population sampled.
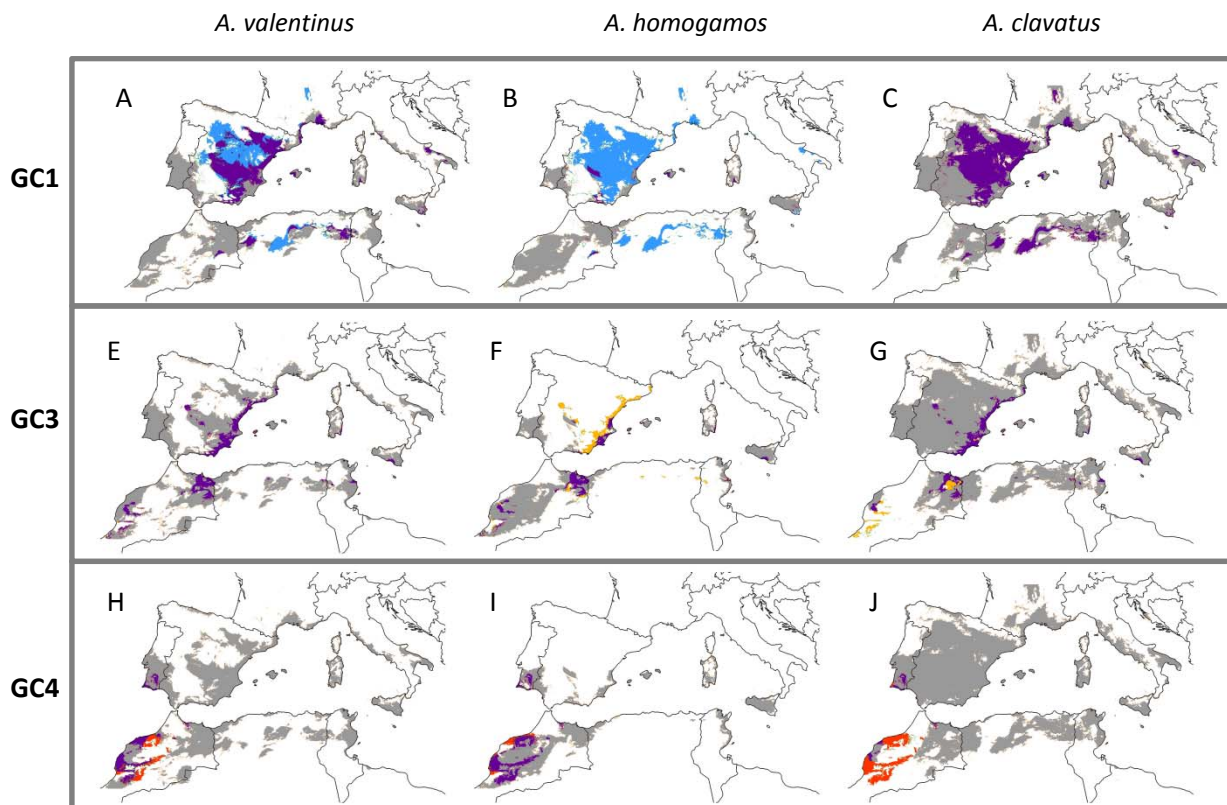
Figure 4. Pairwise potential distributions of the species *A. valentinus*, *A. homogamos* and *A. clavatus* (columns) and the genetic clusters GC1, GC3 and GC4 (rows). Grey color is representative of the species in each case. The genetic clusters are represented with different colors: blue, GC1; yellow, GC3 and red, GC4. Purple color represents overlapping areas between both genetic clusters and species.

GC1 and GC3   **GC1 and GC4**   **GC3 and GC4**
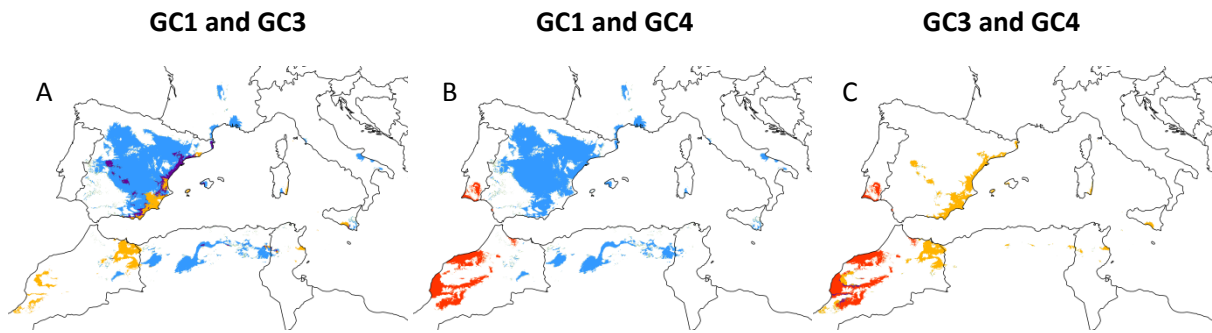
Figure 5. Pairwise potential distributions of the genetic clusters GC1, GC3 and GC4. The genetic clusters are represented with different colors: blue, GC1; yellow, GC3 and red, GC4. Purple color represents areas of overlap between genetic clusters.
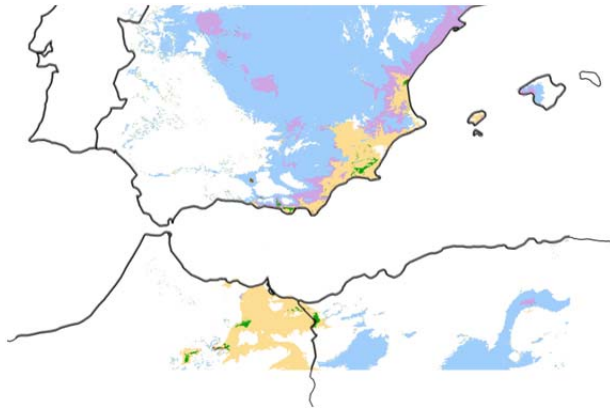
Figure 6. Predicted distribution of GC2 (green) together with the predicted distributions of the genetic clusters GC1 (blue) and GC3 (yellow) and GC1 and GC3 overlapping areas (purple).

SUPPLEMENTARY MATERIAL

Tabla S1. Populations sampled. Species, coordinates, collector number, ID of the populations and number of individuals of the populations sampled.

Species	Latitude	Longitude	collector ID	Population	N
A. homogamos	30.83664444	-8.393558333	IA 2111	1	14
	30.86845556	-8.379269444	IA 2113	2	13
	30.980975	-8.224022222	IA 2114	3	13
	31.25125	-7.977827778	IA 2115	4	14
	31.32925	-7.377277778	AA 71	5	21
	31.37186111	-7.399777778	AA 73	6	1
	31.20309722	-7.864444444	AA 82	8	19
	31.19544444	-8.051972222	AA 86	9	1
	31.29	-8.214055556	AA 88	10	1
	31.3815	-8.126333333	AA 89	11	1
	30.73413889	-7.797555556	AQ 3505	13	1
	31.33194444	-7.408888889	RG 1275	14	1
	32.05544444	-6.544166667	AA 36	15	1
	32.202	-6.29775	AA 39	16	1
	32.37994444	-6.028916667	AA 40	17	1
	32.59869444	-6.064555556	AA 45	18	18
	31.54113889	-7.520388889	AA76	300	1
A. valentinus	34.96405556	-4.400583333	MS 1072	27	1
	34.24933333	-3.828388889	IA 2223	29	1
	34.21786111	-3.592944444	IA 2222	31	1
	34.21994444	-3.386583333	IA 2221	32	1
	34.35033333	-3.302944444	IA 2219	33	1
	34.45927778	-2.986444444	IA 2217	34	1
	34.34944444	-2.897305556	IA 2216	35	1
	34.09602778	-2.731666667	IA 2214	36	20
	34.00497222	-2.033027778	IA 2210	38	1
	34.00111111	-1.986305556	JC 2465	39	1
	34.02994444	-2.52075	AQ 2861	40	1
	34.177	-2.06325	IA 2209	41	1
	34.50733333	-2.050277778	IA 2206	42	1
	34.76052778	-1.938027778	IA 2204	43	20
	34.89338889	-2.016777778	IA 2202	44	1
	34.95169444	-2.107333333	IA 2201	45	1
	34.99772222	-2.170388889	IA 2200	46	1
	35.10425	-2.350361111	IA 2199	47	1
	35.1275	-2.374833333	JC 2442	48	1
	34.94333333	-2.425222222	IA 2198	49	1
	35.12633333	-2.920722222	IA 2195	50	1
	35.17236111	-2.933833333	IA 2194	51	21
	35.21269444	-3.919222222	IA 2183	55	1
	35.19455556	-3.857638889	IA 2185	56	19

	35.21108333	-3.783944444	IA 2186	57	1
	35.24741667	-3.924472222	IA 2182	58	20
	36.77866667	-4.427666667	IA 2124	62	42
	37.16747222	-1.824	IA 2149	73	19
	37.91347222	-0.737027778	IA 2155	76	21
	38.99588889	-0.503888889	IA 2025	84	1
	39.19516667	-0.497638889	IA 2288	85	1
	39.36502778	-0.656388889	IA 2289	86	1
	39.58372222	-0.616972222	IA 2290	87	1
	39.70322222	-0.809555556	IA 2291	88	20
	39.85722222	-0.466055556	IA 2030	89	1
	40.48183333	0.47225	IA 2032	98	1
	40.93513889	0.856777778	IA 2038	103	1
	41.221	1.676055556	IA 2041	104	1
	42.26311111	3.129305556	IA 2059	106	1
	42.29580556	3.038277778	IA 2058	111	1
A. clavatus	42.43600833	-0.521872222	AA 90	12	1
	32.90086111	-5.650722222	AA 48	19	1
	32.7565	-5.67525	AA 49	20	1
	33.43966667	-5.225166667	IA 2234	24	1
	34.12755556	-4.514111111	IA 2228	26	1
	34.19558333	-4.237	IA 2227	28	1
	37.15563056	-5.354397222	IA 2007	59	1
	36.99682778	-5.243672222	IA 2088	60	1
	37.04277778	-4.515083333	IA 2122	61	30
	36.73302778	-4.660333333	IA 2140	63	20
	36.92463889	-3.531305556	IA 2269	66	2
	36.69694444	-3.459166667	AA 1	67	39
	36.74772222	-3.165277778	IA 2158	68	1
	37.01333333	-2.166944444	IA 2009	70	2
	36.80116667	-2.063111111	IA 2161	71	24
	37.12916667	-1.830777778	IA 2148	72	20
	37.61927778	-1.082888889	IA 2152	75	27
	37.91241667	-1.312833333	IA 2147	77	19
	38.63286111	-0.939694444	IA 2285	79	1
	38.71813889	0.061944444	IA 2024	82	1
	39.92141667	-1.127361111	IA 2292	90	1
	40.03394444	-0.924944444	IA 2293	91	1
	40.18058333	-0.704944444	IA 2294	92	1
	40.05088889	-0.145277778	IA 2295	93	1
	40.28927778	-0.04525	IA 2296	94	1
	40.58236111	-0.208555556	IA 2298	96	1
	40.74525	-0.055944444	IA 2299	97	1
	41.05836111	0.441833333	IA 2301	100	1
	41.23569444	0.548416667	IA 2302	101	1
	41.64644444	2.421388889	IA 2045	105	2

42.10888889	3.124722222	IA 2049	107	1
42.12422222	2.956527778	IA 2050	108	1
42.24416667	2.832361111	IA 2055	109	1
42.31930556	2.882916667	IA 2056	110	1
41.66194444	0.166361111	IA 2304	112	1
41.79786111	-0.156444444	IA 2305	113	1
41.49963889	-0.157416667	IA 2306	114	1
41.28263889	-0.326305556	IA 2307	115	1
41.30605556	-0.681916667	IA 2308	116	1
41.34533333	-1.001638889	IA 2309	117	1
42.18323889	-1.619786111	IA 2069	118	1
42.55086111	-2.646777778	LM 4865	120	1
43.02222222	-4.199661111	IA 2172	121	1
42.63766667	-5.423944444	RT 2	122	19
42.60451944	-5.579102778	IA 2010	123	1
41.64099444	-2.842686111	RT 1	124	16
41.13738889	-1.401388889	IA 2310	125	1
41.32691667	-1.64175	IA 2311	126	1
41.31183333	-2.129305556	IA 2312	127	1
41.04047222	-2.45025	IA 2313	128	1
40.98394444	-2.733583333	IA 2314	129	1
40.84536111	-3.462777778	IA 2316	131	1
40.75987222	-3.793205556	IA 2006	132	1
41.18280833	-4.802577778	IA 2273	133	1
40.56436389	-3.712758333	IA 2249	134	1
40.29369444	-3.971666667	IA 2250	135	1
40.13672222	-4.117416667	IA 2251	136	1
39.92844444	-4.021194444	IA 2252	137	1
39.92661111	-5.183916667	IA 2319	138	1
39.90188889	-5.684944444	IA 2320	139	1
39.883	-6.276333333	IA 2321	140	1
39.65175	-4.991694444	IA 2324	141	1
39.68436111	-3.955305556	IA 2253	142	1
39.44208333	-3.811222222	IA 2254	143	1
39.22130556	-3.782333333	IA 2255	144	1
39.00255556	-3.927555556	IA 2256	145	1
38.86563889	-3.696611111	IA 2257	146	1
38.47505556	-3.780080556	IA 2167	147	1
38.50741667	-3.496722222	IA 2146	148	18
38.28263889	-3.590472222	IA 2272	149	1
37.82261111	-3.771	IA 2271	150	1
37.40075	-3.555222222	IA 2270	151	1
37.67664444	-3.564583333	IA 2326	152	1
38.64624722	-2.388922222	IA 2094	153	1
38.74547222	-3.395333333	IA 2258	154	1
38.74261111	-3.024305556	IA 2259	155	1

	39.14336111	-1.762027778	IA 2281	158	1
	39.31361111	-1.923111111	IA 2280	159	1
	39.36811111	-3.19475	IA 2263	160	1
	39.43063333	-2.413188889	IA 2011	161	1
	39.55652778	-3.345972222	IA 2264	162	1
	39.78572222	-2.427527778	IA 2277	163	1
	39.69597222	-3.492305556	IA 2265	164	1
	39.95541667	-3.029722222	IA 2012	165	2
	40.03166667	-3.042694444	IA 2275	166	1
	40.17213889	-3.293611111	IA 2274	167	1
	40.46791667	8.444861111	IA 2005	170	1
	37.01333333	9.21575	AQ 3119	171	1
	38.04222222	13.51416667	TS 421	172	1
A. clavatus and A. valentinus	32.68891667	-5.570777778	AA 50	21	1
	32.69380556	-5.2035	AA 53	22	1
	33.64127778	-4.874583333	IA 2232	25	1
	34.22311111	-3.97025	IA 2224	30	19
	36.71475	-4.270027778	IA 2141	64	21
	36.7305	-4.101666667	IA 2144	65	19
	36.76116667	-2.606138889	IA 2159	69	20
	37.38527778	-1.620611111	IA 2150	74	19
	38.75738889	-0.61375	IA 2286	80	1
	38.60869444	-0.046694444	LM 4434	81	2
	38.98272222	-0.57825	IA 2287	83	1
	40.632	0.281111111	IA 2033	99	3
	41.46030556	0.413972222	IA 2303	102	1
	42.52842778	-2.621158333	IA 2065	119	1
A. radiatus subsp. coronatus	30.34943056	-9.499444444	IA 2109	701	1
	30.65144722	-9.886669444	IA 2108	702	1
	30.77224167	-9.803358333	IA 2107	703	1
	31.49592778	-9.727741667	IA 2102	704	1
	31.53376389	-9.743841667	IA 2105	705	1
A. radiatus subsp. radiatus	36.68125	-4.447888889	IA 2125	802	1
	37.02963056	-7.827452778	IA 2085	803	1
	37.28927222	-7.138866667	IA 2082	804	1
	37.30483611	-6.259816667	IA 2077	805	1
A. monanthos	33.34972222	10.83638889	CA 16266	901	1
	33.72222222	9.7325	CA 16310	902	1
	33.7425	9.984444444	CA 16233	903	1
	33.88638889	10.01805556	CA 16194	904	1
	34.03194444	7.717777778	AH 3957	905	1
	34.33861111	7.670833333	AH 3907	906	1
	34.80555556	8.518888889	JC 3314	907	2
	35.23861111	9.121111111	JC 3267	909	1

Table S2. Frequencies of the populations presenting no amplification, monomorphy and presence of null alleles per species and locus. Na, total number of alleles; N, no amplification; M, monomorphic; null, inferred presence of null alleles according to Van Oosterhout et al (2004).

	A. clavatus				*A. valentinus*				*A. homogamos*			
	Na	N	M	null	Na	N	M	null	Na	N	M	null
locus 9	9	0	0	0.50	5	0	0.44	0	5	0	0.14	0
locus15	6	0	0.40	0.40	1	0.89	0.11	-	0	1.00	0	-
locus17	7	0	0.50	0	2	0	0.89	0	9	0	0	0.14
locus19	10	0	0	0.30	7	0	0	0.56	11	0	0	0.71
locus 20	4	0	0.20	0	1	0	1	-	9	0	0	0.43
locus 21	7	0	0.50	0.10	2	0.33	0.56	-	1	0.71	0.29	-
locus 24	7	0	0.10	0.30	3	0	0.44	0.11	5	0	0.43	0.43
locus D3	8	0	0	0	4	0	0	0	4	0	0	0
average	58	0	0.21	0.30	25	0.15	0.43	0.19	44	0.21	0.11	0.29

Table S3. Substitutions and indels found across microsatellite flanking regions. n rep, number of repetitions of the microsatellite tandem motif. The bp location corresponds to the reference sequence of each loci published in Agudo *et al*. (2013)

|	Allele	n rep	Location (bp) / Polymorphism						
locus 15			31	32	65	70	78	73	80
	97	3	G	A	C	G	CA	A	-
	107	8	C	T	A	T	AT	-	T
locus 21			76			119			
	133	4	G			CTATAGAGCATTAC			
	139	11	T			-			
	142	12	T			-			
locus 24			28			65			
	91	7	A			_			
	105	6	T			AAAAAAATAGGGATTC			

Table S4. Genetic diversity per population and species. N, number of individuals per population. Nh, number of haplotypes. The following parameters represent values on average over all loci: PL, percentage of polymorphic loci; Na, number of alleles; Ne, effective number of alleles; Np, number of private alleles; Ho, observed heterozygosity; Hs, gene diversity; Fis, inbreeding coefficient. The values highlighted in bold represent the result of analyzing the individuals of all populations per species as one cluster.

Species	Population	N	Nh	PL	Na	Ne	Np	Ho	Hs	Fis	
A. homogamos	1	14	12	0.50	1.88	1.37	0.13	0.197	0.38	0.168	ns
	2	13	12	0.75	2.50	1.49	0	0.126	0.44	0.616	
	3	13	13	0.63	2.38	1.49	0	0.241	0.435	0.258	ns
	4	14	14	0.75	2.75	1.88	0	0.306	0.443	0.209	ns
	5	21	20	0.63	2.50	1.71	0.25	0.154	0.453	0.455	
	8	19	18	0.75	2.50	1.17	0	0.192	0.351	0.268	ns
	18	18	17	0.75	3.88	2.29	0.38	0.272	0.533	0.415	
all		**112**	**99**	**0.75**	**5.50**	**2.05**	**1.63**	**0.216**	**0.447**	**0.448**	
A. valentinus	36	20	19	0.50	2.25	1.32	0.13	0.163	0.292	0.252	ns
	43	20	19	0.38	2.13	1.36	0	0.182	0.279	0.127	ns
	51	21	16	0.38	1.63	1.28	0	0.102	0.24	0.429	
	56	19	17	0.50	1.63	1.17	0	0.143	0.256	0.254	ns
	58	20	17	0.38	1.75	1.11	0	0.114	0.166	0.212	ns
	62	42	24	0.75	2.38	1.43	0	0.13	0.288	0.482	ns
	73	19	15	0.38	1.63	1.16	0	0.123	0.189	0.131	ns
	76	21	18	0.25	1.63	1.41	0	0.161	0.193	0.041	ns
	88	20	14	0.25	1.38	1.12	0	0.097	0.144	0.296	ns
all		**201**	**123**	**0.75**	**3.13**	**1.48**	**0.13**	**0.132**	**0.228**	**0.339**	
A. clavatus	61	30	30	1	4.00	2.54	0.13	0.476	0.583	0.182	
	63	20	19	1	3.63	2.07	0	0.329	0.458	0.28	
	67	39	36	0.75	3.38	2.15	0.13	0.292	0.438	0.331	
	71	24	23	0.63	3.00	1.71	0.13	0.307	0.327	0.058	ns
	72	20	20	0.63	2.25	1.55	0	0.241	0.271	0.108	ns
	75	27	27	0.75	3.38	1.83	0.13	0.307	0.292	-0.051	ns
	77	19	18	0.50	2.50	1.86	0	0.277	0.303	0.085	ns
	122	19	19	0.75	2.63	1.56	0.13	0.221	0.303	0.272	ns
	124	16	16	0.88	3.38	2.23	0.13	0.282	0.519	0.456	
	148	18	17	1	3.00	1.95	0.13	0.238	0.492	0.515	
all		**232**	**225**	**1**	**7.25**	**2.78**	**1.75**	**0.317**	**0.598**	**0.47**	
Sympatric populations of *A. valentinus* and *A. clavatus*	30	19	19	1	3.63	2.48	0.38	0.393	0.559	0.296	
	64	21	21	1	3.25	2.03	0	0.269	0.462	0.417	
	65	19	19	0.88	3.38	2.14	0.13	0.252	0.438	0.34	
	69	20	20	0.63	2.75	2.23	0.13	0.336	0.38	0.114	ns
	74	19	16	0.88	2.50	1.63	0	0.163	0.321	0.419	
all		**98**	**93**	**1**	**5.25**	**2.45**	**0.63**	**0.282**	**0.503**	**0.44**	

Table S5. Genetic diversity within the four genetic groups. N, number of individuals; Nh, number of haplotypes; Na, number of alleles; Ne, effective number of alleles; Na, allelic richness; Np, private allelic richness; Ho, observed heterozygosity and Hs, gene diversity.
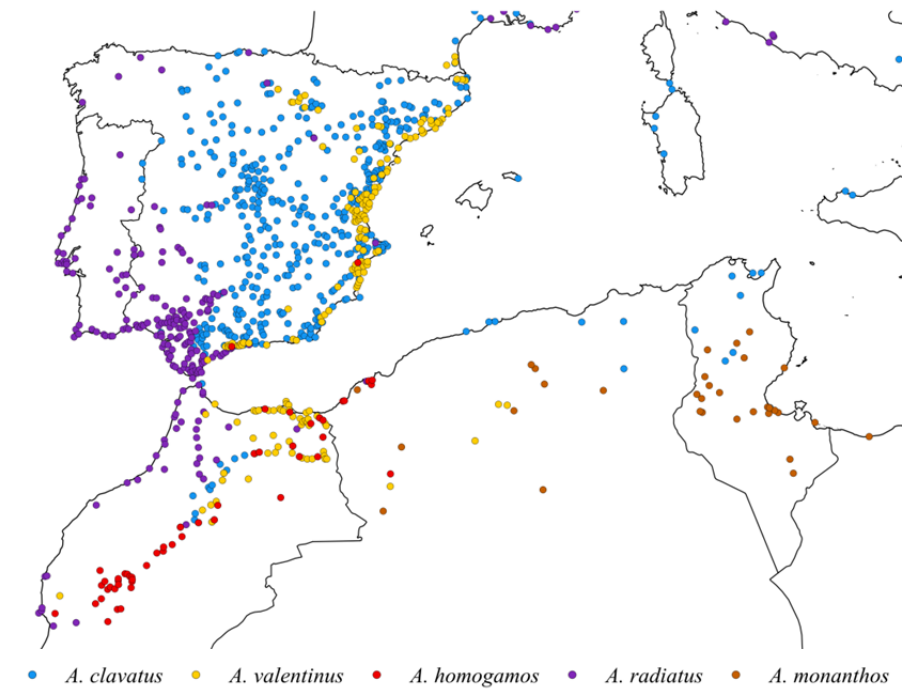
| Group	N	Nh	PL	Na	Ne	Np	Ho	He
GC1	152	150	1.00	6.000	2.806	1.750	0.346	0.560
GC2	160	154	0.88	5.125	2.279	1.000	0.299	0.433
GC3	237	121	0.63	2.500	1.563	0.250	0.124	0.238
GC4	111	97	0.75	5.250	1.954	1.750	0.205	0.385

Suplementary Figures
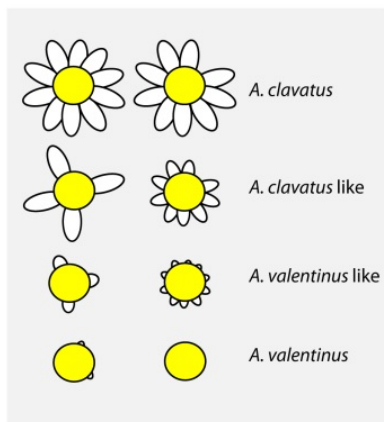
**Figure S1**. Phenotype classification based on flowering head traits.

Figure S2. Location of the populations used as occurrences for species distribution modeling.
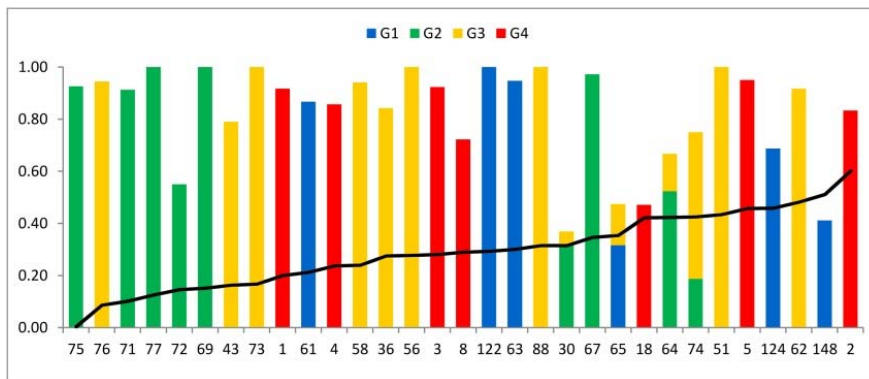
Figure S3. Degree of genetic admixture vs. inbreeding coefficient (*Fis*). Each bar represents the percentage of individuals of membership >0.90 (ordinate axis) per population (abscissa axis). The black line represents the *Fis* values of each population.
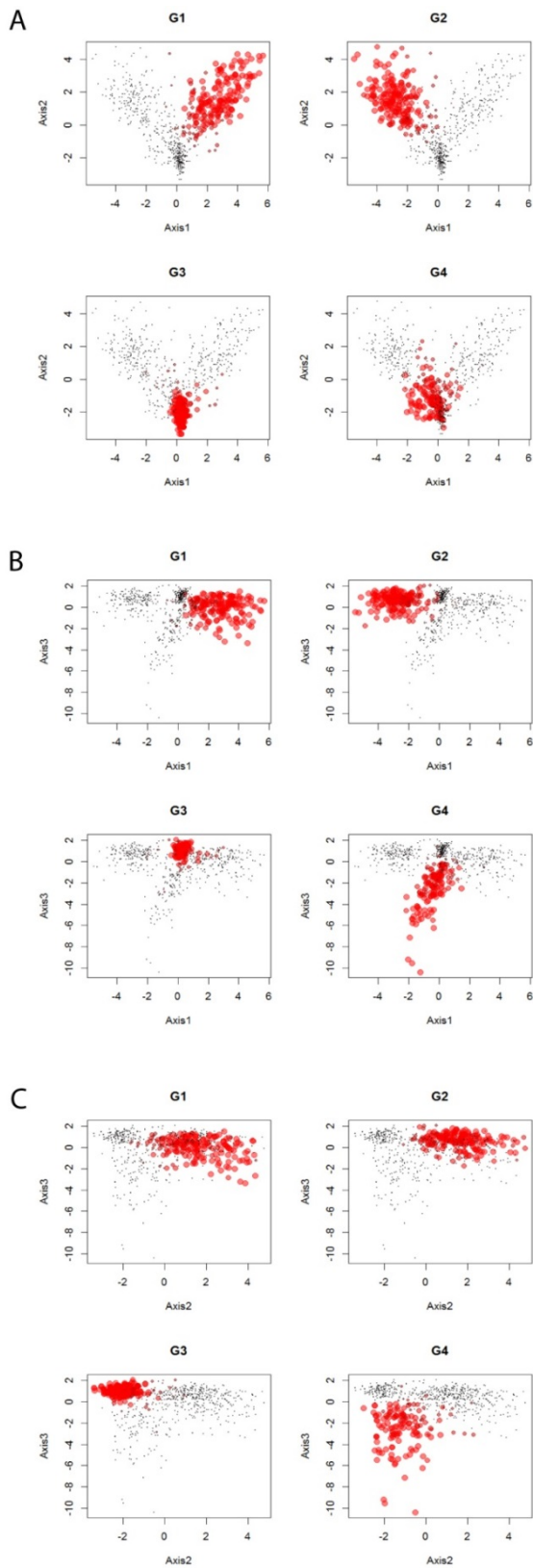
Figure S4. Principal Component Analysis based on microsatellite data. The three eigenvectors with higher eigenvalues are represented as axis combinations. A, axis 1 and 2; B axis 1 and 3; C, axis 2 and 3. Red color points out the samples presenting each of the genetic clusters (GC1, GC2, GC3 and GC4).
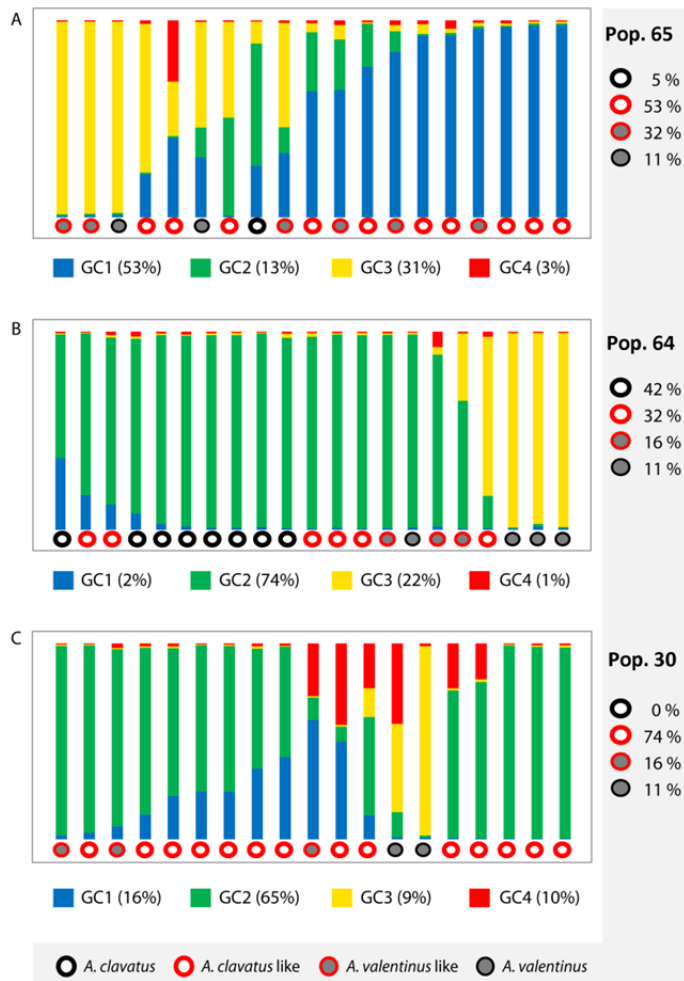
Figure S5. Genetic and phenotypic characterization of three sympatric populations 65 (A), 64 (B), and 30 (C). The percentages of each genetic group and of each phenotype are showed below and at the right side of each panel.
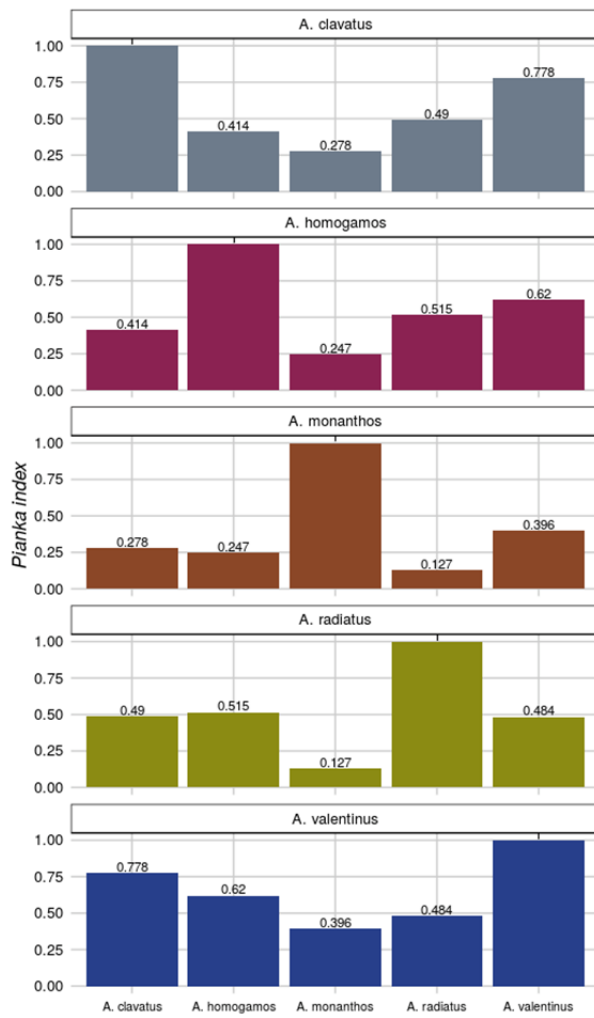
Figure S6. Degree of niche overlap between species according to Pianka index (PI).

CHAPTER THREE: PHYLOGENETIC INFERENCE

3.1. A phylogenetic framework for the western Mediterranean genus *Anacyclus* L. (Anthemideae, Asteraceae)

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Introduction

Phylogenetic hypotheses are one of the most important references to accomplish any study on evolutionary biology (Edwards *& al.,* 2007; Maddison and Schulz 2007; Crawford and Archibald 2017). However, they are not always available for the group of organisms under study. In *Anacyclus*, the most complete published phylogeny makes part of a paper focused on placing other different monospecific genera within the tribe Anthemideae (Oberprieler, 2004). In this work, *Anacyclus* was represented by a total of nine accessions of the internal transcribed spacer (ITS) region of the nuclear ribosomal repeat (nrDNA) of seven out of the nine species recognized by then (Humphries, 1979). Further phylogenetic analyses based on plastid and nuclear markers (Oberprieler *&* Vogt 2000; Watson *& al.*, 2000; Oberprieler, 2004; Himmelreich *& al.*, 2008; Sonboli *& al.*, 2012), were all congruent with the phylogenetic position of the genus *Anacyclus* within the subtribe Matricariinae of the tribe Anthemideae, close to *Heliocauta*, *Achillea*, *Tanacetum,* and *Matricaria*, although including one or few species of the genus.

In the sole monographic work of *Anacyclus* (Humphries, 1979), a total of nine species, six subspecies, and two hybrids were recognized. Subsequent to this monography, a new species, *Anacyclus anatolicus* L.Behçet *&* S.Almanar, was described as new for science for Turkey (Behçet *& al*manar 2004). For our study on the evolution of hybrid zones in *Anacyclus*, we mainly followed this taxonomic treatment with slight modifications based on a revision of the genus for the Iberian Peninsula (Álvarez, unpubl.), in which five species were recognized (i.e., *A. clavatus*, *A. homogamos*, *A. pyrethrum*, *A. radiatus*, and *A. valentinus*) for this territory. One of these species, *A. valentinus*, was considered as being a hybrid between *A. homogamos* and *A. radiatus* by Humphries (1979). Additionally, *Anacyclus inconstans*, a hybrid between *A. homogamos* and *A. clavatus*, also considered by this author, was treated as a synonym of *A. valentinus* in the mentioned revision of Álvarez (unpubl.).

A recent unpublished phylogeny of the genus *Anacyclus* based on plastid and nuclear DNA sequences (Vitales *& al*., unpubl.), revealed that the genus was paraphyletic when the three Eastern Mediterranean species are included. The monophyletic clade of *Anacyclus* was restricted to the five species previously mentioned for the Iberian Peninsula, plus the North African endemic species: *A.*

linearilobus, *A. monanthos*, and *A. maroccanus*. Therefore, the previously published phylogeny based on ITS sequences (Oberprieler 2004) represented actually all species of the *Anacyclus* monophyletic group, although the low sampling both in terms of individuals and molecular markers makes these results inconclusive.

The characterization of the evolutionary history of species diverging through recent radiations was considered being a challenging task (Braun *& Kimball, 2001; Maddison & Knowles, 2006; Townsend, 2007; Knowles & Chan, 2008). On one hand, many molecular regions may not have evolved sufficiently as to contain adequate degrees of polymorphism to generate a bifurcating tree. On the other hand, if diversification occurred rapidly, phylogenetic inference could suffer from additional processes, as the intervals between speciation events being short and effective populations sizes being large would probably lead to conflicts between the gene trees and the overall species tree (Degnan & Rosenberg, 2006). In addition, recently diverged taxa could still be inter-fertile and thus numerous hybridization events could have occurred as a result of secondary contacts. The inclusion of introgressed or hybrid taxa/individuals into a dataset also may produce misleading results as allele migration may either lead to low statistical support values or highly supported, but contradicting gene tree topologies; both contributing to an underestimation of diversification times (Degnan and Rosenberg, 2006; Weisrock & al., 2012; Leaché & al, 2014).

As the recent improvement of sequencing techniques allowed to generate datasets rich in both genes and taxa, most of the phylogeneticists suggested that in concert with careful taxa sampling (e.g. Leaché & al., 2014, Huang & Knowles, 2016), efforts should be directed towards developing better models of sequence evolution (Heath & al., 2008, Nabhan & Sakar, 2011). According to several authors, accurate species trees can be estimated without large numbers of loci (McCormack & al., 2009) by careful species assignment (Leaché & Rannala, 2010) and by sampling more than a single individual per putative taxon (Knowles, 2010; Camargo & al, 2012). However, the need of developing methods allowing to interpret genealogies as networks rather than as bifurcating trees, was suggested by other authors (e.g. Doolittle, 1999; Linder & Rieseberg, 2004), and endorsed by numerous examples of reticulate evolution in different organisms (e.g. Comes & Abbott, 2001; Marhold & Lihová, 2006; Suárez-Santiago & al., 2007). In this regard, the difficulty of distinguishing the effects of incomplete lineage sorting (ILS) from those of hybridization (Buckley & al., 2006;

Holland *& al*., 2008, Joly *& al*., 2009) was still not overcome, despite of the effort of several researches (e.g., Linder, 2004; Huson, 2005; Blanco-Pastor *& al*., 2012; Yamasaki *& al*., 2015; Razkin *& al*., 2016).

Our aim here was to construct a phylogenetic framework based on plastid and nuclear DNA multigene sequence analysis to accomplish evolutionary studies in the species complex *A. clavatus*, *A. valentinus* and *A. homogamos*. The specific objectives were: (i) to know the phylogenetic relationships among the three species of the complex, (ii) to examine the hypothesis of a reticular evolution within the species of the complex, (iii) to detect current and ancient hybridization events between *Anacyclus* species, (iv) to date the divergence of these species over time.

MATERIALS *&* METHODS

Plant Material

Seven out of the eight species that form the monophyletic *Anacyclus* clade (Oberprieler 2009; Vitales *& al.,* unpublished) were represented in the present study. *Anacyclus linearilobus*, an endemic species that grows on coastal dunes in Algeria was not included due to the limited access to material from this population. From the remaining species, a total of 74 individuals from representative localities of their main area of distribution (i.e., one individual per population) were included in the study. The sampling included 11 individuals of each of the species *A. clavatus*, *A. valentinus*, and *A. homogamos*, 10 individuals of *A. monanthos*, 12 of *A. pyrethrum*, 10 of *A. radiatus* subsp. *radiatus*, seven of *A. radiatus* subs. *coronatus*, and two individuals of *A. maroccanus* (Figure 1, Table S1). Amongst the foresaid samples, three individuals of *A. clavatus*, three of *A. valentinus*, one of *A. homogamos*, one of *A. radiatus* subsp. *radiatus*, and one of *A. maroccanus* were sampled from sites in which different species or a notable occurrence of intermediate phenotypes was observed (hereafter "sympatric populations"). Plant material was collected in the field and preserved in silica-gel until DNA extraction or obtained from herbarium specimens.

Ten individuals from genera closely related to *Anacyclus* according to the most up-to-date published phylogeny (Oberprieler, 2004)were incorporated to the sample set to be used as outgroups (Table S1). The monospecific genus *Heliocauta*, considered the closest relative to *Anacyclus* following the results of Oberprieler (2004), was used as outgroup for species-tree reconstruction. In addition to *Helicauta atlantinca* (Litard. & Maire) Humphries, nine representatives of other related genera were included as outgroups for molecular dating analyses: *Achillea tenuifolia* Lam, *Artemisia vulgaris* L*., Ismelia carinata* (Schousb.) Schultz-Bip, *Leucanthemopsis alpine* (L.) Heywood*, Leucanthemum gracilicaule* (Dufour), *Matricaria discoidea* DC*, Santolina rosmarinifolia* L., *Tanacetum coccineum* (Willd.) Grierson and *Tripleurospermum caucasicum* (Willd.) Hayek. By this sampling, all subtribes of Anthemideae according to Oberprieler *& al*. (2007) were included: Matricariinae (*Achillea, Heliocauta, Anacyclus, Matricaria*), Artemisiinae (*Artemisia*), Glebionidinae (*Ismelia*), Leucanthemopsidinae (*Leucanthemopsis*), Leucantheminae (*Leucanthemum*), Santolininae (*Santolina*), Anthemidinae (*Tanacetum, Tripleurospermum*).

Molecular markers

The nuclear regions selected for the present study were a subset of a conserved orthologous set (COS) of low or single-copy markers developed for comparative mapping and phylogenetic analyses in the Asteraceae (Chapman *& al.,* 2007). This subset consisted on the eight markers (i.e., A19, A39, C16, C20, C48, D01, D23, and D35) presenting the higher variation and amplification rates according to 454 labelling requirements after the screening of 35 candidate regions in *Anacyclus*. The selected markers were previously successful in phylogenetic studies in other Anthemideae (e.g. Konowalik *& al*., 2015). Additionally, several chloroplast regions (Shaw *& al*., 2005) were explored for phylogenetic signal (i.e., *trnH-psbA*, *rpS4-trnT*, *trnK*, *trnK-psbA*, *ycf6-trnC*, *trnL*, *trnL-trnF*). Finally, the most informative intergenic spacer regions, *trnL-trnF* and *rpS4-trnT,* presenting nine and four informative sites, respectively, were selected.

DNA extraction and sequencing

Total genomic DNA was extracted from silica-dried leaves collected in the field and from herbarium specimens using the DNEasy Plant Minikit (QUIAGEN, Hilden, Germany). A two-step PCR procedure to prepare templates for the 454 sequencing were performed. In a first PCR reaction, we amplified the target regions with primers characterised by Chapman *& al.,* (2007), which were modified by adding a M13 tail to the forward and a Titanium B motive to the reverse primer. The PCR reactions were performed in a total volume of 10 µl per sample, which contained 2 µl of PCR Buffer, 0.03 mM each of dNTPs, 0.3 pM of each of the primers, 0.01 U/µl of DNA Polymerase and 2 ng/µl of DNA template. The thermocycler conditions used consisted on an initial pre-denaturation at 94°C for 4 min; 5 cycles of 20 seconds at 98ºC, 30 seconds at 64-59°C (the temperature descended 1°C per cycle) and 30 seconds at 72ºC; then 30 cycles of 20 seconds at 98ºC, 30 seconds at 58ºC, and 30 seconds at 72ºC; finally, a conclusive extension of 5 min at 72°C. The second PCR reaction was used to add the Titanium A adaptor and a individual-specific four or ten-letter barcode to the 5´-end of the amplicons. To avoid Taq errors, both subsequent PCR reactions were carried out with proofreading polymerases (KAPA HiFi DNA polymerase for the first and *Pwo* DNA polymerase for the second PCR, respectively; both from Peqlab, Germany). The PCR products were checked by electrophoresis on a 1.5% agarose gel. After purification of amplicons with Agencourt Ampure (Beckman Coulter, USA) magnetic beads and determination of DNA concentrations using the double-stranded DNA high sensitivity assay kit on a Qubit 2.0 fluorometer (Invitrogen, ThermoFischer, U.S.A.), all amplicons were multiplexed into a single probe ensuring their equimolar mixing to obtain an accession- and marker-wise balanced distribution of reads. The resulting probe (a total of 1,148 PCR products) was outsourced for 454 sequencing to an external enterprise (MicroBIOMix, Regensburg, Germany). Additionally, two intergenic spacer regions of the plastid genome (i.e., *trnL-trnF* and *trnT-rps4*) obtained by first generation (Sanger) sequencing were also included. The PCR reactions were performed in a total volume of 10 µl per sample, which contained 2 µl of PCR Buffer, 0.03 mM each of dNTPs, 2 pM of each of the primers, 0.01 U/µl of DNA polymerase and 2 ng/µl of DNA template. The thermocycler conditions consisted on an initial pre-denaturation at 96°C for 4 min; 33 cycles of 20 seconds at 96ºC and 20 seconds at 50°C and a conclusive extension of 4 min at 60°C. The PCR products were subsequently purified with the Sanger sequencing dye terminator removal Agencourt CleanSEQ (Beckman Coulter, USA).

Reads from the 454 sequencing were assigned to accessions and markers using R (R Development Core Team 2008) and the Galaxy web portal (Giardine *& al.*, 2005, Goecks *& al.*, 2010) as described in Griffin *& al.*, (2011). After removing barcodes, M13, and primer sequences (Blankenberg *& al.*, 2010), the quality of reads was assessed and those sequences with Phred scores below 20 in more than 20% of the nucleotide positions were discarded. To identify allelic variation reads were grouped in accessions and marker-wisely aligned with MAFFT v6.833b (Katoh *& al.*, 2002, Katoh *& Toh* 2008). Possible chimeric reads caused by recombination during the PCR were checked in RDP4 (Martin and Rybicki, 2000, Martin *& al.*, 2015). Clusters of reads were then collapsed into consensus sequences (alleles) applying a 20% threshold as criterion to retain intra-allelic polymorphisms by scoring them as IUPAC-coded wobble nucleotide positions. Finally, allelic consensus sequences for all accessions were aligned marker-wisely using MAFFT, the resulting alignments were checked, and edited manually in Geneious v. 7.1.2 (Kearse *& al.*, 2012).

Detection of hybridization signals according to allele sharing

To investigate the genealogy of the alleles per loci we used TCS v1.21 (Templeton *& al.*, 1992, Clement *& al.*, 2000). This software calculates an absolute distance matrix for all pairwise comparisons of the alleles and the probability of parsimony for pairwise differences among alleles resulting in a 95% set of plausible connections forming a network. Gaps were considered as fifth character state (informative indels), so common alleles represented identical sequences. Additional analyses were performed considering the gaps as missing data (uninformative indels). Two datasets were analysed, the first one comprised all samples, and a second one including exclusively the non-sympatric populations.

Phylogenetic inference based on bifurcating trees

A coalescent-based species tree reconstruction method was used to elaborate a phylogenetic hypothesis for the group of species under study and to highlight evidences of hybridization events within *Anacyclus*. We used all accessions from the non-

sympatric populations of *Anacyclus* and one outgroup species (*Heliocauta atlantica*), since it is known that including outgroups is a counterproductive practice when reconstructing phylogenies based on the coalescent model (Drummond & Bouckaert, 2015). We performed this analysis on two different datasets, as the high degree of alleles shared between different species suggested that recent gene flow could have hampered the analysis.

The species tree inference was performed following a hierarchical coalescent method with the program *BEAST (Heled & Drummond, 2010), in which estimations of gene trees and species trees are integrated under the multispecies coalescent model. This method considers each incongruent signal among gene trees as caused by stochastic processes such as ILS, assuming the complete absence of gene flow among species. As *BEAST takes mutational variance into account, it can be computationally difficult when many loci are available (Bayzid & Warnow, 2013). However, according to Bryant & al., (2012), these difficulties can be eased by analysing bi-allelic markers.

The input files for the analyses were prepared in BEAUti v.2.4.3 (Bouckaert & al., 2014). The parameters of the nucleotide substitution models for each marker region were fixed marker-wisely to those found using jModeltest v.2.1.6 (Darriba & al., 2012). Modeltest analyses were run in CIPRES (Miller & al., 2010), and the Akaike information criterion (AIC) was used to select among different models. In order to be able to set the best clock model and the best tree prior for each marker region, we calculated marginal likelihoods via the Path Sampling (PS) method (Beale & al., 2012, 2013) in BEAST v2.4.3. Marginal likelihood was estimated from 100 path steps, each run for one million generations. A difference of more than three log-likelihood units [Kass & Raftery, (1995) considered three log-likelihood units as strong evidence against competing models] was used as threshold for accepting a more parameter-rich model (see the Tables S2 for detailed information on the results of the model selection procedure). The "piece-wise linear and constant root" model was selected for the population size with a prior distribution of 1/x. The same distribution was used for the prior on the birth rate. A lognormal prior distribution was given to the clock rates (in case of a strict clock) and to the uncorrelated lognormal relaxed clock (ucld) means (mean: 0.002; standard deviation: 1). In case of a relaxed clock, a gamma prior distribution (α: 0.54; β: 0.382) was set for the ucld standard deviations. The selection of

the clock models for each locus and species tree was estimated according to maximum marginal likelihoods using stepping stone (SS) sampling. The Yule speciation process was chosen as species tree prior against the birth-death model for both datasets (marginal likelihood: -17958.712 vs. -17996.13 for the first one and -13189.18 vs. -13186.176 for the second one).

Two independent analyses were therefore run for both datasets in the CIPRES portal (Miller *& al.*, 2010), for $8*10^8$ generations sampling every 40,000[th] iteration. Convergence between analyses and the values of the effective sample sizes (ESSs) for all different parameters were checked in Tracer v.1.6 (Rambaut *&* Drummond 2007). Analyses with ESS values above 200 were considered reliable. Output MCMC samples of the species trees were combined with LogCombiner v2.4.3 (Bouckaert *& al.,* 2014) after discarding the 10% of the analyses as burn-in. Finally, the remaining 36,000 trees were used to construct a maximum-clade-credibility tree with a posterior probability limit set to 0.5 using TreeAnnotator v2.4.3 (Bouckaert *& al.,* 2014). Additionally, DensiTree (Bouckaert *&* Heled 2014) was used to generate a cloudogram of the posterior distribution of the complete set of species trees generated with *BEAST, to visually check for conflicting topologies and for potential evidences of hybridization.

Molecular dating

To estimate the age of the most recent common ancestor for *Anacyclus* and the timeframe when diversification processes took place in the genus we used *BEAST for phylogenetic reconstruction based on different datasets including species of *Anacyclus* and the ten species collected as outgroups.

As only one sample from non-sympatric populations of *A. maroccanus* was sampled and nuclear and plastid allele sharing with a different species was observed within this sample, we excluded this species from these analyses to prevent unnecessary artefacts due to unbalanced sample sizes and hybridization signals. Thus, the first data set included all the species of *Anacyclus* sampled (xcept *A. maroccanus*)*,* the species *Heliocauta atlantica*, and the other nine species of closely related genera as outgroups (Table S1).

To minimize the bias produced by the presence of species of hybrid origin in the analysis *A. valentinus* and *A. radiatus* where not included in the second dataset, which comprised only the species *A. clavatus*, *A. homogamos*, *A. monanthos,* and *A. pyrethrum*. Additionally, the outgroup species *Ismelia carinata*, *Leucanthemum gracilicaule,* and *Tanacetum coccineum,* which comprised missing data in four, seven, and four markers, respectively, were excluded from the subsequent two analyses.

To minimize the incongruences due to gene flow among species, two to three individuals per species (a ~30% of the previous dataset) exhibiting no nuclear or chloroplast alleles in common with the extant species were selected. Thereby, the third dataset consisted on two individuals of the species *A. clavatus* (populations 3 and 5), two of *A. homogamos* (populations 23 and 33), three of *A. pyrethrum* (populations 61, 65, and 69), two of *A. monanthos* (populations 51 and 52), and the same outgroup individuals of the preceding dataset.

Input files, parameter and model selection were carried out as detailed in the previous section (see the Tables S3-S5 for detailed information on the results of the model selection), except for the "piece-wise linear and constant root" model, which was selected for the population size with a prior distribution of $1/x$, whereas a uniform distribution (from 0 to 1000) was used for the prior for the birth rate. We use the age of the split between *Artemisia* L. (subtribe Artemisiinae) and the rest of the genera included in the present study as calibration point. The earliest records of *Artemisia* type pollen fossils are from the Lower and Upper Oligocene, in the provinces of Xinjiang and Qinghai, in North-Eastern China (Wang 2004). This allowed us to set the time to the most recent common ancestor (tmrca) prior for a subset of taxa including the whole Eurasian grade and Euro-Mediterranean clade of Anthemideae (all taxa except *Artemisia vulgaris* L.). We applied a log normal prior for this calibration point with an offset of 23.05 Ma (mean: 4, SD: 1.25; 95% HPD: 22.05-44.3 Ma; median: 24.9 Ma).

According to the results from the model selection, a calibrated Yule model was used as species tree prior for the first dataset. Two independent analyses were run in the CIPRES portal for $6*10^8$ generations, sampling every 30,000[th] iterations. The birth-death model (marginal likelihood: -18619.442 vs. -18671.499) was used as species tree

prior for the second dataset. Two independent analyses were run in the CIPRES portal for $3*10^8$ generations, sampling every 30,000th iterations. For the third dataset, a calibrated Yule model (-1449.735 vs. -15349.04 obtained for the birth-death model) was used as species-tree prior. Two independent analyses run in the CIPRES portal for $1*10^8$ generations, sampling every 5,000th iterations.

Convergence between analyses and ESS values were checked following the same procedure detailed in the previous section, as well as the construction of the maximum-clade-credibility tree and the cloudogram of the posterior probability distribution.

Network approach

To elaborate a reticulate evolution hypothesis for the species of *Anacyclus*, we used a network reconstruction method implemented in PhyloNet v.5.6 (Than et al. 2008). We applied the method described by Yu & Nakhleh (2015), which is able to estimate species phylogenies in the presence of both hybridization and incomplete lineage sorting under maximum pseudo-likelihood (MPS). This method infers phylogenetic networks from gene trees, but the number of reticulations has to be specified in advance. For the scope, we selected randomly nine gene trees per locus from those collected during the Bayesian search in *BEAST. These were used as input for five independent MPS analysis, each assuming a different number of reticulations (i.e., one to five). For each analysis, the networks with the highest likelihood score was taken and represented graphically in Dendroscope v.3.5.7 (Huson & Scornavacca 2012).

RESULTS

Sequence divergence

In summary, *A. pyrethrum* and *A. homogamos* were found being the most distantly related according to nuclear markers and presented distant alleles in chloroplast as well. The species *A. maroccanus* was closely related with whether *A.*

pyrethrum or *A. homogamos* in every nuclear marker but exhibited the same chloroplast alleles as found in *A. homogamos*. The species *A. radiatus* was found being distantly related to *A. homogamos* in nuclear markers but it presented alleles closely related to this species among chloroplast. The species *A. valentinus* presented the same chloroplast alleles found in *A. clavatus*, whereas it was closely related to either *A. homogamos*, or *A. clavatus,* or both based on nuclear markers, whereas the latter two species were only related with each other in the presence of *A. valentinus*. Additionally, *A. clavatus* was found to be independently related with *A. monanthos* and *A. radiatus*. The species *A. monanthos* presented chloroplast alleles related to *A. pyrethrum*, *A. clavatus* and *A. valentinus* and was connected to these species and *A. homogamos* among nuclear networks.

Nuclear markers

The complete dataset of this study consisted on a total of 1,607 sequences of lengths ranging between 324 and 600 bp and containing 494 parsimony informative sites. The percentages of pairwise identity and identical sites within the *Anacyclus* alignment were 94.37% and 60.25% on average.

Three nuclear markers (A19, C20, and C48) showed percentages of pairwise identity within *Anacyclus* and *Heliocauta* alignments lower than the average. The markers C20 and C48 exhibited high variation (i.e., 84.1% and 89.8% of pairwise identity, and 20.4% and 34.4% of identical sites, respectively), and no clear pattern to define paralogue sequences was observed. However, the marker A19 presented two clearly divergent groups of sequences (Figure S1), each one represented by all *Anacyclus* species and *Heliocauta atlantica*, and were therefore considered as being two groups of paralogue sequences. Consequently, the two groups of sequences were treated as independent markers (i.e., A19a and A19b) in the subsequent analyses. The alignments of both A19a and A19b reached values of 97.5% and 94.1% of pairwise identity, respectively.

The sequences obtained were clustered in genealogies per marker in which the alleles plausible to make part of the same evolving network were connected together (Figure S2). According to the 95% parsimony credibility limit, the marker A39

produced separated networks per species, whereas the marker A19b clustered all the species in a single network (not shown in Figure S2). The marker C16 (not shown in Figure S2) connected all species excepting *A. pyrethrum* which presented an independent network, except of two alleles (population 61 and 63) that were found inside the network connecting all the other extant taxa. The six remaining markers showed different networks exhibiting varying, marker-specific connections among *Anacyclus* species (Figure S2). Thus, no links between *A. homogamos* and both *A.pyrethrum* and *A. radiatus* were recovered among markers showing differential networks (all excepting 19b and C16) in any case. The species of the complex *A. clavatus*, *A. valentinus,* and *A. homogamos* were clustered together in two marker networks (A19b and D23), whereas *A. valentinus* presented additional connections with *A. clavatus* in C20, C48, and D01, and *A.homogamos* in D35. No connection between *A. homogamos* and *A. clavatus* without the simultaneous inclusion of *A. valentinus* was found. The species *A. clavatus* and *A. valentinus* showed also connections with the remaining species in some markers (Figure S2). Additionally, the accession of *Heliocauta atlantica* was found in an internal node position amongst *Anacyclus* taxa in the networks of markers A19a and A19b, and it was connected to *A. homogamos* in the major network found in C16.

Chloroplast markers

The chloroplast markers *trnT-rps4* and *trnL-F* exhibited a low level of variation, with only four and nine parsimony informative sites, respectively, when all *Anacyclus* and *Heliocauta* sequences were included. In the *trnL-F* intergenic spacer region, all *Anacyclus* accessions showed a characteristic deletion of six bps at the position 170 in the alignment, which was not present in any of the outgroup sequences, including the more closely related *Heliocauta atlantica*.

The haplotypes observed in the two chloroplast markers presented comparable evolutionary patterns and distributions (Figure 2). The ancestral haplotype found in *trnL-F* according to TCS implementation (L1) was the most frequent sampled. However, this haplotype is characterized by an unique nucleotide substitution which is not found in the remaining *Anacyclus* haplotypes, nor in that of *Heliocauta* or the remaining outgroup species. When indels were dismissed, *Heliocauta atlantica* connected with the *Anacyclus* network at L2, making the later the most probable

ancestral haplotype. In the network analysis of the *rpS4-trnT* intergenic spacer region, the haplotype T7 found in *A. monanthos* differs from haplotype T2 in a five bps insertion at position 65 in the alignment, which was also found in *Leucanthemopsis alpina*.

The ancestral character states in *trnL-F* (L2) and *rpS4-trnT* (T2) were found among the species *A. pyrethrum* and *A. monanthos*, as well as halplotypes derived from these ancestral haplotypes in *rpS4-trnT* (T1 and T7, respectively). The main haplotypes observed among *A. homogamos* (L4/T4), *A. radiatus* subsp. *coronatus* (L4/T4) and *A. radiatus* subsp. *coronatus* (L5/T5) at distal positions in the network, suggesting derived positions. The species *A. maroccanus* presented the main haplotype found among *A. homogamos* and *A. radiatus* subsp. *coronatus* (L4/T4).

Allele and haplotype distribution and sharing

Diverging taxa are expected to present different polymorphisms at conserved orthologue loci as a result of mutation, genetic drift and selection during evolution. Although the share of an allele by different species may suggest that gene flow between them occurs, homoplasy and the retention of an ancestral polymorphism cannot be completely dismissed. To evaluate the degree of allele sharing between different species among contact zone areas some individuals from sympatric populations were analysed.

The presence of alleles shared by different species among nuclear markers was scarce (0% to 5% of the total number of alleles per marker). The individuals from sympatric populations presented proportionally higher percentages (a 17-fold range) of allele sharing than those from non-sympatric ones (0.555% and 0.032% on average respectively). In most cases, the sharing was due to an allele present in several individuals of one species and significantly related with the remaining alleles found in that species (i.e., connected in the same genealogic network), also observed within a single individual of a different species, with which there was no connection according to the 95% parsimony criterion. The latter case represented an inconsistent genealogic relationship of these allele respecting the remaining set of alleles of that species. The chloroplast haplotypes of *Anacyclus* were clustered within the same genealogic network in both markers. In this case, allele sharing by different species affected a portion of 38-

68% of the total alleles per marker, although a four-fold range was proportionally observed in individuals from sympatric populations compared to those from non-sympatric ones.

In nuclear markers, the individuals from sympatric populations exhibited/showed a notable allele sharing between *A. clavatus* and both *A. homogamos* and *A. valentinus* (Figure 3A). Moreover, the two *A. clavatus* accessions sampled in the Middle Atlas region (populations 8 and 10) presented alleles found only in *A. homogamos*. One common allele was found being shared between *A. valentinus* and the individual of *A. homogamos* from population 30. Additionally, one population of *A. valentinus* from SE of Iberian peninsula (18) and one of *A. clavatus* from Morocco (8) presented alleles clustered with those of *A. radiatus*, whereas no allele sharing was found between the individual from the sympatric population of the latter species (38) and other ones. Allele sharing between the individual of *A. maroccanus* (73) and the species *A. homogamos* and *A. pyrethrum*, and between one individual of *A. clavatus* from the eastern Iberian Peninsula (population 1) and *A. pyrethrum* (populations 69 and 72) was also found. In plastid markers, the haplotypes found among sympatric populations were the same as the majority of combinations observed in non-sympatric ones, except in the two *A. clavatus* from populations 8 and 10, which presented alleles mostly found in *A. homogamos, A. radiatus* subsp. *coronatus,* and *A. maroccanus* (L4/T4).

Among non-sympatric populations, individuals presenting alleles clustered within different species networks were also observed (Figure 3B). Except for allele sharing between *A. valentinus* and *A. clavatus*, common alleles between different species were inconsistently grouped with the alleles of one of the species involved. Thus, some individuals of *A. valentinus* exhibeted an allele clustered with those of the species *A. homogamos* and *A. pyrethrum*, some of *A. radiatus* with those of *A. clavatus* and *A. pyrethrum*, and some of *A. homogamos* with those of *A. radiatus* and *A. valentinus*. The individual of *A. maroccanus* showed an allele clustering within *A. homogamos*. The only species presenting no allele sharing with a different species was *A. monanthos*.

Among plastid markers (Figure 2), the combination L1/T2, which was present in all the individuals of *A. clavatus* and *A. valentinus*, was also found in one individual of *A. pyrethrum* (population 70), three of *A. monanthos* (57, 58 and 60), one of *A. radiatus coronatus* (46) and one of *A. homogamos* (28). The alleles L4/T4, mainly found within *A. homogamos* and *A. radiatus* subsp. *coronatus*, were also present in one individual of *A. radiatus* subsp. *radiatus* (population 42) in northern Morocco. Similarly, the combination L5/T5, present in most of the *A. radiatus* subsp. *radiatus* individuals, was found in one individual of *A. homogamos* (population 31).

Multilocus species Tree Analysis

The multilocus coalescent analysis generated a species tree (Figure 4) showing a considerable amount of uncertainty of the topologies within *Anacyclus* from the earlier stages of differentiation. These incongruences are visible in a cloudogram of the species trees sampled from the posterior distribution (Figure 4). The most likely topology obtained (blue layout in Figure 4, right) placed the species *A. monanthos* at early-diverging lineages the basal position and the species *A. homogamos* amongthe clade formed by *A. clavatus* and *A. valentinus* and the clade of *A. radiatus*, *A. pyrethrum* and *A. maroccanus*, in which *A. pyrethrum* and *A. maroccanus* are resolved as sister taxa. Alternative topologies (green and red branches in Figure 4, right) suggested a closer relationship between *A. homogamos* and *A. monanthos*, as well as between *A. pyrethrum* and *A. radiatus*. The only nodes supported by high posterior probabilities were the clade containg the species *A. clavatus* and *A. valentinus* and that of the two subspecies of *A. radiatus*.

The independent analysis of nuclear and chloroplast markers in BEAST (Figure S3) showed incongruent probabilities between topologies. The chloroplast data produced an unresolved tree after 2×10^9 generations, likely due to the low degree of polymorphisms of these markers and allele sharing between some species. However, the most likely probabilities found could be represented in a cloudogram by increasing the intensity signal of all sampled trees (Figure S3). Two groupings were clearly differentiated, one probable clade involving *A. pyrethrum*, *A. monanthos*, *A. valentinus* and *A. clavatus*, and a second one grouping *A. homogamos*, the two subspecies of *A. radiatus* and *A. maroccanus*. The analysis of nuclear data generated a tree more highly

resolved than the species tree obtained from both nuclear and plastid data. Among nuclear markers the species *A, monanthos*, *A. homogamos*, *A. pyrethrum*, *A. radiatus* and *A. maroccanus* made part of the same clade with high statistical support, as well as the three latter species with each other (Figure S3).

Molecular Dating based on multispecies coalescent model

Divergence times are often calculated through molecular phylogenies by reconstructing a species tree based on gene trees obtained from multiple loci data. However, gene trees and species trees usually present discrepancies that should be taken into account when estimating the time of speciation events (Nichols, 2001). According to Degnan and Rosenberg (2006) all species tree topologies with five or more taxa, as well as asymmetric topologies with four taxa, present regions in which gene trees were more likely than the consensus species tree. Additionally, gene flow between divergent taxa led to incongruent or spurious topologies in trees obtained by multilocus coalescence approaches (Yu *& al.*, 2012; Weisrock *& al.*, 2012; Leaché *& al*, 2014). Recently, Leaché *& al.,* (2014) reproduced and quantified the effects of allele migration in a simulation study, showing that paraphyletic and ancestral gene flow could result in highly supported sister relationships and underestimations of divergence times, respectively.

To date the diversification of the genus *Anacyclus* and test the effect of hybridization signals on the results obtained, we performed the analysis on three different datasets and represented the cloudograms of the consensus trees sampled to illustrate the degree of conflict among branches. The first analysis included all individuals from six species of *Anacyclus* and ten closely related genera. The species tree obtained presented incongruent topologies among *Anacyclus* species, as well as among outgroup taxa (Figure S4). High levels of topological uncertainty were expected among outgroup taxa, as observed in previous phylogenetic analysis focused on the tribe Anthemideae (e.g. Oberprieler, 2004; Ref). However, the species *Ismelia carinata, Leucanthemum gracilicaule,* and *Tanacetum coccineum* presented missing data in four, seven and four markers, respectively, which could contribute to uncertainty and so we excluded from the subsequent analyses. The species tree obtained dated the origin of the genus at approximately 2.44 Ma, and the split of *Anacyclus* and *Heliocauta* at 9.98 Ma

(Figure S4). Among gene trees, the dating of the last node clustering all *Anacyclus* sequences varied from 8.21 to 13.51 Ma, and the split from *Heliocauta* from 11.90 to 19.57 Ma, except for one marker (D35) in which no outgroup species was clustered with *Anacyclus* with high statistical support and *Heliocauta* presented a deeper split (29.33 Ma).

A second analysis was performed excluding from the dataset the species candidates of been of hybrid origin (*A. valentinus* and *A. radiatus*). By excluding these taxa, the second dataset comprised the species *A. clavatus*, *A. homogamos*, *A. pyrethrum* and *A. monanthos*). The diversification of *Anacyclus* was dated at 3.88 Ma, and the split from *Heliocauta* at 10 Ma (Figure S4). As in the first analysis, significantly higher values were found among gene trees, in which these dates varied from 7.55 Ma to 11.63 Ma and from 11.50 to 19.01 Ma, respectively, with the exception of marker D35 (23.6 Ma).

The third analysis aimed to minimize the incongruences due to hybridization events between the species *A. clavatus*, *A. homogamos*, *A. pyrethrum* and *A. monanthos*. To this purpose, two to three individuals per species (a 30% of the original sample) showing no nuclear or plastid allele sharing with the extant species were selected. No significant effects due to decrease the number of samples per species were expected, as a small number of alleles per species was considered sufficient to accurately reconstruct a tree employing the former methods (Hird *& al.*, 2010; Ence *&* Carstens, 2011, Corl, *&* Ellegren, 2013). The estimated date of diversification of the genus *Anacyclus* was of 5.40 Ma and the split from *Heliocauta* of 10.45 Ma (Figure 5). Similarly to the other two analyses the dates separately obtained per gene tree ranged from 7.36 to 11.26 Ma and from 12.54 to 18.11 Ma, respectively, with the exception of D35 (23.4 Ma).

In summary, the species tree diverging time estimates of *Anacyclus* increased after excluding from the first dataset the species *A. valentinus* and *A. radiatus* and subsequently the individuals in which nuclear or chloroplast allele sharing between different species was observed. Across gene trees, the dates of the statistically supported node comprising all *Anacyclus* sequences were overestimated from the first to the third analysis, presenting respectively averaged values of 11.17, 10.23 and 9.77 Ma. Thus, a reduction in the error between gene and species tree estimates was observed from the first dataset (8.78 Ma) to the second (6.35 Ma) and the third one (4.37 Ma).

The species tree dates for the split of *Anacyclus* and *Heliocauta* were consistently estimated in approximately 10 Ma (9.98-10.45 Ma). For this clade, an overestimation of 5 Ma to 4.5 Main gene trees dates compared to the species tree estimates was repeatedly observed in the three analyses, as well as for the remaining highly supported nodes involving outgroup species.

Network approach

To characterize reticulate evolutionary relationships between species of *Anacyclus*, a network reconstructing method was performed with PhyloNet. This program infers a species network with a specified number of reticulation nodes using maximum pseudo-likelihood and the topologies of the gene trees obtained from the Bayesian gene tree analyses. The most likely hypothesis obtained (Figure 6) among reconstructing networks assuming one to four reticulation events, explained the current diversification in *Anacyclus* as the result of the interaction between the two ancestral lineages, represented by the current species *A. pyrethrum* on one hand and the remaining species on the other. Hence, *A. homogamos* would be the result of gene flow with an ancestor of *A. clavatus,* while *A. clavatus,* would have in turn hybridize with an ancestor of *A. monanthos* in a further stage. The species *A. monanthos* appeared as the result of hybridization between ancestors of *A. homogamos* and *A. pyrethrum* ancestors, and *A. maroccanus* between those of *A. monanthos* and *A. radiatus*.

DISCUSSION

All results presented here suggested that *Anacyclus* is a genus in which is probable that gene flow is extended and presumably Influenced by past and current hybridisation events. In all phylogenetic analyses presented here, a high degree of incongruence between gene trees and species trees, and also among gene trees was observed. Although both incomplete lineage sorting (ILS) and extended gene flow may produce conflicting signals that leads to incorrect or poorly resolved phylogenies, general conclusions supported by other evidence are discussed.

Individuals from sympatric populations contained nuclear alleles clustered within a different species genealogic network at frequencies substantially higher (a 17-fold range) than those observed across individuals from non-sympatric populations, suggesting that the allele sharing observed was mainly due to current or recent gene flow within contact zones. Our results show that gene flow between the species *A. valentinus* and *A. clavatus* on one hand and *A. clavatus* and *A. homogamos* on the other hand, occurred when these species were found in sympatry. Similarly, the presence of gene flow in natural populations between pairs of species presenting overlapping distributions was evidenced, with the exception of *A. homogamos* and *A. pyrethrum.*

The species *A. pyrethrum* and *A. homogamos* were the most distantly related according to nuclear markers and presented distant chloroplast haplotypes as well. Incongruences between plastid and nuclear markers related the species *A. maroccanus* and *A. radiatus* with either *A. pyrethrum* or *A. homogamos*, suggesting a hybrid origin of the first two species. Similarly, the species *A. valentinus* presented the same chloroplast alleles found in *A. clavatus* and nuclear alleles closely related to either *A. clavatus, A. homogamos,* or both, whereas the latter two species were only related with each other in the presence of *A. valentinus*. This pattern would be agreement with the hypothesis of a hybrid origin of *A. valentinus* suggested by Humpries (1979, 1981).

The incongruences among markers produced a high level of uncertainty (Figure 4), which is significant in the position of *A. monanthos*, *A. homogamos*, *A. pyrethrum*, and in a lesser extent in *A. maroccanus*. Therefore, more than the half of species in the genus, presumably involving early-diverging lineages, showed conflicting positions. Only the close relationship between *A. valentinus* and *A. clavatus*, and between both subspecies of *A. radiatus* were free of uncertainty. When independent analyses of nDNA and cpDNA markers were compared (Figure S3), the incongruent position of these four species was evident despite the uncertainty due to the very low sequences variation in the cpDNA analysis.

Paraphyletic gene flow can place parental and hybrid lineages in the same clade in coalescent bifurcating trees, due to misinterpretation of the hybridizing signals as common ancestries (e.g. Yu *& al*., 2012, Leaché *& al*., 2014). The uncertainty observed for the position of *A. homogamos* in the phylogeny of all *Anacyclus* species and *Heliocauta* (Figure 4) could be due to the presence of *A. maroccanus*, *A. radiatus*, and

A. valentinus in the analysis, as these species were at the same time related to other species (*A. pyrethrum* and *A. clavatus*). Similarly, the species *A. pyrethrum*, which should be distantly related to *A. homogamos* and placed at earlier diversification stages according to plastid markers, may be shifted to a recent position due to the inclusion of *A. maroccanus* and *A. radiatus* in the analysis.

Our molecular dating analyses showed that the inclusion of species and individuals presenting conflicting phylogenetic signals among nuclear and plastid markers produced an underestimation of the diverging time of *Anacyclus*, an increase in the error between gene and species tree estimates, higher uncertainty connected with internal nodes and different relationships among species. While a higher number of the ingroup species should contribute to increase the statistical support of the inferred topology (Heat *& al*., 2008), the inclusion of hybridizing taxa will produce an underestimation in divergence times, as well as variations in posterior probabilities and topologies of the species tree obtained (Degnan and Rosenberg, 2006; Weisrock *& al*., 2012; Leaché *& al*, 2014). Additionally, spurious inferences (e.g. Nichols, 2001; Degnan *&* Rosenberg, 2006; McCormack *& al*., 2009; Weisrock *& al*., 2012) and overestimation of divergence times among gene trees (Burbrink *&* Pyron, 2011) would be obtained in the presence of conflicting signals between gene and species trees. The variations of the estimates observed in these results were thus in agreement with different scenarios of hybridization simulated by Leaché *& al.,* (2014).

In the three analyses performed, the estimation of the divergence between *Heliocauta* and *Anacyclus* was consistently dated to an age around 10 Ma. Within *Anacyclus*, more likely relationships and diversification times were obtained after the exclusion of the species *A. maroccanus*, *A. radiatus* and *A. valentinus*. However, the incongruent signals among the remaining species were still high, and thus the dating of *Anacyclus* diversification (5.40 Ma) may be misleading. Although we cannot exclude ILS in our analyses, this pattern could be also explained by different events of hybridization at the early stages of *Anacyclus* diversification, as our network reconstruction for reticulate evolution showed (Figure 5).

Although the origin of *A. valentinus* could not be directly inferred, our results showed in all cases a high support for its close relationship with *A. clavatus*, whereas a close relationship with *A. homogamos* according to nuclear markers was also evidenced.

Additionally, the hypothesis for reticulate evolution (Figure 5) linked *A. valentinus* lineage to both *A. clavatus* and to *A. homogamos*.

In conclusion, incongruence and uncertainty due both to ILS and/or inter-specific gene flow was prevalent within the genus *Anacyclus*, early-diverged lineages. Additional analyses are needed to identify the causes of such incongruence. However, current hybridization in sympatric populations between *A. clavatus* and *A. valentinus*; and between *A. clavatus* and *A. homogamos* was evidenced by the pattern of nDNA markers allele sharing. The species pair *A. clavatus* and *A. valentinus* is more closely related to each other than to *A. homogamos*.

This work illustrates that gene flow in *Anacyclus* species currently occurs between several species in contact zone areas. Additionally, recent and ancient hybridization events between species were evidenced, suggesting that *Anacyclus* displays a reticular mode of evolution and highlighting the need of using network methods to properly infer the evolving history of the genus.

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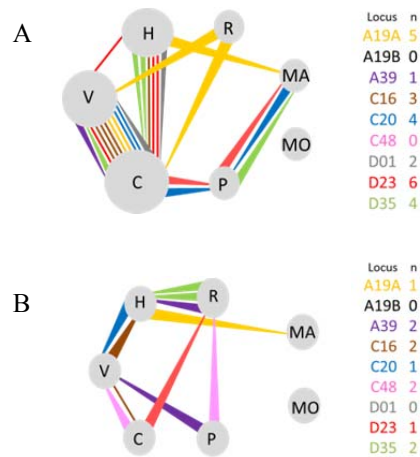
FIGURES
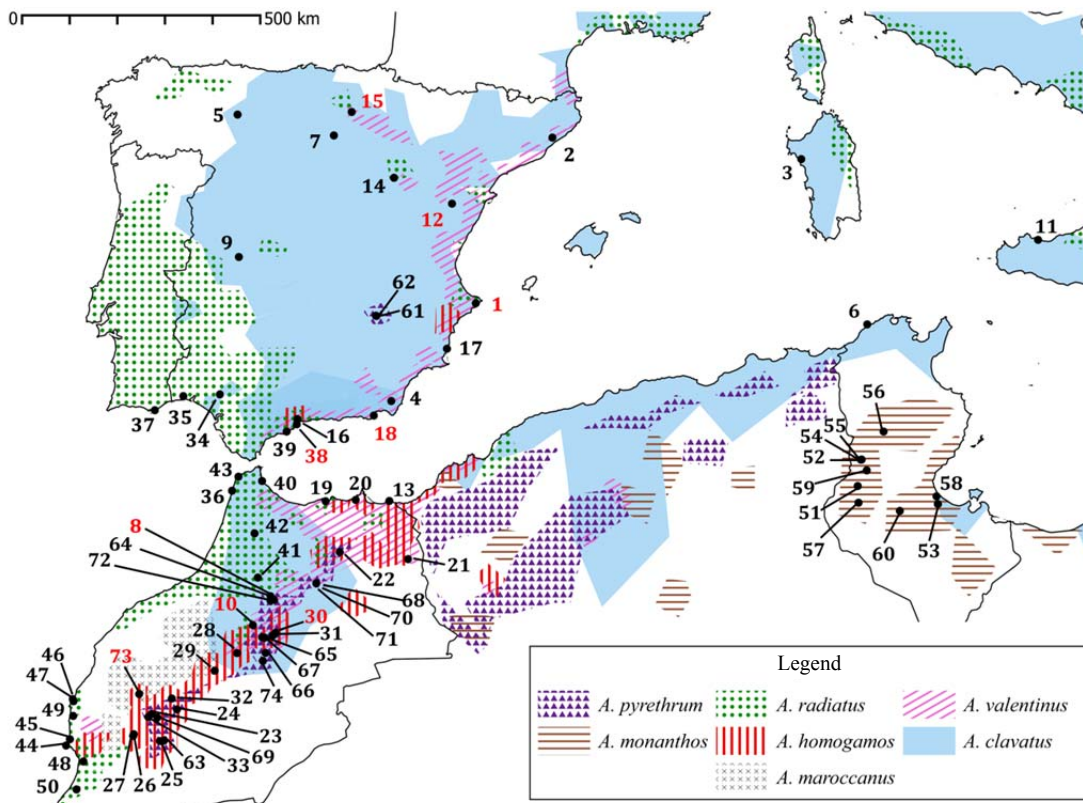
Figure 1. Populations sampled and distribution areas of six *Anacyclus* species. Black circles represent the populations sampled. Numbers correspond to the individual ID sampled as follows: individuals 1 to 11, *A. clavatus*; 12 to 22, *A. valentinus*; 23 to 33, *A. homogamos*; 34 to 43, *A. radiatus* subsp. *radiatus*; 44 to 50, *A. radiatus* subsp. *coronatus*; 51 to 60, *A. monanthos*; 61 to 72, *A. pyrethrum*; 73 and 74, *A. maroccanus*. Numbers in red indicate sympatric populations. The areas drawn represent species distributions according to Humpries (1979).
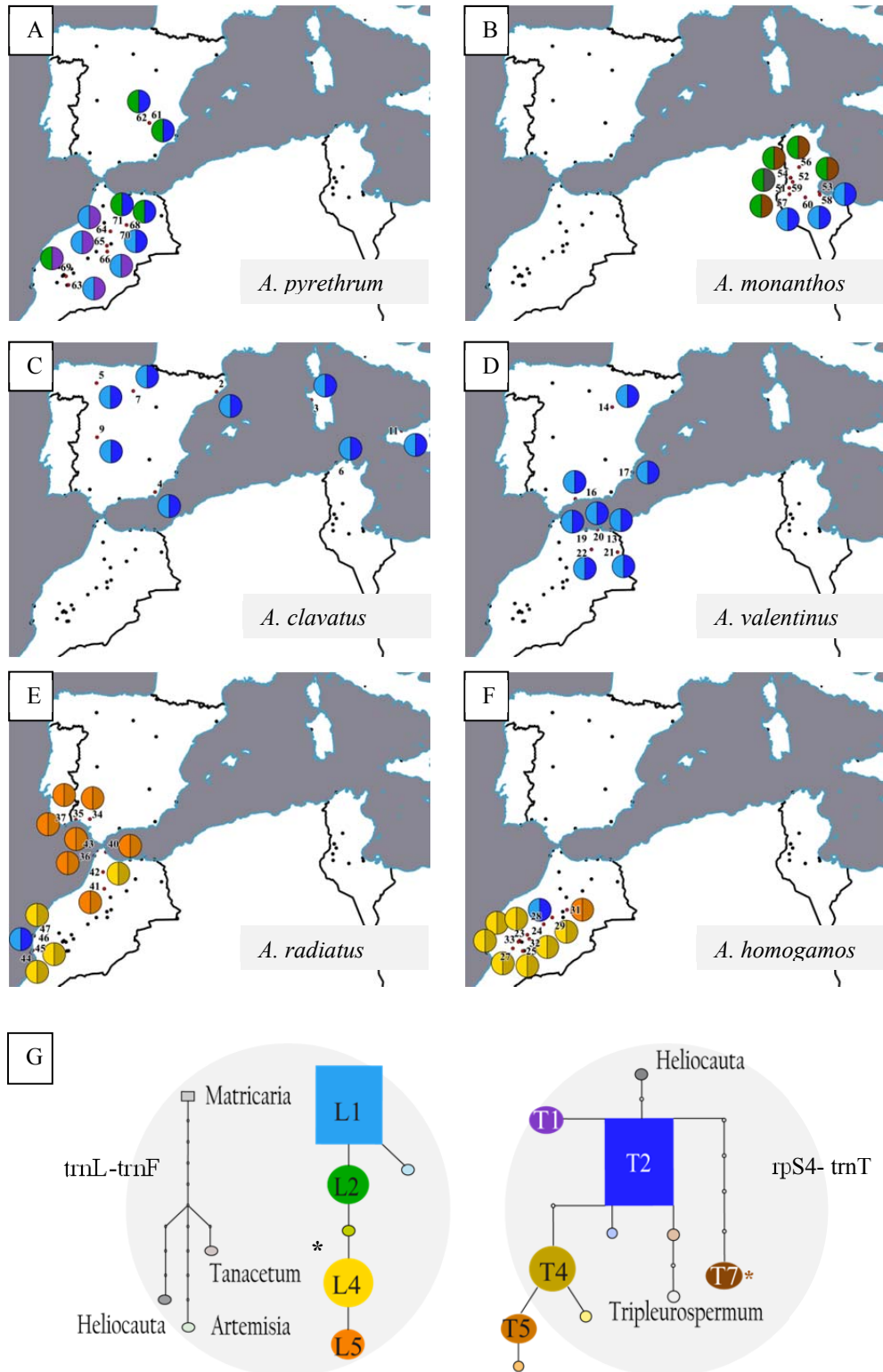
Figure 2. Allele distribution per species and networks according to chloroplast markers. A square represents the predicted ancestral allele and the sizes of each circle or square is in accordance with the number of individuals presenting that allele. Pie diagrams represents that each of the chloroplast alleles found per individual (left, trnL-F; right, trnT-rps4). The black asterisk represents the allele connected with *Heliocauta* when gaps are dismissed. The brown asterisk indicates that this allele is the same as T1 when gaps are dismissed.

Figure 3. Alleles in common among species within nuclear markers. A, allele sharing involving individuals from sympatric populations in the dataset. B, allele sharing among non-sympatric populations. Each color represents a different locus in accordance with the legend, where the number of haplotypes shared between different species per marker (n) is also detailed. The allele sharing is represented by lines in which thicker extremes point out the species presenting alleles parsimoniously clustered with the allele shared among genealogic networks. Sharp extremes represent that the allele shared is not parsimony connected with the remaining alleles of the species hinted, As individuals from both subspecies of *A. radiatus* were generally found within the same networks this species was represented as a single group for simplification. C, *A. clavatus*; H, *A. homogamos*; MA, *A, maroccanus*; MO, *A. monanthos*; P, *A. pyrethrum*; R, *A. radiatus*; V, *A. valentinus*.
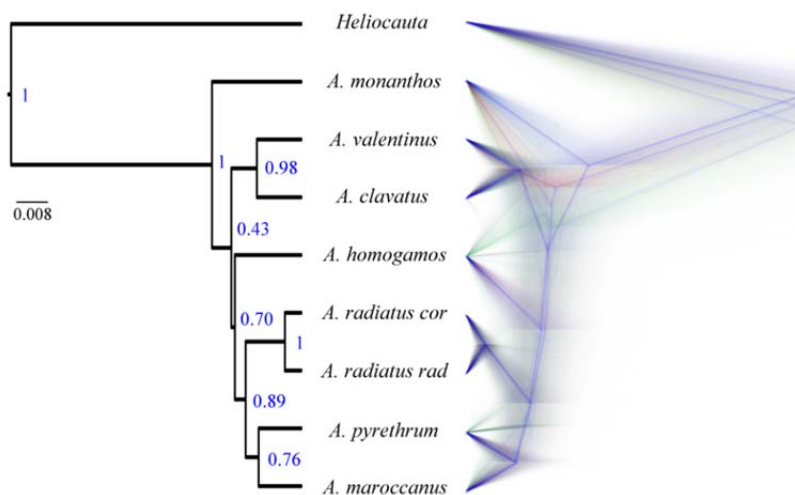
Figure 4. Consensus Tree and Cloudogram of the species tree on two different datasets of *Anacyclus* species and *Heliocauta*. A, result of the analysis performed in the dataset including seven *Anacyclus* species and *Heliocauta*. On the left, the consensus trees generated from two independent runs. Numbers in blue adjacent to nodes allude to posterior probabilities. On the right, cloudograms of the species trees from *BEAST. Frequency of different topologies occurring in the posterior distribution is illustrated with width and intensity of branches. Lines in purple, red and green represent the first, second and third more likely consensus tree topologies.
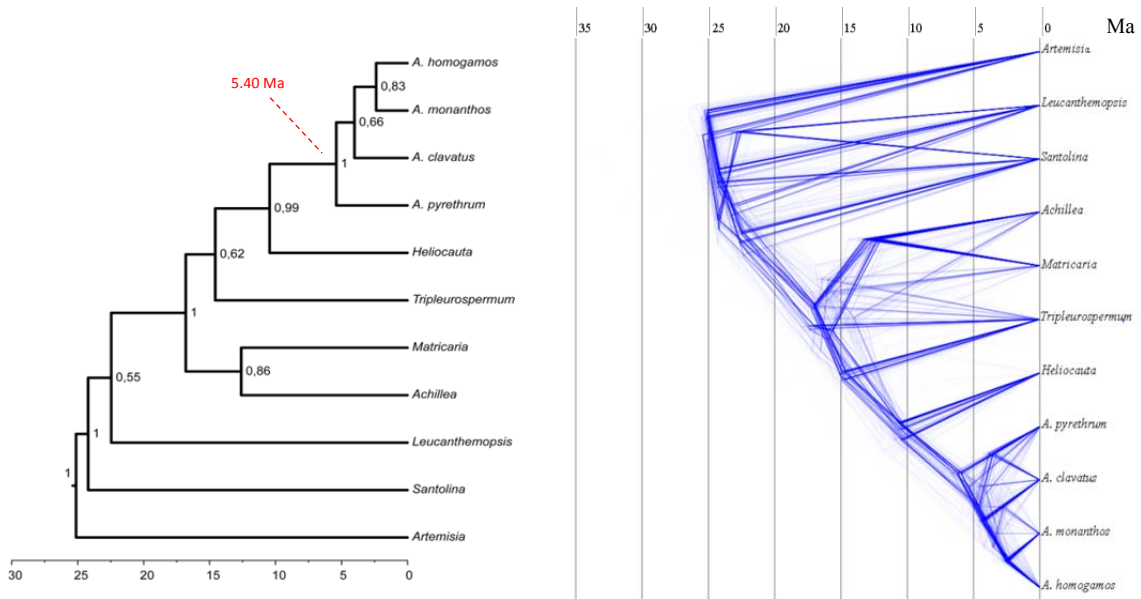
Figure 5. Molecular dating of *Anacyclus* based on nuclear and plastid markers. Consensus trees and posterior probabilities are represented on the left. On the right, representation of all the trees sampled among analyses.
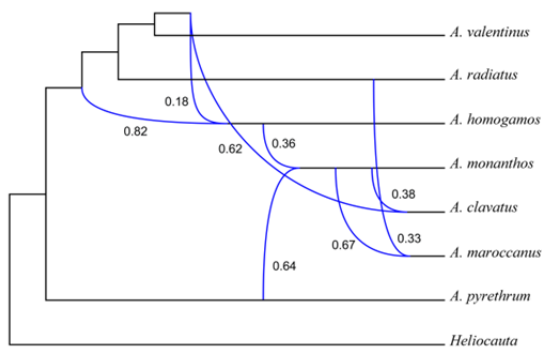
Figure 6. Reticulate evolution hypothesis according to a network reconstruction method. Cladograms represent phylogenetic relationships according to coalescence, while blue lines represent hybridization events between connected taxa. Numbers in A represent the contribution of each putative parental taxa to the hybrid one.

SUPPLEMENTARY MATERIAL

Table S1. Geographic and collection information of the localities sampled. Loc., locality; Col., collection; m, mixed, an asterisk indicates sympatric population. ARM, Armenia; GER, Germany, MOR, Morocco; POR, Portugal; SPA, Spain; TUN, Tunisia.

Species	Loc. label	Latitude	Longitude	Altitude	Col. number	Col. date	m	Location
	1	38.71814	0.0619444	226	IA 2024 (1)	27-VI-2009	*	SPA: Benissa (Alicante)
	2	41.64644	2.4213889		IA 2045 (1)	28-VI-2009		SPA: San Celoní (Barcelona)
	3	40.46792	8.4448611		IA 2005	27-IV-2009		ITA: Cerdeña
	4	37.01333	-2.166944	610	IA 2009	14-V-2009		SPA; Lucainena de las Torres (Almería)
	5	42.60452	-5.579103	917	IA 2010	23-V-2009		SPA: León
A. clavatus	6	37.23342	9.21575	113	AQ 3119	29-III-2009		TUN: Cap Serrat
	7	42.10889	-3.124722	6	IA 2049(1)	29-VI-2009		SPA: L'Escala (Gerona)
	8	33.43967	-5.225167	1322	Ia 2234(1)	14-IV-2012	*	MOR: Azrou
	9	39.90189	-5.684944	271	Ia 2320(1)	31-V-2012		SPA: Navalmoral de la Mata (Cáceres)
	10	32.90086	-5.650722	911	Aa48(1)	6-VI-2012	*	MOR: N8, Khenifra
	11	38.04194	13.514444	120	TS421(2)	24-IV-2012		ITA: Sicilia
A. valentinus	12	40.632	-0.281111		IA 2033 (1)	28-VI-2009	*	SPA: La Sénia (Tarragona)

	13	35.1275	-2.374833	13	JC 2442 (1)	20-VI-2008		MOR: Ras-el-Ma / Oued Moulouya
	14	41.221	-1.676056	43	IA 2041(1)	28-VI-2009		SPA: Vilanova i la Geltrú (Barcelona)
	15	42.52843	-2.621158	516	IA 2065(1)	17-VIII-2009	*	SPA : Elciego (Álava)
	16	36.77867	-4.427667	121	IA 2124(14)	28-III-2011		SPA : La Concepción (Málaga)
	17	37.91347	-0.737028	31	IA 2155(1)	18-IV-2011		SPA : Dehesa de Campoamor (Alicante)
	18	36.76117	-2.606139	7	IA 2159(1)	19-IV-2011	*	SPA : Roquetas del Mar (mixta) (Almería)
	19	35.19456	-3.857639	5	Ia 2185(5)	11-IV-2012		MOR: Al-Hoceima-Nador
	20	35.18794	-3.156722	42	Ia 2192(1)	11-IV-2012		MOR: cerca de Nador
	21	34.00497	-2.033028	929	Ia 2210(5)	13-IV-2012		MOR: Aïn-Benimathar
	22	34.21786	-3.592944	451	Ia 2222(1)	13-IV-2012		MOR: Guercif-Taza
	23	31.25125	-7.977828	1160	IA 2115 (1)	24-V-2010		MOR: Asni
	24	31.33194	-7.408889	2224	RG 1275?	13-VI-2009		MOR: Marrakech / Tensift-Al Haouz
	25	30.73417	-7.7975	1943	AQ 3505	9-VI-2009		MOR, Akaun.
A. homogamos	26	30.83664	-8.393558	1390	IA 2111(4)	24-V-2010		MOR: Chafarni
	27	30.86846	-8.379269	2100	IA 2113(4)	24-V-2010		MOR: Tizi-n-Test
	28	32.05544	-6.544167	1511	Aa36(1)	5-VI-2012		MOR: Azilal / Bin El Ouidane
	29	32.37994	-6.028917	1394	Aa40(1)	5-VI-2012		MOR: N8, El Kshiba

	30	32.69381	-5.2035	1600	Aa53(1)	6-VI-2012	* MOR: R503
	31	32.74144	-5.13775	1727	Aa54(1)	6-VI-2012	MOR: R503, Boumia
	32	31.54114	-7.520389	942	Aa76(1)	8-VI-2012	MOR: N9, 45km to Marrakech
	33	31.19544	-8.051972	950	Aa86(3)	9-VI-2012	MOR: Asni / Ouirgane
	34	37.30484	-6.259817	13	IA 2077 (1)	3-V-2010	SPA: Aznalcázar (Sevilla)
	35	37.28927	-7.138867	23	IA 2082 (1)	3-V-2010	SPA: Cartaya (Huelva)
	36	35.46778	-6.0375	5	JC 3489	9-IV-2009	MOR: Tánger-Tétouan, Asilah
	37	37.02963	-7.827453	4	IA 2085 (1)	4-V-2010	POR: Olhao
	38	36.68125	-4.447889	1	IA 2125 (1)	28-III-2011	* SPA: Málaga playa (Málaga)
A. radiatus subsp. *radiatus*	39	36.55181	-4.696472	35	IA2138	31-III-2011	SPA: Entrerríos (Málaga)
	40	35.63306	-5.325278		IA2179	10-IV-2012	MOR: Tetouan
	41	33.80206	-5.502333	652	IA2238	14-IV-2012	MOR: Boufakrane
	42	34.64361	-5.543417	93	IA2242	15-IV-2012	MOR: Ouazzane
	43	35.73008	-5.877583	-22	IA2247	15-IV-2012	MOR: Tánger
	44	30.65145	-9.886669	4	IA 2108 (1)	22-V-2010	MOR: Tamri-Amesnaz
A. radiatus subsp. *coronatus*	45	30.77224	-9.803358	200	IA 2107 (1)	22-V-2010	MOR: Imessouane-Agadir
	46	31.53376	-9.743842	14	IA 2105	22-V-2010	MOR: Essaouira

47	31.49593	-9.727742	104	IA 2102 (1)	21-V-2010	MOR: Essaouira
48	30.34943	-9.499444	18	IA 2109 (1)	23-V-2010	MOR: Aït-Melloul
49	31.21667	-9.733333	290	vogt11931	22-V-1993	MOR: Haha
50	29.81667	-9.65	180	Cho6355	19-V-1993	MOR. Agadir

A. monanthos	51	34.33861	8.3291667	250	AH 3907	25-III-2009	TUN: gorges de Seldja, Gabès, Metlaoui
	52	34.80556	8.5188889	697	JC 3314	28-III-2009	TUN: Kasserine, Fériana
	53	33.7425	10.015	100	CA 16233	23-III-2009	TUN: Matmata
	54	34.80556	8.502	697	JC 3343	28-III-2009	TUN: Kasserine, Fériana
	55	34.80556	8.5188889	697	JC 3314	28-III-2009	TUN:Kasserine, Fériana
	56	35.23861	9.1211111	560	JC 3267	27-III-2009	TUN:Kasserine, Sbeïtlanas
	57	34.03194	8.2822222	65	AH 3957	25-III-2009	TUN: Gafsa 2 (gorges)
	58	33.88639	10.018056	10	CA 16194	23-III-2009	TUN: Gabés
	59	34.59292	8.5897	420	Cho6962	08-V-1994	TUN: Gafsa
	60	33.74878	9.1573667	150	Cho7141	11-V-1994	TUN: Kebili

	61	38.64625	-2.388922	1240	IA 2095 (1)	18-V-2010	SPA: Peñascosa, Caballerías (Albacete)
A. pyrethrum	62	38.64028	-2.378128	1276	IA 2093 (2)	18-V-2010	SPA: Peñascosa, Malpaso (Albacete)
	63	30.74361	-7.706944	2337-3304	AQ 3531	2009	MOR: Monte Siroua (Jbel Sirwa)

	64	33.36636	-5.150389	1924	IA2233	14-IV-2012		MOR: Meknes
	65	32.65539	-5.392722	1808	AA52	6-VI-2012		MOR: R503, Azarzou
	66	32.36667	-5.383333	2250-2500	Cho3421	03-VII-1989		MOR, Er-Rachidia, Hoher Atlas
	67	32.66667	-5.45	1900-2000	Cho2513	26-IV-1987		MOR, Meknès, Mittlerer Atlas
	68	33.65	-4.166667	2210	Cho1977	26-VI-1989		MOR: Taza, Mittlerer Atlas
	69	31.18333	-7.85	2600-3000	Cho3680	16-VII-1989		MOR, Marrakech, Hoher Atlas
	70	33.65	-4.166667	2040	Cho1963	26-VI-1989		MOR: Taza, Mittlerer Atlas
	71	33.65	-4.166667		Cho1939	26-VI-1989		MOR: Taza, Mittlerer Atlas
	72	33.3579	-5.2303	1900	Cho9263	13-V-1995		MOR: Azrou
A. maroccanus	73	31.63213	-8.252964	61	IA 2096	21-V-2010	*	MOR: Tnine-des-Oudaya
	74	32.21667	-5.45	460	Cho2465	25-IV-1987		MOR: Beni-Mellal
Achillea tenuifolia					Cho10094B	18-VI-202		ARM: Armenia, Vedi,
Artemisia vulgaris					Cho9773			GER: Jena, Botanischer Garten
Heliocauta atlantica				3850	Kreisch 920589	23-VIII-1992		MOR: Toubkal
Ismelia carinata				3850	Kilian 3384B	26-IV-1994		MOR: Agadir
Leucanthemopsis alpina				2380	ST40	18-VII-2010		ARM: Armenia
Leucathemum gracilicaule				296	KK20			SPA: Valencia

Matricaria discoidea		Cho9762B	18-VI-2002	GER: Jena
Santolina rosmarinifolia	1810-1850	Cho1950	01-VII-1989	MOR: Er-Rachidia
Tanacetum coccineum	296	Cho10045B	16-VI-2002	ARM: Sevan - Tsovagyugh
Tripleurospermum caucasicum		Cho10192B	30-VI-2002	ARM: Hayek

Table S2. **Nucleotide substitution models and values examined to molecular clock model selection for phylogenetic inference on seven *Anacyclus* species and *Heliocauta* dataset .**

Parameters / Loci	*A19 a*	*A19 b*	*A39*	*C16*	*C20*	*C48*	*D01*	*D23*	*D35*	*trnL-F*	*trnT*
Alignment length	321	320	379	197	496	468	415	369	335	387	543
Substitution model	TVMef+Γ	TrN	TVM+I+Γ	TPM3uf+I	TPM3uf+Γ	TVM+Γ	TIM2	TIM2+Γ	TPM3uf+Γ	GTR+I	TPM1uf
Strict clock	**-1418.7**	**-1080.714**	**-2596.29**	**-1068.2921**	-3431.547	-2921.103	**-1193.073**	**-2203.598**	-1572.05	**-1665.72**	
Lognormal relaxed clock	-1420.02	-1079.349	-2596.835	-1065.7822	**-3417.086**	**-2918.045**	-1190.783	-2202.753	**-1566.691**	-1666.065	

Table S3. Nucleotide substitution models and values examined to molecular clock model selection for phylogenetic inference on four *Anacyclus* species and *Heliocauta* dataset .

| Parameters / Loci	*A19 a*	*A19 b*	*A39*	*C16*	*C20*	*C48*	*D01*	*D23*	*D35*	*trnL-F*	*trnT*
Alignment length	313	317	355	195	470	450	415	365	327	387	548
Substitution model	TPM3+I	TIM2	TPM3uf+I+Γ	TVM	TPM3uf+Γ	TVM+I	TPM2uf	TrN+Γ	TIM3+I	GTR	TPM1uf
Strict clock	-987.9	-827.115	**-1811.98**	**-765.663**	**-2382.763**	-1951.018	**-990.989**	-1599.31	-1297.892	-1550.621	
Lognormal relaxed clock	**-983.074**	**-817,808**	-1810.397	-765.415	-2381.571	**-1947.421**	-989.445	**-1587.294**	**-1292.538**	**-1518.003**	

Table S4. Nucleotide substitution models and values examined to molecular clock model selection for phylogenetic inference on six *Anacyclus* species, *Heliocauta* and nine outgroup species dataset.

Parameters / Loci	*A19 a*	*A19 b*	*A39*	*C16*	*C20*	*C48*	*D01*	*D23*	*D35*	*trnL-F*	*trnT*
Alignment length	326	320	383	323	520	488	429	380	341	430	588
Substitution model	TPM2uf+Γ	TPM2uf+Γ	TVM+I+Γ	HKY+I	TVM+Γ	TVM+Γ	HKY+I	TIM3+Γ	TPM3uf+I	TVM+Γ	TVM
Strict clock	-1982.394	-1387.003	-3447.048	-1719.394	-4008.01	-3867.767	-2155.774	-2810.167	-2260.722		-2566.293
Lognormal relaxed clock	**-1978.879**	**-1367.851**	**-3441.238**	**-1701.673**	**-3985.473**	**-3862.569**	**-2065.21**	**-2786.239**	**-2244.127**		**-2547.018**

Table S5. Nucleotide substitution models and values examined to molecular clock model selection for phylogenetic inference on four *Anacyclus* species, *Heliocauta* and six outgroup species dataset.

| Parameters / Loci	*A19 a*	*A19 b*	*A39*	*C16*	*C20*	*C48*	*D01*	*D23*	*D35*	*trnL-F*	*trnT*
Alignment length	315	319	362	323	495	478	427	365	337	425	570
Substitution model	TPM+Γ	TPM2uf+Γ	TIM3+Γ	TPM3uf	TPM2uf+Γ	TPM2uf+Γ	HKY+I	TIM3+Γ	TPM3uf+I	TVM+I	TPM1uf
Strict clock	**-1469.141**	-1159.524	**-2298.576**	-1261.251	-3040.798	**-2929.918**	-1930.092	-1890.517	-1880.055		**-2203.578**
Lognormal relaxed clock	-1467.26	**-1135.77**	-2297.295	**-1244.62**	**-3032.228**	-2936.741	**-1867.295**	**-1882.203**	**-1876.613**		-2201.232

Table S6. Nucleotide substitution models and values examined to molecular clock model selection for phylogenetic inference on few individuals of four *Anacyclus* species, *Heliocauta* and six outgroup species dataset.

| Parameters / Loci	*A19 a*	*A19 b*	*A39*	*C16*	*C20*	*C48*	*D01*	*D23*	*D35*	*trnL-F*	*trnT*
Alignment length	318	317	348	321	484	477	427	353	327	425	570
Substitution model	TPM2uf+Γ	TPM2uf+Γ	GTR+Γ	TPM3uf+Γ	HKY+I	TPM2uf+Γ	HKY+I	TIM3+Γ	HKY+I	TVM+I	TPM3uf
Strict clock	**-1102.26**	**-929.259**	**1625.119**	**-979.876**	**-2289.635**	**-2036.139**	-1693.854	**-1222.299**	**-1316.676**		-2033.689
Lognormal relaxed clock	-1105.228	-930.155	1626.059	-979.935	-2291.11	-2035.021	**-1684.7**	-1222.499	-1315.519		**-1989.872**

Supplementary figures
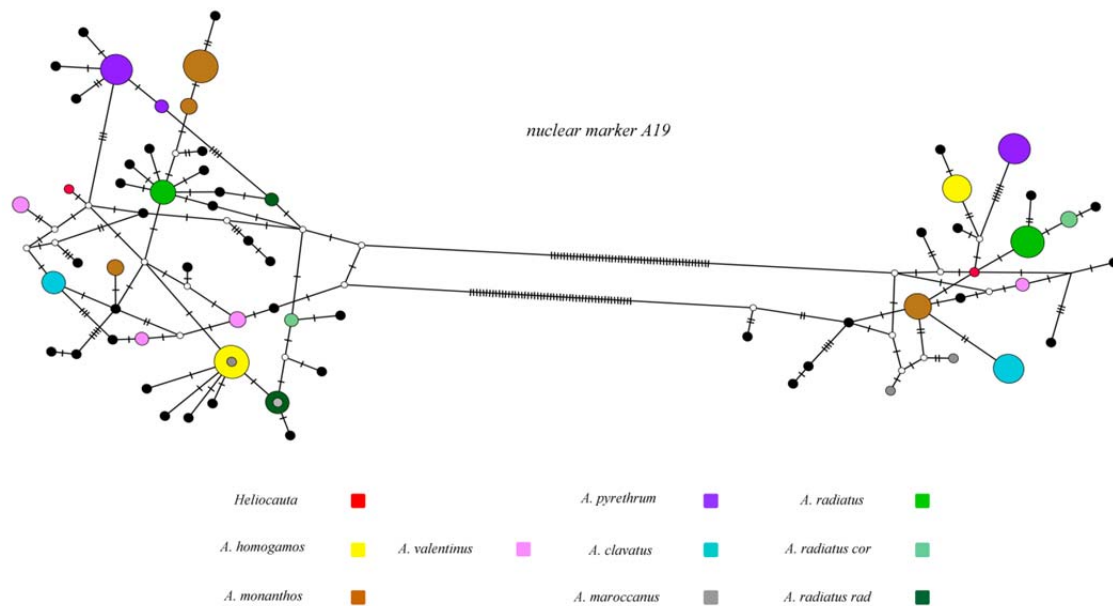
Figure S1. Statistical parsimony networks for the two nuclear markers presenting clear paralogue regions. Circles size correspond to the number of individuals. Hatch marks between nodes represent a mutation. Black circles represent unique alleles.The paralogue regions in A19 were partitioned in two clusters: A19a, at the left of the network, and A19b, at the right.
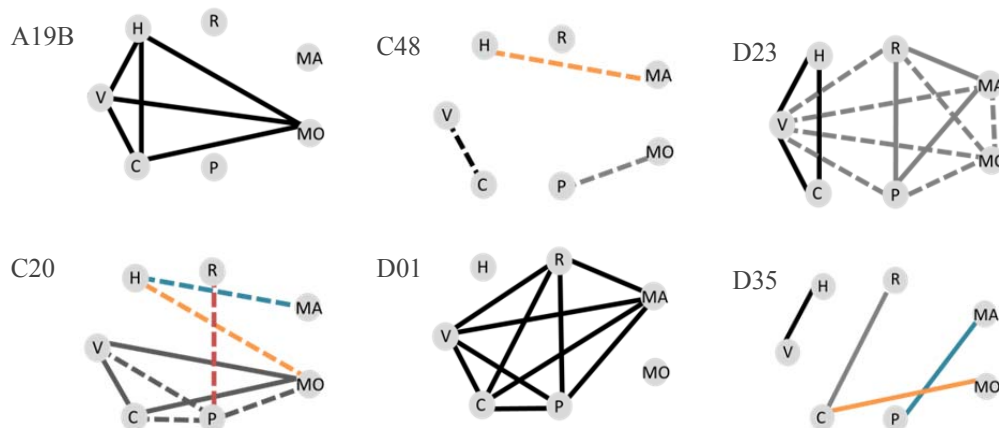
Figure S2. Species connected in parsimony networks according to the 95% credibility limit for six nuclear markers. Different colors represent discrete networks within nuclear regions. Dotted lines represent that the referred species had a minority presence in the network (one or two alleles). R, *A. radiatus*. MA*, A. maroccanus*. MO, *A. monanthos*. P, *A. pyrethrum*. C, *A. clavatus*. V, *A. valentinus*. H, *A. homogamos*. As individuals from both subspecies of *A. radiatus* were generally found within networks this species was represented as a single group for simplification.
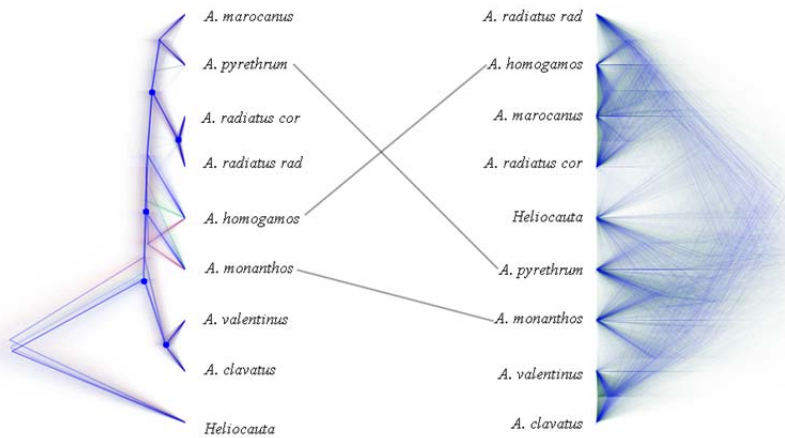
Figure S3. Cloudograms of the species trees obtained by independent analyses of nuclear (left) and plastid (right) markers. Frequency of different topologies occurring in the posterior distribution is illustrated with width and intensity of branches. Lines in purple, red and green represent the first, second and third more likely consensus tree topologies. Blue circles at nodes represent significant posterior probabilities (>0.95). Lines between cloudograms point out the species presenting incongruent connections within the most likely distributions obtained from nuclear and chloroplast markers.

(Figure 4S on the next page)

Figure S4. Molecular dating of *Anacyclus* based on nuclear and plastid markers according to three different datasets. A, six *Anaclyclus* and ten outgroup species, including all individuals from non-sympatric populations. B, four *Anacyclus* and seven outgroup species, including all individuals from non-sympatric populations. C, four *Anacyclus* and seven outgroup species, including 2-3 individuals from populations less likely to contain hybrid signals. Consensus trees and posterior probabilities are represented on the left. On the right, representation of all the trees sampled among analyses.
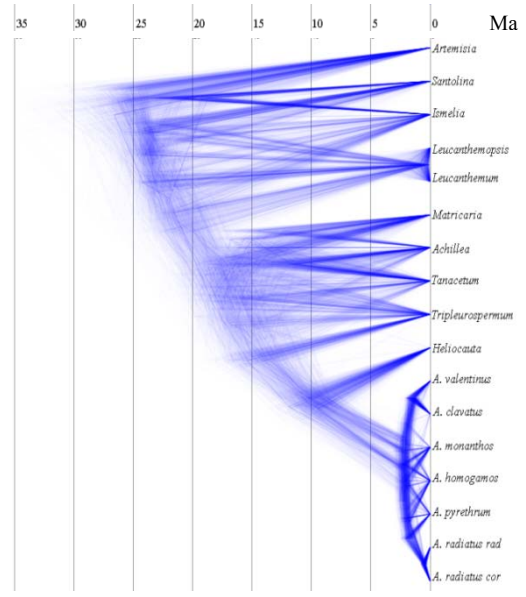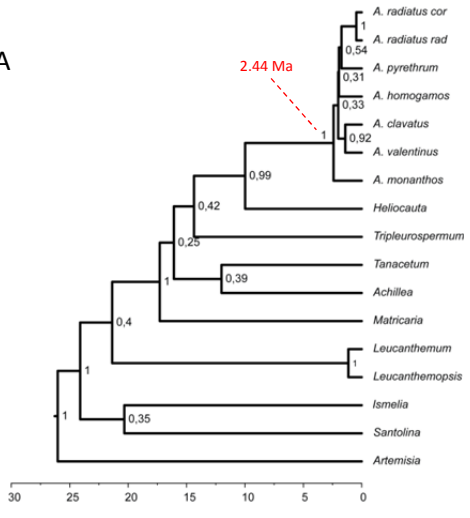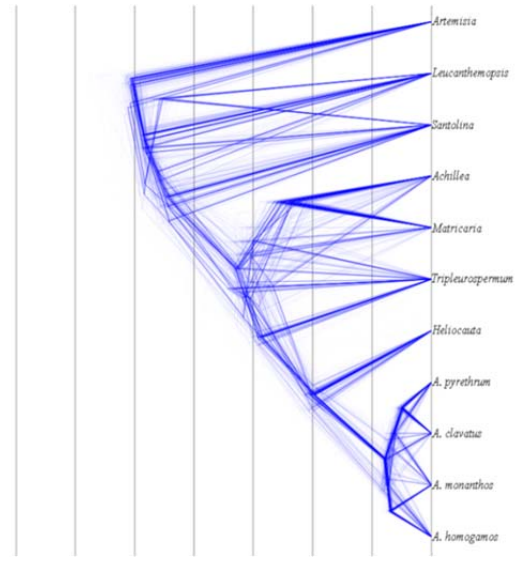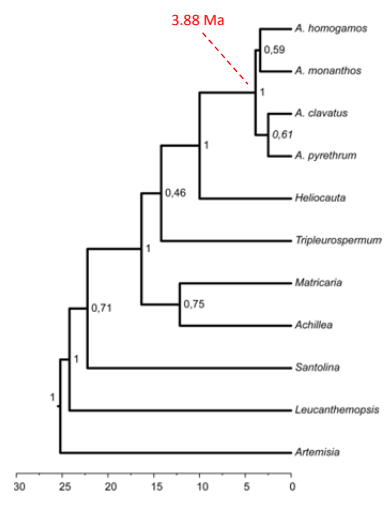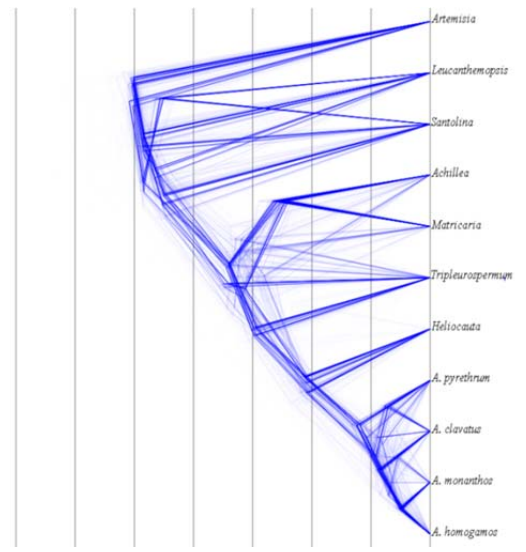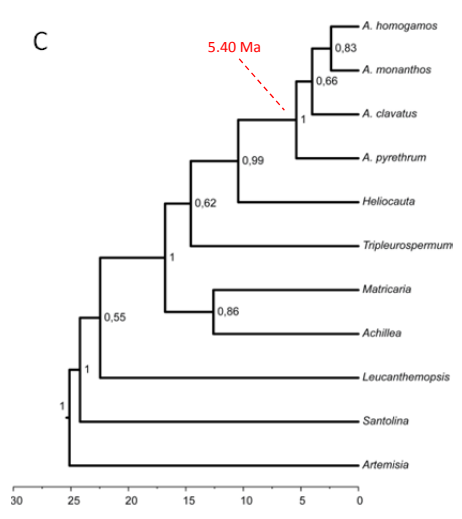
DISCUSIÓN Y CONCLUSIONES GENERALES

Discusión General

Flujo génico actual entre Anacyclus clavatus*,* A. homogamos *y* A. valentinus

Todos los resultados de los diferentes trabajos aportados en esta tesis apuntan en la misma dirección; en todos ellos, es clara la evidencia de flujo génico actual entre las especies de *Anacyclus* que han sido foco de este estudio. Este flujo génico se pone de manifiesto en poblaciones simpátricas que se observan a lo largo de las zonas de contacto o zonas de solapamiento de distribución de especies. Los resultados más sólidos al respecto son los aportados por el análisis de genética de poblaciones (capítulo 2.2), gracias a la identidad genética diferenciada para cada una de las especies del complejo, es decir, cada especie pertenece en un elevado porcentaje de casos a uno o dos grupos genéticos exclusivos. Este hecho, junto con la presencia de individuos con asignación genética mixta de dos o más grupos genéticos en poblaciones simpátricas, indica de manera concluyente la existencia de flujo génico en dichas poblaciones. Del mismo modo que se han observado en estas poblaciones morfologías florales intermedias, también se han observado individuos con tamaños genómicos intermedios (capítulo 2.1), dando lugar a patrones semejantes que indican la existencia de flujo génico natural entre estas especies y la formación de híbridos homoploides. Otra evidencia clara es aportada por el análisis de compartición de secuencias idénticas (alelos), en el capítulo sobre relaciones filogenéticas (capítulo 3), en el cual se muestra un incremento significativo del número de alelos compartidos entre individuos de poblaciones simpátricas respecto de las que no lo son. Estos resultados junto con el hecho de ser todas ellas especies auto-incompatibles e inter-fértiles (capitulo 1.3) y que de hecho comparten los mismos visitantes florales (capítulo 1.2) indican de manera inequívoca la existencia de flujo génico en poblaciones donde estas especies entran en contacto. Los resultados del análisis de visitantes florales (capítulo 1.2) en *A. clavatus* y *A. valentinus* indican que ambas especies son generalistas y que presentan polinizadores que realizan sus visitas independientemente de la morfología del capítulo. Estos patrones irregulares en las visitas suponen la atenuación de los efectos mediados por polinizadores en la selección de uno u otro carácter (Gómez & al., 2008). Sin embargo, se observó un sesgo en el grupo de polinizadores más frecuente en *A. clavatus* y *A. valentinus*, los dípteros, mostraron una mayor interacción con los individuos de capítulos radiados. Así mismo, nuestros resultados indican que las agrupaciones numerosas de *A. clavatus* presentan en simpatría con *A. valentinus* un mayor número de visitas, por lo que la densidad de una u otra morfología puede ser igualmente relevante cuando estas especies entran en contacto. Pese a las aparentes ventajas de los capítulos radiados frente a los discoideos (Lack 1982; Marshall and Abbott 1984; Sun and Ganders 1990; Nielsen et al 2002; Celedón-Neghme et al 2007; Andersson 2008), la pérdida de las flores liguladas ha ocurrido repetidamente en diferentes linajes de la familia, indicando que la ausencia de flores liguladas podría también ser adaptativa

(Bremer and Humphries, 1993; Torices & al, 2011). Por ejemplo, las lígulas podrían resultar llamativas para los predadores de semillas (Fenner & al., 2002), al tiempo que fisiológicamente costosas (Andersson 1999; Andersson 2001; Celedón-Neghme et al 2007; Andersson 2008). Por lo tanto, las poblaciones simpátricas de *A. clavatus* y *A. valentinus* donde fenotipos radiados y discoideos coexisten junto a morfologías intermedias, representan un excelente sistema de estudio para explorar los factores ecológicos que determinan la evolución de las flores liguladas. Es importante destacar que, aunque en muchos casos existen individuos de morfología intermedia en estas poblaciones, muchas otras veces no ocurre así, aunque la identidad genética de dichos individuos sí puede ser mixta o puede llegar a darse el caso de individuos cuyo genotipo no corresponde al fenotipo esperado (ver análisis genético de individuos en poblaciones simpátricas, capítulo 2.2). Esto, puede ser debido al tipo de composición de la población simpátrica, en la que la proporción de especies de ambos parentales, F_1, F_2, retrocruces, etc., puede variar. Estos rangos de variación también se han observado en el tamaño del genoma (capítulo 2.1). Además, los resultados de la caracterización fenotípica de los cruces artificiales entre estas especies, indican que la frecuencia de fenotipo floral (capítulo radiado o no radiado, longitud, anchura y color de la lígula) depende mucho de la "carga genética" de la población, aunque en general, hay una tendencia a mostrar fenotipos radiados (tipo "*clavatus*") en la mayoría de los casos (capítulo 1.3), que incluso puede llegar a observarse cuando dos especies no radiadas como *A. homogamos* y *A. valentinus* se cruzan. Estos resultados explican la gran plasticidad, especialmente en las zonas límite o de solapamiento entre especies, que puede dar lugar a fenotipos nuevos (fenotipo "*trumpet*", Bello et al. 2013) o a asignaciones de especie erróneas. Por lo tanto, aunque la hibridación actual está demostrada, no podemos basarnos exclusivamente en caracteres morfológicos para identificarla, y debemos acudir a marcadores moleculares llevando a cabo un muestreo representativo de cada población, especialmente en las zonas de potencial contacto entre especies.

Origen híbrido de algunas especies y relaciones filogenéticas entre A. clavatus*,* A. homogamos *y* A. valentinus

La existencia de linajes de origen híbrido en el género, sin embargo, no es tan obvia, al menos de manera independiente en los trabajos aquí presentados. Es la visión conjunta de todos ellos lo que sugiere de manera congruente que los procesos de hibridación han jugado un papel muy relevante en la evolución de este género. Los altos niveles de incongruencia mostrados en los análisis filogenéticos (cap zzz) apoyan esta hipótesis, aunque una evaluación más detallada de la cantidad de señal debida a "incomplete lineage sorting" (ILS) es necesaria para poder concluir en qué medida la hibridación de linajes está presente (Buckley & al., 2006; Holland & al., 2008, Joly & al., 2009). De hecho, este ha sido uno de los principales retos en este estudio. Además, se encontraron indicios de flujo génico tanto en los linajes cuya diversificación fue

más antigua como en los más recientes, por lo que el análisis filogenético en este género debe afrontarse desde un enfoque reticular en lugar de mediante relaciones bifurcantes (e.g. Doolittle, 1999; Linder & Rieseberg, 2004). Esta señal es evidente en especies clave como *A. homogamos*, *A. monanthos*, y *A. pyrethrum* dos de las cuales ocupan posiciones basales y más próximas al ancestro común de *Anacyclus*, *Heliocauta atlantica*, y cuyas relaciones filogenéticas según ADN de cloroplasto o nuclear son incongruentes. La incongruencia de la señal que afecta a estos linajes basales impide una estima adecuada del tiempo de divergencia en *Anacyclus*, lo que se refleja en las diferencias de estima dependiendo de la exclusión o no de estas y otras muestras (entre 2.4 y 9 millones de años). Por el contrario, la estima de diversificación del grupo incluyendo *Heliocauta atlantica*, parece mantenerse constante, independientemente de las muestras del análisis (alrededor de 10 millones de años). A pesar de las incongruencias mostradas en este análisis, en todos los casos, dos de nuestras especies objeto de estudio, *A. clavatus* y *A. valentinus*, forman parte del mismo clado, son especies cuyas historias evolutivas han ido de la mano, aunque no podemos descartar que haya habido procesos de hibridación ligados a sus orígenes. Por otra parte, *A. homogamos* sería una especie más alejada de estas otras dos formando parte de otro grupo. Mediante el análisis de compartición de secuencias idénticas (alelos) se llega a la misma conclusión. El número de alelos compartidos (excluyendo poblaciones simpátricas) entre cualquier par de especies es significativo en el caso de *A. clavatus* y *A. valentinus*, mientras que entre cualquiera de éstas y *A. homogamos* es menor. Estos resultados por sí mismos no concluyen sobre el origen híbrido de ninguna de estas especies, aunque tampoco lo niegan. Es necesario avanzar y enlazar otro tipo de evidencias. Nuestra hipótesis inicial (y la de Humphries, 1979) sobre el origen híbrido de *A. valentinus* tiene algunas evidencias que la apoyarían, aunque la proximidad filogenética de uno de sus posibles parentales podría impedir en gran medida una inferencia con los marcadores filogenéticos utilizados. Sin embargo, los resultados sobre aislamiento reproductor (cap. Nnn) entre estas especies estarían totalmente en consonancia con esta hipótesis. Por una parte, existe en cualquier caso un aislamiento reproductor relativo entre las tres especies, pero ese aislamiento es significativamente mayor entre los supuestos parentales, *A. clavatus* y *A. homogamos*, que entre *A. valentinus* y cualquiera de estas dos especies. Por otra parte, la viabilidad de las semillas en *A. valentinus* es inferior a la de las otras dos especies y muy variable, dependiendo del donador de óvulos (efecto materno), lo que podría deberse a un efecto residual de incompatibilidades de algunos tipos de gametos según el modelo Bateson-Dobzhansky-Muller (BDM; Bateson, 1909; Dobzhansky, 1936; Muller, 1942) y el descenso en el eficacia biológica en algunos individuos de esta especie (Cutter, 2012), apoyando su origen híbrido. Además, la hipótesis evolutiva que explica la herencia de la ginomonoecia en este género (capítulo 1.3), implica la existencia de heterocigosis fijada (o principalmente fijada) de uno de los dos loci responsables del control de la expresión de este sistema sexual en *A. valentinus*. Dicha

heterocigosis podría haberse generado a partir de un proceso de hibridación en la formación de este linaje. Del mismo modo, para el mismo locus, también se requiere heterocigosis en *A. homogamos*, aunque en este caso habría menor presión selectiva, ya que no tendría repercusión en el fenotipo floral. El modelo de hipótesis evolutiva de este carácter, recogida en el capítulo nnn, es congruente tanto con el origen híbrido de los linajes de *A. homogamos* y *A. valentinus*, como con la mayor proximidad filogenética entre *A. clavatus* y *A. valentinus* que sugiere el análisis filogenético. Este modelo asume la posibilidad de existencia de desequilibrio en la composición de los tipos de gametos (bien por incompatibilidades BDM o bien por desequilibrios en el muestreo para la mezcla polínica de los cruces). Aunque las horquillas de valores en algunos casos son muy amplias, en prácticamente todos los casos, salvo una excepción, se aceptó el modelo. En esa excepción, se recurre a la falta de fijación de la heterocigosis en *A. homogamos* como explicación alternativa. Esta explicación no es casual. Este mismo fenómeno en *A. valentinus* podría explicar la presencia de individuos de fenotipo "*homogamos*", es decir, hermafroditas, en poblaciones de *A. valentinus* en la península ibérica, y que se han asignados erróneamente como *A. homogamos*. Estos son casos aislados (3-4 pliegos testigo) en puntos muy distantes a lo largo de la costa ibérica mediterránea. Sin embargo, la identidad genética de *A. homogamos* recogida en el capítulo 2.2, no se ha encontrado en este área, donde prácticamente sólo aparecen grupos genéticos de las especies *A. clavatus* y *A. valentinus*. De esta forma, también toma sentido el gran peso que tienen las variables ambientales en la distribución de las especies y de los grupos genéticos, como veremos a continuación, y que también apuntan a óptimos ambientales que solapan entre *A. valentinus* y ambos, *A. clavatus* y *A. homogamos*, mientras que estas dos últimas muestran óptimos más alejados entre sí.

Relevancia de factores ambientales (climáticos) en la distribución de especies, variabilidad genética y fenotípica

El buen ajuste de los modelos de distribución tanto de especies como de grupos genéticos apunta la importancia que las variables climáticas tienen en estas distribuciones (capítulo 2.2). De entre todas las variables utilizadas para los SDM, las que mayor peso tienen en general son las relacionadas con el régimen de lluvias (bien estacionalidad o bien lluvias de invierno), en segundo lugar la isotermalidad parece también relevante en algunas especies. Es muy notable el amplio rango de solapamiento entre especies, también coincidente con los grupos genéticos correspondientes. Al hilo de lo comentado anteriormente, las especies con mayor grado de solapamiento en su óptimo climático son *A. clavatus* y *A. valentinus*, y en segundo lugar *A. homogamos* y *A. valentinus*, seguidos ya de otros pares de especies. La especie cuyo óptimo es más alejada en el sistema es quizá *A. monanthos*, de ambientes áridos de la costa tunecina. Por lo tanto, según los óptimos climáticos, *A. valentinus* ocuparía un lugar intermedio

entre *A. clavatus* y *A. homogamos*, acorde a su posible origen híbrido entre ambas especies. También es importante destacar que la mayor riqueza de diversidad tanto genética como morfológica ocurre en el área de confluencia de diferentes óptimos climáticos, esto es, en el área de clima con mayor influencia Mediterránea a ambos lados del Estrecho de Gibraltar, donde también entran en contacto *A. clavatus* y *A. valentinus* con *A. radiatus* en la península ibérica, y estas tres con *A. homogamos* en el Medio Atlas y Rif en Marruecos. Esta región constituye uno de los puntos calientes de biodiversidad de mundo (Médail & Quézel, 1997). Aunque se han encontrado evidencias de migraciones posteriores a la apertura del Estrecho en algunos organismos (e.g. Castella, 2000; Veith & al. 2004; Carranza & al. 2006), por lo general la dispersión tanto de plantas como de animales por esta vía se ha visto severamente limitada desde el Plioceno (Petit & al. 2005). Aunque los patrones observados en *Anacyclus* son congruentes con una diversificación anterior al rellenado del Mar Mediterráneo (hace ~5.3 millones de años), como se ha propuesto en otras especies mediterráneas (e.g. Vargas & al, 1999; Caujapé-Castells & Jansen, 2003; Kadereit & al. 2005; Ortiz & al., 2008), no puede descartarse que haya podido haber colonizaciones a partir de dispersiones secundarias por medio de diferentes vectores, incluido el ser humano (Cody, 2006, Lavergne & al, 2013).

La relevancia del papel del régimen de lluvias no sólo se ha sugerido por los modelos de óptimos climáticos, si no que parece tener un papel relevante en la dispersión de las semillas en las especies anuales de *Anacyclus*. Los capítulos maduros de las especies del complejo permanecen incluso después de la senescencia total del individuo madre, constituyendo un banco de semillas aéreo (Bastida & al., 2010). Los frutos de estos capítulos son heterocárpicos, y no sólo presentan diferencias morfológicas, sino que también varían en el tiempo de germinación. Los aquenios alados de los verticilos externos presentan, por lo general, tiempos menores de germinación que los del interior, sin alas, permitiendo la propagación de estas semillas durante diferentes ventanas temporales. Los resultados de este trabajo (capítulo 1.1) sugieren que la reserva de semillas en *Anacyclus* se corresponde con la observada en otras especies de Asteráceas de ambientes áridos o semi-áridos, en las que la lluvia es el principal factor desencadenante de la liberación de las semillas (i.e. ombrohidrocoria) y que podría suponer una ventaja adaptativa en ambientes mediterráneos (Gutterman, 1994; Peters & al., 2009). En este sentido, la estructura llamada "alas", que en realidad es una extensión de la cubierta del aquenio, tendría una función facilitadora de la germinación, tal vez aumentando el intercambio de fluido (principalmente agua) entre el embrión y el exterior dando lugar a germinaciones más rápidas, y no como una estructura de dispersión por viento; aunque esto requiere experimentos adicionales para ser probado. Además, la variabilidad observada en los tiempos de germinación (desde 24 horas hasta 90 días) indica que los aquenios de mayor tamaño, aquellos que se liberan en primer lugar y que germinan más rápido, reducen la

probabilidad de dispersión secundaria y por lo tanto su capacidad dispersiva en estos casos; mientras que los aquenios más pequeños, los más internos, tardan más en liberarse y en germinar. Esta característica supone una ventaja adaptativa para los individuos que germinan más rápidamente en los parches más densamente poblados (Dubois & Cheptou, 2012; Orrock & Christopher, 2012).

Otro resultado relevante cuya explicación no hemos podido concluir, pero que bien podría estar relacionado con variación adaptativa a partir de procesos estocásticos de variación o bien a partir de procesos de hibridación fue la obtención de dos grupos genéticos muy divergentes dentro de *A. clavatus*. Esta especie es la que mayor área de distribución ocupa y posiblemente la que mayor rango de variación en su óptimo climático presenta. Estos dos grupos genéticos encontrados en esta especie son los más divergentes de todos según el análisis de estructura genética, más aún que cualquiera de los grupos genéticos indicadores de otras especies. La distribución de estos grupos genéticos tiene una estructura geográfica, uno de ellos, el de distribución más amplia ocupa principalmente zonas del interior de la península ibérica aunque también puede observarse a lo largo de la costa mediterránea. El otro grupo genético es minoritario, y está confinado al SE de la península ibérica y zona próxima en Marruecos, al otro lado del Estrecho. El hecho de que algunas poblaciones simpátricas pertenezcan en su totalidad a este grupo genético sugiere que bien su origen podría tener que ver con procesos de hibridación, o bien que este tipo genético puede dar lugar a una gran plasticidad fenotípica semejante a la que ocurre en poblaciones realmente simpátricas. La modelización de este grupo genético no es concluyente debido al bajo número de poblaciones donde se encontró, y sería interesante aumentar el muestreo para llegar a caracterizar genéticamente toda esta zona y otras de contacto (quizá en Marruecos o en todo el levante y costa NE de la península ibérica) para tener una estima más adecuada de la frecuencia y distribución de este grupo genético. Este patrón es semejante al que arroja el análisis de tamaño de genoma, en el que *A. clavatus* muestra dos grupos de poblaciones, unas hacia el interior de la península ibérica, con un tamaño de genoma mayor, y otras en la costa mediterránea, también a lo largo de zonas de contacto con *A. valentinus*, con un tamaño menor. Ya que estos tamaños del segundo grupo son intermedios entre los del primero y los de *A. valentinus*, al igual que ocurre en las poblaciones simpátricas y semejantes a los de cruces experimentales, se sugiere que estos tamaños hayan podido producirse por hibridación y selección del genotipo en estas áreas de contacto.

Como reflexión final, creo que este trabajo, en su conjunto, aporta una serie de evidencias donde queda claro el papel de la hibridación en la historia de este género y en la dinámica poblacional actual, en la diversidad genética y en la plasticidad fenotípica tan compleja de caracterizar. Asimismo, los factores climáticos parecen haber jugado un importante papel, dando lugar a través de la presión selectiva en cada caso, a los patrones geográficos que

observamos tanto genéticos y genómicos como fenotípicos. Este trabajo deja de manifiesto la importancia de evidencias de todo tipo, no sólo morfológicas y moleculares, que en conjunto dibujan patrones semejantes y apoyan las mismas hipótesis, especialmente en sistemas donde el nivel de ploidía se mantiene homogéneo y donde la hibridación inter-específica puede pasar fácilmente inadvertida.

Conclusiones generales

1.- Las tres especies del complejo *Anacyclus clavatus*, *A. valentinus* y *A. homogamos* tienen identidad genética propia, son entidades biológicas independientes, y por lo tanto se consideran especies bien diferenciadas.

2.- La identificación morfológica de estas tres especies recae exclusivamente en el tipo de sistema sexual (hermafrodita en *A. homogamos*, y ginomonoico en *A. clavatus* y *A. valentinus*), y en la presencia de flores liguladas en *A. clavatus*, que no están presentes en *A. valentinus* y *A. homogamos*.

3.- Las relaciones filogenéticas entre las especies de *Anacyclus* basadas en secuencias de marcadores cloroplásticos y nucleares indican una mayor proximidad entre *A. clavatus* y *A. valentinus* que entre cualquiera de ellas y *A. homogamos*. No obstante, las relaciones de parentesco de *A. homogamos* no pudieron resolverse debido a eventos de hibridación en este linaje o de separación incompleta de linajes (*incomplete lineage sorting*).

4.- En las poblaciones simpátricas de *A. clavatus* y *A. valentinus* en la península ibérica se demuestra la existencia de flujo génico actual, dada la presencia de individuos con genotipos mixtos entre estas dos especies.

5.- En estas poblaciones simpátricas, así como en las zonas donde solapan o entran en contacto las áreas de distribución de estas tres especies existen individuos con morfologías florales intermedias e individuos genéticamente mixtos cuya morfología es semejante a una de las especies parentales. Por lo tanto, la identificación morfológica puede llevar a veces a asignaciones erróneas y es necesaria una caracterización genética si se pretenden abordar estudios evolutivos.

6.- *Anacyclus clavatus* y *A. valentinus* son especies generalistas en cuanto al tipo de polinizadores que las visitan y presentan una mayor frecuencia de visitas de insectos del orden Diptera. En las poblaciones simpátricas estas especies comparten polinizadores, sin embargo los capítulos radiados (*A. clavatus*) recibieron más visitas que los discoideos (*A. valentinus*).

7.- Las tres especies del complejo *A. clavatus*, *A. valentinus* y *A. homogamos* presentan dentro del mismo capítulo frutos de diferente morfología, tamaño y tiempo de germinación, dando lugar a una correspondencia entre heterocarpia y diferentes estrategias de germinación. Los aquenios siguen un patrón posicional en el capítulo que puede estar relacionado con la liberación secuencial de los mismos. Los frutos más externos en el capítulo son los primeros en germinar mientras que los que ocupan las posiciones más internas presentaron ritmos de germinación menores.

8.- Las tres especies del complejo *Anacyclus clavatus*, *A. valentinus* y *A. homogamos* son auto-incompatibles, e inter-fértiles, aunque la bajada significativa de la fertilidad en las líneas híbridas implica cierto grado de aislamiento reproductivo entre ellas.

9.- Del análisis de la herencia de los sistemas sexuales en cruces (F_1, F_2) y retrocruces entre *A. clavatus*, *A. homogamos* y *A. valentinus*, se infiere por primera vez epistasia doble recesiva entre dos loci implicados en la expresión de la ginomonoecia. Sin embargo, tanto el número de flores femeninas, como la longitud y anchura de las lígulas requiere otros loci, cuya interacción génica es aún desconocida.

10.- La estructura genética de las especies estudiadas muestra un claro patrón geográfico, que según muestran la modelización de nicho en cada caso, está correlacionado con variables climáticas, principalmente con el régimen de precipitaciones y la isotermalidad.

11.- Existe un alto grado de solapamiento de nicho entre *A. clavatus* y *A. valentinus*, y en menor medida entre ésta última y *A. homogamos*. Sin embargo, el solapamiento de nicho entre grupos genéticos característicos de *A. valentinus* y *A. homogamos* es mayor que entre estas y los de *A. clavatus*.

12.- La especie que alberga mayor diversidad genética es *A. clavatus*, en la que a su vez se han identificado dos grupos genéticos divergentes y con distribución geográfica diferenciada, mientras que la especie genéticamente más homogénea y con menor diversidad es *A. valentinus*.

13.- El área geográfica donde el solapamiento, tanto de especies y grupos genéticos como de sus óptimos climáticos, es mayor es la región de Estrecho de Gibraltar en ambos continentes, que es también donde mayor diversidad genética y morfológica se puede encontrar en una misma población.

14.- El tamaño de genoma entre poblaciones de *A. clavatus* y *A. valentinus* es suficientemente diferente para permitir una diferenciación clara entre ellas. Sin embargo, esta diferenciación no es posible entre las poblaciones de *A. clavatus* y las de *A. homogamos*. Tanto en las poblaciones simpátricas de *A. clavatus* y *A. valentinus* como en las F_1 de sus cruces artificiales se observan

tamaños de genoma variables e intermedios a estas dos especies, lo que confirma la existencia de híbridos homoploides entre estas especies.

15.- La gran variabilidad en el tamaño de genoma encontrada en *A. clavatus* muestra un patrón marcadamente geográfico semejante al observado en sus grupos genéticos.

16.- Aunque muchos resultados están en consonancia con el origen híbrido de *A. valentinus*, como el análisis de su biología reproductiva, su posición filogenética, su proximidad reproductiva a los posibles linajes parentales y la segregación de caracteres florales en los cruces, no se ha encontrado la huella genética (marcador molecular) que de manera concluyente indique su origen híbrido y los posibles linajes implicados.

17.- La falta de señal filogenética clara no sólo afecta a *A.valentinus*. La incongruencia entre marcadores moleculares engloba a todas las especies del género, lo que sugiere que la hibridación, junto con la separación incompleta de linajes (*incomplete lineage sorting*), podría ser un fenómeno frecuente en *Anacyclus*, tanto a tiempo presente como pasado, y cuya evolución se refleja mejor en un modelo reticular.

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GENERAL CONCLUSIONS

General conclusions

1. The three species of the complex *Anacyclus clavatus*, *A. valentinus* and *A. homogamos* have their own genetic identity, are independent biological entities and thus are considered well-defined species.

2. Morphological identification of these three species exclusively depends on the type of sexual system (hermafrodite in *A. homogamos*, and gynomonoecious in *A.clavatus* and *A. valentinus*), and in the presence of ligulate flowers in *A. clavatus* and its absence in both *A. valentinus* and *A. homogamos*.

3. The phylogenetic relationships between these species based on sequences of plastidial and nuclear DNA markers indicate that *A. clavatus* and *A. valentinus* were more closely related than any of them and *A. homogamos*. Nevertheless, the phylogenetic relationships of *A. homogamos* were not resolved due to hybridization events in its lineage or to incomplete lineage sorting.

4. The presence of current gene flow in sympatric populations of *A. clavatus* and *A. valentinus* of the Iberian Peninsula is evidenced, given the presence of individuals with mixed genotypes of these two species.

5. In sympatric populations, as well as in the overlapping areas or contact zones between these three species, individuals with intermediate morphologies and individuals genetically mixed presenting the morphology of one of the species were observed. Thus, the morphological identification can lead to incorrect assignments. Genetic characterization is therefore required to address any evolutionary study in this system.

6. *Anacyclus clavatus* and *A. valentinus* are generalist species respecting the type of pollinators visiting them, presenting a higher visitation frequency of insects from the Diptera order. In sympatric populations, these species share pollinators, although radiate capitula (*A. clavatus*) received more visits than the discoid ones (*A. valentinus*).

7. The three species of the complex *A. clavatus*, *A. valentinus* and *A. homogamos* present fruits with different morphologies, size and germination time within the capitulum, which result in a correspondence between heterocarpy and different germination strategies. The achenes followed a positional pattern within the capitulum, which may

be related to their sequential release. The outermost fruits are the first ones germinating whereas the innermost fruits presented larger germination times.

8. The three species of the complex *Anacyclus clavatus*, *A. valentinus* and *A. homogamos* are self-incompatible and inter-fertile, although the significant fertility decrease in the hybrid lines implies a certain degree of reproductive isolation between them.

9. The analysis of the sexual system inheritance in crosses (F_1, F_2) and backcrosses between *A. clavatus*, *A. valentinus* and *A. homogamos*, indicated a double recessive epistasis between two loci involved in the expression of the gynomonoecy. However, the number of female flowers, as well as the ligule length and width require additional loci, whose interaction still remains unknown.

10. The genetic structure of the studied species showed a clear geographic pattern, which was according to niche modelling correlated with climatic variables, mainly related with the precipitation regime and isothermality.

11. There is a high degree of niche overlapping between *A. clavatus* and *A. valentinus*, and in a lower degree between the later and *A. homogamos*. However, the niche overlapping between genetic groups of *A. valentinus* and *A. homogamos* is higher than between any of these and *A. clavatus*.

12. *Anacyclus clavatus* is the species harbouring a higher genetic diversity, in which two divergent genetic groups with differentiated geographic distributions were found; whereas *A. valentinus* was the species more genetically homogeneous.

13. The geographic area where the species, genetic groups and climatic optima show a higher overlapping is the Strait of Gibraltar region at both continents, where a higher within-population morphological and genetic diversity can be also found.

14. Genome size between *A. clavatus* and *A. valentinus* is sufficiently different as to allow a clear differentiation between them. However, this differentiation is not possible between *A. clavatus* and *A. homogamos* populations. In both sympatric populations of *A. clavatus* and *A. valentinus* and F_1 artificial hybrids, variable and intermediate genome sizes were observed, which agrees with the existence of homoploid hybrids between these species.

15. The great variability of genome size observed in *A. clavatus* shows a markedly geographic pattern, which is similar to the pattern observed in their genetic groups.

16. Although much of the results are in agreement with the hybrid origin of *A. valentinus*, such as the analysis of its reproductive biology, its phylogenetic relationships, its reproductive proximity to its two putative parental lineages, or the segregation of floral characters in the experimental crosses; it has not been found the genetic print (molecular marker) that conclusively indicate its hybrid origin and the possible lineages implicated.

17. The lack of a clear phylogenetic signal does not only affect *A. valentinus*. Incongruence among molecular markers comprises all species in this genus, suggesting that hybridization, together with incomplete lineage sorting could be frequent events in *Anacyclus*, and both at the present and past times, and thus its evolution would be better explained by a reticulate model.

APPENDIX: Microsatellite primers in *Anacyclus clavatus* and closely related species

Microsatellite primers in the weedy annual herb *Anacyclus clavatus* (Asteraceae) and four closely related species[1]

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- *Premise of the study:* Nuclear microsatellite primers were developed for the weedy herb *Anacyclus clavatus* to study the genetic structure of hybrid zones with closely related taxa in the western Mediterranean Basin, where different floral phenotypes are present.
- *Methods and Results:* We obtained two microsatellite libraries using next-generation sequencing and Sanger sequencing of cloned restriction fragments. A total of 13 polymorphic and 11 monomorphic loci were identified in three Iberian populations of *A. clavatus*. The primers amplified di- and trinucleotide repeats with 1–8 alleles per locus. Most primers also amplified in *A. homogamos*, *A. monanthos*, *A. radiatus*, and *A. valentinus*.
- *Conclusions:* These results indicate the utility of these markers in *A. clavatus* for population genetic and hybridization studies as well as their applicability across the genus.

Key words: *Anacyclus clavatus*; Asteraceae; hybridization; population genetics; weeds.

Anacyclus L. (Anthemideae, Asteraceae) is a Mediterranean genus of mostly weedy annual herbs with approximately 12 species distributed in North Africa, southern Europe, and the Middle East (Humphries, 1979; Oberprieler et al., 2007). This genus is characterized by an extraordinarily large variation in floral symmetry (Bello et al., 2013). This diversity is especially remarkable in areas where two to three species coexist. *Anacyclus clavatus* (Desf.) Pers. is present throughout the distribution area of the genus. The species cohabits with *A. homogamos* (Maire) Humphries, which is mainly restricted to inland areas of Morocco and Algeria, and *A. valentinus* L., which mostly occurs in coastal areas across all of the western Mediterranean Basin. Based on the phenotypes obtained by artificial crosses among these species, intermediate floral phenotypes were interpreted as hybrids (Humphries, 1981), although there are no molecular data supporting this hypothesis. We developed nuclear microsatellite markers for *A. clavatus* to investigate its genetic diversity, population structure, and gene flow among closely related species in hybrid zones.

METHODS AND RESULTS

Two different methods were used to obtain microsatellite libraries for *A. clavatus*. For the first microsatellite library, silica-dried leaves of 10 individuals

of *A. clavatus* from Miraflores de la Sierra (40°47′34.53″N, 3°44′1.85″W) were sent to Genetic Identification Services (GIS; Chatsworth, California, USA) for DNA isolation and sequencing of cloned enriched restriction fragments following Jones et al. (2002). A voucher (*Álvarez 2173*) was deposited at the herbarium of the Royal Botanic Garden–Consejo Superior de Investigaciones Científicas (CSIC; MA). Recombinant plasmids were produced by ligating restriction fragments from *A. clavatus* DNA into the *Hin*dIII site of the pUC19 plasmid. The fragments were enriched for CA, GA, AAC and ATG microsatellite motifs, and ligation products were introduced into *E. coli* strain DH5α (ElectroMaxJ, Invitrogen, Carlsbad, California, USA) by electroporation. After transformation and recovery in super optimal broth with catabolite repression (SOC; Invitrogen), cells were incubated on Bluo-Gal/isopropyl-β-D-1-thiogalactopyranoside (IPTG)/ampicillin LB (BIA-LB) agar plates. To select insert fragments longer than 300 bp, white colonies were screened by PCR and subsequently sequenced. One hundred twenty-one sequences containing microsatellites were received from GIS, for which PCR primers were designed using DesignerPCR version 1.03 (Research Genetics, Huntsville, Alabama, USA). The second microsatellite library was prepared by Genoscreen (Lille, France) with the 454 GS FLX (Roche Diagnostics, Meylan, France) high-throughput DNA sequencer (Malausa et al., 2011). Total genomic DNA was extracted from silica-dried leaves of eight individuals of *A. clavatus* from Estación de Cártama (36°43′58.09″N, 4°39′37.02″W) using a modified cetyltrimethylammonium bromide (CTAB) method described in Doyle and Doyle (1987). A voucher (*Álvarez 2140*) was deposited at MA. Genomic DNA was fragmented and enriched with TG, TC, AAC, AAG, AGG, ACG, ACAT, and ACTC motifs. A total of 27,006 high-quality sequences were obtained. Analysis of these sequences with QDD software (Meglécz et al., 2009) revealed 2341 sequences with microsatellite motifs, for which 115 primer pairs were designed.

A total of 83 primer pairs, of which 42 were obtained by GIS and 41 by Genoscreen, were tested by PCR using 90 individuals from three populations of *A. clavatus*, in which 30 individuals were collected from Antequera (37°02′34.00″N, 004°30′54.30″W), 30 from Cartagena (37°37′09.04″N, 001°04′58.04″W), and 30 from Los Escullos (36°48′04.02″N, 002°03′47.02″W). Vouchers (*Álvarez 2122*, *Álvarez 2152*, and *Álvarez 2161*) were deposited at MA. Total genomic DNA was extracted from silica-dried leaves using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). PCRs were performed in a total volume of 20 μL, which contained 1× PCR Buffer, 2 mM MgCl₂,

TABLE 1. Characteristics of 13 polymorphic microsatellite primers developed in *Anacyclus clavatus*.[a]

Locus[b]	Primer sequences (5′–3′)		Fluorescent dye	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
D3	F:	GAAGGTGTGTCAAAAGGGTATT	NED	$(GAT)_8$	194–209	55	KF418743
	R:	AAGCAACAAATGAAGAGAGAGG					
8	F:	TCTTTACAAAGACCAGACGCC	6-FAM	$(AC)_7$	84–96	56	KF418722
	R:	GCTAGGCACCTAGGTTAATCACTT					
9	F:	CCATGAATATCATTCTTCCGTG	NED	$(CTT)_9$	77–96	56	KF418723
	R:	CGCGGAAGGTGTAATAGAGTC					
15	F:	TCATAATTACCCACCAACAGC	PET	$(AC)_{10}$	97–109	55	KF418724
	R:	GCCATTTTTGTTTGATTTCAATAG					
16	F:	ACTTGATAATTGATAAAACCACGGA	6-FAM	$(TG)_7$	86–96	56	KF418725
	R:	CCTAGAACATCAGACGCCAA					
17	F:	GAAGCTTTCTTAAGGTTTCTTCTTG	NED	$(TGT)_9$	129–153	55	KF418726
	R:	TCATTTGAATCTCATCATAGGAAA					
18	F:	TCACCAAATACTTCCCGAGC	VIC	$(TC)_8$	101–115	55	KF418727
	R:	ACTTTTGATCGAGCAATCCG					
19	F:	TTACCCGACTTGCTGAAAGG	PET	$(AAC)_6$	148–160	55	KF418728
	R:	CCTTGCGTATTTGCACTCCT					
20	F:	AGCTTACATTACAAGCCATGC	VIC	$(CA)_7$	89–97	55	KF418729
	R:	GAGGGTTTGGTTTGATTTGC					
21	F:	TCTTACCTGTTCCTTAGATCTTATTCA	6-FAM	$(CAA)_{11}$	120–137	55	KF418730
	R:	TGATTTGAATTTTCTAATGCTGC					
24	F:	CACGATCACTTTTCGATACTTACA	6-FAM	$(CT)_7$	89–105	56	KF418731
	R:	AATTTGCGGCTGTGGTAAAG					
27	F:	GGGTAGGTTTAACCATGGGG	NED	$(GA)_8$	185–191	55	KF418732
	R:	TGACGATACATCCAAAGTATCCC					
28	F:	AAAACACCTATCCACAATATGACC	VIC	$(AGA)_8$	263–278	56	KF418733
	R:	AGTATCTTGTCTAGAGACACTCTTCCC					

Note: T_a = annealing temperature.

[a] All values are based on 90 samples from three South Iberian populations.

[b] Locus D3 was obtained by sequencing of cloned enriched restriction fragments and the remaining were obtained by next-generation sequencing.

0.2 mM each of dNTPs, 0.4 µM each of primers, 0.6 U of *Taq* DNA Polymerase (Bioline USA, Canton, Massachusetts, USA), and 40 ng of DNA template using the following thermocycler conditions: an initial denaturation step at 94°C for 2 min; followed by 35 cycles of 1 min at 94°C, 1 min at 54–56°C, 2 min at 72°C; and a final extension of 10 min at 72°C. The PCR products were separated by electrophoresis on a 3% agarose gel to select those primer pairs that amplify fragments of the expected sizes and that might show allelic variation. A total of 24 primer pairs were selected as candidates to evaluate polymorphic loci. Forward primers of each pair were marked with 6-FAM, VIC, NED, or PET fluorescent dyes (Table 1). PCR products were analyzed with Peak Scanner Software version 1.0 (Applied Biosystems, Foster City, California, USA).

A total of 13 loci were polymorphic (Table 1), whereas 11 were monomorphic (Appendix 1). We estimated the mean number of alleles per locus, observed and expected heterozygosities, and Hardy–Weinberg equilibrium (HWE; Table 2) with GenAlEx version 6.3 (Peakall and Smouse, 2006). Tests for linkage disequilibrium between markers in each population were performed using FSTAT version 2.9.3.2 (Goudet, 1995). In the Antequera population, the number of alleles per locus ranged from two to six, and the observed and expected heterozygosities were 0.316–0.667 and 0.278–0.745, respectively. In the Los Escullos population, the number of alleles ranged from one to seven, and the observed and expected heterozygosities were 0.000–0.826 and 0.000–0.631, respectively. Loci 15, 17, and 21 were monomorphic in this population. In the Cartagena population, the number of alleles ranged from one to eight, and the observed and expected heterozygosities were 0.000–0.955 and 0.000–0.774, respectively. Loci 17 and 21 were monomorphic in this population. Significant deviation from HWE ($P < 0.05$) was seen for loci 8, 9, 15, 18, and 21 in the Antequera population, for loci 20 and 24 in the Los Escullos population, and for loci 20 and 27 in the Cartagena population. No significant departures from linkage disequilibrium ($P > 0.05$) were detected for any pair of loci. Cross-amplification was performed for these 13 polymorphic loci in *A. homogamos*, *A. monanthos*

TABLE 2. Results of initial primer screening of polymorphic loci in three populations of *Anacyclus clavatus*.

	Antequera				Los Escullos				Cartagena			
Locus	A	H_o	H_e	HWE[a]	A	H_o	H_e	HWE[a]	A	H_o	H_e	HWE[a]
D3	3	0.630	0.510	0.535 ns	4	0.588	0.631	0.605 ns	8	0.600	0.591	1.000 ns
8	3	0.538	0.447	0.000 ***	2	0.250	0.219	0.450 ns	2	0.542	0.395	0.069 ns
9	3	0.320	0.574	0.009 **	2	0.308	0.260	0.354 ns	2	0.120	0.180	0.096 ns
15	5	0.346	0.700	0.002 **	1	0.000	0.000	–	2	0.034	0.034	0.925 ns
16	5	0.591	0.594	0.675 ns	4	0.560	0.566	0.420 ns	5	0.571	0.556	0.926 ns
17	4	0.500	0.515	0.912 ns	1	0.000	0.000	–	1	0.000	0.000	–
18	4	0.316	0.633	0.008 **	7	0.750	0.580	1.000 ns	4	0.800	0.580	0.898 ns
19	5	0.500	0.646	0.377 ns	7	0.462	0.478	0.303 ns	6	0.696	0.774	0.980 ns
20	2	0.333	0.278	0.327 ns	4	0.826	0.591	0.002 **	3	0.955	0.542	0.000 ***
21	6	0.667	0.745	0.001 ***	1	0.000	0.000	–	1	0.000	0.000	–
24	4	0.517	0.583	0.329 ns	4	0.273	0.550	0.008 **	3	0.083	0.081	0.997 ns
27	4	0.389	0.454	0.379 ns	4	0.667	0.584	0.713 ns	2	0.647	0.438	0.049 *
28	3	0.409	0.344	0.693 ns	3	0.091	0.088	0.997 ns	3	0.190	0.177	0.972 ns

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium probabilities.

[a] Deviations from HWE were not statistically significant (ns) and statistically significant at *$P < 0.05$, **$P < 0.01$, and ***$P \leq 0.001$.

(L.) Thell., *A. radiatus* Loisel., and *A. valentinus*. All loci, except locus 15 in *A. radiatus*, amplified successfully within the expected allele size in all species.

CONCLUSIONS

Here we report on a set of polymorphic microsatellite markers for *A. clavatus*. Amplification success for most of these markers in almost half of the species of *Anacyclus* extends their potential usefulness to the entire genus. These markers will be useful for investigating the genetic structure, gene flow patterns, and mating system of *A. clavatus* across its distribution and especially in hybrid zones with closely related species.

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APPENDIX 1. Characteristics of 11 monomorphic microsatellite primers developed in *Anacyclus clavatus*.[a]

Locus[b]	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	GenBank accession no.
A9	F: TCAGTGACTTTAGAAAGGTAGTAAGGA R: CTCATGTGGGGTGTTCCTCT	$(GT)_{13}AT(GT)_8$	170	54	KF418744
A121	F: TCTCGCTACTCCCGCTTTAC R: GCAGGATCACTTAAAGGATATCAG	$(ACA)_{14}$	237	54	KF418738
A123	F: TCAGTGACTTTAAAAGGTAGTAAGGA R: TAAGTGCTCCACACCCATGT	$(GT)_{15}$	165	54	KF418739
C101	F: GCATAAACCTTCGGAATCTCA R: ATGGTGACAATCGTGGTAACC	$(TTG)_9$	119	54	KF418740
D8	F: TTCCTTTGCCTCTTTCTTGG R: GTTCCCGACTGTGGTCTCTC	$(ATC)_7$	193	55	KF418745
D101	F: ACTCCATGACCGAAGAGGTG R: GACACTTGTGGTCCCTCGAT	$(TCA)_2TCT(TCA)_5$	238	54	KF418741
D103	F: ATGGTGGTGGAGCATAGG R: GAGGACGAGGATGATGAGA	$(TCA)_{19}$	289	54	KF418742
4	F: TTCTCCATTTTCTTTGATCTTGG R: GGGACGTATGTACTCACCTTCG	$(TTG)_7$	145	56	KF418734
23	F: CCATGTTATGGATTCACTTAGTAAAAG R: CCATATGTTGGAAGGGGTGT	$(AG)_7$	141	55	KF418735
25	F: GGAGGGGTTGGATTCTCATA R: GAGGAGTTCTTAGTGAGATGTTGG	$(CT)_8$	94	55	KF418736
30	F: GGTGGTCTTGGTAAATGAAAGA R: TGAGGGGTTGAGGTTCTTGT	$(GA)_7$	122	55	KF418737

Note: T_a = annealing temperature.

[a] All values are based on 90 samples of three South Iberian populations.

[b] Loci in italics are those obtained by sequencing of cloned enriched restriction fragments and the remaining are those obtained by next-generation sequencing.