UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE ECOLOGÍA

BIOLOGÍA EVOLUTIVA

HIBRIDACIÓN NATURAL Y ESTRUCTURA GEOGRÁFICA DE *Tithonia tubaeformis y Tithonia rotundifolia* (ASTERACEAE)

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTOR EN CIENCIAS

PRESENTA:

ALFREDO LÓPEZ CAAMAL

TUTOR PRINCIPAL DE TESIS: DR. EFRAÍN TOVAR SÁNCHEZ
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MÉXICO, CD. MX. **JUNIO, 2018**

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UNAM
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COORDINACIÓN

OFICIO CPCB/492/2018

Asunto: Oficio de Jurado para Examen de Grado.

Lic. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted, que el Subcomité de Ecología y Manejo Integral de Ecosistemas, del Posgrado en Ciencias Biológicas en su sesión ordinaria del día 16 de abril de 2018, aprobó el jurado para la presentación del examen para obtener el grado de **DOCTOR EN CIENCIAS** al alumno **LÓPEZ CAAMAL ALFREDO** con número de cuenta **301844888** con la tesis titulada: **"HIBRIDACIÓN NATURAL Y ESTRUCTURA GEOGRÁFICA DE *Tithonia tubaeformis* y *Tithonia rotundifolia* (ASTERACEAE)"**, bajo la dirección del **DR. EFRAÍN TOVAR SÁNCHEZ:**

Presidente:	DR. JUAN SERVANDO NÚÑEZ FARFAN
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Suplente:	DR. RICARDO REYES CHILPA
Suplente	DR. ANTONIO GONZÁLEZ RODRÍGUEZ

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARA EL ESPIRITU"
Cd. Universitaria, Cd. Mx., a 11 de junio de 2018

DR. ADOLFO GERARDO NAVARRO SIGÜENZA
COORDINADOR DEL PROGRAMA

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Dr. Daniel Ignacio Piñero Dalmau
Dr. Zenón Cano Santana

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A mis padres

A mis hermanos

ÍNDICE

RESUMEN

Las causas de la distribución actual de la variación genética obedecen a factores tanto históricos como contemporáneos. En una gran variedad de especies, se ha observado que las oscilaciones climáticas ocurridas en el Pleistoceno han tenido un efecto sobre la distribución de éstas, reduciendo sus poblaciones significativamente durante las épocas glaciales. Al finalizar cada época glacial, ocurrieron en muchos casos expansiones poblacionales a partir de refugios, incluyendo contacto secundario y la formación de zonas de hibridación entre linajes divergentes. Generalmente, estos eventos dejan una huella genética en las poblaciones, la cual se puede inferir mediante el uso de marcadores moleculares. En esta tesis, se exploraron estas causas próximas e históricas involucradas en la distribución de la variación genética en dos especies asociadas a ambientes perturbados: *Tithonia tubaeformis* y *T. rotundifolia* (Asteraceae). En primera instancia, se utilizaron marcadores moleculares (microsatélites nucleares) y químicos (metabolitos secundarios) para explorar los niveles de hibridación así como el fenotipo de los individuos híbridos en cuatro zonas de hibridación contemporáneas en México. Los análisis genéticos mostraron que en todas las zonas de hibridación estudiadas se encuentran individuos con ancestría mixta, existiendo retrocruzas putativas hacia ambas especies progenitoras. Por su parte, el análisis químico mostró que los individuos puros de las especies progenitoras presentan un perfil químico diagnóstico, mientras que los híbridos muestran un mosaico en la expresión de los metabolitos secundarios. Algunos híbridos mostraron aditividad o dominancia, mientras que otros (retrocruzas hacia *T. tubaeformis*) mostraron un nuevo perfil químico, el cual presenta un compuesto que no se había reportado previamente. Estos resultados sugieren que la hibridación podría impactar la distribución de la variación genética si ésta se presenta extensamente. Posteriormente, se analizó la estructura geográfica de ambas especies utilizando microsatélites de cloroplasto y se infirió la historia poblacional de las especies con la ayuda de modelos de distribución. Los resultados

sugieren que las especies han tenido una historia evolutiva muy distinta. Por un lado, *T. rotundifolia* mostró zonas de idoneidad climática hacia las costas del Pacífico durante el último máximo glacial, mientras que *T. tubaeformis* lo hizo hacia el centro del país en el Altiplano y la Faja Volcánica Transmexicana. Los análisis moleculares revelaron que las poblaciones colectadas en las zonas de idoneidad climática descritas para *T. rotundifolia* se encuentran estructuradas. Sin embargo, una barrera común a un gran número de taxa (el Istmo de Tehuantepec) no es el principal responsable de la estructuración. Por lo tanto, se sugiere una historia compleja de *T. rotundifolia*. Por otro lado, *T. tubaeformis* mostró altos valores de diversidad genética en las poblaciones aledañas a las zonas de idoneidad climática, sugiriendo que la heterogeneidad ambiental asociada al centro del país debido a una intensa actividad geológica puede estar implicada en este patrón. Asimismo, se encontró que las poblaciones ubicadas hacia el Istmo de Tehuantepec y Centroamérica mostraron una importante reducción de la diversidad genética. Lo anterior sugiere una migración desde el altiplano de México hacia Centroamérica después del último máximo glacial. Por último, debido a que usamos los mismos loci para ambas poblaciones, se buscó la existencia de haplotipos compartidos entre especies. A pesar de que encontramos cinco haplotipos compartidos, se sugiere que una evolución convergente o la retención de caracteres plesiomórficos, más no la hibridación, puede estar implicada en su presencia. Además, dichos haplotipos compartidos se encuentran entre poblaciones geográficamente distantes. En conclusión, se puede mencionar que ambas especies tuvieron una influencia de las oscilaciones climáticas Pleistocénicas, conduciendo al aislamiento de sus poblaciones durante las épocas glaciales. Al terminar las épocas glaciales, se sugiere una migración a partir de estos refugios, conduciendo al contacto secundario entre *T. tubaeformis* y *T. rotundifolia* y a la formación de zonas de hibridación.

ABSTRACT

The current distribution of genetic variation is due to historic and contemporary processes. In a number of species, it has been reported that Pleistocene climatic oscillations affected its distribution. Populations were significantly reduced during glacial ages and population expansions from these refugia have been reported, leading to secondary contact and the formation of hybrid zones between previously isolated lineages. Usually, these events leave a genetic imprint, which can be inferred through molecular markers. In this thesis, we explored proximal and historic processes involved in the current distribution of two Neotropical weedy species: *Tithonia tubaeformis* and *T. rotundifolia* (Asteraceae). As a first step, molecular (nuclear microsatellites) and chemical (secondary metabolites) markers were used to explore hybridization between these species at four hybrid zones in Mexico. The genetic analyses showed that hybrid individuals were found at all four hybrid zones, showing probable backcrosses toward both parental species. Chemical analyses showed that pure parental individuals showed a diagnostic chemical profile, while hybrids showed a mosaic of secondary metabolite expression. Some hybrids displayed additivity or dominance. However, some admixed individuals (backcrosses toward *T. tubaeformis*) showed a new chemical profile, where a previously unreported compound was found. These results show that hybridization may impact the current distribution of the genetic variation of these species. Next, we analyzed the geographic structure of both species through chloroplast microsatellite markers, and population history was inferred with species distribution models. Results suggested that both species had a quite different history. In one hand, *T. rotundifolia* showed climatic suitability areas at the Pacific coast during the last glacial maximum, while *T. tubaeformis* did it at the center of the country. Molecular analyses revealed that *T. rotundifolia* populations collected at climatic stability areas were genetically structured. However, a common geographic barrier to a number of taxa (Isthmus of Tehuantepec) was not the main driver of

population structure. Thus, a complex population history is invoked. On the other hand, *T. tubaeformis* populations showed high levels of genetic diversity when collected near climatic stability areas, suggesting that the heterogeneity caused by an intense geological activity may explain this pattern. However, populations collected at the Isthmus of Tehuantepec and Central America showed a significant reduction of genetic diversity. This suggests a north-south migration following the last glacial maximum. Lastly, shared haplotypes were found between these species. It is proposed that either convergent evolution or the retention of plesiomorphic characters better explain the observed pattern rather than hybridization: shared haplotypes occurred between geographically distant populations. In conclusion, we found that both *T. tubaeformis* and *T. rotundifolia* were affected by Pleistocene climatic oscillations followed by population expansion from refugia leading to secondary contact between *T. tubaeformis* and *T. rotundifolia* and hybrid zone formation.

INTRODUCCIÓN GENERAL

Hibridación natural y sus consecuencias

La hibridación natural es un fenómeno frecuente en la naturaleza el cual puede ocurrir a un nivel intra- o inter-específico. Una de las tantas definiciones de hibridación considera a esta fenómeno como la cruza entre individuos de dos poblaciones o grupos de poblaciones, los cuales son distinguibles en al menos un carácter heredable (Arnold, 1997). A pesar de que su papel en la evolución fue altamente debatido por uno de los zoólogos más importantes de su época (Mayr, 1963), actualmente se acepta que la hibridación es un fenómeno evolutivamente importante en animales (Dowling y Secor, 1997; Norris et al., 2015; Dowling et al., 2016) y más aún en plantas (Rieseberg, 1997; Whitney et al., 2010). De hecho, el estudio formal de la hibridación natural comenzó a mediados del siglo pasado con los trabajos botánicos de Anderson (1949), Heiser (1947) y Stebbins (1959), entre otros. A pesar de no contar con evidencia suficiente, estos trabajos enfatizaron en las ventajas adaptativas de la hibridación. En particular, Anderson y Stebbins sugirieron que la hibridación introgresiva puede facilitar el origen y transferencia de adaptaciones entre las poblaciones participantes, lo que ha sido comprobado para algunos casos de plantas, animales y microorganismos (Rieseberg et al., 2003; Arnold, 2004; Stuckenbrock, 2016). Sin embargo, las consecuencias de la hibridación y la hibridación introgresiva son varias y pueden ser tanto positivas como negativas para la integridad genética de los taxa participantes. Dentro de las consecuencias que pueden ser consideradas como 'positivas' en términos de diversidad podemos mencionar la especiación (Payseur y Rieseberg, 2016), el origen de nuevas características en los híbridos (caracteres transgresivos; Kagawa y Takimoto, 2018) y a la transferencia de adaptaciones (introgresión; Jeong et al., 2014). En algunos casos, estas consecuencias pueden proveer a algunas poblaciones o especies con la capacidad de expandir su área de distribución geográfica o bien, pueden conducir a radiaciones adaptativas (Stankowski y Streisfeld, 2015).

Mucha de la literatura existente sobre hibridación considera solamente las consecuencias positivas para las especies participantes. Sin embargo, ésta puede tener una serie de consecuencias que pueden causar una disminución de la diversidad. En este sentido, existen ejemplos en los que la hibridación introgresiva puede poner en riesgo de extinción a algunas especies con distribución restringida (Todesco et al., 2016). La extinción vía hibridación ha sido un tema que ha cobrado especial importancia en el contexto del cambio global. Debido al rápido incremento de las temperaturas medias y a los cambios en los regímenes de precipitación, muchos modelos suponen una migración tanto altitudinal como latitudinal de la biota (Gómez et al., 2015). Estos eventos, así como la introducción de especies exóticas, conducirían al contacto entre linajes recientemente divergentes resultando en procesos de hibridación y eventualmente, dependiendo de la arquitectura genética de las poblaciones participantes, a su potencial extinción o a la fusión de las especies cuando existen débiles barreras reproductivas (Gómez et al., 2015).

Existen otros eventos que favorecen el contacto o la simpatría entre poblaciones o especies cercanas filogenéticamente. Además de los disturbios producto de las actividades humanas ya mencionados en el párrafo anterior, existen otros que pueden afectar la frecuencia con la que ocurre la hibridación. Estos disturbios (p. ej., la tala, agricultura, construcción de caminos) favorecen el contacto entre especies relacionadas y permiten el establecimiento de individuos híbridos. Los ambientes perturbados son altamente inestables y heterogéneos, por lo que Anderson (1948) consideraba que el contacto entre linajes divergentes y el establecimiento de individuos híbridos era probable en esos ambientes. Recientemente, se ha encontrado que la asociación entre la ocurrencia de hibridación y la presencia de ambientes alterados producto de actividades humanas es significativa (Todesco et al., 2016). Esta asociación es evidente sobre todo cuando la hibridación cesa al terminar los disturbios a causa de actividades humanas. En este sentido, Heiser (1979) documentó tres zonas de hibridación entre especies de malezas pertenecientes a Asteraceae

(*Helianthus divaricatus* y *H. microcephalus*). En la década de 1940, el autor encontró que los híbridos entre estas especies ocurrían en tres sitios con frecuente actividad humana (construcción de caminos, pastoreo). Después de 22 años, encontró que dos de estos sitios habían recuperado las condiciones pre-disturbio, presentando individuos de *Helianthus* similares a los fenotipos de las especies progenitoras, con la aparente ausencia de individuos híbridos. Mientras tanto, en el otro sitio los disturbios, y por lo tanto la hibridación, eran constantes.

Como se puede observar, el papel de la hibridación natural en plantas ha sido muy importante en la evolución vegetal. Sin embargo, la constante presencia de disturbios producto de las actividades humanas favorece la simpatría entre linajes filogenéticamente cercanos, llevando a la formación de zonas de hibridación en donde se puede encontrar una amplia variabilidad genética y fenotípica.

Zonas de hibridación, modelos: ¿contacto primario o contacto secundario?

Las zonas de hibridación son regiones en las que dos poblaciones genéticamente distintas se encuentran, cruzan y producen híbridos fértiles o parcialmente fértiles (Barton y Hewitt, 1985). La extensión y la ubicación de las zonas de hibridación son muy variables. Por lo tanto, la importancia de los factores que se encuentran involucrados en el mantenimiento de las zonas de hibridación ha sido debate de la comunidad científica durante varias décadas (Barton y Hewitt, 1985; Harrison, 1993; Arnold, 2006). Existen diferentes modelos que predicen la variación dentro de las zonas de hibridación. En estos modelos, la importancia relativa de la selección en contra de los individuos híbridos, de la dispersión y del flujo génico difiere, dando lugar a distintas predicciones acerca de las consecuencias de la hibridación.

Existen algunos modelos de zonas de hibridación que son considerados *independientes del ambiente*, los cuales sugieren que las zonas de hibridación son mantenidas por un balance entre la

dispersión de los individuos de las especies progenitoras hacia la región de solapamiento y la selección en contra de los individuos híbridos (Barton y Hewitt, 1985). Estos modelos sugieren que los individuos híbridos tienen una menor adecuación en relación a los individuos de las especies progenitoras sin importar el ambiente en el que éstos se encuentren (selección endógena; Arnold, 2006). En este caso, se supone que la inferioridad de los individuos híbridos está dada por el rompimiento de una combinación de alelos co-adaptados, resultando en híbridos inviables o infértiles. Bajo estos modelos, la hibridación será un proceso transitorio, representando los últimos pasos de la especiación que conducirán al reforzamiento de las barreras reproductivas entre las especies participantes (Hoskin et al., 2005).

Por otra parte, los modelos *dependientes del ambiente* consideran la interacción genotipo – ambiente, siendo el factor que determina el mantenimiento de las zonas de hibridación un gradiente en la intensidad de la selección natural. Este gradiente de selección es producto de la heterogeneidad ambiental y por lo tanto, estos modelos suponen que la adecuación de los híbridos será relativa al ambiente en el que éstos se establezcan (selección exógena). El modelo de Superioridad Híbrida (*Bounded hybrid superiority model*; Moore, 1977) considera que los individuos híbridos presentan una mayor adecuación en ambientes intermedios a los de las especies progenitoras. Estos ambientes intermedios pueden ser el resultado de disturbios antropogénicos; los híbridos pueden establecer poblaciones grandes en estos ambientes mientras que el disturbio sea persistente. Sin embargo, el modelo predice que a medida que los disturbios sean eliminados, los individuos híbridos serán reemplazados por individuos de las especies progenitoras involucradas (Moore, 1977).

Además de la descripción de los factores que se encuentran involucrados en el mantenimiento de las zonas de hibridación, existen otras dificultades que emergen al estudiarlas. Uno de los principales problemas radica en la identificación de estas zonas. Los individuos híbridos

presentan una amplia variación en cuanto a su genotipo y fenotipo, por lo que actualmente existe una amplia variedad de herramientas para la identificación de éstos (ver Capítulo I). Sin embargo, cualquiera que sea la herramienta empleada en la identificación de individuos híbridos, una característica que ha sido empleada históricamente en la identificación de zonas de hibridación es la presencia de clinas abruptas y concordantes para varios marcadores, ya sean genéticos o fenotípicos (Endler, 1977; Harrison, 1993). Sin embargo, la presencia de una clina de un solo marcador no necesariamente es originada por un proceso de hibridación: la variación a través de un gradiente ambiental puede generar intensidades de selección diferenciales que dan origen a un patrón similar (Endler, 1977). Por lo tanto, el uso de múltiples marcadores en las zonas de hibridación putativas es considerado importante para la identificación de éstas.

El origen de las zonas de hibridación es otro tema importante en su estudio. De forma general se ha considerado que las zonas de hibridación contemporáneas son el resultado de un contacto secundario entre linajes que se diferenciaron en alopatría o bien, de un contacto primario entre dos linajes que se diferenciaron en parapatría. A finales del siglo pasado, el origen de las zonas de hibridación se infería a partir de los patrones de variación de los marcadores empleados. En la mayoría de estos estudios (ver Harrison, 1993), se concluía que las zonas de hibridación se originaban a partir de un contacto secundario, ya que el aislamiento geográfico era considerado un prerrequisito para la diferenciación y la especiación. Sin embargo, como hizo notar Endler (1977), las inferencias sobre el origen de una zona de hibridación a partir de los patrones de variación son aventuradas, ya que el contacto primario y secundario puede producir patrones de variación idénticos. Por ello, durante esta etapa del estudio de las zonas de hibridación, se le dio poca atención al papel de los eventos históricos en la formación de las zonas de hibridación (Hewitt, 2011).

El entendimiento de las zonas de hibridación en un contexto histórico nos permite hacer inferencias sobre el funcionamiento y el futuro de éstas (Hewitt, 2011). Los avances en la

tecnología del ADN y en los modelos teóricos han proporcionado a los investigadores de herramientas con las cuales sugerir la existencia de un contacto primario o secundario entre especies cercanamente relacionadas. Asimismo, la descripción de la historia poblacional de las especies participantes nos permite inferir el efecto de las oscilaciones climáticas Pleistocénicas y los eventos geológicos sobre la biota de cierto lugar o región. En particular, los avances en la paleoclimatología, en el modelado de la distribución de especies, así como el surgimiento de la disciplina de la filogeografía han sido de gran importancia para inferir la historia poblacional de las especies implicadas en eventos de hibridación.

Inferencia de la historia poblacional

Marcadores moleculares y filogeografía

Los patrones actuales de la distribución de la biota han sido objeto de fascinación por los naturalistas desde hace varios siglos y muchos intentos por explicar dichos patrones han sido llevados a cabo a lo largo del tiempo. Las hipótesis acerca de las causas de éstos generalmente involucran factores ecológicos o condiciones ambientales (Beebee y Rowe, 2008). Sin embargo, se ha sugerido que los factores históricos a los que han estado sujetas las poblaciones son igualmente importantes (Hewitt, 2011). En este sentido, varios naturalistas han sugerido el papel de las glaciaciones Pleistocénicas y de algunos eventos geológicos en la distribución contemporánea de las especies. Sin embargo, al no contar con los elementos teóricos y metodológicos, sus hipótesis difícilmente podían ponerse a prueba. No fue hasta finales del siglo pasado que los avances teóricos y metodológicos permitieron la implementación de varios análisis capaces de poner a prueba estas hipótesis. En este sentido, Avise et al. (1987) introdujo el término filogeografía. Esta disciplina se desprende de la biogeografía, y se encarga de estudiar los principios y procesos involucrados en la distribución geográfica de los linajes génicos. En otras

palabras, la filogeografía estudia los procesos históricos que podrían ser responsables de la distribución geográfica de un taxón. A pesar de que teóricamente cualquier marcador genotípico o fenotípico puede emplearse en los estudios filogeográficos, los marcadores neutros de ADN son los que se ocupan primordialmente para este fin.

Actualmente existe una gran cantidad de marcadores moleculares con los cuales se puede estudiar la estructura y diversidad genética de las poblaciones. Dichos marcadores poseen distintas tasas de mutación, por lo que dependiendo del marcador que se emplee, distintos eventos históricos podrán ser inferidos. La molécula que fue utilizada en los primeros estudios filogeográficos fue el ADN mitocondrial (ADNmt; Avise et al., 1979). La principal ventaja del ADNmt es que presenta una herencia uniparental, por lo que no existe recombinación. Además, la tasa de mutación de ésta molécula es elevado, por lo que frecuentemente presenta variación entre individuos y poblaciones. Inicialmente, los estudios filogeográficos empleaban marcadores en ADNmt en animales. En contraste, el ADNmt de las plantas presenta una baja tasa de mutación a nivel nucleotídico, mientras que presenta cambios rápidos en el arreglo de sus genes. Por lo tanto, el ADNmt en plantas es de poca utilidad en estudios poblacionales.

En plantas, el ADN de cloroplasto (ADNcp), presenta una tasa de mutación mayor a la del ADNmt (Provan et al., 2001). Por lo que es la molécula que se ha empleado en un gran número de estudios poblacionales. A diferencia de los animales, las plantas tienen la capacidad de 'mover' sus genes de dos formas distintas durante su desarrollo. En primer lugar, los genes pueden desplazarse previo a la fecundación a través del polen. Una vez lograda la fecundación, los genes pueden desplazarse aún más a través de las semillas. Por lo tanto, al presentar una herencia materna en la mayoría de las angiospermas, el estudio del ADNcp nos revelará la historia del linaje materno. Además, la dispersión de las semillas generalmente es mucho menor a la del polen, por lo que la divergencia entre poblaciones será más marcada en el ADNcp en comparación con el ADN nuclear

(Ennos, 1994). Aunado a lo anterior, debido a su menor tamaño efectivo poblacional en relación con el ADN nuclear, los efectos de la deriva génica serán mayores en el ADNcp, por lo que al estudiar éste, se encontrará una mayor divergencia entre poblaciones comparado con los marcadores nucleares.

Los microsatélites han sido marcadores populares para el estudio de la genética de poblaciones de un amplio rango de taxa. Los microsatélites (SSR) son secuencias cortas repetidas en tándem, generalmente de 1 a 6 bases de longitud, que se encuentran ampliamente distribuidas en el genoma (Thuillet et al., 2002). En particular, los microsatélites de cloroplasto (cpSSR) son una fuente importante de información para los estudios filogeográficos. A pesar de que recientemente se han desarrollado nuevas herramientas de secuenciación, la vigente popularidad de los cpSSR radican en su alta tasa de mutación ($10^{-2} – 10^{-6}$ mutaciones por locus por generación; Thuillet et al., 2002), dando lugar a un elevado polimorfismo. Además, son útiles para muchas especies para las cuales existen pocos recursos genómicos disponibles (Jaramillo-Correa et al., 2015). Sin embargo, la hipervariabilidad de los microsatélites no siempre es positiva. Uno de los principales problemas que existen al estudiar regiones microsatélites es la probabilidad de encontrar homoplasias, es decir, que dos fragmentos sean idénticos en estado (en cuanto a su tamaño en pares de bases) más no por descendencia. No obstante, el uso de varios loci y la elevada diversidad alélica de los microsatélites compensan los problemas de evolución por homoplasia (Estoup et al., 2002), haciéndolos útiles para describir la dinámica de la genética poblacional y la divergencia de clados hacia el pasado reciente (Pleistoceno tardío – Holoceno, Hewitt, 2001).

La implementación de varios marcadores moleculares y de varias herramientas metodológicas propias de la filogeografía (construcción de redes de haplotipos [Avise et al., 1987], pruebas de neutralidad [Tajima, 1989; Fu, 1997], análisis de clados anidado [Templeton, 1998], entre otros) han permitido describir la ubicación de los refugios pleistocénicos así como las rutas

de colonización post-glaciales en varias regiones del mundo. En este sentido, Europa ha sido una de las regiones mejor comprendidas (Taberlet et al., 1998; Hewitt, 1999). Debido a que durante el último máximo glacial (UMG, hace 22,000 años aprox.), la mayor parte de Europa quedó cubierta por hielo, la inferencia sobre los sitios que actuaron como refugios pleistocénicos fue relativamente sencilla: éstos debieron ubicarse en las regiones más cálidas ubicadas en las penínsulas sureñas. De manera interesante, la ubicación de los refugios durante el UMG y las rutas de colonización post-glaciales son coincidentes para varios taxa con diferente capacidad de dispersión. Por lo tanto, se han logrado reconstruir los efectos de las oscilaciones climáticas sobre la biota en el Viejo Mundo.

Los procesos ocurridos durante la expansión poblacional ocurrida después del UMG en Europa son igualmente interesantes. Durante el UMG, las poblaciones quedaron reducidas en distintos refugios durante varios miles de años, conduciendo a la diferenciación debido a un sorteo de linajes y a la acumulación de mutaciones entre refugios. Una vez que las épocas glaciales cesaron y se dio la expansión poblacional, distintos linajes intraespecíficos o distintas especies presentaron un contacto secundario, dando lugar a zonas de hibridación bien delimitadas. Las zonas de hibridación producto de las oscilaciones climáticas son coincidentes en algunas regiones de Europa (p. ej. los Alpes Suizos), conduciendo a las llamadas zonas de sutura (Remington, 1968; Swenson y Howard, 2004). Así, los estudios de la distribución de la variación genética han probado ser una poderosa herramienta para describir las rutas de colonización después del UMG.

Filogeografía y modelos de distribución de especies

La filogeografía fundamenta sus análisis e inferencias en el uso de marcadores moleculares. Sin embargo, los enfoques interdisciplinarios y los avances en los algoritmos para definir los sitios de idoneidad climática han favorecido la incorporación de los modelos de distribución de especies

(SDM, por sus siglas en inglés) en los análisis que tratan la estructura geográfica de las poblaciones así como en los análisis filogeográficos (Alvarado-Serrano y Knowles, 2014). Los SDM utilizan datos independientes a los derivados por los marcadores moleculares, por lo que pueden ser utilizados para generar o probar hipótesis acerca de la distribución de la variación genética. Además, los SDM pueden ser desarrollados tanto para las condiciones actuales como para las históricas, de manera que son una herramienta invaluable para explorar los efectos de las oscilaciones climáticas sobre la distribución de la variación genética contemporánea.

Los SDM son utilizados generalmente como una herramienta con la cual se puede evaluar visualmente la concordancia entre la distribución de la variación genética y las zonas de idoneidad climática para un taxón dado. Por ejemplo, la divergencia entre dos linajes cercanos geográficamente podría indicar un aislamiento histórico. En este caso, los SDM podrían soportar esta hipótesis al llevar a cabo una evaluación visual de las proyecciones históricas de distribución utilizando modelos paleoclimáticos. En el caso de que las proyecciones de distribución presenten una disyunción, se podría sugerir un efecto de las oscilaciones climáticas en la distribución de la variación genética (Moussalli et al., 2009).

Por otro lado, los SDM pueden ser utilizados para generar hipótesis que después pueden ser probadas mediante los marcadores moleculares (p. ej. Knowles et al., 2007). Asimismo, los SDM pueden ser utilizados para identificar áreas de estabilidad climática para cierta especie a lo largo del tiempo histórico. Con este último enfoque, se ha podido inferir junto con los análisis genéticos, la ubicación y el número precisos de refugios pleistocénicos en varios grupos de especies (Waltari et al., 2007).

La familia Asteraceae

La familia Asteraceae comprende especies herbáceas, leñosas, anuales y perennes que presentan una distribución global (excepto en la Antártida), por lo que es considerada uno de los principales componentes de la flora a nivel mundial (Judd et al., 2002; Funk et al., 2005). El número total de especies de esta familia no está aún descrito con precisión. Sin embargo, se ha sugerido que presenta entre 22,000 y 30,000 especies, representando aproximadamente el 10 % de las angiospermas (Jeffrey, 2007). Esta familia es monofilética, y se caracteriza por presentar inflorescencias primarias que constituyen una cabezuela pseudántica, presentando un involucro de brácteas que asemejan a un cáliz (Villaseñor, 1993, Funk et al., 2005). Los representantes de Asteraceae pueden encontrarse en cualquier tipo de hábitat. No obstante, son menos frecuentes en bosques tropicales húmedos, siendo comunes en ambientes perturbados. Es importante señalar que a pesar de que generalmente se les considera malezas, la mayoría de estas especies presentan una distribución restringida, por lo que se encuentran en alguna categoría de riesgo (Funk et al., 2005).

En México, la familia Asteraceae contribuye de manera importante a la riqueza florística del país. Se ha calculado que existen cerca de 3,000 especies agrupadas en 340 géneros, de los cuales, 63% son endémicos. Es por ello que desde hace más de un siglo, México es considerado un centro de diversificación de la familia (Bentham, 1873; Villaseñor, 1993). Algunos autores han propuesto que la intensa actividad geológica ocurrida durante el Paleogeno y el Neogeno ha sido importante en la diversificación de esta familia en México (Villaseñor, 1990). Los registros fósiles de Asteraceae en México datan del Mioceno (Graham, 1976). Durante esta época existió una intensa actividad geológica, dando como resultado la emergencia de varias cadenas montañosas (p.ej. la Faja volcánica Transmexicana), así como una intensa actividad volcánica. Se ha sugerido que estos eventos favorecieron la diversificación de la familia Asteraceae en México, ya que crearon una gran heterogeneidad ambiental permitiendo el establecimiento de colonizadores

primarios y al mismo tiempo aislando sus poblaciones, permitiendo su diferenciación (Villaseñor, 1990).

Actualmente, muchas especies pertenecientes a la familia Asteraceae presentan una amplia distribución geográfica. Mani y Saravanan (1999) sugieren que su alta capacidad reproductiva así como sus eficientes procesos de polinización y de dispersión de semillas son responsables de su amplia distribución geográfica. A pesar de esto, los estudios sobre la biología reproductiva en esta familia son escasos debido a la dificultad que representa el manejo de sus estructuras reproductoras. Sin embargo, algunos autores han encontrado que los principales órdenes de insectos que llevan a cabo la polinización en esta familia son Hymenoptera, Diptera, Lepidoptera y Coleoptera, siendo la abeja (*Apis mellifera*) el polinizador con el mayor número de visitas (Torres y Galetto, 2008). Asimismo, se ha sugerido que aunque esta familia frecuentemente presenta una incompatibilidad esporofítica (Lafuma y Maurice, 2007), existen una gran cantidad de especies anuales de Asteraceae que exhiben cierto grado de auto-compatibilidad (Ferrer et al., 2004; Torres y Galetto, 2008).

El género *Tithonia* Desf. ex Jussieu

El género *Tithonia* (Asteraceae, Heliantheae) incluye un conjunto de especies nativas de México y Centroamérica. El género incluye 12 especies (Turner, 2015) anuales y perennes, las cuales se agrupan en dos secciones: *Mirasolia* y *Tithonia* (La Duke, 1982). *Tithonia* ha sido relacionado históricamente con los géneros *Helianthus*, *Viguiera* y *Gymnolomia* (Bentham y Hooker, 1873; La Duke, 1982). Estudios filogenéticos llevados a cabo por Schilling y Panero (1996) empleando ADN nuclear y ADNcp han demostrado que el género *Viguiera* es probablemente el grupo hermano de *Tithonia*. Sin embargo, los mismos autores encontraron una incongruencia entre las filogenias resultantes de marcadores de ADNcp y nucleares (ITS). Las filogenias derivadas de marcadores

nucleares (ITS) soportan la idea de que el género *Tithonia* es monofilético, lo que fue sugerido por La Duke (1982) utilizando caracteres morfológicos y fitoquímicos. No obstante, las filogenias derivadas con ADNcp muestran un origen polifilético de *Tithonia*, presentando algunas especies del género (*T. rotundifolia, T. diversifolia, T. koelzii* y *T. thurberi*) una relación con especies del género *Simsia*. De forma interesante, una de estas especies (*T. rotundifolia*) mostró un genotipo idéntico al de *Simsia* (Schilling y Jansen, 1989). La incongruencia entre las filogenias encontradas sugiere un evento de evolución reticulada en el origen de *Tithonia* y *Simsia*. En este sentido, se ha sugerido que los eventos de hibridación y de captura citoplásmica entre *Tithonia* y *Simsia* ocurrieron antes de su divergencia (Schilling y Panero, 1996). Por lo tanto, estos autores mencionan que la evolución reticulada en *Tithonia* es un ejemplo excepcional de las consecuencias evolutivas de la hibridación en plantas.

La Duke (1982) realizó una clasificación infragenérica del género *Tithonia*. Este autor dividió al género en dos grandes secciones: *Tithonia* y *Mirasolia*. Dentro de la sección *Tithonia* se encuentra una sola serie (*Tithonia*) en la cual se ubican dos de las especies con la mayor área de distribución geográfica: *Tithonia tubaeformis* (Jacq.) Cass y *Tithonia rotundifolia* (Miller) S. F. Blake. A pesar de que La Duke no las consideró especies hermanas, sí las consideró muy cercanamente relacionadas, e incluso consideró que existen híbridos naturales entre estas especies (La Duke, 1982). Sin embargo, Schilling y Panero (1996) encontraron que estas especies se ubican en clados muy divergentes (basados en ADNcp), sugiriendo que cada una de éstas tiene su propia historia evolutiva: *T. rotundifolia* tiene indicios de evolución reticulada con *Simsia*.

Flujo génico en *Tithonia*: *T. tubaeformis* y *T. rotundifolia*

A pesar de que los estudios filogenéticos apenas mencionados sugieren la existencia de eventos de hibridación históricos en la divergencia de las especies de *Tithonia*, los estudios sobre flujo génico

contemporáneo entre estas especies son escasos, y en algunas ocasiones, anecdótico. Las primeras sugerencias de hibridación en el género involucran a *T. tubaeformis* y *T. rotundifolia*. En un estudio de viabilidad de polen, Hauser y Morrison (1964) encontraron híbridos putativos entre estas especies, los cuales mostraban una marcada reducción en la viabilidad del polen con respecto a individuos de las especies progenitoras. Asimismo, existen reportes de hibridación intergenérica entre *T. rotundifolia* y *Helianthus annuus* (Cristov y Panayotov, 1991; Reyes-Valdés et al., 2005; Gómez-Martínez et al., 2010; Luévanos-Escareño et al., 2010; Encheva et al., 2014). A pesar de que estos estudios tienen un interés agronómico, sirven para ilustrar la posibilidad de cruzas intergénicas dentro de Heliantheae así como la presencia de débiles barreras reproductivas en la tribu.

Recientemente, la hibridación natural entre *T. tubaeformis* y *T. rotundifolia* ha sido estudiada. Estas especies son malezas importantes, las cuales pueden formar poblaciones grandes a la orilla de caminos o en los cultivos (Castillo et al., 2007). Sin embargo, ambas especies presentan una divergencia en sus preferencias de hábitat. *Tithonia rotundifolia* se encuentra exclusivamente en ambientes xéricos por debajo de los 1,000 m s.n.m. asociados a bosques tropicales secos. Por su parte, *T. tubaeformis* presenta una tolerancia ecológica más amplia. Por un lado, esta especie se distribuye preferentemente en bosques templados de *Quercus* o *Pinus – Quercus* (La Duke, 1982). No obstante, también puede encontrarse asociada a matorrales ubicados en el altiplano de México así como en algunas partes de la selva baja caducifolia (López-Caamal et al., 2018). Es por ello que existen poblaciones simpátridas entre estas especies. En estos sitios, Tovar-Sánchez et al. (2012) estudiaron poblaciones mixtas de *T. tubaeformis* y *T. rotundifolia* mediante RAPD. Los autores encontraron que en las poblaciones mixtas, existen individuos con ancestría mixta, sugiriendo la existencia de hibridación entre estas especies. Asimismo, López-Caamal et al. (2013) encontraron una elevada diversidad morfológica en esos mismos sitios

simpátridos, sugiriendo que la hibridación puede ser parcialmente responsable de los patrones observados.

Presentación de tesis

Esta tesis tiene como principal objetivo el estudio de la hibridación natural entre *Tithonia tubaeformis* y *T. rotundifolia*. Para este fin, existen una gran cantidad de herramientas genéticas, morfológicas y químicas. Debido a esto, el **Capítulo I** está dedicado a revisar los principales marcadores utilizados en la determinación de individuos híbridos. Asimismo, se enfatiza en la necesidad de utilizar varios marcadores en la detección de zonas de hibridación. En consecuencia, en el **Capítulo II** se presenta el estudio de cuatro zonas mixtas contemporáneas entre *T. tubaeformis* y *T. rotundifolia* utilizando marcadores de ADN (microsatélites nucleares) así como marcadores químicos (flavonoides y lactonas sesquiterpénicas).

Los **Capítulos III** y **IV** se enfocan en el estudio de la estructura y diversidad genética de *T. rotundifolia* y *T. tubaeformis*. En estos capítulos se utilizaron marcadores microsatélites de cloroplasto así como modelos de distribución de especies con el fin de inferir la historia poblacional de ambas especies. Asimismo, el uso de los mismos loci de cpSSR permitió comparar los haplotipos de ambas especies y explorar la existencia de haplotipos compartidos entre ambas especies progenitoras. El uso de SDM para *T. tubaeformis* y *T. rotundifolia* proyectados a diferentes escenarios históricos así como el uso de los mismos loci de cpSSR nos permitió explorar si la hibridación ha jugado un papel importante en la estructura geográfica de ambas especies.

CAPÍTULO I

Genetic, morphological, and chemical patterns of plant hybridization

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Genetic, morphological, and chemical patterns of plant hybridization

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Abstract

Natural hybridization is a frequent phenomenon among vascular plants. Hybridization is considered an important evolutionary force since it may lead to (1) an increase of the intraspecific genetic diversity of the participating populations, (2) the creation of new species, (3) species extinction through genetic assimilation, and (4) the generation of highly invasive genotypes. Because of the importance of plant hybridization in evolution, it is of great importance to accurately identify hybrid individuals. In this review, we give a general historical background of the study of plant hybridization. Also, we review some of the tools employed for hybrid recognition and their pattern of expression in hybrid individuals (morphological, chemical, chromosome number, and DNA fingerprinting techniques). We emphasize that even when chromosome number, morphological characters, and chemical characters are of limited use for hybrid recognition in the absence of DNA fingerprinting techniques, their exploration may give insights of the ecological performance of hybrids. This is of special importance when hybridization leads to evolutionary novelty in the form of polyploidy, transgressive character expression, or the expression of new secondary metabolites not present in the parental species.

Keywords: Chromosome number; DNA fingerprinting; Hybrid performance; Hybrid phenotype; Invasive species; Secondary metabolites

Introduction

Natural hybridization is recognized as an important evolutionary process in plants, animals and fungi (Mallet 2005; Schwenk et al. 2008; Paun et al. 2009; Whitney et al. 2010). Indeed, hybridization has been considered the rule rather than the exception at least in plants. During the recent decades, a number of studies related with the role of natural hybridization in shaping earth's biodiversity have been published (Wiesseman 2007). In this sense, it has been found that interspecific hybridization may have an important effect shaping the genetic diversity of a single population (Arnold 2006). Also, hybridization may be the cause of extinction of endemic species or rare plant populations (Levin and Francisco-Ortega 1996). In contrast, hybridization may lead to the creation of new species (Soltis & Soltis 1993; Arnold 2006). Beyond the species level, hybridization may impact the arthropod community structure associated with hybridizing populations (Tovar-Sánchez and Oyama 2006) and lastly, it may affect some ecosystem processes (e.g. nutrient cycling due to the secondary metabolite composition of hybrids; e.g. Driebe & Whitham 2000).

Recently, the movement of species far from their original distribution range has promoted the emergence of a number of invasive species (Mooney and Cleland 2001). Besides the concern that this species have in conservation biology due to their high competitive ability, this species may also cause the extinction of closely related species with restricted distribution range through introgressive hybridization (Vilà et al. 2000). Also, as hybridization creates new allelic combinations in the offspring, it has been proposed that hybridization may enhance the invasive capacity of an invasive species through gene introgression (Schierenbeck and Ellstrand 2009). As plant hybridization may have profound effects on the genetics and ecology of the participating species, the correct identification of hybrid individuals is of prime importance as a first step to specify which processes occur within hybridizing populations.

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In this review, we aim to give a brief historical background of the study of plant hybridization as well as show the principal outcomes for the involved populations and their hybrid offspring. Then, due to the importance of hybrid individual identification, we explore several of the tools (morphological, chemical, DNA based markers) that have been employed for this purpose. We discuss their advantages and limitations in the recognition of hybrid individuals as well as the pattern of expression of each marker in hybrids.

Review

Historical background of interspecific hybridization

Plant hybridization has been important to humans since the Neolithic era, when the domestication of plants and animals began (Zirkle 1935). However, the formal study of plant hybridization was retarded due to the lack of knowledge about plant sexuality. Although hybridization between some species certainly occurred during domestication, these events passed unnoticed as there was no knowledge of pollen function. However, some cultures noticed that staminate flowers should be in close proximity to pistillate flowers in order to produce fruits in dioecious plants (Roberts 1929). For example, Babylonians were aware that hand pollination was necessary for reproduction of dioecious palms, identifying 'male' and 'female' plants. However, they did not recognize sex in plants because they had not a clear idea of fertilization. Also, as Babylonians cultivated palms to harvest the edible fruit rather than the seeds, the breeding of new plants was merely a matter of chance and thus, sexual reproduction remained unknown (Roberts 1929; Zirkle 1935).

Until the seventeenth century, the discussion about the existence of sex in plants was largely based on philosophical and moral discussions, with little experimental evidence. For example, Tabernaemontanus (1731) and other authors observed that grains of different colors occurred on the same ear of maize. However, they did not understand the mixture of grains, ascribing it to the creation of God in order to astonish the botanists of the time (Zirkle 1934). It was until 1694 that Camerarius proved experimentally that pollen is necessary for seed development, thus recognizing plant sexuality. Although he perceived the possibility of fertilizing a female plant with the pollen from another species, he never conducted hybridization experiments (Roberts 1929).

The first intentional hybrid produced is ascribed to Thomas Fairchild involving crosses between *Dianthus* spp. (Zirkle 1935). Although there is no record of the experiments by Fairchild himself, Bradley (1717) summarizes these experiments, noting that the hybrids were alike a 'mule' since they yielded no seeds. Also, Linnaeus performed several experimental crosses between *Tragopogon* species in 1759. He found that hybrids sometimes have intermediate morphologies between the parental species (Roberts 1929). Additionally, Haartman also found that interspecific hybrids between *Thalictrum* sp. were intermediate regarding the number of stamens and pistils (Haartman 1764). So, by the mid of the eighteenth century, although poorly understood, plant hybridization was known as a phenomenon that could yield sterile plants with intermediate phenotypes.

However, it was Kölreuter who began with the systematic study of plant hybridization. This author performed crosses between *Nicotiana paniculata* and *N. rustica* (Kölreuter 1761). As a result from these crosses, he made important conclusions about plant hybridization. First, he observed that first generation (F1) hybrids usually exhibit pollen malfunction, describing them as 'botanical mules'. Second, Kölreuter made an important observation regarding ecological factors in the formation of hybrid individuals. He noted that hybrids were likely to occur when two closely related allopatric species are brought into sympatry by means of anthropogenic disturbance; resulting in hybrids with morphological intermediacy. When the hybrids obtained by Kölreuter were backcrossed with the parental species, the phenotype of the latters was recovered. He considered that hybrid sterility and the reversion to the parental phenotypes were a mean by which the preexisting harmony of nature could be conserved, considering hybrids as 'unnatural procreations' (Kölreuter 1761). Thus, he considered that hybridization was an ephemeral phenomenon with little importance, reinforcing the ideas of special creation of species prevailing at that time (Roberts 1929). By this time, all hybridization experiments were conducted by hand pollination. However, Kölreuter also noted that insect pollination occurred in a number of plants including the genus *Iris*, *Nicotiana*, *Oenothera* among others. Later, Sprengel (1793) described in great detail insect pollination in plants. Also, Sprengel found that the maturation of stamens and pistils within a same flower could exhibit temporal isolation. With this evidence, he suggested that these were mechanisms that maximize cross fertilization in plants, also noting that hybridization between different species pair could occur through insect pollination. Sprengel's work was influential to several authors such as Darwin (1859).

At the beginnings of nineteenth century, hybridization was largely used as a source of variation for plants of agronomical or ornamental importance. However, at this time, hybridization was mainly seen as a way to prove if two different plants deserve the species status (Roberts 1929). When plants of two presumably different species were crossed and fertile offspring was produced, then the two parental individuals were considered as varieties of a single species. On the contrary, if two plants were intercrossed and the resulting hybrid was sterile, the

plants were considered as belonging to different species (e.g. Godron 1863). Most of the naturalists of the 19th century considered sterility as a criterion for species status and hybrids were considered sterile by definition. This view was challenged by Hebert (1847) as he recognized that a continuum between species and varieties exists, with no discrete limits among them.

Following Hebert ideas, Naudin (1863) carried out interspecific crosses between *Datura laevis* and *Datura stramonium*. He observed that almost all hybrids were fertile, allowing him to grow further hybrid generations beyond F1. Naudin described the hybrids between *Datura* species as morphologically variable, exhibiting a 'disordered variation'. Sometimes they were morphologically intermediate and others they exhibited morphologies similar to either parental species. He also noted that F1 hybrids were always morphologically intermediate and were very similar among themselves, while later generation hybrids exhibited great variation. Other authors such as von Gärtner (1827), Knight (1799) and Nägeli (1865) also noticed this pattern. However, although they made similar inferences about character segregation described in 1865 by Mendel, they lack a mechanism to explain such variation. A very important finding in plant hybridization is the work by Mendel (1865). Despite of its importance, Mendel's paper was published in an obscure journal and thus, it passed unnoticed to the scientific community. However, the rediscovery of Mendel work by de Vries (1900), Correns (1900) and Tschermak (1900) set the foundation for modern plant genetics.

A couple years after the rediscovery of Mendel's laws, Sutton (1902) identified individual chromosomes in cells of *Brachystola magna* undergoing meiosis. He also noticed that the number of chromosomes was consistent in cells of a certain species, but the number of chromosomes varied among organisms. However, the most important discovery made by Sutton was that chromosomes were stable elements that pass from generation to generation, identifying the chromosomes as the genetic material responsible for Mendelian inheritance. This observations lead Sutton formulate the Chromosomal Theory of Inheritance, which provided the mechanism underlying Mendel's laws (Sutton 1902, 1903).

Once the mechanism of segregation of characters was discovered, plant hybridization became a very important mean to obtain new crop varieties. However, the evolutionary importance of hybridization was scarcely discussed. At the beginnings of the 20th century, there were important discoveries that suggested the role of hybridization in evolution. Wingë (1917) showed through theoretical studies that new and stable species could arise by the duplication of the chromosome number of a hybrid individual (i.e., allopolyploidy). Nowadays, hybrid speciation through allopolyploidy is considered a prominent process within flowering plants (Soltis and Soltis 1993). Later, Müntzing (1930) proposed a second mechanism in which hybridization may produce new and stable species. Müntzing proposed that later generation hybrids may, by chance, lead to new combinations of alleles due to chromosome rearrangements producing populations that are homozygous for a unique combination of chromosome sterility factors (Müntzing 1930; Rieseberg 1997).

Also in the 20th century, Anderson and his colleagues emphasized on the creative role of plant hybridization in evolution (Anderson 1949). Anderson suggested that, in natural conditions, hybrids may backcross toward their parental species, thus increasing the genetic diversity of the participating populations and contributing to adaptive evolution. Hybridization by this time was considered as a very frequent phenomenon in flowering plants (Stebbins 1959). In the last decades, the high number of molecular markers used to document hybridization have permitted to confirm several of the hypothesis proposed by Anderson. However, it has been also demonstrated that hybridization may have other consequences for the participating populations and their offspring (see further section).

Hybridization, species concept, and phylogeny reconstruction

As shown in the above section, several of the early investigators used hybridization as a way to prove or deny the species status of two supposedly different plant species. This criterion shows the difficulty in species' definition. This everlasting debate is exacerbated by genetic exchange (i.e., hybridization) between two different species. Although a discussion of the species concept is beyond the scope of this review, interspecific hybridization necessarily requires a consideration of species concept.

The biological species concept is one of the most widely used (Mayr 1942). This concept considers that species are a group of potentially or actually interbreeding organisms, emphasizing that the development of reproductive barriers is the main process by which species are defined (Mayr 1942, 1963). Mayr considered that hybrid zones were always stable resulting from a secondary contact. If the hybrids within this hybrid zones were fertile, then he considered that the two species should be considered subspecies. Alternatively, Mayr considered that hybrids always are sterile exhibiting a lower fitness and even when backcrosses may occur, they were considered unfit genotypes that were discarded by natural selection. Thus, hybridizing populations were seen as an intermediate step of speciation (Dobzhansky 1940, 1970). The study of hybrid zones were important in order to clarify the steps in speciation that yield to complete reproductive isolation between taxa (i.e., reinforcement). Although reinforcement is an important consequence of natural hybridization, it is not the only one; hybrid speciation,

introgression and genetic assimilation may also occur. So, how can we recognize these consequences and still consider the participating taxa as different species?

One way to achieve this is by relaxing the definition of hybridization itself. Hybridization is commonly considered as the cross fertilization between individuals of different species. However, a broader definition considers hybridization as the cross fertilization of individuals from populations that are distinguishable on the basis of one or more heritable characters (Harrison 1990; Arnold 1997). In the same sense, introgression is typically considered as the movement of genes between species through repeated backcrossing. A broader definition of introgression involves the movement of genes between genetically distinguishable populations (Rieseberg and Carney 1998). These definitions of hybridization and introgression have the advantage of not relying upon any species concept. Thus, under this definition hybridization may occur between species, subspecies or between differentiated populations of a single species.

Another way to recognize hybridizing taxa as species is relying upon the cohesion species concept as described by Templeton (1989). This concept arose in order to overcome the limitations of the biological species concept to recognize asexual organisms as well as those involved in syngameons. Under this concept, a species is defined as 'the most inclusive group of organisms having the potential for genetic and/or demographic exchangeability' (Templeton 1989). This definition includes the ecological and genetic characteristic of the species, which gives as a result a cohesive group. The genetic characteristics that this concept takes into account include, of course, hybridization and genetic exchange between divergent lineages. Also, this concept does not consider hybridizing taxa as a subspecific taxa. This concept takes into account hybridization as part of the evolutionary history of a certain taxon (Templeton 1989).

We may also recognize hybridizing species when relaxing the biological species concept. In this regard, Rieseberg and Carney (1998) define a biological species as "groups of interbreeding populations that are 'genetically isolated' rather than 'reproductively isolated' from other such groups". According to the authors, this definition allows the recognition of hybridizing taxa as different species. The authors consider that most hybrid zones act as a barrier to genetic exchange among species, thus each species will preserve its genetic integrity, permitting their differentiation by genetic means (Rieseberg and Carney 1998).

Besides the problem of the species concept that should be employed to define hybridizing taxa, the genetic exchange between differentiated species may also pose a problem in systematics during the phylogeny reconstruction of a certain lineage (Arnold 2006). In this sense, there are two approaches when including hybrids in phylogeny reconstruction. The first of these approaches employ explicitly the phylogeny reconstruction as a tool for detecting hybridization or as a tool to prove the hybrid origin of a species (van Ramsdonk et al. 2000; Koontz et al. 2004; Soltis et al. 2008). Also, hybridization could be suggested to occur between a species pair when an incongruence in the topology of the phylogenies derived from different data sets occur (nuclear vs. cytoplasmic markers; Rieseberg and Soltis 1991; Pelser et al. 2010). However it is emphasized that phylogeny incongruence may be the result of processes other than hybridization such as incomplete lineage sorting (Comes and Abbott 2001; Pelser et al. 2010). Despite this difficulty, in these studies there is an *a priori* knowledge of hybrid taxa or putative hybrid individuals.

The second approach, deals with the real problem when including hybrids in a phylogeny. In this case, there is no *a priori* knowledge of the inclusion of hybrids in the analysis. It has been proposed that the inclusion of hybrids may impact importantly the topology of the phylogeny, distorting the hypothesized relationships between non-hybrid taxa (Rieseberg et al. 1995). However, under certain conditions, this is not necessarily the case. For instance, McDade (1990, 1992) tested the impact of the inclusion of F1 hybrids of several species of *Aphelandra* in the topology of phylogenies employing morphological data. He concluded that the inclusion of hybrids between closely related species do not alter the topology of the phylogeny, while the inclusion of hybrids between distantly related species within the genus have a very important impact in the topology of the phylogeny and distort the relationships between non hybrid-taxa (McDade 1990, 1992). Also, she found that hybrids are usually grouped in a clade where one of the parental species occurs, but rarely with both (McDade 1992). In a similar fashion, Soltis et al. (2008), evaluated the impact of the inclusion of hybrid's polymorphic sequences of ribosomal DNA (rDNA) spacers in the topology of phylogenies of *Tragopogon*, *Glycine* and *Rubus*. In general the authors found that the inclusion of hybrids do not disrupt the overall topology of the phylogenies, conserving the relationships between non-hybrid taxa. However, McDade (1992) and Soltis et al. (2008) outline that cladistics analysis based on rDNA spacers and morphology may be an unreliable method to distinguish between hybrid and non-hybrid taxa, making necessary the use of additional data to unveil the phylogeny of a group. Therefore, the authors suggest that hybridization may be a source of error during cladistic analysis (McDade 1992; Soltis et al. 2008).

Outcomes of plant hybridization

Natural hybridization is nowadays recognized as a frequent phenomenon among vascular plants. Whitney et al. (2010) studied the frequency and patterns of interspecific hybridization across eight floras. The authors found that

40.4% of the families and 16.2% of the genera studied reported at least one hybrid. However, the consequences of these hybridization events may have a variety of consequences for the hybridizing taxa and their hybrids. These consequences depend on the environmental conditions (i.e., degree of disturbance), the local abundance of the parental species and the genetic structure of the participating species (Levin and Francisco-Ortega 1996; Rhymer and Simberloff 1996; Arnold 2006). A remarkable consequence of plant hybridization is the high frequency of hybrid species reported (Rieseberg 1997). It has been suggested that up to 11% of flowering plants may be directly the result of hybridization events owing to the high levels of polyploidy found in angiosperms (Arnold 2006). Also, there are several studies that report the occurrence of speciation via hybridization in which the newly formed species shows the same ploidy of the parental species (Rieseberg 1997).

However, in several studies it has been found that although hybridization may occur between a species pair, the resulting first generation hybrids (F1) exhibit a low reproductive success measured as pollen fertility (e.g. Campbell et al. 2003), leading to 'botanical mules' (sensu Kölreuter). The production of these unfit F1 has been proposed as a mechanism of reinforcement of the reproductive barriers between the participating species due to selection against hybrid genotypes (Marshall et al. 2002). However, in most cases, the fitness of hybrid individuals appears to be dependent on the environment in which they establish. In this sense, a number of studies (Levin and Francisco-Ortega 1996; Lamont et al. 2003; Lihová et al. 2007; Tucker & Behm 2011) have reported a high occurrence of hybrids in environments with a high degree of disturbance (along roadsides, crops, sites with recent volcanic activity, etc.). The role of disturbance in plant hybridization is twofold:

1. A major isolating mechanism between potentially hybridizing species is a divergence in the habitat preferences between species (Arnold 2006). Disturbance usually breaks down this isolating mechanism by creating environments where both species can establish and reproduce.
2. Once hybridizing species meet, hybrid genotypes may be produced in the absence of other isolating mechanisms (i.e. incompatibility). In this disturbed environments, certain hybrid genotypes may exhibit a similar or higher fitness than both parental species (Mallet 2005). Thus, it has been considered that disturbance is a prerequisite to the formation of hybrid zones (e.g. Rieseberg and Gerber 1995).

Once F1 individuals are formed, they may act as a bridge whereby alleles may cross from one species to another through repeated backcrossing with the parental species (i.e., introgression). If the rate of backcrossing is limited and the abundance of the parental species is similar, introgression may lead to an increase of the intraspecific genetic diversity of the parental species, which may enable them to colonize new areas (e.g., Caraway et al. 2001). However, when the abundance of the parental species differ considerably, the introgression towards the less abundant species may lead to the loss of its genetic integrity, leading to its extinction through the process known as 'genetic assimilation' (Levin and Francisco-Ortega 1996; Meyerson et al. 2010). In this regard, the introduction of species far from its native range has resulted in the formation of invasive species (Vilà et al. 2000; Petit et al. 2004; Schierenbeck and Ellstrand 2009). In addition to the threat of invasive species to local biodiversity due to their high competitivity, they may also pose a serious threat to endemic or rare populations of species by means of genetic assimilation, especially in environments with a high degree of disturbance. Also, it has been proposed that hybridization may enhance the invasive behavior of certain species, leading to highly competitive genotypes with increased invasive behavior (Schierenbeck and Ellstrand 2009). As shown above, natural hybridization may have a number of consequences that may affect either positively (e.g., increased allele diversity, speciation) as well as negatively (e.g., extinction through genetic assimilation, increased invasiveness of certain genotypes) the genetic diversity of a taxon or the species composition of a certain community. Thus, as a first step in the study of this processes, it is of chief importance to make a correct identification of hybrid individuals. In the following sections, we discuss the morphological, chemical and DNA based markers used for hybrid identification.

Morphological character expression in hybrids

Traditionally, taxonomist rely upon intermediate morphology of hybrids compared to their parental species for their identification. An intermediate morphology was an intuitively assumed characteristic of hybrids as these characters were supposed to be in polygenic control with simple additive effects (Rieseberg et al. 2007). In fact, most of the methodologies employed in the past century were designed to detect this intermediate morphology. Within these methodologies, Anderson (1949) and other influential authors employed methods such as pictoralized scatter diagrams (Anderson 1949), multivariate analysis such as principal component analysis (Wagner 1969) and the character count procedure (Wilson 1992). All the authors suggest that these methodologies should be employed with a large data set with as much morphological characters as possible (Anderson 1949; Wilson 1992). Also, it was supposed that the correlation between

the morphological characters in the parental individuals will be conserved in hybrid individuals. This 'character coherence' of morphological traits, was considered a diagnostic feature of hybrid individuals (Anderson 1949). However, when morphological intermediacy or character coherence were lacking in hybrid individuals, it was rarely discussed.

Later, it was recognized that morphological character intermediacy is a poor predictor of hybrid ancestry by several reasons:

1. Morphological characters are usually correlated. Thus, the number of available characters is much reduced.
2. As any other phenotypic character, the morphological expression in hybrids is highly dependent on the environment. In this sense, the same genotype may exhibit a wide array of morphologies (intermediate or not) if grown in different conditions.
3. Also, morphological intermediacy may be originated by processes other than hybridization. For example, some individuals of closely related species may exhibit morphological intermediacy if these species retain plesiomorphic character states of their ancestral population, conducing to an erroneous interpretation of hybridization (Rieseberg 1995; Judd et al. 2002; Arnold 2006).

Also, from the genetic point of view there are also explanations from a deviation of morphological intermediacy. In a revision of 46 studies exploring morphological character expression in hybrids, Rieseberg and Ellstrand (1993) pointed out that F1 hybrids expressed 44.6% of intermediate characters, while 45.2% of the characters in hybrids were similar to either parental species. Lastly, 10.2% of the characters showed values beyond the range of the parental species (transgressive characters). The high frequency of parental values in hybrids may be the result of only one ore few loci having a dominant (rather than codominant) effect on a certain morphological character in hybrids. However, the most outstanding result is the presence of ca. 10% of transgressive character in F1 individuals (Rieseberg and Ellstrand 1993). In advanced hybrid generations (F2, backcrosses) the percentage of transgressive characters was ca. 30%. The occurrence of the transgressive characters have been ascribed to an increased mutation rate in hybrids, the complementary action of new allele combinations in hybrids, reduced developmental stability, epistatic effects, heterosis, among others (Grant 1975; Voigt and Tischler 1994; Rieseberg et al. 1999; Bell and Travis 2005). As several of this explanations are not genetically inherited traits (epsitasis, heterosis, developmental instability), they account for a small fraction of the variation. So, it is assumed by a number of authors that transgressive character expression is the result of a complementary action of genes (Rieseberg et al. 2003; Bell and Travis 2005; Stelkens and Seehausen 2009).

Complementary gene action assumes that the parental species have fixed alleles with opposing effects within the hybrid (Rieseberg et al. 1999). This is, transgressive character expression in hybrids results comes from loci interactions between alleles having opposing effects on phenotypes within each parental species but have reinforcing effects (i.e., complementation) in hybrids (Bell and Travis 2005; Table 1). This model of transgressive character inheritance explains the larger amount of transgression found in later generation hybrids compared to F1 hybrids, where intermediate character expression is more frequent due to an additive effect of the parental loci (Table 1).

The frequency of transgressive character expression in hybrids seems to be the rule in plants. From a survey of 113 studies reporting hybrid phenotypic values, Rieseberg et al. (1999) found that only three of these failed to report at least one transgressive character. Also, of 579 morphological traits measured in these 113 studies, 58% exhibited transgressive values. Due to this unpredictable morphological expression in hybrids, some authors have considered that these are of limited value in detecting hybridization (Rieseberg and Ellstrand 1993; Hardig et al. 2000, Arnold 2006), and additional markers should be employed to make robust hypothesis of hybridization.

Secondary metabolite expression in hybrids

Given that morphological markers are of limited importance as tools for hybrid recognition, other markers have

Table 1 Hypothetical case of transgressive segregation of a quantitative morphological trait due complementary gene action

Locus	Phenotypic value				
	Species A	Species B	F1	Transgressive F2 hybrids	
1	+1, +1	−1, −1	+1, −1	+1, +1	−1, −1
2	+1, +1	−1, −1	+1, −1	+1, +1	−1, −1
3	−1, −1	+1, +1	+1, −1	+1, +1	−1, −1
4	−1, −1	+1, +1	+1, −1	+1, +1	−1, −1
Net phenotypic value	0	0	0	+8	−8

been historically employed for this purpose. Among these, the secondary metabolite composition of hybrids was considered as a more reliable tool. In the mid 1950's and until 1980, the use of this markers was thoroughly employed in phylogenetic and in taxonomic studies of several species (La Duke 1982; Rieseberg and Ellstrand 1993). Zobel (1951) was the first to apply the secondary chemistry in hybrid recognition. However it was with the studies of Alston and Turner (1962) that this approach was widely recognized.

The secondary metabolite composition in hybrids was thought to be a reliable tool for hybrid identification because it was supposed that they had a simple inheritance mechanism (i.e. oligogenic control with Mendelian segregation ratios). However, as shown later, hybrid secondary metabolites usually have more complex patterns of inheritance in hybrids both qualitatively and quantitatively (Orians 2000; Cheng et al. 2011). The main secondary metabolites studied in hybrids have been phenolic, terpenoid, alkaloid, isothiozyanates and flavonoid compounds. Of these, the flavonoid compounds have been the most studied compounds due to their high variability and stability (Crawford and Giannasi 1982; Rieseberg and Ellstrand 1993).

Qualitative variation and its genetic basis in hybrid secondary metabolite expression

Rieseberg and Ellstrand (1993) compile 24 studies about plant secondary metabolite expression in hybrids. As a result of this revision, the authors found that first generation hybrids (F1) usually exhibit a complementary expression of the secondary metabolites present in the parental species. That is, the hybrid usually expresses both parental secondary metabolites. Rieseberg and Ellstrand (1993) found that 67.7% of F1 hybrids exhibit this complementary pattern, while 27% lack at least one secondary metabolite present in the parental species and 5.2% express new metabolites which are not present in their progenitors.

More recently, Cheng et al. (2011) complemented the revision of Rieseberg and Ellstrand (1993) by incorporating the patterns found by Orians et al. (2000) and other studies done until 2011. The findings of these authors follow the same general pattern above mentioned. In total they revised the expression of 1,112 secondary metabolites and their expression in hybrids: 70.3% of these metabolites were present in both the parental species and their hybrids, 24.2% of the metabolites were lacking in hybrid progeny and 5.5% were new metabolites not present in the parental species.

It has been proposed that the expression of metabolite secondary compounds is regulated by one or few genes exhibiting dominance/recessivity (where dominance is given by the expression of a secondary metabolite) which follow the Mendelian segregation ratios. This pattern of inheritance explain the complementarity found in hybrid metabolite composition. However, it is clear that deviations of this general pattern exist. This deviations may be explained in several ways:

1. The lack of a parental secondary metabolite in hybrids may be due to the polymorphism in the loci controlling the expression in parental individuals. If the parental individuals of a hybrid are heterozygous for a gene controlling the expression of a secondary metabolite, their hybrid progeny may or not exhibit such metabolite (according to Mendelian inheritance).
2. Also, the lack of a secondary metabolite in hybrids may be due to the elongation of the biosynthetic pathway. The elongation in a certain pathway may yield a secondary metabolite which is an intermediary to another pathway, being rapidly converted to the next compound in the pathway.
3. The expression of new secondary metabolites may be due to the obstruction of a biosynthetic pathway in hybrids. If this occurs, the accumulation of intermediary compounds that are only transient in the parental species will be evident in hybrids (Rieseberg and Ellstrand 1993; Orians 2000; Firn and Jones 2003; Cheng et al. 2011).

Because of this, in large populations where hybridization occurs, it is expected that a large qualitative variation of secondary metabolite compounds will occur. This high variability of secondary metabolite has been observed in a number of studies (e.g., Hallgren et al. 2003; Kirk et al. 2004; Oberprieler et al. 2010; Oberprieler et al. 2011).

Quantitative variation and its genetic basis in hybrid secondary metabolite expression

As with the qualitative variation, the quantitative patterns of the secondary metabolite expression in hybrids is variable. Orians (2000) evaluated the concentration of secondary metabolites found in hybrids in relation to the levels present in the parental individuals (5 studies). As a result, the author found that 33% of the 96 secondary metabolites were expressed at similar concentrations of the parents, 29% showed intermediate concentrations in hybrids, 19% were at higher concentrations in hybrids (overexpressed) and 14% showed lower concentrations than their parents (underexpression). Recently, Cheng et al. (2011) extended this revision by adding 7 studies published until 2010. The authors found a similar pattern: most of the metabolites are expressed at similar concentrations of one or both parents or at intermediate concentrations (51.6 and 28.2% respectively). Also, some metabolites are overexpressed (11.5%) or underexpressed (8.7) in hybrids.

The genetic regulation of the quantitative expression of secondary metabolites in hybrids is controlled by more than one gene with dominant, overdominant or epistatic effect within a locus (Cheng et al. 2011). The effects of this genes usually affect the expression of the enzymes involved in the pathway of secondary metabolites. If the enzyme is overexpressed, so will happen to the secondary metabolites. Although the metabolite secondary concentration of hybrids is rarely used as a criterion for hybrid identification, it may impact plant-herbivore interactions and other ecological processes (see further discussion).

Genetic data employed for hybrid recognition: chromosome number and DNA fingerprinting techniques

Besides phenotypic characters, hybrid recognition relies nowadays in the genetic data of individuals. In this sense, one approach that was thoroughly used as an important evidence of hybridization during the 20th century was the chromosome number of putative hybrid individuals. This approach supposes that hybrid individuals always undergo an instant duplication of the chromosome complement (i.e. allopolyploidy; Harlan and deWet 1975). Several well known cases of allopolyploidy include hybrids between *Tragopogon* spp (Ownbey 1950; Roose and Gottlieb 1976), *Spartina* spp (Strong and Ayres 2013), and species belonging to the Hawaiian silversword alliance (Barrier et al. 1999; Lawton-Rauh 2003) among others. This tool became popular because the analysis of the chromosome number in hybrids was supposed to be relatively easy; hybrids will display a duplication of the whole chromosome complement when compared with the putative parental species. Also, the discovery of allopolyploidy was quite important, since it provided a mechanism that lead to reproductive isolation between hybrids and its parental species (Winge 1917; Ownbey 1950). Indeed, polyploidy is nowadays considered a prominent speciation mechanism in flowering plants (Levin 1983; Ramsey and Schemske 1998; Soltis et al. 2010).

However, in some cases it may be difficult to distinguish between autopolyploidy (i.e., polyploids that arise within a single population of a single species; Grant 1981) and allopolyploidy (i.e., polyploids derived from hybridization between two different species; Ramsey and Schemske 1998), rendering the chromosome number as an unreliable tool for hybrid detection (Comai 2005). This difficulty arises because intermediate states between autopolyploidy and allopoliploidy may occur, making difficult the differentiation between these categories. It has been proposed that 'segmental allopolyploids' may occur, in which some chromosomes may exhibit a pairing similar to those of allopolyploids during meiosis, and other chromosomes have a similar pairing to autopolyploids (Stebbins 1950). Although no clear examples of the occurrence of segmental allopolyloids can be mentioned (Soltis et al. 2010), this condition explains some segregation ratios found in nature (Stift et al. 2008). Besides this problem, the differentiation between auto- and allopolyploids pose some methodological difficulties. In order to establish whether an individual is an auto- or an allopolyploid, an exploration of the segregation ratios at many loci as well as the absence or presence of multivalents during meiosis should be observed (Soltis et al. 2010). This techniques are expensive and time-consuming, making them ineffective to determine the status of putative hybrid individuals.

As mentioned above, the chromosome number of putative hybrids may give insights of hybridization in some instances. However, when it is used in the absence of additional data (morphological or genotypic), the frequency of hybridization may be underestimated. This occurs because hybrids may exhibit the same chromosome number than the parental species (i.e., homoploid hybrids; Mallet 2007, Abbott et al. 2010). Indeed, despite that allopolyploidy is considered a prominent mode of speciation within angiosperm, homoploid hybrid speciation has been recorded in a number of species (Gross and Rieseberg 2005; Abbott et al. 2010). Because of this, the chromosome number of hybrids is not a reliable tool when used in the absence of additional data, however it may provide robust hypothesis of hybridization when morphological or DNA fingerprinting techniques are employed (e.g., Newaskar et al. 2013)

Due to the complex pattern of expression of phenotypic data and the unreliable data provided by chromosome number counts in putative hybrids, DNA fingerprinting techniques are now the most used tool for hybrid identification. The advantages that these markers have over phenotypic traits are: 1) they are present in a large number within the genome, 2) they usually exhibit independence, 3) as these markers are supposed to be located in non-coding regions, they are selectively neutral, and 4) their inheritance is strictly under Mendelian segregation ratios (Rieseberg and Wendel, 1992). Also, these markers have been useful detecting different hybrid classes (F1, F2, backcrosses and later generation hybrids) when Bayesian based analyses such as STRUCTURE (Pritchard et al. 2000), NEWHYBRIDS (Anderson and Thompson 2002), BAPS (Corander and Marttinen 2006) and GENECLASS (Piry et al. 2004) have been employed.

However, there is a wide variety of DNA fingerprinting techniques employed for hybrid recognition. In Table 2, we mention the main DNA fingerprinting techniques, their mode of inheritance and degree of polymorphism. Within the non-based PCR techniques the Restriction Fragment Length Polymorphism (RFLPs) is the most recognized technique (Jeffreys et al. 1985). This technique is based on the activity of bacterial restriction enzymes. These enzymes identify specific palindrome sequences, producing fragments with variable dimensions. This

Table 2 Comparison of several DNA-based markers used for hybrid identification

Marker	Degree of polymorphism	Inheritance	Reproducibility	Technical requirements
RAPD	Medium	Dominant	Low	Low
RFLP	Medium	Codominant	High	High
AFLP	Medium	Dominant	High	High
SSR	Medium	Codominant	Medium	Medium
SNP	High	Codominant	High	Medium

RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; AFLP, amplified fragment length polymorphism; SSR, single sequence repeats; SNP, single nucleotide polymorphism.

fragments are then visualized on gel electrophoresis (Jeffreys et al. 1985). The variation in the length of restriction fragments are due to changes (point mutations or translocations) in the sequence recognized by the enzymes. Although RFLP are highly reproducible and polymorphic, this technique is time consuming. Also, the use of radioactive agents and the need for large quantities of sample DNA make this technique unpopular nowadays.

Several PCR-based markers overcome some of the difficulties of RFLPs. PCR based markers are less time consuming techniques, and the initial DNA sample could be minimal. Also, some of these techniques require no previous knowledge of sequence of the organism under study. Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) are two of these markers (Williams et al. 1990; Vos et al. 1995). RAPD markers are generated through the random amplification of genomic DNA using short primers (10 base pair). The use of short primers is necessary to increase the probability that they are able to find homologous sequences suitable for annealing. The polymorphism in RAPD fragments is produced by rearrangements or deletions at the primer binding sites in the genome (Williams et al. 1990). However, the major problem with this technique is its poor reproducibility, usually exhibiting different banding pattern when carried out under slightly different PCR conditions (Williams et al. 1990; Hadrys et al. 1992). AFLP are a much reproducible technique which combine RAPD and RFLP. In this technique, restriction fragments of the sample DNA are obtained. Then, the fragments are amplified through random PCR amplification (Vos et al. 1995). The main limitation of both RAPD and AFLP fragments is that both have a dominant pattern of inheritance. That is, the character is recorded as the presence of a specific band in gel electrophoresis (dominant state) or the absence of such band (recessive state).

A different approach to arbitrary PCR amplification consists in the amplification of target regions of a genome through specific primers. These kind of DNA fingerprinting techniques have become very popular due to the ease and accessibility of sequence technology. A popular technique relies in the polymorphism of Microsatellite or Simple Sequence Repeats (SSR). SSR consist of sequences of repetitions, comprising short motifs generally between 2 and 6 base-pairs long (Kalia et al. 2011). The polymorphism within a specific locus is due to the variation in length of the microsatellite sequence, which depends on the number of repetitions of the basic motif. These markers have several advantages compared to AFLP, RAPD and RFLP. SSR have a codominant inheritance (i.e., the heterozygote state for a locus can be assessed), they are highly abundant in the genome, they exhibit a high allelic diversity and their amplification is highly reproducible (Kalia et al. 2011). However, SSR require extensive knowledge of DNA sequence of the target species in order to design specific primers. Also, the presence of null alleles (i.e., a point mutation in the primer annealing site may cause the amplification failure of a specific microsatellite locus) is thought to be frequent, leading to an underestimation of allele diversity (Chybicki and Burczyk 2009).

The techniques mentioned so far have been widely employed for hybrid recognition (Blair and Hufbauer 2010; Meyerson et al. 2010; Travis et al. 2010; Zalapa et al. 2010; Saad et al. 2011; Tovar-Sánchez et al. 2012). However, the new technological advances in sequencing technology have provided with new markers with an almost unlimited amount of variation based on DNA sequences. The main problem with classical protocols employing previous markers such as RAPD, SSR, AFLP, and RFLP is that they have a low availability of markersb that discriminate between closely related species with low resolution and identify later generation hybrids (Twyford and Ennos 2012). Although a recent hybridization event can unequivocally detected with as few as four markers (Boecklen and Howard 1997), the correct assignment of individuals to hybrid categories (F1, F2, backcrosses) will require the exploration of 24 to 48 independent codominant loci (Vähä and Primmer 2006). Also, it is sometimes difficult to find diagnostic or species-specific markers (Howard et al. 1997) which differentiate between hybridizing species using the traditional PCR based methodologies. This diagnostic markers are the most powerful markers used to detect late generation introgression (Hohenlohe et al. 2011).

These limitations in most PCR based DNA markers have conducted to the development of new markers that assess the sequence of a complete subsection of a genome without the need of previous knowledge of the target sequence. In particular the new technique known as Next-Generation Sequencing (NGS) generates a large quantity of nucleotide sequence data from complex nucleic acid populations (Metzker 2010). NGS is considered an emerging technique which is thought to improve our knowledge about hybridization and introgression because no prior knowledge of the sequence of the target organisms is required (Hohenlohe et al. 2011). In particular, the vast amount of markers for each of the parental hybridizing populations may increase considerably (up to ca. 300), compared to the traditional PCR based methods above mentioned (Twyford and Ennos 2012).

Why should we employ several different markers for hybrid identification?

In the previous sections, we discussed the principal tools for hybrid identification. It is clear nowadays that DNA fingerprinting techniques are the most reliable tool for hybrid identification compared to morphological and secondary metabolite markers. The main advantages of DNA molecular markers are principally their neutrality and the nearly unlimited number in which they can be found within the genome. As mentioned earlier, morphological character expression is considered complex and in some cases unpredictable (Rieseberg et al. 1999; Hardig et al. 2000). Meanwhile, secondary metabolites have a more predictable inheritance mechanism, with F1 hybrids exhibiting both secondary metabolites of the parental species (Rieseberg and Ellstrand 1993; Orians 2000; Cheng et al. 2011). However, obtaining the chemical profile of hybrids is time consuming and is technically difficult. Also, it is a poor predictor of hybrid ancestry in later generation hybrids (Cheng et al. 2011). Finally, chromosome number may provide information about the hybrid origin of individuals when these exhibit allopolyploidy, however, sometimes hybrids exhibit a homoploid condition compared to its parental species (Abbott et al. 2010). Also, the chromosome count of putative hybrids and parental individuals may be expensive and time consuming.

So, if DNA fingerprinting techniques are the best tools for hybrid recognition, why should researchers continue using other markers such as morphological, chemical profiles and chromosome number in hybridization related studies? Some authors propose that besides DNA fingerprinting, other markers should be employed in order to have a vast amount of evidence to confirm the hybridization hypothesis between two taxa (e.g. Hardig et al. 2000). Also, it has been found that these additional markers may unveil hybrid individuals that go undetected when using problematic DNA markers such as RAPD or AFLP (e.g., Kirk et al. 2012). However, the main reason to study phenotypic and chromosomal traits in hybrids is that they may give insights of their ecological performance. This is of special importance when hybrids exhibit novel or transgressive characters compared to their parental species.

In this regard, the expression of transgressive morphological characters have been proposed as a mechanism of speciation when ecological divergence occurs between the parental species and their hybrids. In this regard, Schwarzbach et al. (2001) studied the character expression of the hybrid species *Helianthus anomalus* and their parental species; *H. annuus* and *H. texanus*. The hybrid species has a very distinct ecological preference than their parental species. *H. anomalus* occurs mainly in sand dune habitats. As a first step, the hybrid species was determined as such employing DNA based markers (Rieseberg 1991). But later, Schwarzbach et al. (2001) accounted its morphological variation. The authors measured 41 morphological traits. They found that *H. anomalus* was intermediate in 2.4% of the characters, while parental and transgressive characters accounted for 56.1% and 41.5%. The authors suggest that the high frequency of transgressive character expression of *H. anomalus* may have facilitated its ecological divergence. This hypothesis is reinforced as many of this transgressive characters are consistent with adaptations for other sand dune plants (Schwarzbach et al. 2001).

Besides the role of hybridization in speciation above mentioned, natural hybridization has also been proposed as a process involved in the evolution of invasive genotypes due to its potential in generating evolutionary novelty (Schierenbeck and Ellstrand 2009). The transgressive character expression in some hybrid genotypes has been proposed as one of the mechanisms that enhance invasiveness in some populations (Ellstrand and Schierenbeck 2000).

On the other hand, the secondary metabolite expression in hybrids may affect he herbivore-plant interaction. In this sense, the quantity and the type of the secondary metabolites present in hybrid individuals may reduce the palatability to herbivores (Orians 2000). In terms of the qualitative variation, the expression of new metabolites in hybrid could deter nonadapted herbivores, both generalists and specialists thus generating resistant plants. Whether these hybrids remain resistant might depend upon the relative abundance of the plant. Chew and Courtney (1991) argue that if the abundance of a host plant producing a novel chemical is highly variable over time, herbivores will be unable to track the plant and adapt to that chemical. In terms of quantitative variation hybrids may exhibit a higher concentration of secondary metabolites. In general, it has been proposed that a high concentration of secondary metabolites give resistance

to hybrid individuals (Orians 2000). However, a single chemical might stimulate, deter or have no effect in the activity of an herbivore (Orians 2000). Thus, the quality and quantity of hybrid secondary metabolites may influence the arthropod associated community and the herbivore-plant interaction.

However it has been suggested that hybrid secondary metabolite composition may alter processes at the ecosystem level. For example, Driebe and Whitham (2000) evaluated the leaf litter decomposition rate of *Populus angustifolia*, *P. fremontii* and their hybrids. In general, the authors found that F1 hybrids showed all secondary metabolites present in the parental species. The leaf litter decomposition rate of F1 hybrids was intermediate between both parental species. However, they found that backcross hybrids showed higher levels of condensed tannins than both pure parental individuals and F1 hybrids, which lead to a slower decomposition rate of the leaf litter. The authors suggest that if hybridization occurs in broad geographic areas, the nutrient availability in the soil could be seriously altered due to the altered decomposition rate, modifying whole ecosystem processes (Driebe and Whitham 2000).

Regarding chromosome number, several hybridization related studies continue to study the cytogenetic of putative hybrid individuals (e.g. Suárez-Santiago et al. 2011). The approach of these studies may answer several important questions about hybrid performance. First, hybridization may produce allopolyploids that are intersterile to both parental species and may yield to new species (Comai 2005). On the other hand, if hybrids are homoploid, they may backcross toward parental species and lead to introgression (e.g., Peffley and Mangum 1990). However, although homoploid hybrids may be interfertile with both parental species, homoploid hybrid speciation may occur. Homopoid hybrid speciation may occur in two ways; 1) homoploid hybrids may exhibit new characteristics (i.e., transgressive characters) that enable them to colonize new areas, leading to an ecological divergence from parental species or 2) hybrids may exhibit new chromosome or genetic sterility barriers that lead to reproductive isolation from their parental species (Abbott et al. 2010). Homoploid hybrid speciation has been proposed to produce several species such as *H. anomalus* (Sapir et al. 2007), *H. paradoxus* (Lexer et al. 2003) and *H. deserticola* (Gross et al. 2003).

Also, allopolyploids may have also important features that yield to evolutionary novelty. Allopolyploids exhibit some advantages compared to their diploid parental species. For instance, polyploids usually exhibit heterosis, causing them to be more vigorous than their parental species (Chen 2013). Also, due to the chromosome doubling in allopolyploids, hybrids exhibit gene redundancy which provide them of several advantages. First, gene redundancy in allopolyploids provide them with a protection against recessive deleterious alleles and recessive mutations (Comai 2005). Second, allopolyploids are able to diversify gene function by modifying the redundant copies of genes. This may lead to advantageous alleles that may increase their fitness (Prince and Pickett 2002; Adams et al. 2005). Finally, allopolyploids usually show an increase of asexual reproduction that enable them to increase their population even in the absence of sexual mates (Comai 2005). *Spartina anglica* is a remarkable example of the advantageous characteristics offered by allopolyploidy (see Strong and Ayres 2013). Due to the importance of allopolyploidy and homoploidy in the ecological performance of hybrids, it is not trivial to study the chromosome number of putative hybrid individuals.

As shown in a previous section, morphological markers, chemical markers and chromosome number are not reliable tools for hybrid identification when used in the absence of DNA fingerprinting techniques. However, the evaluation of these markers is of importance due to the evolutionary novelty that may arise via hybridization. Hybrids may exhibit transgressive character expression in morphological characters, new secondary metabolites and enhanced genetic variability due to allpoliploidy. This evolutionary novelty may result in new species if ecological divergence occurs or it may lead to an increase of hybrid resistance to herbivores or pathogens. Although DNA fingerprinting techniques are the most reliable tool for hybrid identification, the use of additional markers will continue to be employed as they give insights of the ecological performance of hybrids.

Conclusions

In this review we explored the utility and/or mode of inheritance of the main markers that have been historically employed for hybrid identification: morphological and chemical markers as well as chromosome number and DNA fingerprinting techniques. While morphological characters were thoroughly employed during the last century as the main marker for hybrid recognition, nowadays it is known that their pattern of inheritance is complex and usually unpredictable. Also, during the last century, the plant secondary metabolite composition emerged as a more reliable tool than morphological markers, however, their high costs, their low polymorphism and complex inheritance made them also unreliable tools for hybrid recognition in the absence of other markers. As both chemical and morphological markers are phenotypic traits, their expression in hybrids is highly dependent of the environment, reducing their utility to detect hybridization under natural conditions. Also, the chromosome number of putative hybrids is a criterion that may exhibit some disadvantages. Some hybrids may exhibit allopolyploidy, while others may be

homoploid hybrids. Thus, hybridization rates may be underestimated. Additionaly, the techniques employed during chromosome count may be expensive and time-consuming. In this regard, DNA fingerprinting appears as a much better option for hybrid recognition due to their high availability in the genome, their neutrality and the ease with which large amounts of data may be obtained.

It is undeniable that DNA fingerprinting techniques are the best option for hybrid recognition. When correctly employed, they are able to detect later generation hybrids with high reliability. However, while DNA-based markers are the prime marker when studying whether or not hybridization occurs between a species pair and its frequency, other important questions arise about the fate that hybrids will have under natural conditions: How can we recognize these hybrids under field conditions? Will they exhibit a higher resistance/tolerance to pathogens? Will they possibly exhibit a higher fitness than pure parental individuals? These questions may be answered using a cytological approach as well as molecular and secondary metabolite markers. Whether hybridization is carried out under natural or greenhouse conditions, these questions may be very important issues. The relevance of these questions is exacerbated when considering the global changes occurring on earth; global warming, deforestation, and the introduction of exotic species favor the sympatry between species that are previously allopatric. As hybridization introduces much more genetic diversity than mutation alone, it may confer hybrids with advantageous characteristics that may alter significantly the ecosystems where they grow.

So, while DNA fingerprinting techniques are really useful to unveil the existence and frequency of natural hybridization, the use of other markers may give insights of the ecological performance of hybrids.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
Both authors participated in the review topic design, in the data analyses and in the manuscript writing. Also, both authors read and approved the final version of the manuscript.

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CAPÍTULO II

Hybridization between *Tithonia tubaeformis* and *T. rotundifolia* (Asteraceae) evidenced by nSSR and secondary metabolites

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ORIGINAL ARTICLE

Hybridization between *Tithonia tubaeformis* and *T. rotundifolia* (Asteraceae) evidenced by nSSR and secondary metabolites

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Abstract
Hybridization has a number of ecological and evolutionary consequences by either increasing intraspecific genetic diversity or by altering morphological characters and secondary chemical content of recombinant individuals. In this paper, we reanalyzed through nSSR and secondary metabolites four mixed stands between *Tithonia tubaeformis* and *T. rotundifolia* previously studied with RAPD markers. We amplified nSSR regions to classify individuals in mixed stands as pure or admixed individuals. Then, we explored the chemical profile of each individual in pure and mixed stands by scoring the presence/absence of one abundant flavonoid unique to *T. tubaeformis* and two sesquiterpene lactones unique to *T. rotundifolia*. Bayesian analysis of SSR data revealed the presence of pure and admixed individuals in all but one mixed stand, where no pure *T. tubaeformis* individuals were found. Also, contrary to previous RAPD analysis, we identified a significant number of backcrosses toward *T. tubaeformis* in two mixed stands. Regarding secondary chemical profiles, pure *T. tubaeformis* and *T. rotundifolia* showed characteristic chemical profiles, while admixed individuals showed a mosaic of chemical profiles; some individuals exhibited additivity, while most individuals identified as backcrosses showed dominance. However, some individuals identified as backcrosses toward *T. tubaeformis* lacked parental compounds, and a new chemical profile was recorded. A new flavonoid (5,3′,4′-trihydroxy-6,7,8-trimethoxyflavanone) was found in these individuals exhibiting the new chemical profile. We suggest that the presence of admixed individuals with novel combinations of secondary metabolites may increase their fitness due to their phytotoxicity and also by the protectant activities against insect herbivores and environmental stress.

Keywords Chemical profile · Flavonoid · Microsatellites · Secondary metabolites · Sesquiterpene lactone

Introduction

Hybridization is a frequent phenomenon in plants (Whitney et al. 2010). Although hybrids are genetically intermediate compared to parental individuals, their phenotypic expression is more complex as they may exhibit intermediate, parental-like or transgressive character expression (Rieseberg and Ellstrand 1993; Bell and Travis 2005; López-Caamal and Tovar-Sánchez 2014). These changes in phenotypic expression may have a series of ecological and evolutionary consequences. For instance, it may impact the arthropod and fungal communities associated with hybridizing populations (Whitham et al. 1994; Bangert et al. 2008; Pérez-López et al. 2016), inducing a shift of herbivore distribution. Ultimately, plant hybridization may have cascading effects in ecosystems by altering some processes when there is a change in the secondary metabolite content of hybrids (Driebe and Whitham 2000). Therefore, during the

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last decades, hybridization has been the focus of ecological and evolutionary research.

Due to their importance, the recognition of hybrid individuals is of prime importance in hybridization-related studies. For that purpose, a variety of markers such as morphological, anatomical and secondary metabolites markers have been historically employed (Zobel 1951; Stuessy 1990; Wilson 1992). However, since DNA based markers rely on genotypic data, they are considered the most powerful markers to detect first and later generation hybrids. Simple sequence repeats (SSR; Valencia-Cuevas et al. 2015), amplified fragment length polymorphism (AFLP; Eidesen et al. 2015), random amplified polymorphic DNA (RAPD; López-Caamal et al. 2014) and single-nucleotide polymorphism markers (SNP; Lamaze et al. 2012) have been employed in detecting and describing the genetic structure of hybrid zones. However, the use of DNA markers that are able to distinguish the heterozygote state (e.g., SSR) is clearly preferred over dominant markers such as RAPD and AFLP since they may exhibit low reproducibility as well as difficulties in their interpretation (Avise 2004).

Plant secondary metabolites (PSM) are a series of structurally diverse compounds that have an important role in plant defense and/or signaling (Wink 2003). Due to their oligogenic control, PSM have a far more predictable inheritance than morphological characters, which are usually under polygenic control and the appearance of transgressive characters is common (Rieseberg and Ellstrand 1993; Orians 2000; Cheng et al. 2011; López-Caamal et al. 2013). First-generation ($F1$) hybrids usually have an additive pattern in both the type and quantity of the PSM present in the parental species (Orians 2000; Cheng et al. 2011). For that reason, a number of researchers have employed PSM in addition to molecular markers to identify hybrid individuals in natural populations (e.g., Georgescu et al. 2016). Usually, PSM are complementary to DNA markers as they may provide support for hybrid ancestry of individuals that are equivocal solely under DNA marker analysis (Kirk et al. 2004; Oberprieler et al. 2011). However, it is outlined that PSM are used only as a complement and their sole use for hybrid identification is not suggested (Hardig et al. 2000; Kirk et al. 2004). Lastly, hybrid's secondary chemistry is important as it may be responsible for the success of hybrid populations due to the expression of novel combinations of PSM that promote the defense against herbivores (Whitehead and Bowers 2013).

Natural hybridization is common within Asteraceae, and it is thought to play a significant role in some genera where species of hybrid origin and widespread introgression have been identified (Scascitelli et al. 2010; Brennan et al. 2012; Mameli et al. 2014). Recently, a DNA marker (RAPD) analysis revealed hybridization between *Tithonia rotundifolia* (Mill.) Blake and *T. tubaeformis* (Jacq.) Cass. in Mexico (Tovar-Sánchez et al. 2012). These species are aggressive colonizers of disturbed habitats such as roadsides or croplands, becoming invasive species in their exotic range (Juárez and Cazón 2003; Muoghalu and Chuba 2005). *Tithonia tubaeformis* and *T. rotundifolia* have noticeable morphological and chemical differences when distributed in allopatry (La Duke 1982; Chagas-Paula et al. 2012). However, populations of both species meet at intermediate habitats in Mexico, yielding $F1$ hybrids and probable backcrosses toward *T. rotundifolia* (Tovar-Sánchez et al. 2012) with transgressive morphological phenotypes (López-Caamal et al. 2013). Although dominant markers may give rough estimates of hybridization between taxa (Boecklen and Howard 1997), there is a lack of information of the hybridization process between these species as revealed by codominant markers. Besides testing the hybridization hypothesis, the use of codominant markers will allow to estimate with greater accuracy the gene flow between *T. tubaeformis* and *T. rotundifolia*. In this paper, we use nuclear microsatellite (nSSR) and PSM markers to (1) compare the patterns of hybridization described by Tovar-Sánchez et al. (2012) employing RAPD data with those obtained in the present work using codominant markers, (2) describe the genetic structure of populations of *T. rotundifolia* and *T. tubaeformis*, (3) establish the chemical profiles of pure individuals of *T. rotundifolia* and *T. tubaeformis* and determine whether admixed individuals exhibit novel combinations of secondary compounds or their chemical profiles are not affected by hybridization. The presence or absence of the main PSM of both *T. tubaeformis* and *T. rotundifolia* was used to delineate chemical profiles due to the complex relationship of quantitative composition with environment, whereas qualitative composition is likely to be genetically controlled (Orians 2000).

Materials and methods

Study species, population sampling and plant material collection

Tithonia tubaeformis and *T. rotundifolia* are diploid ($n = 17$), self-incompatible species within Asteraceae exhibiting a ruderal annual herbaceous habit in Mexico and Central America (La Duke 1982). These species show noticeable morphological and chemical differences when distributed in pure stands. For instance, *T. tubaeformis* is easily recognizable for its dentate leaves and its linear pubescent phyllaries with yellow ligules. On the other hand, *T. rotundifolia* exhibits 3–5 lobed leaves with lanceolate and softly pubescent phyllaries. *Tithonia rotundifolia* shows orange ligules throughout most of its range, although Central American populations show yellow ligules (Fig. 1; La Duke 1982).

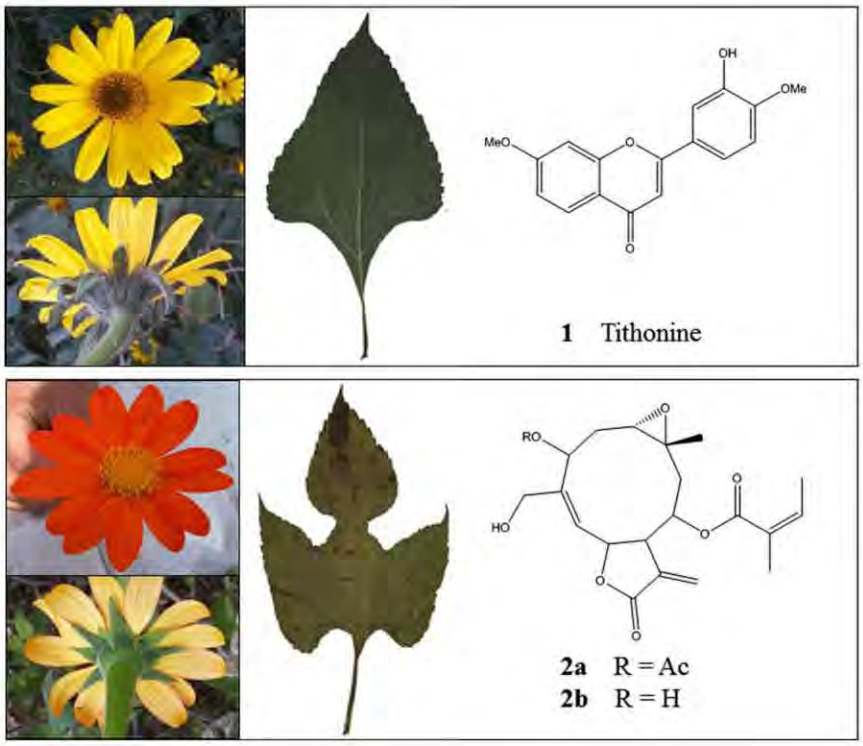

Fig. 1 Inflorescences and leaves of *Tithonia tubaeformis* (**a**) and *T. rotundifolia* (**b**). Marker compounds found in pure stands of *T. tubaeformis* (**1**) and *T. rotundifolia* (**2a**, **2b**). **1** = tithonine, **2a** = 3-acetyl-15-hydroxyleptocarpin, **2b** = 15-hydroxyleptocarpin. (*Tithonia rotundifolia* photographs were provided by Mauricio Mora-Jarvio)

In the same way, these species show clear differences in their PSM. *Tithonia tubaeformis* synthesize mainly flavonoids, and *T. rotundifolia* specially sesquiterpene lactones. For instance, it has been reported that *T. tubaeformis* produces two aglycone flavonoids (geraldol and tithonine), two glycosylated flavonoids (quercetin-3-glucoside, quercetin-3-rutinoside), one unknown chalcone, and one sesquiterpene lactone (orizabine). Meanwhile, *T. rotundifolia* synthesizes 34 sesquiterpene lactones, and five glycosylated flavonoids, such as quercetin and kaempferol-3-glucosides, quercetin and kaempferol-3-rutinosides and patulitirin (La Duke 1982; Pérez et al. 1984; Chagas-Paula et al. 2012 and references therein). Both species also exhibit a divergence in habitat preferences: *T. tubaeformis* usually establishes in mesic habitats above 1000 m above sea level (a. s. l.) (Table 1), while *T. rotundifolia* prefers xeric habitats associated with tropical forests below 1000 m a. s. l. Hauser and Morrison (1964) provided early evidence of hybridization between these species based on pollen fertility analysis. Later, Tovar-Sánchez et al. (2012) used RAPD markers to evaluate hybridization between these species. Authors found that hybridization occurs at intermediate altitudes with a probably asymmetrical gene flow toward *T. rotundifolia*.

In this paper, we employed the same individuals and localities studied by Tovar-Sánchez et al. (2012). *Tithonia tubaeformis* pure stands were collected at ca. 2000 m a. s. l. in the Faja Volcánica Transmexicana (FVT) biogeographic region; *T. rotundifolia* pure stands were collected ca. 1000 m a. s. l. at the Depresión del Balsas (DB) biogeographic regions. Meanwhile, mixed stands were collected at DB or at Sierra Madre del Sur (SMS) biogeographic regions (Fig. 2). The locality of Cerro de Piedra studied by Tovar-Sánchez et al. (2012) was excluded due to a lack of plant material to carry out genetic and chemical analyses. We analyzed 20 individuals per mixed stand and 20 individuals for each pure parental species ($N = 120$; Table 1). From each individual, we collected all individual's leaves; young leaves were preserved in liquid nitrogen and were further used for genetic

Table 1 Sampling localities of pure and mixed stands of *Tithonia tubaeformis* and *T. rotundifolia* in Mexico

Locality	Lat N	Long W	Altitude (m a. s. l.)	Sample size
Tithonia tubaeformis				
Jardín Botánico	19° 19′	99° 11′	2321	10
Caseta	19° 14′	99° 08′	2439	10
Tithonia rotundifolia				
Vicente Aranda	18° 34′	99° 13′	889	10
Guadalupe de Allende	18° 26′	98° 21′	1127	10
Mixed stands				
Huautla	18° 28′	99° 00′	1080	20
Zacacoyuca	18° 18′	99° 30′	863	20
Teloloapan	18° 24′	99° 41′	1020	20
Corral de Piedra	17° 45′	97° 44′	1830	20

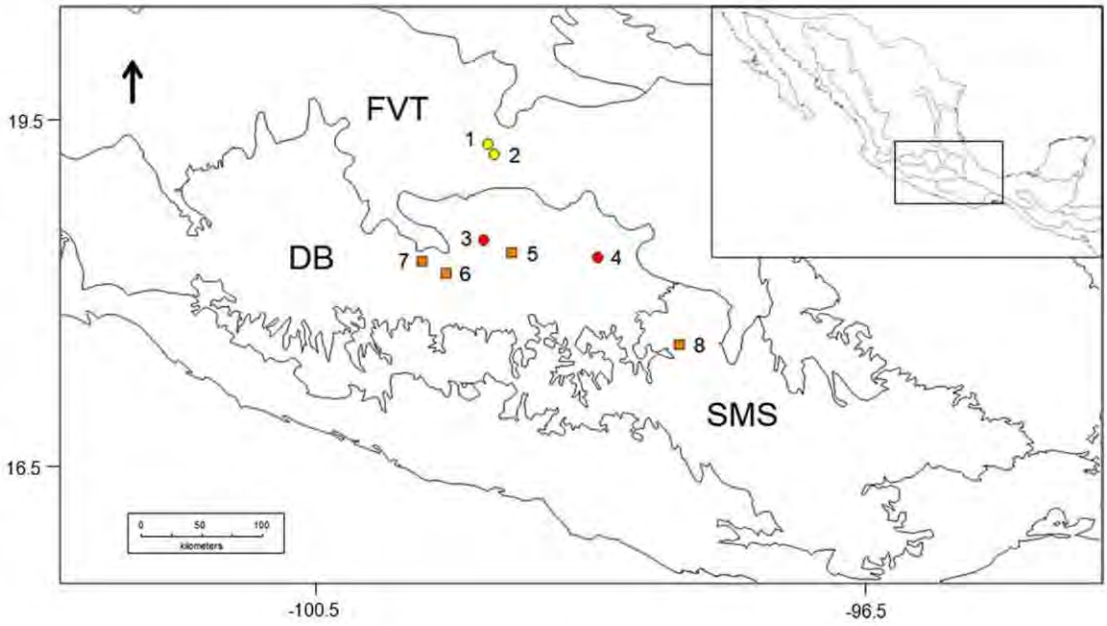

Fig. 2 Sampling localities for *Tithonia tubaeformis* (*1* = Jardín Botánico, *2* = Caseta), *T. rotundifolia* (*3* = Vicente Aranda, *4* = Guadalupe Allende) and mixed stands (*5* = Huautla, *6* = Zacacoyuca, *7* = Teloloapan, *8* = Corral de Piedra). Solid lines represent biogeographic regions. *FVT* Faja Volcánica Transmexicana, *DB* Depresión del Balsas, *SMS* Sierra Madre del Sur. See text for details

analyses. The rest of the leaves were air-dried and stored until secondary metabolite analysis.

Genetic analysis

Genomic DNA was extracted from 120 mg of fresh leaf tissue using the DNeasy Plant Minikit (Qiagen, Valencia, CA, USA). We employed PCR to amplify nuclear microsatellite regions using primers designed for *Helianthus annuus* (Paniego et al. 2002). We initially tested for microsatellite polymorphism with a subset of individuals of both *T. rotundifolia* and *T. tubaeformis* using 15 primers. However, only five yielded polymorphic loci for both species (Ha49, Ha80, Ha140, Ha357, Ha360). PCRs were carried out in a volume of 10 µL following the conditions described in Paniego et al. (2002). Fragments were genotyped in an ABI 3500 (Applied Biosystems) capillary sequencer using GeneScan-500 ROX as an internal size standard. Fragment analysis and scoring were carried out in Geneious v. 9.1.5 (Biomatters). In order to eliminate scoring errors, large allele dropout, stuttering and to evaluate amplification quality and the presence of null alleles, microsatellite data were evaluated in Micro-Checker (van Oosterhout et al. 2004). Also, linkage disequilibrium between all pair of loci was tested in Arlequin v. 3.5.2.2 (10,000 permutations; Excoffier and Lischer 2010).

We calculated genetic diversity measures for each locus of both parental species. Genetic differentiation between pure stands of each species was not significant ($F_{ST} = 0.03$, $P > 0.05$ for *T. tubaeformis*, and $F_{ST} = 0.04$, $P > 0.05$ for *T. rotundifolia* pure stands), so pure stands of the same species were pooled in all analysis. For each locus, we calculated: number of alleles (Na), frequency of null alleles (NAl), observed heterozygosity (H_O), expected heterozygosity (H_E), and the inbreeding index (F_{IS}) with Fstat v. 2.9.3.2 (Goudet 2002), FreeNa (Chapuis and Estoup 2007) and Arlequin. Finally, the ability of the microsatellite dataset to discriminate between individuals of *T. tubaeformis* and *T. rotundifolia* was tested by first calculating pairwise F_{ST} (with and without the correction for null alleles described by Chapuis and Estoup 2007) and R_{ST} values (significance tested after 10,000 iterations) in FSTAT and Arlequin and then by a principal coordinate analysis (PCoA) based on individual genetic distances as implemented in GenAlEx v. 6.5 (Peakal and Smouse 2006, 2012).

Assignment of hybrid and parental categories

The software STRUCTURE 2.3.4 (Pritchard et al. 2000) was used to estimate the contribution of *T. tubaeformis* and *T. rotundifolia* to each putative hybrid genome. STRUCTURE assigns individuals to K populations (genetic clusters) based on their multilocus genotypes (Pritchard et al. 2000; Falush et al. 2003; Hubisz et al. 2009). For each individual, an admixture coefficient (q) is calculated. This coefficient represents the proportion of an individual's genotype that originates from a given population. First, we employed this

software to establish the value of K in pure and mixed populations of *T. tubaeformis* and *T. rotundifolia*. This analysis was performed with 300,000 MCMC with an initial burn-in period of 20,000, which demonstrated to be sufficient for the parameters to reach convergence. We used the admixture model with correlated allele frequencies and the LOCPRIOR option off. Five independent replicates were performed for each value of K estimated. The K values estimated ranged from 1 to 6, assuming no genetic structure ($K = 1$) to complete differentiation among sampled localities ($K = 6$). K was estimated following the Evanno method (Evanno et al. 2005) in Structure Harvester (Earl and vonHoldt 2012). The q values from the five runs were merged in CLUMPP (Jakobsson and Rosenberg 2007), and the results were visualized in DISTRUCT (Rosenberg 2004).

Since $K = 2$ was estimated in the above analysis, and all individuals of *T. rotundifolia* and *T. tubaeformis* were assigned to either one or the other cluster, we next evaluated the ability of the microsatellite dataset to distinguish between multiple hybridization events. The program Hybridlab (Nielsen et al. 2006) simulates crosses between populations by calculating the allele frequencies and by randomly drawing one allele at each locus of each parental populations defined. We employed all individuals from pure stands of *T. tubaeformis* and *T. rotundifolia* to generate 50 simulated hybrid genotypes from each of the following categories: (a) First-generation hybrids ($F1$), (b) backcrosses (BC) of $F1$ with *T. rotundifolia*, and (c) BC of $F1$ with *T. tubaeformis*. We only intended to identify these hybrid classes because of the low number of polymorphic loci found for *T. tubaeformis* and *T. rotundifolia*. Then, we applied Bayesian clustering analysis to the simulated dataset using STRUCTURE. Ten replicates were made with $K = 2$ with a burn-in period of 20,000 and 300,000 MCMC. The results of all five runs were merged in CLUMPP and the mean q values were obtained for each hybrid category. $F1$ hybrids are expected to have a q value of 0.5, while backcrosses are expected to have q values of 0.75 or 0.25. Lastly, pure parental individuals are expected to have q values higher than 0.80 (Vähä and Primmer 2006; Henriques et al. 2016). We employed a cutoff of $q = 0.9$ to assign pure *T. tubaeformis* or *T. rotundifolia* individuals in order to avoid incorrect assignments of "pure" individuals as hybrids (Vähä and Primmer 2006). With these analyses, we intended to assign individuals in the real dataset as hybrids or pure parental individuals. Then, we used these labels to study the chemical profile of all individuals in pure and mixed stands.

Secondary metabolite analysis

We initially isolated the most abundant and marker compounds for each species, one for *T. tubaeformis* (**1**), and two for *T. rotundifolia* leaves' (**2a**, **2b**); these compounds can clearly distinguish each species by thin-layer chromatography (TLC) analysis after heating and spraying the developed plate (Silica Gel 60) with an oxidizing solution (cerium sulfate 1% in sulfuric acid 2N).

In order to identify the most abundant and marker compounds for each species, we initially pooled the leaves of several individuals of each species collected at pure stands. The leaves of *T. tubaeformis* or *T. rotundifolia* were dried at room temperature, grinded to a fine powder, and extracted three times with a mixture of hexane-CH_2Cl_2–MeOH (1:1:1). Next, the extracts of each species were pooled, filtrated and concentrated under vacuum with a rotary evaporator. Extracts of *T. tubaeformis* (27 g) and *T. rotundifolia* (1 g) were subjected to column chromatography (Silica Gel 60, mesh 70–230, Merck) eluting with hexane, and mixtures of increasing polarity of hexane + ethyl acetate. All fractions were analyzed through TLC.

Isolation of marker compound from *Tithonia tubaeformis*

Fractions 176–232 eluted from column chromatography with hexane–EtOAC (55:45) afforded an abundant (170 mg) yellow solid, with Rf = 0.65 in TLC (mobile phase: CH_2Cl_2-acetone 3:1), which revealed as a blue spot under UV light (254 nm) and orange after spraying with the cerium sulfate solution. The isolated compound showed an identical ^{1}H-NMR and mass spectra with those reported for the flavonoid tithonine (compound **1**; Fig. 1) (Rao et al. 2002) previously isolated from *T. tubaeformis* (Correa and Cervera 1971), *T. brachypappa*, and *T. thurberi* (La Duke 1982).

Isolation of marker compounds from *Tithonia rotundifolia*

Fractions 131–132 eluted from column chromatography with hexane–EtOAC (50:50) yielded crystalline incolorous needles (15 mg), with Rf = 0.49 in TLC (mobile phase: CH_2Cl_2-acetone 3:1). The isolated compound showed an identical ^{1}H-NMR and mass spectra with those of the sesquiterpene lactone 3-acetyl-15-hydroxyleptocarpin (compound **2a**; Fig. 1) previously isolated from *T. rotundifolia* (Pérez et al. 1984). Fractions 133–134 eluted with hexane–EtOAC (25:75) yielded a colorless resin (10 mg), showing an Rf = 0.27 in TLC (mobile phase: CH_2Cl_2-acetone 3:1). Its ^{1}H-NMR spectrum matched that of 15-hydroxyleptocarpin (compound **2b**; Fig. 1) previously isolated from *T. rotundifolia* (Pérez et al. 1984). Both sesquiterpene lactones (**2a**, and **2b**) revealed as gray spots after treatment with the cerium sulfate solution.

Once the marker metabolites of each parental species were identified and corroborated as easily identifiable through TLC, each individual plant collected at the mixed

stands was analyzed. For this purpose, 2 g of grinded leaves of each plant was extracted three times using 25 mL of a mixture of hexane-CH_2Cl_2-MeOH (1:1:1). The solvent mixture was eliminated in a rotary evaporator, the concentrated extract was diluted to a final concentration of 25 mg/mL, and 3uL were applied to TLC plate, eluting with CH_2Cl_2-acetone (3:1) as mobile phase. Extracts of parental species and pure compounds were applied at the same concentration, TLC plates were revealed with UV light, and the cerium sulfate solution, where the presence/absence of each metabolite was recorded. If a compound different than **1**, **2a** or **2b** was detected by TLC in admixed individuals, it was isolated through preparative TLC using chloroform–methanol (96:4) as mobile phase. Then, its structure was elucidated through ^{1}H-NMR and electron impact mass spectrometry (EI-MS).

Results

Genetic structure of *Tithonia tubaeformis* and *T. rotundifolia* pure stands

The microsatellite dataset showed no evidence of large allele dropout or stuttering. *Tithonia rotundifolia* microsatellite loci showed no evidence of null alleles. On the other hand, *T. tubaeformis* showed no evidence of null alleles except for locus Ha357 (frequency = 0.10). However, populations of both species conform to Hardy–Weinberg expectations overall loci (Table 2). We found no evidence of linkage disequilibrium between any loci pair, and thus, all five loci were retained in subsequent analyses. *Tithonia rotundifolia* showed higher estimates of genetic diversity (Na = 7.0, $H_O = 0.75$) than *T. tubaeformis* (Na = 5.4, $H_O = 0.68$). The microsatellite dataset was able to distinguish between the populations of both species; pairwise F_{ST} values between *T. rotundifolia* and *T. tubaeformis* were estimated as $F_{ST} = 0.139$ (uncorrected, $P < 0.05$), $F_{ST} = 0.131$ (corrected, $P < 0.05$) and $R_{ST} = 0.87$ ($P < 0.05$). Also, PCoA separated both species' individuals into discrete groups with minimal overlap between them (Fig. 3). The first two principal coordinates explained up to 31.26% of the variation in the original dataset.

Assignment of hybrid and parental categories

Bayesian clustering analysis implemented in STRUCTURE of pure and mixed stands of *Tithonia tubaeformis*

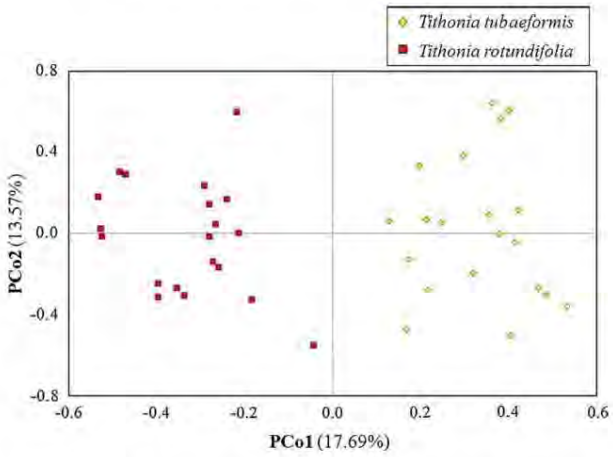

Fig. 3 Principal coordinate analysis based on genetic distances of *Tithonia tubaeformis* and *T. rotundifolia* pure stands using five microsatellite nuclear loci

Table 2 Estimates of genetic diversity of *Tithonia tubaeformis* and *T. rotundifolia*

Species	Locus	*Na*	Allele size (bp)	*NAl*	H_E	H_O	F_{IS}
Tithonia tubaeformis	Ha49	5	203–211	0	0.79	0.77	0.05
	Ha80	6	165–177	0	0.75	0.68	−0.07
	Ha140	5	133–141	0.001	0.6	0.63	0.07
	Ha357	6	120–134	0.1	0.5	0.71	**0.32**
	Ha360	5	158–186	0	0.6	0.64	0.09
	TOTAL	5.4			0.64	0.68	0.09
Tithonia rotundifolia	Ha49	8	175–189	0	0.95	0.83	−0.11
	Ha80	6	145–183	0	0.8	0.65	−0.21
	Ha140	7	139–157	0.01	0.75	0.78	0.12
	Ha357	7	80–94	0.06	0.65	0.76	0.17
	Ha360	7	170–190	0	0.75	0.74	0.01
	TOTAL	7			0.78	0.75	0.003

N number of individuals, *Na* number of alleles, *NAl* frequency of null alleles, H_E expected heterozygosity, H_O observed heterozygosity, F_{IS} fixation index

Significant departures from Hardy–Weinberg expectations are indicated in bold, $P < 0.05$. All five loci were assessed in 20 individuals for each parental species

and *T. rotundifolia* showed that $K = 2$ (Online Resource 1) with both clusters represented entirely by individuals of one species or the other. All individuals of pure stands of *T. rotundifolia* and *T. tubaeformis* had values of $q > 0.9$ and thus were considered as pure individuals. However, all mixed populations included both pure and admixed individuals (Fig. 4). Bayesian analysis in mixed populations identified 35% of admixed individuals in Corral de Piedra, 40% in Teloloapan, 45% in Huautla and 57% in Zacacoyuca. Also, pure parental individuals of both species were found in all, but one population (Teloloapan), where no pure *T. tubaeformis* individuals were identified.

Following STRUCTURE analysis, simulated *F*1 hybrid genotypes generated in HybridLab showed $q = 0.49 \pm 0.01$ (mean ± SD), BC toward *T. rotundifolia* showed $q = 0.26 \pm 0.02$ and BC toward *T. tubaeformis* $q = 0.73 \pm 0.02$ (Fig. 5). Due to the overlap between the q values of the simulated hybrid categories, microsatellite dataset was not able to fully discriminate between hybrid categories (Fig. 5). Thus, assignment of individuals with q values between 0.27 and 0.67 is equivocal, either representing *F*1 or BC individuals. However, BC toward *T. rotundifolia* showed no overlap with other hybrid categories between q values of 0.68 and 0.89. In the same way, BC toward *T. tubaeformis* showed no overlap with other categories between q values of 0.11 and 0.28 (Fig. 5). Thus, individuals in mixed and pure stands were labeled as either pure *T. rotundifolia* or *T. tubaeformis*, "hybrids" (when assignment to any hybrid category is equivocal), "BC toward *T. rotundifolia*" or "BC toward *T. tubaeformis*" (Table 3). We found that the localities of Huautla and Teloloapan showed higher percentages of BC toward *T. rotundifolia* (15 and 20%, respectively), while localities Zacacoyuca and Corral Piedra showed higher percentages of BC toward *T. tubaeformis* (35 and 10% respectively). This labels were used in secondary metabolite analysis (Table 3).

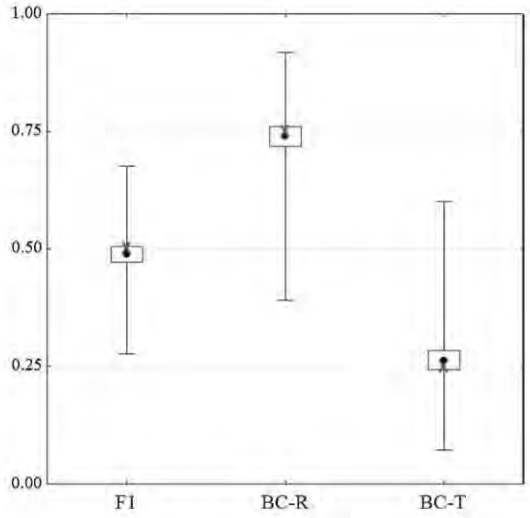

Fig. 5 Clustering results of simulated data of hybrids between *Tithonia tubaeformis* and *T. rotundifolia* after Bayesian analysis implemented in STRUCTURE. Vertical axis denotes q values relative to *T. tubaeformis* ancestry. Boxes represent the standard error of the q values for the simulated data and whiskers the range of the data. Green crosses indicate expected q values for each hybrid category. *F*1 = *T. tubaeformis* × *T. rotundifolia*, BC-R = F1 × *T. rotundifolia*, BC-T = *F*1 × *T. tubaeformis*

Secondary metabolite analysis

TLC analysis of individuals collected at pure stands revealed that all *T. tubaeformis* individuals exhibited compound **1**, while all *T. rotundifolia* individuals exhibited both compounds **2a** and **2b** (Fig. 6). All three compounds were easily identified through TLC (Fig. 6) and thus were used as diagnostic markers for each species. Each species exhibit a different and predominant active secondary metabolism pathway. In the case of *T. tubaeformis*, the phenylpropanoid pathway leads to the biosynthesis of the major flavonoid thitonine (**1**). On the other hand, in *T. rotundifolia*, the mevalonic acid pathway leads to the biosynthesis of two abundant

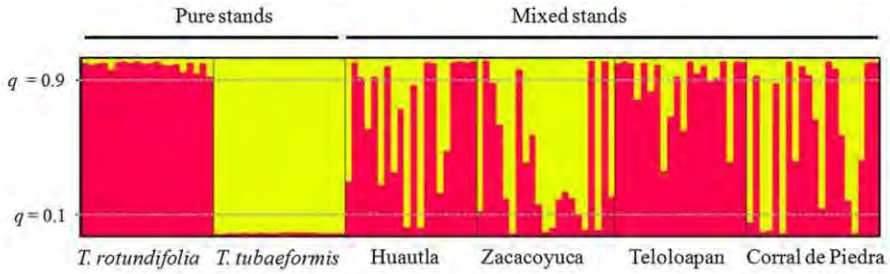

Fig. 4 Bayesian clustering of individuals of pure (20 individuals for each species) and mixed stands (20 individuals per mixed stand) of *Tithonia tubaeformis* and *T. rotundifolia* based on five nuclear microsatellite markers using STRUCTURE ($N = 120$). Each vertical bar represents an individual, and each population is separated by a black vertical line. Dashed line represents the cutoff value of pure species individuals ($q = 0.1$ and 0.9)

Table 3 Percentage of parental and admixed individuals identified after Bayesian analysis in four mixed stands of *Tithonia tubaeformis* and *T. rotundifolia* in Mexico

Mixed stand	Pure parental individuals (%)		Admixed individuals (%)		
	T. tubaeformis	*T. rotundifolia*	Hybrids	BCT	BCR
Huautla	10	45	25	5	15
Zacacoyuca	25	20	15	35	5
Teloloapan	0	60	20	0	20
Corral de Piedra	25	40	20	10	5

Hybrids admixed individual with equivocal assignments, *BCT* and *BCR* putative backcrosses toward *T. tubaeformis* and *T. rotundifolia* identified after comparing their *q* values with those obtained through simulations

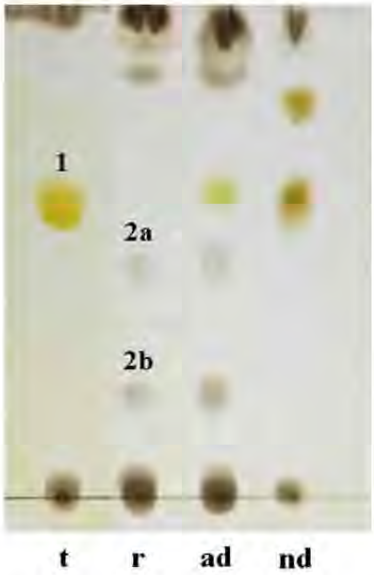

Fig. 6 Thin-layer chromatography [CH_2Cl_2-acetone (3:1) as mobile phase] of crude extracts of *Tithonia tubaeformis* (t), *T. rotundifolia* (r), and hybrids with additive (ad) and new (nd) chemical profiles. **1** = tithonine, **2a** = 3-acetyl-15-hydroxyleptocarpin, **2b** = 15-hydroxyleptocarpin, **3** = 5, 3′,4′-trihydroxy-6,7,8-trimethoxyflavanone. See text for details

sesquiterpene lactones with 15-hydroxyleptocarpin skeleton (**2a**, **2b**).

TLC analysis of mixed stands revealed that individuals identified as either pure *T. tubaeformis* or *T. rotundifolia* by the genetic analysis showed the characteristic chemical markers of the pure species, either compounds **1** (flavonoid), or **2a** + **2b** (sesquiterpene lactones), respectively (Table 4, Fig. 6). All plants genetically determined as "BC toward *T. rotundifolia*" exhibited the predictable sesquiterpene lactones **2a** + **2b**. Most individuals identified as "BC toward *T. tubaeformis*" showed the presence of the flavonoid **1** as expected. However, six plants at Zacacoyuca exhibited a new chemical profile (ND) composed by two yellow spots with Rf = 0.75 and 0.65, the former was isolated through preparative TLC and its structure was elucidated (see below), while the latter is similar to tithonine (**1**), although darker under UV light and with cerium sulfate solution (Table 4, and Fig. 6).

Table 4 Percentage (number) of individuals with compounds **1**, **2a**, **2b** and new chemical profile (ND) for hybrid and parental individuals of *Tithonia tubaeformis* and *T. rotundifolia* identified after the genetic analysis in four mixed stands in Mexico (1 = tithonine, 2a = 3-acetyl-15-hydroxyleptocarpin, 2b = 15-hydroxyleptocarpin, ND = new chemical profile. A total of 20 individuals at each mixed stand were analyzed

Genetic assignment	Chemical profile			
	1	**2a + 2b**	**1 + 2a + 2b**	**ND**
Huautla				
T. tubaeformis	100 (2)	–	–	–
T. rotundifolia	–	100 (9)	–	–
Hybrid	60 (3)	–	40 (2)	–
BC-tubaeformis	100 (1)	–	–	–
BC-rotundifolia	–	100 (3)	–	–
Zacacoyuca				
T. tubaeformis	100 (5)	–	–	–
T. rotundifolia	–	100 (4)	–	–
Hybrid	100 (3)	–	–	–
BC-tubaeformis	12.5 (1)	–	–	87.5 (6)
BC-rotundifolia	–	100 (1)	–	–
Teloloapan				
T. tubaeformis	–	–	–	–
T. rotundifolia	–	100 (12)	–	–
Hybrid	50 (2)	–	25 (1)	25 (1)
BC-tubaeformis	–	–	–	–
BC-rotundifolia	–	100 (3)	–	–
Corral de Piedra				
T. tubaeformis	100 (5)			
T. rotundifolia	–	100 (8)	–	–
Hybrid	75 (3)	25 (1)	–	–
BC-tubaeformis	100 (2)	–	–	–
BC-rotundifolia	–	100 (1)	–	–

Regarding plants genetically determined as hybrids, all individuals collected at Zacacoyuca showed the presence of compound **1**. In Huautla, 40% of individuals displayed an additive profile (**1** + **2a** + **2b**), indicating the synthesis of a mixture of parental compounds, while the remaining 60% of individuals produced compound **1**. In Teloloapan, 25% of individuals exhibited the additive profile, but the remaining hybrids had either compound **1** (50%) or the

new chemical profile ND (25%). Lastly, in Corral de Piedra, 75% of individuals synthesize **1,** while the remaining 25% produce compounds **2a** + **2b.** (Table 4, and Fig. 6).

The compound with Rf = 0.75 found in admixed individuals exhibiting the new chemical profile (ND) was isolated through preparative TLC (mobile phase: chloroform–methanol 96:4), yielding 2.5 mg of an orange resin. The ^{1}H-NMR spectrum ($CDCl_3$, 700 MHz) of this compound revealed a flavanone-like skeleton with signals attributable to the heterocyclic ring (C-ring) protons (Alavez-Solano et al. 2000): δ 2.85 dd, 1H, J = 2.8, 16.8 Hz, H-3 eq; 3.06 dd, 1H, J = 12.6, 17.5 Hz, H-3ax; 5.33 dd, 1H, J = 2.8, 12.6 Hz, H-2. Also, the ^{1}H-NMR spectrum showed signals for three methoxy groups (δ 3.79 [s, 3H], 3.85 [s, 3H], 4.08 [s, 3H]), and three aromatic protons (δ 6.89 and 6.87 [both dd, 1H each one, J = 8.4, and 2.1 Hz], 7.01 [*d*, 1H, J = 2.1 Hz]), as well as one signal attributable to a chelated hydroxyl group in position 5 of the A-ring (δ 11.76 [s, 1H]). EI-MS analysis showed the molecular ion peak at *m/z* 362, which corresponds to the molecular formula $C_{18}H_{18}O_8$. It also revealed fragments consistent with the retro Diels–Alder reaction. The peaks at *m/z* 226 (fragment A) and *m/z* 136 (fragment B) correspond to a cleavage in the C-ring of flavonoids at carbons 1,3. The peak at *m/z* 226 corresponds to the ion containing the A-ring with a chelated hydroxyl group at position 5 and three methoxy substitutions. The base peak was shown at *m/z* 211, which indicates the loss of a CH_3 group in [A]. Meanwhile, the peak at *m/z* 136 corresponds to the fragment containing the B-ring with two *ortho*-hydroxyl groups and three ABX coupled hydrogens. All in all, the compound may correspond to the undescribed 5,3′,4′-trihydroxy-6,7,8-trimethoxyflavanone (**3**). The ^{1}H-NMR and EI-MS spectra of **3** are similar to those of sideritoflavone isolated from *Baccharis patens* (Asteraceae) (Silva et al. 1985) and *Sideritis leucantha* (Labiateae) (Tomas et al. 1979), but compound **3** lacks the characteristic double bond of a flavone in the C-ring.

5,3′,4′-trihydroxy-6,7,8-trimethoxyflavanone. ^{1}H-NMR (700 MHz, $CDCl_3$) δ: 2.85 (dd, J = 2.8 and 16.8, 1H), 3.06 (dd, J = 12.6 and 17.5, 1H), 3.79 (s, 3H), 3.85 (s, 3H), 4.08 (s, 3H), 5.33 (dd, J = 2.8 and 12.6, 1H), 6.87 (dd, J = 2.1 and 8.4 Hz, 1H), 6.89 (dd, J = 2.1 and 8.4 Hz, 1H), 7.01 (d, J = 2.1 Hz), 11.76 (s, 1H). EI-MS *m/z* (%): 362 (71) M^+ [$C_{18}H_{18}O_8$], 226 (87) [$C_{10}H_{10}O_6$], 211 (100) [$C_9H_7O_6$], 183 (36), 136 (35) [$C_8H_8O_2$].

Discussion

Tithonia tubaeformis and *T. rotundifolia* are two composite annual species that have been suggested to hybridize in natural conditions (Hauser and Morrison 1964; Tovar-Sánchez et al. 2012). The purpose of this paper was to compare the patterns of hybridization revealed by nSSR and PSM with those obtained with dominant markers (RAPD) by Tovar-Sánchez et al. (2012). However, as outlined by several authors (e.g., Henriques et al. 2016), an important step in hybrid identification is the evaluation of the suitability of the nSSR dataset in distinguishing between the parental species as well as between multiple hybridization events (Henriques et al. 2016). Accordingly, we initially studied the genetic variation of pure stands of both *T. tubaeformis* and *T. rotundifolia*. Next, we evaluated the suitability of the nSSR dataset to distinguish between several hybrid generations. Finally, the qualitative chemical profiles of pure and admixed individuals identified through the genetic analysis were assessed.

Genetic variation of *Tithonia tubaeformis* and *T. rotundifolia* pure stands

Pure stands of both *Tithonia* species studied here showed high heterozygote frequencies overall loci (H_E = 0.64 and 0.78 for *T. tubaeformis* and *T. rotundifolia*, respectively; Table 2). Also, populations of both species showed no significant deviations of the Hardy–Weinberg equilibrium expectations overall loci. However, locus Ha357 showed a significant deficit of heterozygotes for *T. tubaeformis* (F_{IS} = 0.32). This finding can be partially explained by the presence of null alleles at this locus rather than selfing (Table 2). In this regard, both *T. tubaeformis* and *T. rotundifolia* have been suggested to be self-incompatible species (La Duke 1982; Muoghalu and Chuba 2005). Also, H_E values (0.64–0.78) found in pure stands of *Tithonia* sp. are similar to those reported in several outcrosser sunflower species using expressed sequence tag-SSR markers [0.58 for *Helianthus annuus* (Ellis et al. 2006) and 0.68 for *H. porter* (Gevaert et al. 2013)]. Some other characteristics of *T. tubaeformis* and *T. rotundifolia* that likely explain this high genetic diversity are insect pollination (such as *Apis mellifera*), zoochory, anthropochory, and a high reproductive effort (Muoghalu and Chuba 2005). Populations of *T. tubaeformis* and *T. rotundifolia* usually consist of several hundred of individuals with at least 5 inflorescences per plant that are pollinated by *Apis mellifera*, several Lepidoptera and other generalist pollinators (López-Caamal, personal observation). However, a detailed reproductive biology study for *T. tubaeformis* and *T. rotundifolia* is still lacking (but see Muoghalu and Chuba 2005).

Reproductive output for *Tithonia* species is usually high (ca. 17,000 achenes per plant of *T. rotundifolia*; Muoghalu and Chuba 2005). Also, due to their weedy habitat, *T. tubaeformis* and *T. rotundifolia* seem likely to be dispersed by means of anthropogenic activities as suggested for wild sunflowers (Asch 1993; Mandel et al. 2011). Altogether, these data suggest that these *Tithonia* species are obligate

outcrossers with high genetic flow between populations. However, whether populations of these species show high genetic flow and/or substructuring along their native range is currently under research.

Genetic characterization of admixed and parental individuals

We initially tested the ability of the microsatellite data to differentiate between individuals of both parental species. Pairwise population differentiation revealed high genetic structure between pure stands of *T. tubaeformis* and *T. rotundifolia* for both the infinite allele ($F_{ST} = 0.13$, $P < 0.001$) and the stepwise mutation model ($R_{ST} = 0.87$, $P < 0.001$). Striking differences between F_{ST} and R_{ST} values are accounted by large differences of nSSR allele sizes between species (Table 2).

Regarding hybrid identification, we found a similar pattern to that reported by Tovar-Sánchez et al. (2012) using RAPD data; however, some differences exist. For instance, these authors found no pure *T. tubaeformis* individuals in two stands (Huautla and Teloloapan) with low frequencies of backcrosses toward *T. tubaeformis* overall localities. Given these findings, Tovar-Sánchez et al. (2012) claimed that gene flow between *T. tubaeformis* and *T. rotundifolia* is mainly unidirectional toward the latter species, suggesting that some degree of incompatibility exists when *T. rotundifolia* acts as pollen donor. However, the present nSSR analysis showed that the gene flow direction is idiosyncratic. Localities Zacacoyuca and Corral de Piedra showed greater gene flow toward *T. tubaeformis*, while the remainder localities exhibited a higher percentage of backcrosses toward *T. rotundifolia*. Differences between RAPD and nSSR patterns are expected, however, given that codominant markers and simulations with the actual microsatellite data set of *T. tubaeformis* and *T. rotundifolia* were used (as suggested by several authors; Leong Pock Tsy et al. 2013; Henriques et al. 2016), we believe the patterns found in this study to be more reliable than previously reported.

Genetic identification of hybrids through SSR shows two major pitfalls: allele size homoplasy and the number of loci employed. Regarding allele size homoplasy, several researchers point out that SSR evolution is complex and some loci do not conform completely to the infinite allele (IAM) or stepwise mutation model (SMM) (van Oppen et al. 2000; Estoup et al. 2002). This suggests that SSR markers might not be suitable for describing hybridization unless conformance to SMM of SSR loci is proven (van Oppen et al. 2000). However, *T. tubaeformis* and *T. rotundifolia* exhibited striking differences in allele sizes, so although it may occur, allele size homoplasy between these species is not expected to significantly affect the observed patterns. Also, previous RAPD analysis shows some concordance with the present SSR analysis, suggesting that hybridization between these species takes place under natural conditions.

As outlined by Boecklen and Howard (1997), the power to infer genealogical classes of multilocus genotypes is dependent on the number of loci employed. When using five loci (as in the present study), the probability that a first-generation BC has alleles of only one species is 3%, whereas it is 24% for a second generation BC, 51% for a third generation BC, and so on (Boecklen and Howard 1997). This limitation in the number of loci may therefore cause a misinterpretation of late generation hybrids as pure parental individuals. Due to these caveats, we only intended to identify $F1$ and first-generation BC hybrid individuals between *T. tubaeformis* and *T. rotundifolia*. The identification of later generation hybrids may require the exploration of a significantly higher amount of nSSR loci (Vähä and Primmer 2006).

Besides the genetic evidence of hybridization between *T. tubaeformis* and *T. rotundifolia*, the ecological context that promotes interspecific gene flow is worth to mention. First, flowering times for *T. tubaeformis* and *T. rotundifolia* show a temporal overlap in November, so the potential for gene flow between these species exists (La Duke 1982). However, the phenological spatial heterogeneity in flowering times found throughout Mexico may limit hybridization between these species at a number of localities (López-Caamal, personal observation). Also, both *T. tubaeformis* and *T. rotundifolia* show differences in their habitat preferences; the former inhabits temperate regions (usually above 1000 m a. s. l.), while the latter does it at xeric tropical habitats (usually below 1000 m a. s. l.). Due to their weedy habit, these species meet at intermediate altitudes in heavily disturbed habitats where large populations establish (Muoghalu and Chuba 2005). The frequent disturbance due to human activities (i.e., agriculture) found throughout their range, may create habitats where individuals of both species establish, promoting mixed stands between *T. tubaeformis* and *T. rotundifolia*. Evidence suggests that hybridization between *T. tubaeformis* and *T. rotundifolia* is likely when individuals of both species coincide in space and time. However, the factors that promote the establishment of certain hybrid genotypes (BC toward *T. tubaeformis* or *T. rotundifolia*) are yet to be studied.

PSM profile of pure and hybrid individuals between *Tithonia tubaeformis* and *T. rotundifolia*

In this work, we aimed to determine the chemical profiles of pure and admixed *Tithonia* sp. individuals. Although both species can potentially synthesize flavonoids and sesquiterpene lactones, *T. tubaeformis* is characterized by producing mainly flavonoids, while *T. rotundifolia* is a sesquiterpene lactone producer (Pérez et al. 1984; Chagas-Paula et al. 2012). As a first step in this study, we isolated the major

constituents for both *T. tubaeformis* and *T. rotundifolia*; then, we evaluated their utility as marker compounds. In this regard, the expression of a number of PSM has been found to be plastic (Metlen et al. 2009). Biotic and abiotic pressures such as nutrient availability, herbivory or the presence of mutualists may influence the qualitative and quantitative variation of PSM (Metlen et al. 2009). Given these difficulties, we carefully explored the PSM composition of both *T. tubaeformis* and *T. rotundifolia*. We isolated their main constituents [the flavonoid tithonine (**1**) for *T. tubaeformis* and the sesquiterpene lactones 3-acetyl-15-hydroxyleptocarpin (**2a**) and 15-hydroxyleptocarpin (**2b**) for *T. rotundifolia*; Figs. 1 and 6] and evaluated their utility as marker compounds. We found that all individuals genetically determined as pure *T. tubaeformis* or *T. rotundifolia* at pure and mixed stands showed the characteristic marker compound of that species. This finding suggests that, although quantitative variation among individuals may exist, the presence/absence of compounds **1**, **2a** and **2b** is a diagnostic character of *T. tubaeformis* and *T. rotundifolia* regardless of the environmental pressures. Flavonoids and sesquiterpene lactones are produced by different biosynthetic routes [phenylpropanoid (Falcone Ferreyra et al. 2012) or mevalonic acid (Padilla-González et al. 2016) pathways, respectively]; therefore, the presence/absence of these compounds in admixed individuals may reveal the stimulation of certain metabolic pathways as the result of hybridization.

Both flavonoids and sesquiterpene lactones are important PSM with a number of biological functions (Padilla-González et al. 2016). Flavonoids have a number of roles in plant protection; they provide tolerance to high UV irradiation, low temperatures (Clé et al. 2008; Olsen et al. 2009), nitrogen starvation (Løvdal et al. 2010), and act to deter herbivory (Gould and Lister 2006). The high production of the flavone tithonine by *T. tubaeformis* may be an adaptation/response to the habitat where it establishes. This species is mainly adapted to open areas of temperate montane forests where fully exposure to sunlight is frequent. Also, *T. tubaeformis* is considered an ancestral species within series *Tithonia* (La Duke 1982) with a stock of flavonoids that exhibit diverse chemical structures (chalcones, flavonols, flavones, aurones; La Duke 1982). On the other hand, *Tithonia rotundifolia*, the most derived species within the genus, exhibits a loss in flavonoid diversity and complexity (glycosylated flavonols) with high interpopulation variation in flavonoid content (La Duke 1982). Instead, this species primarily produces sesquiterpene lactones (Pérez et al. 1984). Sesquiterpene lactones have been recognized by their protective activity against insect herbivory by either impairing herbivore development or affecting insect mortality (Padilla-González et al. 2016). Also, these compounds have a phytotoxic activity toward a number species (Padilla-González et al. 2016). *Tithonia rotundifolia* is associated with tropical dry forests with a marked rainy and dry season. Increased competition for light and herbivory is common in the rainy season, so a high sesquiterpene lactone production in this species may be an important adaptation.

While pure parental individuals were fixed either with the presence of tithonine flavonoid (**1**) or sesquiterpene lactones (**2a**, **2b**), admixed individuals between *T. tubaeformis* and *T. rotundifolia* exhibited a diverse array of PSM content (Table 4). Although hybrids were initially considered to show an additive inheritance of parental PSM (Rieseberg and Ellstrand 1993), recent research shows that hybrid expression is complex and far from intermediate (e.g., Orians 2000; Kirk et al. 2004). In this study, we found that admixed individuals between *T. tubaeformis* and *T. rotundifolia* showed a mosaic of PSM expression; three out of 34 admixed individuals showed additivity. Backcross individuals exhibited the characteristic chemical profile of the species with which they share more genetic similitude (i.e. dominance), and also, a new chemical profile was found in some individuals labeled as BC toward *T. tubaeformis*. These results are in accordance with previous reports of secondary metabolite expression in hybrids (i.e., Orians 2000), where a high qualitative variation in PSM expression in admixed individuals has been reported.

The presence of admixed individuals with an additive pattern of *T. tubaeformis* and *T. rotundifolia* PSM suggests that certain metabolic pathways in hybrids may be stimulated. Flavones and flavonols share the precursor naringenin (a flavanone), though flavone synthesis requires FSI and FSII (flavone synthase I and II; Winkel-Shirley 2001). On the other hand, sesquiterpene lactone production relies on the enzyme COS (costunolide synthase) to generate costunolide, a central molecule in sesquiterpene lactone biosynthesis (Padilla-González et al. 2016). The presence of both the flavone tithonine and the sesquiterpene lactones **2a** and **2b** in hybrid individuals suggests the inheritance of genes coding for specific enzymes in the phenylpropanoid and mevalonic acid pathways. However, the low PSM additivity found in *Tithonia* hybrids suggests that these individuals exhibit a fitness reduction due to disruption of coadapted gene complexes (Arnold 2006).

The presence of compounds not identified in the parental species indicates additional modification of metabolic routes. In this study, we found that some individuals genetically determined as BC toward *T. tubaeformis* exhibited a new chemical profile (ND). The isolation and spectroscopic analysis of this chemical profile through ^{1}H-NMR and EI-MS allowed the identification of a compound with a flavanone skeleton (**3**; Fig. 6). The relevance of this finding is twofold. First, flavanones were unreported within *Tithonia*, where the presence of flavones, flavonols, chalcones and aurones is known (see La Duke 1982; Chagas-Paula et al. 2012). Second, as noted by Orians (2000), the presence of

a new compound in hybrid individuals may reflect the elongation or the obstruction of a biosynthetic pathway. During flavonoid biosynthesis, flavanones are the precursors of both flavonols and flavones (Winkel-Shirley 2001). Whether the presence of the flavanone is the result of an obstruction of the metabolic pathway leading to its accumulation (due to disruption of enzymatic complexes FSI and FSII) or a result of the environmental pressure in which the admixed plants grow is in need of further research. However, the mosaic in PSM denotes that hybridization leads to chemical novelty in *Tithonia*. In the same way, López-Caamal et al. (2013) found morphological novelty in mixed stands of *T. tubaeformis* and *T. rotundifolia*.

Hybridization between *T. tubaeformis* and *T. rotunidifolia* may have a number of ecological and evolutionary implications. For instance, both species exhibit a weedy habitat, establishing large populations at disturbed habitats. Due to the frequent disturbances (agriculture, road construction) found throughout their native range, the opportunities for sympatry between these species are increasing. If hybrids displaying phenotypic novelty are frequent, they may impact the herbivore distribution due to their novel PSM chemical profiles. Human activities may also be affected by hybrids; *T. tubaeformis* and *T. rotundifolia* establish in roadsides and croplands. Specifically, both species aggressively colonize the edges of crops such as *Zea mays*. Phytotoxic activity for both *T. tubaeformis* and *T. rotundifolia* has been detected (Juárez and Cazón 2003; Otusanya and Ilori 2014), so the exploration of phytotoxic activities and fitness of *Tithonia* hybrids are in need of research.

Tithonia tubaeformis and *T. rotundifolia* have been introduced outside their native range where they have developed as serious invaders in a number of countries (Juárez and Cazón 2003; Muoghalu and Chuba 2005). In their exotic range, *T. tubaeformis* and *T. rotundifolia* displace native species and disrupt human activities. Given the phenotypic novelty arising from hybridization between *T. tubaeformis* and *T. rotundifolia*, hybrid genotypes with a greater invading capacity compared with the parental species may occur. So, hybridization between *T. tubaeformis* and *T. rotundifolia* should be prevented in their exotic range.

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Compliance with ethical standards

Conflict of interest The authors declare there is no conflict of interest.

Information on Electronic Supplementary Material

Online Resource 1. ΔK, mean and standard deviation of LnP(K), and Ln'(K) values for each K simulated with Bayesian clustering in STRUCTURE as calculated in Structure Harvester following the Evanno et al. (2005) method for mixed stands of *Tithonia tubaeformis* and *T. rotundifolia*.

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CAPÍTULO III

Species distribution modelling and cpSSR reveal population history of the Neotropical annual herb *Tithonia rotundifolia* (Asteraceae)

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ABSTRACT

Premise of the study: The impacts of the geological events and Pleistocene climatic fluctuations have been studied recently in several Neotropical taxa. However, the geographic structure of annuals associated to tropical dry forests has been seldom studied. Such studies will enable a better understanding of the historical climatic oscillations in the Neotropics. In this paper, we explored the geographic structure and population history of the annual herb *Tithonia rotundifolia*.

Methods: We sampled a total of 175 individuals from 19 populations of *T. rotundifolia*. Species distribution modelling and six microsatellite chloroplast loci were used to infer its population history. We identified areas of historical climatic suitability and then, we tested if genetic structuring among these areas occurs.

Key results: Haplotypes showed strong phylogeographical structure. Historical climatic suitability areas were found along the Pacific coast, however, a gap was found at the Isthmus of Tehuantepec (IT). Although Bayesian analysis showed population structuring, AMOVA revealed that the IT is not its main driver. Instead, a subdivision into a higher number of regions showed higher F_{CT} values. Also, populations at the east of the IT showed evidence of recent population expansion and migration in a south-north direction.

Conclusions: Pleistocene climatic fluctuations partially explain the geographic structure of *T. rotundifolia*. However, life-history characteristics such as limited seed dispersal and the patchy distribution of suitable habitats explain the high haplotype diversity and population substructuring and diversity. Lastly, the absence of geographic structure of some haplotyes may indicate long distance dispersal, or hybridization with the closely related *T. tubaeformis*.

Keywords: Isthmus of Tehuantepec; Mesoamerica; Mexico; phylogeography; Pleistocene climatic change; tropical dry forest

INTRODUCTION

The Neotropics are among the most biologically rich regions of the world with nearly 90 000 flowering plant species (Kier et al., 2009; Antonelli and Sanmartín, 2011). This high level of biodiversity is the result of a complex geological and climatic history (Gutiérrez-García and Vázquez-Domínguez, 2013), rendering the Neotropics a very interesting area to study evolutionary processes such as migration and extinction. The northern limit of the Neotropical region, comprised by southern Mexico and Central America, has been the focus of a number of population genetic and phylogeographical studies of a diverse array of taxonomic groups (Tovar-Sánchez et al., 2008; Gutiérrez-Rodríguez et al., 2011; Twyford et al., 2013; Ornelas et al., 2013; Chávez-Pesqueira and Núñez-Farfán, 2016; Rodríguez-Correa et al., 2017). These studies have shown that the partition of genetic diversity is the result of complex geological and climatic changes during the Pleistocene (mainly as the result of glacial ages) and the Neogene – Paleogene geological events. Although some general patterns have been deduced, the response of the biota to the climatic/geological fluctuations seems to depend on the life-history traits of each species (e.g. Twyford et al., 2013). In order to identify common patterns, additional studies integrating molecular, ecological and paleoclimatic data of species distributed along the Neotropics are needed (Ramírez-Barahona and Eguiarte, 2013).

The patterns of genetic diversity in the Neotropics have been influenced by the appearance of physical barriers derived from the intense pre-Pleistocene geological activity. Although no consensus of these barriers have emerged, some studies show strong support for the Isthums of Tehuantepec (IT), the Nicaraguan Depression (ND), the Polochic-Motagua-Jocotán (PMJ), and the Isthums of Panama (IP) as elements that participate in the phylogeographic structure in a number of Neotropical taxa (Gutiérrez-García and Vázquez-Domínguez, 2013 and references therein; Rodríguez-Correa et al., 2017). The IT, a portion of lowlands that separates

the south-central Mexican highlands from the highlands of Chiapas, has been continuously described as an important barrier to gene flow for several species (Ornelas et al., 2013). Following its formation (ca. 6 Mya), the IT experienced marine incursions during the Pliocene and the Pleistocene (Morrone, 2006). As a result, populations at either side of the IT show significant genetic structure (Ornelas et al., 2013).

In addition to the physical barriers to gene flow, the climatic fluctuations during the Pleistocene have influenced significantly the distribution of biota and the partitioning of their genetic diversity (Hewitt, 2000). However, the paleoecological and molecular data yields contrasting scenarios of the effects of climatic fluctuations on the Neotropical biota during the Pleistocene glaciations (Ramírez-Barahona and Eguiarte, 2013). In this regard, some authors consider that rainfall was not significantly affected during these climatic fluctuations (Van Der Hammen, 1961), so tropical forests have gone relatively unaffected by climatic variability during the Pleistocene (moist forest hypothesis, Ramírez-Barahona and Eguiarte, 2013; Fine and Ree, 2006). On the other hand, some authors consider that glaciations affected rainfall, leading to drier and cooler conditions in areas previously occupied by tropical forests. As a result, tropical forests were drastically compressed during the Last Glacial Maximum (LGM, 23 – 18 Kyr BP), surviving in refugia with warmer and wetter conditions (dry refugia hypothesis) (Toledo, 1982). Interestingly, evidence suggests that grasslands and tropical deciduous forests expanded at the expense of tropical forests. This expansion of tropical deciduous forests during the LGM is supported by empirical and palinological evidence (Toledo, 1982; Caetano et al., 2008; Pennington et al., 2009; Poelchau and Hamrick, 2011). So, if tropical deciduous forests expanded during the LGM, it may be expected that large stability areas arose during this time, reducing genetic structuring among populations in areas with a constant climatic suitability during the Pleistocene.

Recent research in plant population history in the Neotropics focuses on montane temperate trees such as oaks and conifers (Jaramillo-Correa et al., 2008; Jardón-Borbolla et al., 2011), cloud forest (Twyford et al., 2013, Ornelas et al., 2013) or tropical forest biota (Chávez-Pesqueira and Núñez-Farfán, 2016). However, less attention has been paid to the tropical deciduous forest vegetation in the northern Neotropics (but see Arias et al., 2010). Also, compared to perennials, the spatial genetic structure of annual plants has been less studied. Due to their life history traits, annual plants show some characteristics that have a strong effect on their genetic diversity (Hamrick and Godt, 1996). Particularly, the low mutation rate found in annuals (Linhart, 2000) and their short generation time may produce different structure and levels of genetic diversity compared to perennial species. In addition, the failure to reproduce in a year due to environmental adversity is usual in annuals, so a higher number of bottlenecks and a stronger genetic structure are expected compared to perennials. Given these characteristics, the study of the genetic variation of widespread annual plants distributed throughout the tropical deciduous forests will contribute to our overall understanding of the climatic fluctuations during the Quaternary in the northern Neotropics.

Tithonia rotundifolia (Mill.) S.F. Blake (Asteraceae) is an annual herbaceous species native to Mexico and Central America (La Duke, 1982). *Tithonia rotundifolia* is easily recognized by its moderately pubescent 3 – 5 lobed leaves, lanceolate phyllaries and orange ligules, however, some Central American populations exhibit yellow ligules (La Duke, 1982). This species exhibits a weedy habitat, establishing large populations at croplands and roadsides associated to tropical deciduous forests mainly at the Pacific coast of Mexico and Central America. Although no detailed reproductive biology studies of *T. rotundifolia* are available, it has been suggested to be an autoincompatible species (Muoghalu and Chuba, 2005; López-Caamal et al., 2018). Also, the large achenes (11.5 × 2.45 mm; López-Caamal et al., 2013) and the presence

of two large awns suggest either epianthropochory (Vibrans, 1999) or barochory, which partially explain the patchy distribution of its populations. In this study, we employ chloroplast microsatellites (cpSSR) and species distribution modelling (SDM) to assess the spatial distribution of genetic variation along the native range of *T. rotundifolia*. Also, we infer the demographic history of its populations through neutrality tests and we intended to explain the causes of the population structuring due to the geographical and/or the climatic fluctuations during the Pleistocene. Through SDM, we aim to identify areas of historical climatic stability for *T. rotundifolia*. Once identified, we expect a high genetic differentiation among these areas due to the limited seed dispersal ability of *T. rotundifolia*. Specifically, we ask whether the IT have left a genetic signature in populations of *T. rotundifolia*.

MATERIALS AND METHODS

Plant collection, DNA extraction and cpSSR amplification—We collected young and undamaged leaves from 175 individual plants of *T. rotundifolia* in its native range (Figure 1, Table 1). Foliar tissue was stored in liquid nitrogen (for plants collected in Mexico) or was silica-gel dried (Central American plants) until DNA extraction. A total of six to 15 plants were collected in each of 19 localities (hereafter referred as "populations"). All sampled populations were monospecific and no other *Tithonia* related species was found.

DNA extraction was carried out following a modified version of the cetyl trimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). DNA was diluted to a final concentration of 10 ng/μL and a subset of individuals were used to test polymorphism using 12 pairs of mononucleotide cpSSR universal primers (ccmp1, ccmp2, ccmp3, ccmp4, ccmp6, ccmp7, ccmp10, NTCP2, NTCP4, NTCP9, NTCP13, and NTCP18; Bryan et al., 1999; Wills et al., 2005). Reactions were carried out in a volume of 10 μL following the conditions described by

Wills et al. (2005). Six primer pairs yielded reproducible bands and showed polymorphic fragments in at least 30% of individuals: ccmp1, ccmp2, ccmp4, ccmp7, NTCP9 and NTCP18. Forward primers were then labelled with either HEX, 6-FAM or NED fluorophores and PCR reactions were carried out in a volume of 8 μL following Wills et al. (2005). Fragment analysis of PCR products was carried out in an ABI 3500 (Applied Biosystems, Foster City, California, USA) automatic sequencer using GeneScan-500 ROX as internal size standard. Finally, allele scoring was carried out in GeneMarker version 2.7.0 (Softgenetics, Inc., USA).

Genetic analyses—Haplotypes were defined as a unique combination of alleles for the six cpSSR employed. In order to characterize the genetic diversity of each *T. rotundifolia* population, we recorded the number of haplotypes and the number of private haplotypes. The haplotypic richness corrected for sample size and the Nei's unbiased genetic diversity index (Nei, 1973) were estimated with the Haplotype Analysis software ver. 1.05 (Eliades and Eliades, 2009). Also, the mean within-population (h_S) and total gene diversity (h_T) were calculated with cpSSR (Pons and Petit, 1996). Lastly, the average distances among individuals within populations following a stepwise mutation model (Morgante et al., 1998; Goldstein et al., 1995) was calculated. These parameters were estimated for each population, and also, we estimated them in a regional level, separating populations East and West of the Isthums of Tehuantepec (IT).

In order to identify signs of population demographic expansion, we calculated the F_S statistics (Fu, 1997). A population with a recent demographic expansion shows a star-shaped genealogy, which shows short internodes and long terminal branches (Slatkin and Hudson, 1991). A large negative value of the F_S statistics indicates sudden population growth. To perform F_S calculations in ARLEQUIN, cpSSR were coded as binary data: the number of repeats of the largest variants were coded with '1's, while shorter variants were replaced by '0's (as in Heuertz

et al., 2010). The existence of phylogeographic structure within populations of *T. rotundifolia* was inferred by testing for significant differences between G_{ST} and R_{ST}. R_{ST} takes into account genetic distances between haplotypes, so a significant value of $R_{ST} > G_{ST}$ indicates that phylogenetically related haplotypes occur more often within populations than expected by chance (Pons and Petit, 1996). Genetic differentiation indices (G_{ST} and R_{ST}) for populations east and west of the IT were also calculated. These tests were carried out as implemented in CpSSR 2.0 (Pons and Petit, 1996) after 1000 permutations.

Bayesian analysis of population structure implemented in BAPS ver 6.0 (Corander et al., 2008a) was used to explore the geographical structure of the genetic variation of *T. rotundifolia*. We preferred this Bayesian approach rather than STRUCTURE because BAPS incorporates spatial information of sampled localities. So, under a spatial model, the genetic structure is estimated supposing that the structure of a certain population depends on that of adjacent areas. We performed a spatial genetic mixture analysis with linked loci (Corander et al., 2008b) and the numbers of groups (K) was set to 2 – 19. Five replicates were performed for each K value.

In order to determine the relationships between haplotypes, haplotype connections lengths were calculated in ARLEQUIN ver 3.5.2.2 (Excoffier et al., 2005), and the resulting minimum spanning network was visualized in GEPHI ver 0.9.2 (Bastian et al., 2009) using the Force-Atlas force-based algorithm.

The Monmonier's maximum difference algorithm implemented in BARRIER ver. 2.0 (Manni et al., 2004) was used to infer the geographical location of the main genetic discontinuities between populations. BARRIER creates a map where each collecting site is connected with Delaunay triangulation and then, the Voronoi tessellations are projected. The Monmonier's maximum difference algorithm identifies a barrier along the Voronoi tessellations when provided with a pairwise distance matrix of sampled populations. For this purpose, we

employed the Goldstein's genetic distance ($\delta\mu^2$) matrix (Goldstein et al., 1995). In order to provide significance of the identified barriers, 100 bootstrapped matrices were computed using Microsatellite Analyser software (Dieringer and Schlötterer, 2003). The number of barriers identified by the software was set from 1 to 5. We only reported barriers with a support > 45%.

In order to test for different historical scenarios of population structuring of *T. rotundifolia*, we performed several analyses of molecular variance (AMOVA). First, populations were treated as a single group, revealing how the genetic variation is partitioned among and within populations. Then, in order to test for the historical scenario of the IT acting as a dispersal barrier for *T. rotundifolia*, we performed a hierarchical AMOVA in which populations were grouped either east or west of the IT. This allowed us to test the proportion of Φ_{ST} accounted by the IT and also, to describe the way the genetic variability is partitioned among populations within groups and within populations (Excoffier et al., 2005). Lastly, we performed an additional AMOVA in which populations were grouped according to the genetic discontinuities identified by BARRIER. A total of 10000 permutations were performed to determine the significance of the estimates for each AMOVA performed. Also, a Mantel test was used to test if isolation by distance (IBD) occurs in the dataset. For this purpose, we calculated the pairwise genetic differentiation of populations assuming a stepwise mutation model using Goldstein's $\delta\mu^2$ (Goldstein et al., 1995). We then correlated the $\delta\mu^2$ pairwise population distance matrix with the log-transformed pairwise geographic distance matrix. The significance of the estimators was calculated with 1000 permutations in IBD (Isolation by distance) software ver. 1.52 (Bohonak, 2002). We performed a Mantel test overall populations and also, we did a separate test for populations east and west of the IT.

Species distribution modelling—Species distribution models (SDMs) for *Tithonia rotundifolia* were built for present-day conditions, the Last Glacial Maximum (LGM; 21 kyr BP) and the Last Interglacial (LIG; 120 kyr BP) periods with MaxEnt v 3.4.1 (Phillips and Dudík, 2008). Occurrence data for *T. rotundifolia* was obtained from the GBIF repository database (Global Biodiversity Information Facility; http://www.gbif.org) and from deposited specimens at MEXU, EAP, LAGU and FCME herbaria.

Occurrence points were carefully checked for duplicates and points with doubtful coordinates were eliminated. In order to eliminate autocorrelation of data, we performed the following procedure. We generated a climate heterogeneity map with all 19 current bioclimatic variables (downloaded from www.worldclim.com at a spatial resolution of 30 arc-seconds; Fick and Hijmans, 2017) as implemented in SDMtoolbox 2.1 (Brown et al., 2017) in ArcMap 10.5. (ESRI, 2017). Then, we spatially filtered the occurrence points at 10, 30 and 50 km^2 at zones with high, medium and low climate heterogeneity respectively (Boria et al., 2014). In order to reduce variable correlation, we extracted the bioclimatic data for each occurrence point and we retained the more general variable when a pair of variables was significantly correlated at $r < 0.7$. Following this procedure, the dataset of *T. rotundifolia* was reduced to six bioclimatic variables (Annual mean temperature [BIO1], Mean diurnal range [BIO2], Isothermality [BIO3], Annual precipitation [BIO12], Precipitation seasonality [BIO15] and Precipitation of warmest quarter [BIO18]) and 174 presence records. All procedures were performed in ArcMap 10.5.

SDMs were estimated in MaxEnt ver 3.4.1. The modern distribution of *T. rotundifolia* was modelled 10 times. The convergence threshold was left at default values, however; the maximum number of iterations was increased to 1000. Each time a SDM was estimated, a different random 75% of the presence records was used to train the model and the remaining 25% was used to test it. The area under the receiver operating characteristic (ROC) curve (AUC) and

jackknife tests of variable importance were compared among all runs to estimate model quality and homogeneity of results. The SDMs were then projected onto palaeoclimate scenarios: LGM and LIG. For LGM, we used two models: the Model for Interdisciplinary Research on Climate (MIROC) and the Community Climate System Model (CCSM; Collins et al., 2006). Both LGM and LIG (Otto-Bliesner et al., 2006) palaeoclimate data were downloaded from the WorldClim database (www.worldclim.com; Hijmans et al., 2005). The logistic output was set for present-day conditions and for each historical scenario. Then, for each SDM we obtained a binary data matrix from each period: the fixed cumulative value 1 logistic threshold (FCV1) was applied. FCV1 was used because it has shown to be an appropriate threshold in other Neotropical species analyzed with palaeoecological methods involving SDM (Rodríguez-Correa et al., 2017) and also, because a high proportion of presences was estimated adequately. Each thresholded SDM was overlapped in order to identify areas of historical climatic suitability for *T. rotundifolia*. Then, we compare if genetic discontinuities match those of the climatic suitability areas identified by the SDMs.

RESULTS

Genetic analyses—The six cpSSR markers were successfully amplified in all 175 individuals and polymorphism was found at all loci. The genotypic matrix is included as Appendix S1 (see Suplemental Data with this article). Five to nine alleles were detected per loci and 32 alleles were identified at the six loci. These alleles combined into 60 haplotypes, with 2 to 6 haplotypes per population (Table 1). However, a large number of these haplotypes were private (86%) and several populations exhibited only private haplotypes (e.g. Los Mezcales, El Rocío, Juchitán; Table 1). Overall populations, haplotypic richness (*Rh*) ranged from 1.00 – 3.39. Meanwhile, all populations showed high values of Nei's unbiased genetic diversity (*He* = 0.59 – 0.93). *Tithonia rotundifolia* showed high values of within- population (h_S = 0.74) and total gene

diversity (h_T = 0.98). The overall mean genetic distance between individuals (D^2_{sh}) was 4.76. However, two populations showed high values of D^2_{sh}: Nuxca (20.74) and Barra Copalita (24.84) (Table 1). The test for recent demographic expansion using Fu's F_S showed no evidence of population expansion in all but one population (Juchitán). Genetic differentiation overall populations was G_{ST} = 0.25 (unordered alleles) and R_{ST} = 0.68 (ordered alleles). R_{ST} was significantly higher than G_{ST} ($P < 0.001$), indicating the presence of a phylogeographic structure in *T. rotundifolia*.

At the regional level (East – West the IT), populations west the IT showed a higher number of haplotypes, *Rh*, *He*, D^2_{sh} and mean within-population (h_S = 0.74) and gene total diversity (h_T = 0.99) values compared to populations east the IT (h_S = 0.73, h_T = 0.95) (Table 1). Although large negative values of F_S statistics were found for populations at either side of the IT, only the populations east the IT showed a significant value (F_S = - 6.64, $P < 0.02$) (Table 1). Also, populations at the east showed G_{ST} = 0.21 and R_{ST} = 0.47 ($R_{ST} > G_{ST}$, $P < 0.001$), while populations at the west showed G_{ST} = 0.24 and R_{ST} = 0.66 ($R_{ST} > G_{ST}$, $P < 0.001$).

The overall spatial structure of genetic diversity estimated with BAPS revealed that the best partition contained 9 clusters. Interestingly, these clusters formed distinct geographical groups and two main clusters were identified; one main cluster was identified at the west of IT (Cluster A) and the other was found at the east of the IT (Cluster G) (Figure 1a). The remainder clusters were located at peripheral populations of Clusters A and G. For instance, the northernmost populations sampled in Mexico (Los Mezcales and El Rocío) were designated as Cluster B, while the populations close to the IT were designated as either Clusters E or F. A population at northern Mexico was designated as a separate cluster (Cluster C, Figure 1a). Meanwhile, the southernmost populations located in Nicaragua were identified as Cluster I and

the northern population of Honduras as Cluster H. Lastly, the population in central Mexico (Vicente Aranda) was identified as a separate cluster (Cluster 9) (Figure 1a).

The haplotype minimum spanning network showed a complex scenario (Figure 1b). In general, haplotypes showed an overall low frequency; most of them were private (86%), and only eight haplotypes were shared between populations. Interestingly, the shared haplotypes with the highest frequencies (0.07 – 0.12) occurred between populations at the east of the IT. The remainder haplotypes showed frequencies < 0.04. Haplotypes belonging to the clusters A and B (according to BAPS) occurred mainly in a single branch, while populations west of the IT (clusters F, G, H, I) occur in a highly divergent branch of the network, suggesting long-term isolation of these clusters (Figure 1b). However, some haplotypes occurring in populations from Northern Mexico, populations close to the IT and the population located in central Mexico (pop. 9) showed haplotypes closely related to those of Central American populations (Figure 1b). Also, the haplotype with the highest number of connections (9) corresponds to an individual located in Guatemala (population 13) (Figure 1b).

BARRIER suggested that four main genetic discontinuities occur along the distribution of *T. rotundifolia*. Two genetic barriers (both with 86% bootstrap) were located at northern Mexico, separating population 4 and 9 from surrounding populations. Two additional barriers separated the populations at the IT. One of these barriers (between populations 7 and 8) showed 86% of support while the other barrier (between populations 11 and 13) showed 56% of support (Figure 1a).

The analysis of molecular variance with populations treated as a single group revealed that the highest proportion of variance is located among populations with both F_{ST} (57.22%) and R_{ST} (69.31%) (Table 2). Hierarchichal AMOVA with groups defined as populations at east and west of the IT revealed that a considerable proportion was explained by differences among

groups (16.34%, for F_{ST} and 34.30% for R_{ST}). However, the highest proportion of variance was located among populations within groups (44.29% for F_{ST} and 40.16% for R_{ST}), while the proportion of variance was lower within populations (Table 2). Lastly, hierarchichal AMOVA with populations grouped according to the genetic discontinuities identified by BARRIER showed that, for R_{ST}, the highest proportion of variance was located among groups (65.53%), while the least proportion was located among populations within groups (8.94%). Meanwhile, for F_{ST}, the highest proportion of variance was located within populations (39.71%), while variance among populations within groups and among groups showed 32.37% and 27.91% of variation respectively.

The Mantel test for IBD overall populations was significant, indicating restricted seed dispersal in *T. rotundifolia* ($Z = 2256.08$, $r = 0.34$, $P < 0.001$). A Mantel test showed that IBD occurs between populations located west of the IT ($Z = 791.46$, $r = 0.25$, $P < 0.05$), while populations at the east of the IT showed no evidence of IBD ($Z = 76.49$, $r = 0.06$, $P > 0.05$).

Species distribution modelling—SDM with present-day conditions showed a good performance of the model as revealed by the AUC values (AUC = 0.94, SD = 0.003). The variable that showed the largest relative contribution was BIO15 (34.6%), followed by BIO3 (32.9%), BIO1 (18.3%), BIO12 (11.3%), BIO2 (2.3%) and BIO18 (0.6%).

SDMs of *T. rotundifolia* for present-day conditions reveal that suitable climates occur continuously in the Neotropical regions of Mesoamerica such as the Pacific Lowlands Province (PLP) from northern Mexico to Nicaragua, the Balsas Basin Province (BBP), portions of the Veracruzan (VP) and the Yucatán Peninsula Provinces (YPP) and in northern and southern parts of the Puntarenas-Chiriqui Province (PCP) (Figure 2d). SDMs revealed a reduction of the climatic suitability during the LGM. In particular, under the MIROC model, a gap of climatic

suitability occurred at the IT and in the Nicaraguan depression. Also, suitable conditions on the Balsas Basin Province disappear during this period (Figure 2b). In a similar fashion, SDMs under the CCSM model predicted a gap of suitable conditions at the IT during the LGM. However, climatic suitability was found in the Nicaraguan Depression (Figure 2c). Lastly, during the LIG a drastic reduction in climatic suitability was found (Figure 2a). When all models are considered together, we found that the region showing a constant climatic suitability for *T. rotundifolia* during LIG, LGM and present-day conditions is the PLP. However, the climatic suitability has been interrupted by the IT and the Nicaraguan Depression (Figure 1a).

DISCUSSION

In this paper, we explored the population history of *Tithonia rotundifolia*, an annual herb widely distributed in the tropical dry forests of the Northern Neotropics. For this purpose, we employed both molecular data (cpSSR) and species distribution modelling (SDM). As cpSSR show relatively low mutation rates (Provan et al., 2001) and are maternally inherited in most angiosperms (Wills et al., 2005), its study provides insights of ancient seed dispersal. Meanwhile, SDM projections enable us to identify areas of historical climatic suitability, allowing the generation of hypothesis regarding the effects of Pleistocene climatic oscillation. Our results suggest that the population history of *T. rotundifolia* is complex and not completely driven by historical barriers common to a number of taxa (e.g. the Isthmus of Tehuantepec). However, regions of historical climatic suitability revealed by SDM showed population structuring, suggesting a partial role of the glacial/interglacial cycles in shaping the geographic structure of *T. rotundifolia*.

Genetic diversity and population differentiation—Spatial genetic diversity and structure of annual herbaceous species have been seldom explored in the northern Neotropics. Due to their short life span, reduced seed dispersal (Vibrans, 1999) and big population sizes, we expected a higher diversity and high population differentiation due to genetic drift in weedy annuals such as *T. rotundifolia* when compared to perennials despite low mutation rates at chloroplast loci (Linhart, 2000). Accordingly, we found that within-population (h_S = 0.74) and total gene diversity (h_T = 0.98) was higher in *T. rotundifolia* compared to other studies of Neotropical perennial species employing cpSSR. For instance, these values were higher than those reported by Twyford et al. (2013) for the perennial herb *Begonia heracleifolia* (h_S = 0.44, h_T = 0.93) and those found for *Quercus sapotifolia* (h_S = 0.44, h_T = 0.93) and *Q. insignis* (h_S = 0.44, h_T = 0.93) by Rodríguez-Correa et al. (2017). Gene diversity in *T. rotundifolia* was much higher than that reported for Old World species (e.g. Heuertz et al., 2010; Vrancken et al., 2009), which is in accordance with previous studies that found a general pattern of richer gene diversity in Neotropical species compared to high-latitude species.

While gene diversity was higher compared to other Neotropical species, population differentiation of *T. rotundifolia* was similar (G_{ST} = 0.25, R_{ST} = 0.68) to that reported for other species (G_{ST} = 0.52, R_{ST} = 0.737; Twyford et al., 2013). However, the number of haplotypes found in this study was considerably higher (60). Interestingly, most of these haplotypes were private (86%) and only eight haplotypes exhibiting low frequencies were shared between populations. Previous studies employing cpSSR found 39 haplotypes in *B. heracleifolia* (85% were private haplotypes). Meanwhile, Rodríguez-Correa et al. (2017) found 28 and 34 haplotypes for *Q. insignis* and *Q. sapotifolia* respectively. Regarding annual species, Vrancken et al. (2009) found 20 cpDNA haplotypes in *Rhinanthus angustifolius* in Europe. The high number of haplotypes and their low frequency found in this study may be the result of the life-history

characteristics of *T. rotundifolia*. This species establish large populations with a patchy distribution consisting of hundreds of individuals at disturbed sites in tropical deciduous forests. Restricted seed dispersal has been suggested (Vibrans, 1999), although long distance dispersal by humans cannot be ruled out. Due to the large population sizes, restricted seed dispersal and annual habit of *T. rotundifolia*, a new allele originating in a population will hardly become dominant and will be likely to be lost by genetic drift. Under this scenario, new alleles will exhibit low frequencies and a large number of haplotypes will appear, which also explain the high within-population gene diversity found. This scenario has been proposed for other annual species with rapid fluctuations in population density (Vrancken et al., 2009; Ameloot et al., 2006). Additional and nonexclusive hypothesis regarding the high genetic diversity found in *T. rotundifolia* involve historical and contemporary processes such as the persistence of large climatic suitability areas since the LIG and hybridization with the closely related *T. tubaeformis*. These hypothesis are discussed in the next section.

Geographic structure and historical dispersal barriers—Analyses revealed that *T. rotundifolia* showed signs of phylogeographic structure ($R_{ST} > G_{ST}$). In order to test several hypotheses that explain such pattern, we conducted a series of analyses. First, SDM revealed that historical climatic suitability areas for *T. rotundifolia* were located throughout the Pacific Lowlands Province. However, a gap in climatic suitability was found close to the IT. Also, population structuring at areas of historical climatic suitability at either side of the IT (clusters A, B and G, Figure 1a) and a highly supported (86%) genetic discontinuity identified by BARRIER close to the IT suggest a role for this region as a dispersal barrier (Ornelas et al., 2013). Because of this, the historical scenario that accounts for the IT as a dispersal barrier of *T. rotundifolia* was tested. The IT is a valley separating the Central Mexican highlands and the Chiapas Highlands

acting as an important barrier in a number of plant and animal taxa (Bryson et al., 2011; Gutiérrez-Rodríguez et al., 2011; Ornelas et al., 2013). Since its formation in the Miocene (6 Mya), several marine incursions (Barrier et al., 1998) and climatic oscillations during the Pleistocene (Gutiérrez-Rodríguez et al., 2011) have led to population structuring at either side of the IT. However, we found through AMOVA that even when a significant proportion of the variation in *T. rotundifolia* was explained by grouping populations East and West the IT, the highest variation was found among populations within groups rather than between groups (Table 2). This result shows that although the glacial/interglacial cycles around the IT had a role in the geographic structure of *T. rotundifolia*, the IT is not the main driver of its genetic variation, suggesting a more complex scenario.

An alternative scenario consisted in grouping populations according to the genetic discontinuities found through BARRIER. Following this procedure, AMOVA calculations based on R_{ST} showed that the highest percentage of variation was explained by differences among groups (Table 2). However, the same analysis based on F_{ST} calculations showed the opposite pattern; the highest percentage of variation was explained by differences within populations and among populations within groups (Table 2). As R_{ST} is based on the stepwise mutation model and F_{ST} on allele identity, the differences found in AMOVA results are due to the occurrence of a number of closely related haplotypes within a certain region (as seen in the minimum spanning network, Figure 2b) leading to higher F_{CT} values when using R_{ST}. All in all, more variation was explained when populations were divided at a finer scale. As outlined by several authors (Twyford et al., 2013; Poelchau and Hamrick, 2013; Ornelas et al., 2013), the complex population structuring found in some Neotropical taxa suggests that Pleistocene climatic fluctuations responses are dependent on the life-history characteristics of the species. For instance, the large climatic stability areas found at either side of the IT since the last interglacial

(21 Kyr BP) and the absence of significant F_S values at the population level suggest overall large effective population sizes of *T. rotundifolia*. However, its restricted seed dispersal and the patchy availability of suitable habitats (i.e. forest edges, disturbed habitats) may have promoted population differentiation, resulting in the appearance of a number of closely related haplotypes within a region following range expansion. The haplotype minimum spanning network supports this hypothesis, as closely related haplotypes with a low frequency generally occur within a region.

The aforementioned scenario is especially true for the populations at the west of the IT, where significant isolation by distance and absence of signs of recent population expansion were detected at the population and regional level, suggesting population equilibrium (Carnaval et al., 2009). However, populations at the east of the IT showed significant F_S values at the regional level and non-significant isolation by distance, suggesting a recent population expansion from a refuge located at the historical climatic suitability area. For instance, the population at the Yucatán Peninsula Province (population 12) may have originated recently from this refuge through long distance dispersal by humans. However, the minimum spanning network showed that the haplotype with the highest number of connections was located at Guatemala (population 13). This haplotype showed connections with haplotypes belonging to populations at the IT, suggesting a south-north migration towards the IT followed by population differentiation.

Although the minimum spanning network showed overall geographic structure, some haplotypes belonging to a cluster west to the IT (Cluster A in Figure 1a) were grouped with haplotypes at the east of the IT. Also, populations at the north of the PLP (populations 4 and 9) showed haplotypes more closely related to those of Central American populations. Several explanations may account for the lack of geographic structure of some cpSSR haplotypes: long distance dispersal by humans, the retention of plesiomorphic haplotypes, and hybridization with

the closely related and widespread *T. tubaeformis* (López-Caamal et al., 2018). In order to discriminate between these hypotheses, further research should study additional organelle and nuclear DNA sequences and also, compare the phylogeographic patterns of *T. rotundifolia* with those of *T. tubaeformis*.

Population history studies of plants associated to tropical dry forests in the northern Neotropics are scarce. Historical ecology of these environments is poorly known. Palinological records in tropical dry forests are scarce because pollen grains are easily damaged by corrosion (Anderson and Van Devender, 1995; Berrio et al., 2006), so records extending back to the Pleistocene are lacking for the Pacific coast of Mexico. However, due to the apparent ecological preference of *T. rotundifolia* to tropical dry forests, our results suggest that tropical dry forests had large stability areas since the LIG at the pacific coasts of Mexico and Central America, with signs of a south-north migration of *T. rotundifolia* following the LGM. In a similar fashion, Arias et al. (2010) suggested south-north migrations of the tree *Jacaratia mexicana* and also, they found a similar population structuring. These authors found four main clades: a northern pacific, central, southern pacific and southeastern clade in Mexico. The split of these clades date from the late Pliocene to the late Pleistocene, suggesting both geological and climatic fluctuations in the geographic structuring of *J. mexicana* (Arias et al., 2010). Altogether, the results of *J. mexicana* and *T. rotundifolia* suggest that tropical dry forest biota has been influenced by both climatic and geological fluctuations leading to south-north migrations following the LGM. However, additional studies of species with different life-history characteristics are needed to describe the general response of the tropical dry forest biota due to historical and climatic fluctuations.

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TABLE 1. Population name, sample site and size (*n*) and geographic location of samples included in this study. The number of haplotypes, private haplotypes (PA), rarefacted haplotype richness (*Rh*), Nei's unbiased genetic diversity (*He*), mean genetic distance between individuals (D^2_{sh}) and historical demography estimates (Fu's *F* statistics) for each population is shown. Significant values for Fu's *F* statistics ($P < 0.02$) are indicated in bold.

Population	Sample site	*n*	Latitude	Longitude	Haplotype no.	PA	*Rh*	*He*	D^2_{sh}	*F*
West of the Isthums of Tehuantepec		79			33		32.00	0.97	19.50	-6.45
1	Los Mezcales, Colima	8	19.16	-103.76	3	3	1.96	0.75	0.57	0.97
2	El Rocío, Colima	8	18.74	-103.72	3	3	1.75	0.68	3.47	2.92
3	Barra de Nexpa, Michoacán	8	18.10	-102.82	2	1	1.00	0.54	0.09	0.86
4	Joluta, Guerrero	8	18.00	-101.97	2	2	1.00	0.57	0.10	0.96
5	Nuxca, Guerrero	10	17.21	-100.78	5	4	3.33	0.89	20.74	3.31
6	El Ciruelo, Oaxaca	8	16.34	-98.23	5	5	3.39	0.89	8.23	0.95
7	Barra Copalita, Oaxaca	8	15.81	-96.04	3	3	1.93	0.71	24.83	6.64
8	Juchitán, Oaxaca	8	16.44	-95.01	6	6	3.93	0.93	0.74	**-2.82**
9	Vicente Aranda, Morelos	13	18.56	-99.21	5	5	2.38	0.71	0.99	-0.36
East of the Isthmus of Tehuantepec		96			27		23.83	0.91	7.64	**-6.63**
10	Tierra y Libertad, Chiapas	6	16.38	-93.86	3	1	2.00	0.73	0.59	0.53
11	El Tanque, Chiapas	6	16.71	-93.52	4	2	3.00	0.87	1.12	-0.32
12	Mérida, Yucatán	12	21.01	-89.65	3	1	1.47	0.59	0.27	0.39
13	Flores Costa Cuca, Quetzaltenango	8	14.65	-91.80	3	2	1.93	0.71	1.40	1.72
14	El Resbaladero, Santa Ana	9	13.98	-89.44	4	2	2.56	0.81	7.89	2.98
15	Guaymango, Ahuachapán	9	13.75	-89.84	5	2	2.67	0.72	1.69	-0.55
16	Ajuterique, Comayagua	14	14.4	-87.68	5	4	2.50	0.74	4.30	1.77
17	San Lorenzo, Valle	15	13.42	-87.43	5	2	2.37	0.70	0.99	-0.26
18	León, León	9	12.39	-86.84	6	2	3.57	0.89	9.89	0.57
19	Villa Unión, Masaya	8	12.00	-86.13	4	2	2.25	0.64	2.52	0.61

TABLE 2. Analysis of molecular variance (AMOVA) of *Tithonia rotundifolia* populations treated as a single group (a), divided into populations at east and west the Isthums of Tehuantepec (b) and into clusters divided by genetic discontinuities identified by BARRIER (c) using F_{ST} and R_{ST} values.

Source of variation	d.f.	SS	Variance component	Percentage of variation	Fixation indices
a) Populations treated as a single group					
F_{ST}					
Among populations	18	195.18	1.09	57.22	
Within populations	156	127.47	0.81	42.78	$\Phi_{ST} = 0.57$*
Total	174	322.65	1.90		
R_{ST}					
Among populations	18	2551.71	14.74	69.31	
Within populations	156	1018.23	6.52	30.69	$\Phi_{ST} = 0.69$*
Total	174	3569.95	21.67		
b) East-West the IT					
F_{ST}					
Among groups	1	38.32	0.33	16.34	$\Phi_{CT} = 0.16$*
Among populations within groups	17	156.86	0.91	44.29	$\Phi_{SC} = 0.52$*
Within populations	156	127.47	0.81	39.36	$\Phi_{ST} = 0.60$*
Total	174	322.65	2.07		
R_{ST}					
Among groups	1	845.18	8.76	34.30	$\Phi_{CT} = 0.34$*
Among populations within groups	17	1706.53	10.26	40.16	$\Phi_{SC} = 0.61$*
Within populations	156	1018.23	6.52	25.54	$\Phi_{ST} = 0.74$*
Total	174	3569.95	25.53		
b) BARRIER grouping					
F_{ST}					
Among groups	4	97.69	0.57	27.91	$\Phi_{CT} = 0.25$*
Among populations within groups	14	97.49	0.66	32.37	$\Phi_{SC} = 0.49$*
Within populations	156	127.47	0.81	39.71	$\Phi_{ST} = 0.62$*
Total	174	322.65	2.05		
R_{ST}					
Among groups	4	2164.91	16.75	65.53	$\Phi_{CT} = 0.65$*
Among populations within groups	14	386.79	2.28	8.94	$\Phi_{SC} = 0.25$*
Within populations	156	1018.23	6.52	25.57	$\Phi_{ST} = 0.74$*
Total	174	3569.95	25.57		

Note: * = $P < 0.001$

FIGURE 1. *Tithonia rotundifolia* populations sampled (A). Numbers represent populations as described in Table 1 and colors depict cluster structuring following BAPS. Thick black lines represent genetic discontinuities identified by BARRIER. Colors in the base maps represent SDM overlapping; yellow indicates overlap of all four models, indicating historical climatic suitability areas. Solid thin lines represent biogeographic provinces (Morrone 2014; Löwenberg-Neto 2014). PLP = Pacific Lowland Province, BBP = Balsas Basin Province, SMS = Sierra Madre del Sur Province, VP = Veracruzan Province, YPP = Yucatán Peninsula Province, CHP = Chiapas highlands Province, PCP = Puntarenas-Chiriqui Province. The haplotype minimum spanning network is shown in (B). Colors indicate the clustering identified by BAPS, while the size of each node represents haplotype frequency. Only connection lengths ≥ 5 are shown.

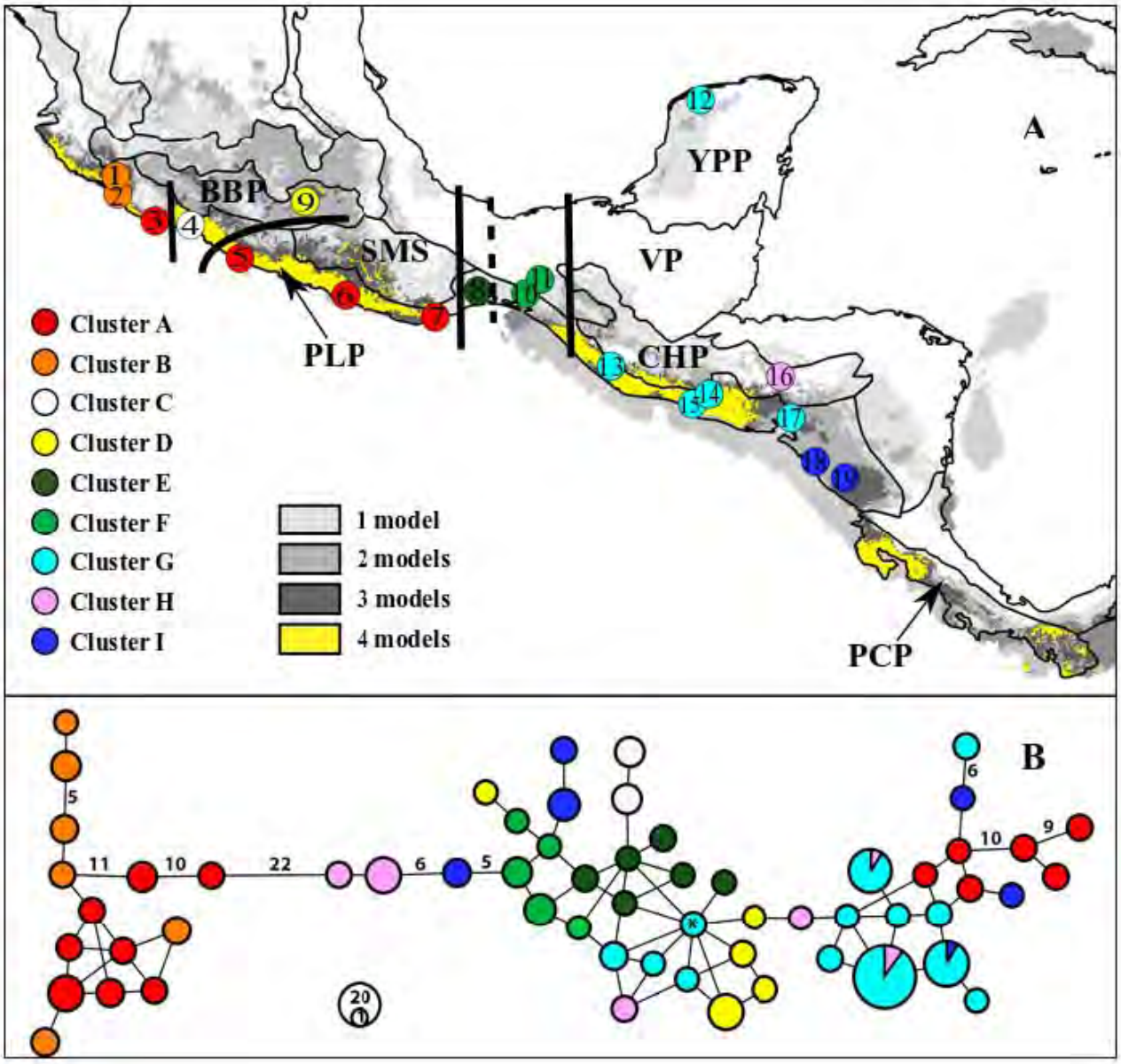
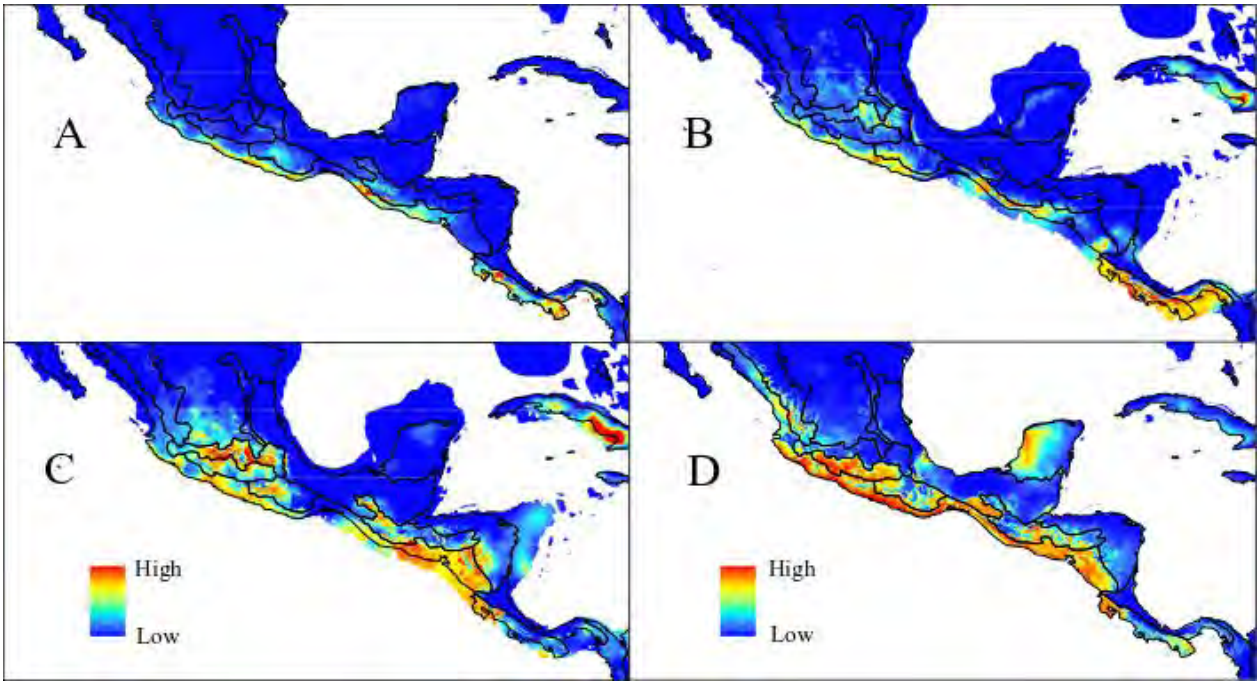

A
YPP
VP
BBP
SMS
PLP
CHP
PCP
Cluster A
Cluster B
Cluster C
Cluster D
Cluster E
Cluster F
Cluster G
Cluster H
Cluster I
1 model
2 models
3 models
4 models
B

FIGURE 2. Logistic output of species distribution modelling of *Tithonia rotundifolia* during the LIG (A), LGM under the MIROC (B) and CCSM (C) model and present-day conditions (D). Solid lines depict the biogeographical provinces as described in Figure 1.



CAPÍTULO IV

Estructura geográfica de *Tithonia tubaeformis* (Asteraceae) revelada a través de cpSSR y modelos de distribución de especies

Alfredo López-Caamal, Efraín Tovar-Sánchez

RESUMEN

Premisa del estudio: Los efectos de los cambios climáticos Pleistocénicos han sido poco estudiados en plantas anuales neotropicales. Este trabajo explora la estructura geográfica de *Tithonia tubaeformis* utilizando marcadores moleculares. Asimismo, se explora si la hibridación con *T. rotundifolia* ha tenido un efecto en la distribución de su variación genética.

Métodos: Seis regiones de microsatélites de cloroplasto fueron amplificadas en 136 individuos repartidos en 17 localidades de *T. tubaeformis*. Se realizaron modelos de distribución de especies para establecer hipótesis de los efectos de los cambios climáticos históricos sobre la distribución de *T. tubaeformis*. Los patrones obtenidos en *T. tubaeformis* fueron comparados con los de *T. rotundifolia* y se exploró la existencia de haplotipos compartidos entre estas especies.

Resultados clave: Los haplotipos de *Tithonia tubaeformis* mostraron la existencia de una estructura filogeográfica ($R_{ST} > G_{ST}$). Se encontraron zonas de idoneidad climática durante el último máximo glacial en el norte de México y en la Faja Volcánica Transmexicana. Asimismo, se observó una reducción de la diversidad genética en las poblaciones periféricas ubicadas en el sur de México y Centroamérica.

Conclusiones: Se sugiere la existencia de refugios durante las épocas glaciales en el norte de México y en la Faja Volcánica Transmexicana a partir de los cuales existió una migración norte – sur después de las oscilaciones climáticas Pleistocénicas. Debido al bajo número de haplotipos compartidos entre especies y a que éstos se encuentran presentes entre poblaciones muy lejanas de *T. tubaeformis* y *T. rotundifolia* se sugiere que la hibridación no ha tenido un efecto importante en la historia de estas especies.

Palabras clave: Filogeografía, Haplotipos compartidos, hibridación, México, microsatélites.

INTRODUCCIÓN

La flora del Neotrópico es excepcionalmente diversa y contiene un gran número de especies. La gran diversidad que presenta esta región se debe en parte a su heterogeneidad ambiental producto de una intensa actividad geológica durante el Neógeno y a las oscilaciones climáticas ocurridas durante el Pleistoceno. Debido a esto, la región norte del Neotrópico ha sido objeto de un gran número de estudios filogeográficos y de estructura genética en varios grupos de organismos durante los últimos años. En particular, se han estudiado un gran número de vertebrados (Vázquez-Miranda et al., 2009; Bryson et al., 2011; Werneck et al., 2012; Gutiérrez-García y Vázquez-Domínguez, 2013) así como de plantas vasculares (Cavers et al., 2003; Lemes et al., 2010; Gutiérrez-Rodríguez et al., 2011; Ornelas et al., 2013; Rodríguez-Correa et al., 2017). En algunos casos, se ha demostrado que las oscilaciones históricas climáticas han tenido un papel fundamental en la estructuración genética de las poblaciones de algunas especies (Ornelas et al., 2013).

En la región norte del Neotrópico se han identificado algunas barreras históricas comunes a la dispersión o al flujo génico en varias especies. Por ejemplo, se ha identificado que el Istmo de Tehuantepec (IT) actúa como una barrera para un gran número de especies. Sin embargo, de acuerdo a Ornelas et al. (2013), el sitio geográfico y el tiempo de divergencia de los linajes encontrados al este y oeste del IT varía de acuerdo a la historia de vida de la especie estudiada. Lo anterior sugiere que las características inherentes a cada especie en cuanto a su capacidad de dispersión juegan un papel tan importante como los factores históricos climáticos y geológicos en la estructuración poblacional.

En este sentido, existen estudios en los que no se encuentra evidencia de estructuración genética debido a las barreras apenas mencionadas. Twyford et al. (2013) analizaron la estructura geográfica de *Begonia heracleifolia* en los bosques mesófilos de México. Los autores

encontraron que ni la barrera geográfica del IT, ni las hipótesis de refugios Pleistocénicos propuesta por Toledo (1982) explican la estructuración de sus poblaciones. En cambio, los autores atribuyen estos resultados a la baja dispersión de las semillas de *B. heracleifolia* y a la sobrevivencia de poblaciones en refugios restringidos durante el último máximo glacial (UMG). Resultados similares fueron encontrados por Chávez-Pesqueira y Núñez-Farfán (2016) al estudiar poblaciones silvestres de *Carica papaya*. Sin embargo, los autores atribuyen la ausencia de estructura filogeográfica a la dispersión a larga distancia de sus semillas. Asimismo sugieren que algunos disturbios recientes, como la destrucción del hábitat y posiblemente la dispersión a larga distancia por el hombre pueden explicar la ausencia de estructura filogeográfica en *C. papaya*.

La ausencia de una marcada estructura filogeográfica tiene otras posibles causas. En particular, la hibridación interespecífica puede enmascarar la huella genética que los procesos históricos dejan en las poblaciones de las especies participantes. En este sentido, varios trabajos han reportado haplotipos compartidos empleando marcadores citoplásmicos entre especies cercanamente relacionadas (Belahbib et al., 2001; Tovar-Sánchez et al., 2008; Heuertz et al., 2010; Nevill et al., 2014; Ramos-Ortiz et al., 2016). Lo anterior sugiere que la hibridación puede tener un papel importante en la distribución de la variación genética de las poblaciones. Por ejemplo, Nevill et al. (2014) estudiaron tres especies de *Eucalyptus* con diferentes tolerancias ecológicas. Los autores encontraron que estas especies presentan un patrón filogeográfico en común, así como una mayor diversidad genética en las áreas propuestas como refugios durante el UMG. Más aún, en las áreas propuestas como refugios, los autores encontraron haplotipos compartidos entre las tres especies, indicando procesos de hibridación históricos en estas regiones (Nevill et al., 2014)

Aun cuando varios trabajos reportan la presencia de haplotipos compartidos entre especies cercanamente relacionadas, la presencia de éstos no necesariamente denota un proceso de

hibridación; el sorteo incompleto de linajes y la simpatría entre linajes divergentes son causas igualmente probables. En especial, la simpatría entre linajes divergentes es una explicación con especial relevancia para los estudios que emplean microsatélites de cloroplasto (cpSSR), en donde la homoplasia es frecuente (Provan et al., 2001). Debido a esto, los estudios que buscan determinar si la hibridación ha tenido un papel importante en la estructuración de especies cercanamente relacionadas deben comparar la estructura geográfica de las especies así como inferir la historia de sus poblaciones utilizando datos paleoecológicos (Vrancken et al., 2009; Nevill et al., 2014). De esta forma, será posible identificar zonas históricas de simpatría en donde la hibridación pudo haber ocurrido.

El género *Tithonia* Desf. ex Jussieu (Asteraceae) contiene 11 especies anuales y perennes que se distribuyen en México y Centroamérica (La Duke, 1982). Se ha propuesto que este género se originó en la región norte del Neotrópico (Blake, 1921; La Duke, 1982). Dentro de *Tithonia*, las especies anuales que presentan la mayor área de distribución son *Tithonia tubaeformis* (Jacq.) Cass y *Tithonia rotundifolia* (Miller) S. F. Blake. En el capítulo III se analizó la estructura geográfica de *T. rotundifolia*, una especie que se distribuye preferentemente en la costa del Pacífico de México y Centroamérica asociada a ambientes perturbados en bosques tropicales caducifolios. Para este fin, se estudiaron 6 loci de cpSSR así como modelos de distribución de especies. Se encontró que la distribución de la variación genética es explicada en parte por las oscilaciones climáticas ocurridas durante el Pleistoceno. Sin embargo, algunas características de historia de vida, como la baja dispersión de sus aquenios y la disponibilidad discontinua de hábitats pueden explicar la distribución de su variación genética.

Este capítulo se enfoca en el análisis de la estructura geográfica de *Tithonia tubaeformis*. Esta especie es una maleza frecuente en el centro de México, generalmente asociada a cultivos de maíz (Vibrans, 1999) y a ambientes perturbados en los bosques templados por encima de los

1 000 m s.n.m (La Duke, 1982). Sin embargo, es posible encontrar poblaciones de *T. tubaeformis* asociadas a matorrales y bosque tropical caducifolio por debajo de los 1 000 m s.n.m. (La Duke, 1982, López-Caamal, observación personal). En el bosque tropical caducifolio, ambas especies forman poblaciones mixtas, produciendo híbridos de primera generación y retrocruzas hacia ambas especies progenitoras (López-Caamal et al., 2018).

Este capítulo tiene como objetivo describir la estructura geográfica de *T. tubaeformis* utilizando los mismos marcadores cpSSR empleados en el capítulo anterior para *T. rotundifolia*. Asimismo, se relaciona la estructura geográfica encontrada con las oscilaciones climáticas ocurridas en el Pleistoceno con el fin de encontrar patrones en común entre estas especies. Sin embargo, dado que las características de la historia de vida de *T. tubaeformis* son muy similares a las de *T. rotundifolia*, esperamos una estructuración compleja de sus poblaciones debido a su baja dispersión de sus aquenios y su distribución en ambientes antropogénicos. Por otro lado, con el fin de evaluar el papel de la hibridación en la distribución de la variación genética entre *T. tubaeformis* y *T. rotundifolia* se busca encontrar posibles zonas de simpatría histórica mediante el uso de modelos de distribución de especies. Si la hibridación entre *T. tubaeformis* y *T. rotundifolia* ha ocurrido de manera constante en un contexto histórico, se espera que en dichas regiones se presenten haplotipos compartidos entre especies.

MATERIALES Y MÉTODOS

Especie de estudio—*Tithonia tubaeformis* (Asteraceae) es una maleza anual de hasta 3 metros de altura. Esta especie es fácilmente distinguible por sus flores liguladas amarillas, sus filarias lineares a oblongas altamente pubescentes y sus hojas enteras. *Tithonia tubaeformis* establece poblaciones de cientos de individuos a la orilla de caminos y cultivos asociados los bosques templados del centro de México (La Duke, 1982; Vibrans, 1999). Sin embargo, a

diferencia de *T. rotundifolia*, que aparenta tener una estrecha tolerancia ecológica, *T. tubaeformis* puede encontrarse asociada también a matorrales (principalmente en el altiplano) y bosque tropical caducifolio (La Duke, 1982). Aunque no existen estudios detallados sobre la biología reproductiva de esta especie, se ha observado que la polinización es llevada a cabo por polinizadores generalistas como lepidópteros y *Apis mellifera* (López-Caamal, observación personal). Por su parte, dado el peso y tamaño de sus aquenios, se presume una baja capacidad de dispersión. Los aquenios de *T. tubaeformis* son muy similares a los de *T. rotundifolia*, presentando un pappus fusionado y dos aristas largas que suponen barocoria y/o epiantropocoria (Vibrans, 1999). Sin embargo, debido a que *T. tubaeformis* se presenta a la orilla de cultivos, su dispersión a larga distancia por el hombre no se descarta.

Tithonia tubaeformis y *T. rotundifolia* forman poblaciones mixtas en el centro de México. Recientemente, López-Caamal et al. (2018) encontraron híbridos y posibles retrocruzas hacia ambas especies progenitoras. Asimismo, en estas zonas mixtas se ha encontrado novedad fenotípica en la forma de caracteres morfológicos transgresivos (López-Caamal et al., 2013) así como de nuevos metabolitos secundarios (López-Caamal et al., 2018)

Modelos de distribución de especies—Los modelos de distribución de especie (SDM) para *T. tubaeformis* fueron realizados con el programa MAXENT ver 3.4.1 (Phillips y Dudík, 2008). Se realizaron modelos para las condiciones actuales así como proyecciones al último interglacial (UIG) y el último máximo glacial (UMG). Para las condiciones actuales (1970 – 2000), se utilizaron 19 variables bioclimáticas reportadas por Fick y Hijmans (2017) a una resolución de 30 arc-segundos (~ 1 Km; disponible en www.worldclim.com). Los datos de presencia de *T. tubaeformis* se obtuvieron de la base de datos GBIF (Global Biodiversity Information Facility; http://www.gbif.org), los cuales fueron complementados con los ejemplares

depositados en los herbarios MEXU, LAGU, FCME y EAP así como de las localidades colectadas para el presente trabajo.

Con el fin de eliminar la autocorrelación espacial de los datos de presencia de *T. tubaeformis*, se generó un mapa de heterogeneidad climática utilizando las 19 variables bioclimáticas con SDMtoolbox 2.1 (Brown et al., 2017) en ArcMap 10.5 (ESRI, 2017). Una vez realizado esto, se filtraron los datos de presencia a 10, 30 y 50 km^2 en zonas de alta, media y baja heterogeneidad climática (Boria et al., 2014). Asimismo, para reducir la correlación de las variables climáticas, se extrajeron los valores de cada variable para cada punto de presencia de *T. tubaeformis*. Posteriormente, se eliminó la variable más específica cuando un par de variables presentaba $r < 0.7$. Las variables que fueron retenidas para el SDM bajo las condiciones actuales fueron: la temperatura media anual (BIO1), media del rango diurno (BIO2), isotermalidad (BIO3), precipitación anual (BIO12), precipitación del mes más cálido (BIO14) y la estacionalidad de la precipitación (BIO15). Por su parte, el número de puntos de presencia empleados para la modelación fueron 377.

El SDM calculado en MAXENT para las condiciones actuales de *T. tubaeformis* fue modelado 10 veces utilizando los 377 puntos de presencia y las seis variables bioclimáticas apenas mencionadas. Una vez realizada la modelación actual, el SDM resultante fue proyectado en distintos modelos paleoclimáticos: UIG y UMG. Las variables bioclimáticas para el UIG se obtuvieron de www.worldclim.org (Hijmans et al., 2005; Otto-Bliesner et al., 2006). Mientras que para los escenarios paleoclimáticos del UMG se utilizaron dos modelos: el Community Climate System Model (CCSM; Collins et al., 2006) y el Model for Interdisciplinary Research on Climate (MIROC). A pesar de que ambos modelos simulan las condiciones climáticas durante el UMG, el modelo CCSM asume un mayor descenso en la temperatura en comparación con MIROC (Otto-Bliesner et al., 2006).

Para la modelación de las condiciones actuales como de los escenarios paleoclimáticos, se utilizaron los valores predeterminados por MAXENT. Sin embargo, el número máximo de iteraciones se incrementó a 2000 con el fin de permitir la convergencia del modelo. Para cada caso se realizaron 10 modelaciones independientes en las cuales se empleó el 75% de los puntos de presencia para entrenar el modelo y el 25% restante para probar el modelo. Una vez llevadas a cabo las 10 modelaciones, se utilizó el índice AUC (Area under the receiver operating characteristic curve) como una medida del desempeño relativo del modelo (Phillips y Dudík, 2008). Por su parte, la salida logística del promedio de las 10 modelaciones fue empleado para representar las áreas de idoneidad climática en cada escenario climático. Asimismo, se utilizó el umbral Fixed Cumulative Value 1 para tener una salida binomial de cada uno de los escenarios (condiciones actuales, UMG y UIG). Al sobrelapar los cuatro modelos, se pretende encontrar regiones de estabilidad histórica climática para *T. tubaeformis*.

Por otro lado, con el fin de conocer si *T. tubaeformis* y *T. rotundifolia* han compartido regiones de idoneidad climática durante el tiempo histórico, realizamos comparaciones entre los SDM para *T. rotundifolia* obtenidos en el capítulo III con los que se obtuvieron en el presente capítulo. Para cada escenario histórico, realizamos un sobrelapamiento de los modelos con el fin de encontrar regiones en las que ambas pudieron haber entrado en simpatría a lo largo del tiempo.

Colecta de tejido foliar, extracción de ADN y amplificación de cpSSR—El tejido foliar de 136 individuos pertenecientes a 17 localidades (4 a 13 individuos por localidad) de *T. tubaeformis* fue colectado a lo largo de su área de distribución nativa (Figura 1a, Tabla 1). El tejido foliar fue preservado en nitrógeno líquido hasta su procesamiento. La extracción de ADN genómico fue llevada a cabo siguiendo una modificación del protocolo propuesto por Doyle y Doyle (1987). Brevemente, el tejido vegetal fue pulverizado con nitrógeno líquido y previo a la

lisis celular, se realizó una limpieza del tejido utilizando el buffer STE. Posteriormente se llevó a cabo la lisis celular con el buffer CTAB 2x adicionado con PVP-40 1%, una extracción con cloroformo-alcohol isoamílico, y por último, una precipitación con etanol absoluto. El ADN genómico de cada individuo fue cuantificado y diluido a una concentración final de 10 ng/uL.

Para la amplificación de regiones microsatélites de cloroplasto se eligieron los mismos loci que fueron empleados para el estudio de la estructura geográfica de *T. rotundifolia* (Capítulo III): ccmp1, ccmp2, ccmp4, ccmp7, NTCP9 y NTCP18 (Bryan et al., 1999; Wills et al., 2005). Una vez comprobado que los oligonucleótidos para estos loci dieran lugar a productos de PCR polimórficos para *T. tubaeformis*, se procedió al marcaje de éstos con los fluoróforos NED, 6-FAM y/o HEX. Las reacciones fueron llevadas a cabo en un volumen final de 8 uL bajo las condiciones descritas por Wills et al. (2005). El análisis de fragmentos fue llevado a cabo mediante electroforesis capilar en un secuenciador automático ABI 3500 (Applied Biosystems, Foster City, California, USA) utilizando Genscan-500 ROX como estándar. La estimación del tamaño de cada fragmento se realizó con ayuda del programa GeneMarker ver. 2.7.0 (Softgenetics, Inc., USA).

Análisis genéticos—En este trabajo se definió a un haplotipo como una combinación diferente de alelos en los seis loci de cpSSR evaluados. Para cada una de las poblaciones se calculó el número de haplotipos, el número de haplotipos privados (PA), la riqueza haplotípica (*Rh*), el índice de diversidad genética de Nei (*He*) y la distancia promedio entre individuos dentro de cada población de acuerdo a un modelo de mutación paso a paso (D^2_{sh}; Morgante et al., 1998) utilizando HAPLOTYPE ANALYSIS ver. 1.05 (Eliades y Eliades, 2009). Asimismo, se calculó el promedio de la diversidad intrapoblacional (h_S) y la diversidad total (h_T). Por su parte, para calcular la diferenciación genética entre poblaciones y para evaluar la existencia de un patrón

filogeográfico en *T. tubaeformis*, se calcularon los índices G_{ST} y R_{ST}. Posteriormente, se realizó una prueba de permutación para conocer si R_{ST} es significativamente mayor a G_{ST}, lo cual indica una estructura filogeográfica de los haplotipos muestreados. Esta comparación fue llevada a cabo con CpSSR ver. 2.0 (Pons y Petit, 1996). Asimismo, para identificar señales de reciente expansión poblacional se calculó el estadístico F_S (Fu, 1997). Un valor negativo y estadísticamente significativo indica una reciente expansión poblacional. Esta prueba fue llevada a cabo con ARLEQUIN ver. 3.5 codificando a los haplotipos de acuerdo a lo propuesto por Heuertz et al. (2010). Por su parte, utilizando el mismo software se generó una red de pasos mínimos de los haplotipos muestreados (*minimum spanning network*). La red de haplotipos fue visualizada con el programa GEPHI ver 0.9.2 (Bastian et al., 2009).

El análisis de la estructura poblacional fue llevado a cabo utilizando el enfoque bayesiano implementado en BAPS ver. 6.0 (Corander et al., 2008). El enfoque de este programa permite llevar a cabo un análisis espacial de la estructura genética, en el cual se provee al algoritmo de la información geográfica de cada población. Para cada uno de los valores de *K* evaluados (2 - 17) se llevaron a cabo 5 réplicas independientes, eligiendo el valor óptimo de *K* de acuerdo a los valores de *logmls* para cada *K*.

Con el fin de encontrar las posibles barreras genéticas a lo largo de la distribución de *T. tubaeformis*, se utilizó el programa BARRIER ver. 2.2 (Manni et al., 2004). Este programa tiene el propósito de buscar barreras correlacionadas con las tasas más altas de diferenciación en la matriz de distancias genéticas utilizando el algoritmo de máxima diferenciación de Monmonier (Monmonier, 1973). Para esto, se generaron 100 matrices de distancias genéticas ($\delta\mu^2$; Goldstein et al., 1995) utilizando el software Microsatellite Analyser (Dieringer y Schlötterer, 2003). Se calcularon hasta 5 barreras, sin embargo, solamente se reportan aquellas con un soporte igual o mayor a 45%.

Un Análisis de Varianza Molecular (AMOVA) fue llevado a cabo para conocer cómo se encuentra dividida la variación genética dentro y entre poblaciones utilizando F_{ST} y R_{ST}. En un primer análisis, todas las localidades muestreadas fueron consideradas como un único grupo. Sin embargo, realizamos un análisis adicional en el que las localidades fueron agrupadas de acuerdo a las barreras genéticas identificadas por BARRIER. Para ambos análisis, se realizaron 10 000 permutaciones para estimar la significancia de los estimadores de diferenciación. Por último, se compararon los haplotipos de *T. tubaeformis* y *T. rotundifolia* con el fin de identificar si existen haplotipos compartidos; la hibridación sería una causa posible de dichos haplotipos compartidos.

RESULTADOS

Modelos de distribución de especies—El modelo de distribución de especies para las condiciones actuales mostró un buen desempeño de acuerdo a los valores del índice AUC (0.92, SD 0.003). Por otro lado, al utilizar las seis variables con el menor coeficiente de correlación, se observó que la variable con el mayor porcentaje de contribución al modelo fue BIO3 (41.9%), seguida de BIO15 (37.4%) y BIO (15.4%) mientras que el resto de las variables mostró un bajo porcentaje de contribución al modelo (0.5 – 3.5%).

Para las condiciones actuales, el modelo mostró que las áreas de idoneidad climática se encuentran principalmente en la zona centro de México. En particular, se observó que existen áreas de idoneidad climática en las regiones montañosas templadas de Mesoamérica (Sierra Madre Oriental [SMOc], Sierra Madre Oriental [SMOr], Sierra Madre del Sur [SMS], Faja Volcánica Transmexicana [FVT], y en las zonas Montañosas de Chiapas [CH]) (Figura 1a). Sin embargo, también existen áreas de idoneidad climática en la Cuenca del Balsas (CB) así como en la Provincia del Pacífico (PLP). Por su parte, las proyecciones a los distintos escenarios paleoclimáticos indicaron una migración hacia el Norte durante el UMG para ambos modelos

estudiados (MIROC y CCSM). En general, se observaron áreas de idoneidad climática en algunas porciones de la SMS y de la FVT, encontrándose la más extensa en la región del Altiplano. Por último, durante el UIG se observó una reducción importante en las áreas de idoneidad climática para *T. tubaeformis*, encontrándose solamente en la porción oriental de la FVT y en la parte norte del Altiplano y la SMOc (Figura 1a).

La comparación de los SDM de *T. tubaeformis* y *T. rotundifolia* mostraron que durante el UIG no se encontraron áreas de idoneidad climática en común para ambas especies (Figura 2a). Sin embargo durante el UMG, se encontró que ambas especies compartieron áreas de idoneidad climática en la porción central de la SMS, en la FVT y en la región sur del atiplano (Figura 2b, 2c). Esto sugiere que la simpatría entre estas especies puede datar del UMG. Por último, al comparar los SDM para las condiciones actuales se observaron áreas de idoneidad climática compartida entre *T. tubaeformis* y *T. rotundifolia* en la CB, la PLP, la SMS y en algunas porciones de la Península de Yucatán (Figura 2d).

Análisis genéticos—Los seis loci de regiones cpSSR fueron amplificados exitosamente en los 136 individuos muestreados. En total se encontraron 37 alelos, los cuales dieron lugar a 69 haplotipos repartidos en las 17 localidades muestreadas. Cada localidad mostró de dos a nueve haplotipos, siendo la mayoría (~82%) haplotipos privados. Es interesante notar que la mayoría de los haplotipos privados, los valores más altos de riqueza haplotípica (1.73 – 3.0), diversidad genética de Nei (0.75 – 1.0) y de D^2_{sh} (26.13) se encuentran en las poblaciones al oeste del Istmo de Tehuantepec (IT), mientras que las poblaciones al este del IT muestran valores más bajos (*Rh* = 0.93 a 1.6, *He* = 0.53 a 0.7, D^2_{sh} = 12.67) (Tabla 1). Por su parte, se encontró que el valor de h_S = 0.81 mientras que h_T = 0.98. Asimismo, los análisis mostraron valores no significativos de F_S,

indicando la ausencia de señales de reciente expansión demográfica para *T. tubeformis* en todas las poblaciones, excepto en Guadalajara (Tabla 1).

Los valores de los índices de diferenciación fueron $G_{ST} = 0.17$ y $R_{ST} = 0.53$, siendo R_{ST} significativamente mayor que G_{ST} ($P < 0.0001$), indicando la presencia de una estructura filogeográfica. Los análisis de estructura poblacional llevados a cabo con BAPS mostraron que la mejor partición contiene nueve clusters. Varios de estos clusters están representados por una sola población, presentándose en el centro de México (clusters C – G). Mientras tanto, los demás clusters se encuentran en el Altiplano mexicano (cluster A y B), en la región cercana al IT (cluster H) y en Centroamérica (Cluster I). La red de haplotipos mostró una baja estructura geográfica de los haplotipos. Solamente los haplotipos encontrados en los clusters H e I identificados por BAPS se encuentran formando dos haplogrupos distintos. Sin embargo, los demás clusters no muestran un patrón claro, encontrándose en varias ramas de la red de haplotipos.

El análisis llevado a cabo con BARRIER encontró que existen cuatro discontinuidades genéticas en la distribución de *T. tubaeformis*. La barrera con el mayor soporte (72%, Barrera I en Figura 1) ocurre separa a la población 9 ubicada en el extremo oriental de la FVT de aquella presente en una porción central de la FVT y de la población ubicada en la SMS. La segunda barrera con el mayor soporte (70%) se encuentra separando las poblaciones del Altiplano y de la FVT de forma este-oeste (Barrera II en Figura 3). Por su parte, la Barrera III presentó un soporte del 69%. Esta barrera se encuentra cerca del IT, separando a las poblaciones de Oaxaca y Chiapas de las poblaciones centroamericanas (Figura 3).

El análisis de varianza molecular (AMOVA) tomando a todas las localidades como solo grupo reveló el mayor porcentaje de la variación tomando F_{ST} es explicado por la varianza dentro de las poblaciones (58.87%), mientras que para R_{ST} se encuentra entre poblaciones (53.45%)

(Tabla 2). Por otro lado, al realizar la regionalización de las localidades de acuerdo a las discontinuidades genéticas calculadas por BARRIER, se pudo observar que, al realizar los cálculos con F_{ST}, la varianza dentro de las poblaciones explica el mayor porcentaje. Mientras, la varianza entre grupos y entre poblaciones dentro de las poblaciones explica un menor porcentaje de la variación. En contraste, al utilizar R_{ST} se encontró que la variación entre grupos explica el mayor porcentaje de la varianza (55.53) (Tabla 2). Por último, se encontró que *T. tubaeformis* y *T. rotundifolia* presentan cinco haplotipos compartidos (Tabla 3). En la mayoría de los casos los haplotipos se comparten entre poblaciones lejanas; solamente un haplotipo es compartido en la misma región (haplotipo 3, Tabla 3). Sin embargo, el número de individuos que comparten dicho haplotipo es bajo (2 individuos).

DISCUSIÓN

Los trabajos que evalúan la estructura geográfica de especies herbáceas anuales son escasos. En este trabajo se exploró la distribución de la variación genética de *T. tubaeformis* utilizando seis loci de cpSSR y se compara con aquella reportada en el capítulo anterior para *T. rotundifolia*. Asimismo, con el fin de realizar hipótesis de los efectos de las oscilaciones climáticas ocurridas durante el Pleistoceno sobre la variación genética de *T. tubaeformis*, se llevaron a cabo SDM para diferentes tiempos históricos (UIG, UMG). Posteriormente, se discute si la distribución de la diversidad genética coincide con los modelos de distribución llevados a cabo. Por último, se exploró si existe evidencia de eventos históricos de hibridación entre *T. tubaeformis* y *T. rotundifolia* tomando en cuenta las áreas de idoneidad reveladas por los SDM y los haplotipos compartidos entre ambas especies.

***Diversidad genética y diferenciación poblacional en* T. tubaeformis—**Los estudios que aborden la estructura genética de especies anuales en el Neotrópico son escasos. Sin embargo, como se hizo notar en el capítulo III, *T. rotundifolia* mostró una mayor diversidad genética (h_S = 0.74 y h_T = 0.98) con un mayor número de haplotipos (60 haplotipos) comparado con otras especies arbóreas y herbáceas perennes estudiadas en el norte del Neotrópico (Twyford et al., 2013; Rodríguez-Correa et al., 2017). Por su parte, al analizar la diversidad genética de *T. tubaeformis* (h_S = 0.81 y h_T = 0.98) encontramos valores similares a los reportados para *T. rotundifolia*. De la misma forma encontramos que *T. tubaeformis* presenta un número de haplotipos similiar al de *T. rotundifolia* así como de haplotipos privados (80%). Como se hizo notar en el capítulo III, la gran cantidad de haplotipos que presentan estas especies puede estar relacionado con el corto tiempo generacional así como con el gran tamaño poblacional que presentan. En otros trabajos se ha encontrado un patrón similar en cuanto al número de haplotipos y la diversidad genética de especies anuales comparado con especies perennes en Europa (Vrancken et al., 2009).

***Estructura geográfica e historia poblacional de* Tithonia tubaeformis—**En este trabajo, se realizaron inferencias acerca de la historia poblacional de *T. tubaeformis*. Para generar hipótesis robustas, se utilizaron datos paleoclimáticos a partir de los cuales se generaron modelos de distribución de especies para el Último Interglacial (UIG) y el Último Máximo Glacial (UMG). Este enfoque ha sido sugerido por varios autores (Buckley et al., 2009; Lawson, 2010; Alvarado-Serrano y Knowles, 2014), y es considerado un buen punto de partida para considerar los efectos de las oscilaciones climáticas sobre la distribución de las especies. Como puede apreciarse en la Figura 2, los efectos que tuvieron los diferentes escenarios climáticos sobre *T. tubaeformis* ocasionaron que las zonas de idoneidad climática se ubicaran principalmente en el

Altiplano así como en la Faja Volcánica Transmexicana (FVT) y la Sierra Madre del Sur (SMS). A pesar de que *T. tubaeformis* es una especie que puede encontrarse en una variedad de tipos de vegetación, incluyendo matorrales y bosque tropical caducifolio, ésta se encuentra asociada principalmente a bosques templados de *Quercus* o *Quercus – Pinus* (La Duke, 1982). Las reconstrucciones paleoclimáticas sugieren que durante el UMG la parte norte de México, incluyendo el altiplano, fueron más frías y húmedas de lo que son actualmente. Lo anterior permitió el establecimiento de extensos bosques de *Pinus, Juniperus* y bosques mixtos que se extendieron desde el Pleistoceno tardío hasta el Holoceno temprano (Meyer, 1973; Metcalfe et al., 2000). Asimismo, el registro fósil sugiere que el régimen actual de lluvias en el norte de México se estableció en el Holoceno medio o tardío (van Devender, 1990; Spaulding, 1991). La ocurrencia de zonas de estabilidad climática para *T. tubaeformis* en el altiplano durante el UMG sugiere que ésta se encontró asociada a dichos bosques templados. La presencia de una alta diversidad genética en las zonas norteñas de México (Tabla 1) y los modelos de distribución de especies sugieren que éstas actuaron como refugios para *T. tubaeformis* durante las épocas glaciales Pleistocénicas.

Los modelos de distribución de especies realizados para *T. tubaeformis* sugieren que además del Altiplano, la FVT actuó como otro sitio de estabilidad climática, particularmente la porción oriental de ésta (Figura 2). Las reconstrucciones paleoecológicas sugieren que la FVT ha presentado una gran variabilidad climática, oscilando entre períodos cálidos y húmedos, y fríos y secos (Metcalfe et al., 2000). Asimismo, la intensa actividad volcánica ocurrida a lo largo de la FVT desde el Mioceno hasta la actualidad ha sido parcialmente responsable de la gran heterogeneidad que ocurre en esta zona (Gómez-Tuena et al., 2007), generando patrones complejos de estructuración genética en una serie de organismos (Bryson et al., 2011; Ruiz-Sánchez y Specht, 2013; Pérez-Crespo et al., 2017; Rodríguez-Gómez et al., 2018). A pesar de

que el tipo de vegetación más frecuente en la FVT ha sido el bosque mixto de *Pinus* y *Quercus*, se sabe que durante el Pleistoceno tardío la porción oeste fue más húmeda y la porción este más árida de lo que es actualmente (Metcalfe, 2006), por lo que algunos autores han propuesto la regionalización de la FVT en una porción este, central y una oeste (Corona et al., 2007; Gómez-Tuena et al., 2007). La estructuración poblacional encontrada en *T. tubaeformis* es compleja en la FVT. Cada una de las poblaciones muestreadas a lo largo de la FVT corresponde a un cluster genético distinto de acuerdo al análisis llevado a cabo por BAPS. Asimismo, el análisis de varianza molecular mostró que una subdivisión de las localidades en varios grupos explica el mayor porcentaje de variación entre grupos (Tabla 2), mientras que las barreras geográficas identificadas por BARRIER muestran una subdivisión de las localidades colectadas en la FVT (Figura 3). La estructuración de *T. tubaeformis* en la FVT puede deberse a la intensidad actividad volcánica registrada en la región, creando hábitats disponibles para algunas especies de Asteraceae que se ven favorecidas bajo algún grado de disturbio (Villaseñor, 1990). Debido a la alta actividad volcánica en la FVT, se sugiere que existió una disponibilidad de hábitats distribuida de forma heterogénea en la región, creando poblaciones de *T. tubaeformis* entre las cuales existió una baja tasa de migración.

Por otra parte, debido a la estrecha relación de los haplotipos de las poblaciones de *T. tubaeformis* colectadas en las zonas aledañas a la porción oeste de la FVT (poblaciones 5, 3, 6 y 10) evidenciadas en la red de haplotipos y a la ausencia de barreras geográficas inferidas por BARRIER entre estas poblaciones, sugerimos que éstas pudieron haberse originado a partir de un refugio ubicado en la FVT a partir del cual hubo una expansión después de las oscilaciones climáticas del Pleistoceno. Sin embargo, una explicación adicional a la estructuración poblacional encontrada en *T. tubaeformis* está relacionada con las actividades humanas. La zona centro de México ha sido de preferencia para los asentamientos humanos desde las épocas precolombinas.

En estos sitios, la presencia de polen de *Zea mays*, el cual indica la presencia de actividades agrícolas, mostró un aumento significativo hace 2700 años (Metcalfe et al., 2000; Bhattacharya y Byrne, 2016). Debido a que *T. tubaeformis* es una maleza que frecuentemente se encuentra en los cultivos de *Z. mays* (Vibrans, 1999, 2016), el transporte a larga debido a las actividades humanas es altamente probable a pesar de la inherente baja tasa de dispersión de sus aquenios (Vibrans, 1999). Por lo tanto, la compleja estructuración de *T. tubaeformis* en el centro de México puede deberse tanto a factores históricos climáticos como a factores ocurridos recientemente en el Holoceno debido al establecimiento de grandes poblaciones humanas.

La modelación de distribución de especies sugiere una migración de *T. tubaeformis* desde el Altiplano y la FVT hacia las porciones sur de México y Centroamérica después del UMG. Si existió una migración con dirección norte – sur después del UMG se esperaría que los sitios recientemente colonizados presenten una baja diversidad genética. Esta reducción en la diversidad genética en poblaciones periféricas se ha observado en varios estudios sobre la historia poblacional de especies de ambientes templados (Sewell et al., 1996; Hewitt, 2001; Gugger et al., 2011). Este patrón puede emerger cuando existen refugios aislados durante los máximos glaciales, originando poblaciones poco diversas a partir de éstos (Sewell et al., 1996). Sin embargo, este mismo patrón puede originarse debido al efecto '*leading-edge*' (Hewitt, 2001). Este modelo supone que las poblaciones presentarán un decremento paulatino de la diversidad genética hacia las zonas de recolonización debido a repetidos efectos fundadores (Hewitt, 2001). En *T. tubaeformis* se observó que los valores más bajos de diversidad genética se encontraron en las poblaciones al este del Istmo de Tehuantepec (Tabla 1). Estos resultados sugieren que hubo una colonización hacia estas zonas después de las oscilaciones climáticas del Pleistoceno, resultando en una baja diversidad genética en las poblaciones Centroamericanas, las cuales se encuentran en la periferia de la distribución de *T. tubaeformis* (poblaciones 15, 16 y 17; Tabla 1).

Asimismo, la presencia de una barrera geográfica delimitando las poblaciones mexicanas y centroamericanas sugiere la diferenciación entre éstas después de la expansión de su área de distribución.

¿La hibridación entre* T. tubaeformis *y* T. rotundifolia *tiene un papel en la distribución de su variación genética?—*Tithonia tubaeformis*** y ***T. rotundifolia*** forman al menos cinco zonas de hibridación contemporáneas en la Cuenca del Balsas y en la Sierra Madre del Sur (López-Caamal et al., 2018). Los modelos de distribución de ambas especies mostraron que bajo las condiciones actuales, ambas especies comparten zonas de idoneidad climática en la Cuenca del Balsas así como en porciones de la Sierra Madre del Sur, la Provincia del Pacífico, (particularmente en la zona del Istmo de Tehuantepec) y en la Península de Yucatán (Figura 2d). Estas zonas de solapamiento coinciden con la presencia de zonas de simpatría entre ambas especies (López-Caamal, observación personal). Por su parte, ambas especies presentaron áreas compartidas de idoneidad climática durante el UMG, particularmente en la FVT, en la SMS y en zonas del Altiplano (Figuras 2b y 2c). Mientras tanto, durante el UIG ambas especies mostraron una distribución restringida, en la que no presentaron zonas comunes de idoneidad climática (Figura 2a). Estos resultados sugieren que estas especies han sufrido de contracciones y expansiones de su área de distribución a lo largo del tiempo, conduciendo a zonas de contacto secundario. Varios estudios han demostrado que durante los periodos de contacto secundario producto de un cambio climático, se puede dar la simpatría entre linajes divergentes (Hewitt, 2001; Belahbib et al., 2001; Nevill et al., 2014), conduciendo a una mayor diversidad genética y a la presencia de haplotipos compartidos entre especies en los sitios propuestos como refugios pleistocénicos (e.g. Nevill et al., 2014). A pesar de que *T. rotundifolia* y *T. tubaeformis* comparten zonas extensas de idoneidad climática durante el UMG, encontramos un bajo número de haplotipos compartidos.

Además, dichos haplotipos se encuentran entre poblaciones de *T. rotundifolia* y *T. tubaeformis* que en algunos casos se encuentran separadas por más de 400 km, por lo que es poco probable que la hibridación sea responsable de la presencia de su aparición. Por lo tanto, la evolución convergente y la retención de un polimorfismo ancestral (sorteo incompleto de linajes) son otras alternativas para la aparición de haplotipos compartidos entre *T. tubaeformis* y *T. rotundifolia* (Palme et al., 2004). A pesar de que los datos de este trabajo no permiten discriminar entre estas dos opciones, se sugiere que la hibridación no ha jugado un papel importante en la distribución de la variación genética de las poblaciones de *Tithonia*. Aparentemente, las zonas de hibridación detectadas tienen un origen reciente, y pueden estar relacionadas con los constantes disturbios producto de las actividades humanas. Específicamente, debido a que estas especies son malezas asociadas a cultivos (principalmente de *Zea mays*; Vibrans, 1999), las actividades agrícolas permiten la creación de ambientes en los que ambas especies pueden entrar en contacto y formar zonas de hibridación. Sin embargo, el estudio de un mayor número de localidades de *T. tubaeformis* y *T. rotundifolia* con un mayor número de marcadores permitirán evaluar de forma más precisa el papel de la hibridación en la distribución de su variación genética.

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TABLA 1. Poblaciones de *Tithonia tubaeformis* muestreadas para este estudio. Para cada una se muestra el número de individuos (*n*), la latitud, longitud, el número de haplotipos, el número de haplotipos privados (*PA*), así como la riqueza haplotípica (*Rh*), la diversidad genética de Nei (*He*), la distancia intrapoblacional (D^2_{sh}) y el estadístico *F* de Fu

Población	Sitio de colecta	AMOVA regionalization	*n*	Latitud	Longitud	No. de Haplotipos	PA	*Rh*	*He*	D^2_{sh}	*F*
1	Fresnillo, Zacatecas	a	6	23.12	-102.82	3	1	1.80	0.80	20.80	4.54
2	Guadalupe, Zacatecas	a	4	22.75	-102.45	4	1	3.00	1.00	20.67	0.25
3	Valle de Guadalupe, Jalisco	b	10	21.03	-102.59	8	6	2.73	0.96	10.62	-1.32
4	San Miguel de Allende, Guanajuato	a	8	20.89	-100.75	8	7	3.00	1.00	18.76	-2.22
5	Guadalajara, Jalisco	b	10	20.71	-103.48	9	6	2.87	0.98	6.48	**-3.94**
6	Panindícuaro Michoacán	b	10	19.97	-101.77	6	6	2.37	0.89	24.80	2.08
7	Contepec, Michoacán	a	9	19.88	-100.14	6	5	2.38	0.89	26.13	1.65
8	Chamilpa, Morelos	b	10	18.97	-99.23	7	6	2.50	0.91	7.47	-0.35
9	Ciudad Mendoza, Veracruz	a	8	18.81	-97.18	6	5	2.57	0.93	19.76	0.79
10	Alcaraces, Colima	b	9	19.36	-103.56	4	3	1.73	0.75	0.33	-0.82
11	Chilpancingo, Guerrero	b	13	17.60	-99.52	9	8	2.52	0.91	4.89	-2.27
12	Salina Cruz, Oaxaca	c	6	16.21	-95.20	4	1	2.00	0.80	1.33	-0.27
13	Puerto Arista, Chiapas	c	8	15.94	-93.80	3	0	1.41	0.68	1.04	1.53
14	Jiquipilas, Chiapas	c	5	16.69	-93.64	3	0	1.60	0.70	12.67	2.6
15	El Aguacate, Sacatepéquez	d	6	14.60	-90.62	2	0	0.93	0.53	3.20	4.18
16	Escuela Agrícola Panamericana, Fco. Morazán	d	8	13.98	-86.98	2	0	0.97	0.57	3.43	5.23
17	San Buenaventura, Fco. Morazán	d	6	13.88	-87.20	2	1	0.93	0.53	3.20	4.18
	TOTAL / MEDIA (EE)		136			69	56	2.08 (0.71)	0.81 (0.16)	10.92 (9.02)	**-24.27**

TABLA 2. Análisis de varianza molecular (AMOVA) para las poblaciones de *T. tubaeformis* tratadas como un solo grupo (a) y agrupadas según las barreras geográficas detectadas por BARRIER (b).

Fuente de variación	g.l.	SC	Componente de varianza	Porcentaje de variación	Índices de fijación
a) Poblaciones tratadas como un solo grupo					
F_{ST}					
Entre poblaciones	16	120.09	0.79	41.13	
Dentro de las poblaciones	119	136.08	1.14	58.87	$\Phi_{ST} = 0.41$*
Total	135	256.17	1.94		
R_{ST}					
Entre poblaciones	16	2368.21	16.75	53.45	
Dentro de las poblaciones	119	1736.86	14.59	46.55	$\Phi_{ST} = 0.53$*
Total	135	4105.04	31.35		
b) Agrupamiento de acuerdo a BARRIER					
F_{ST}					
Entre grupos	3	69.85	0.63	29.86	$\Phi_{CT} = 0.29$*
Entre poblaciones dentro de los grupos	13	50.24	0.33	16.00	$\Phi_{SC} = 0.22$*
Dentro de las poblaciones	119	136.08	1.14	54.14	$\Phi_{ST} = 0.45$*
Total	135	256.17	2.11		
R_{ST}					
Entre grupos	3	1990.47	20.46	55.53	$\Phi_{CT} = 0.55$*
Entre poblaciones dentro de los grupos	13	377.73	1.79	4.87	$\Phi_{SC} = 0.10$*
Dentro de las poblaciones	119	1736.83	14.59	39.60	$\Phi_{ST} = 0.60$*
Total	135	4105.44	36.85		

TABLA 3. Haplotipos compartidos entre las poblaciones de *T. tubaeformis* analizadas en el presente capítulo con aquellas de *T. rotundifolia* analizadas en el capítulo III. Se muestra el tamaño de cada locus de microsatélite de cloroplasto. El nombre de las localidades de *T. tubaeformis* es igual al de la Tabla 1, mientras que para *T. rotundifolia*, las poblaciones corresponden a la Tabla 1 presentada en el Capítulo 3.

Haplotipo	Alelos (pb)						Localidades	
	ccmp1	ccmp2	ccmp4	ccmp7	NTCP9	NTCP18	*T. tubaeformis*	*T. rotundifolia*
1	136	203	115	114	266	172	9	10
2	136	204	115	114	267	172	5, 4	11
3	136	204	115	115	268	172	12	13
4	136	204	116	114	268	172	9	14
5	136	204	116	116	270	172	1, 2	16

FIGURA 1. Salida logística de los modelos de distribución de especies para *Tithonia tubaeformis* (a) durante el Último Interglacial (UIG), el Último Máximo Glacial (UMG) bajo dos distintos modelos (CCSM y MIROC) y en las condiciones acutales. Se muestra también el sobrelapamiento de los modelos (b). En verde se muestran las zonas de idoneidad climática desde el UMG y en amarillo, las zonas de idoneidad climática desde el UIG. 1 = Sierra Madre Oriental, 2 = Altiplano, 3 = Sierra Madre Oriental, 4 = Provincia del Pacífico, 5 = Faja Volcánica Transmexicana, 6 = Cuenca del Balsas, 7 = Sierra Madre del Sur, 8 = Península de Yucatán, 9 = Altos de Chiapas (Morrone, 2014).

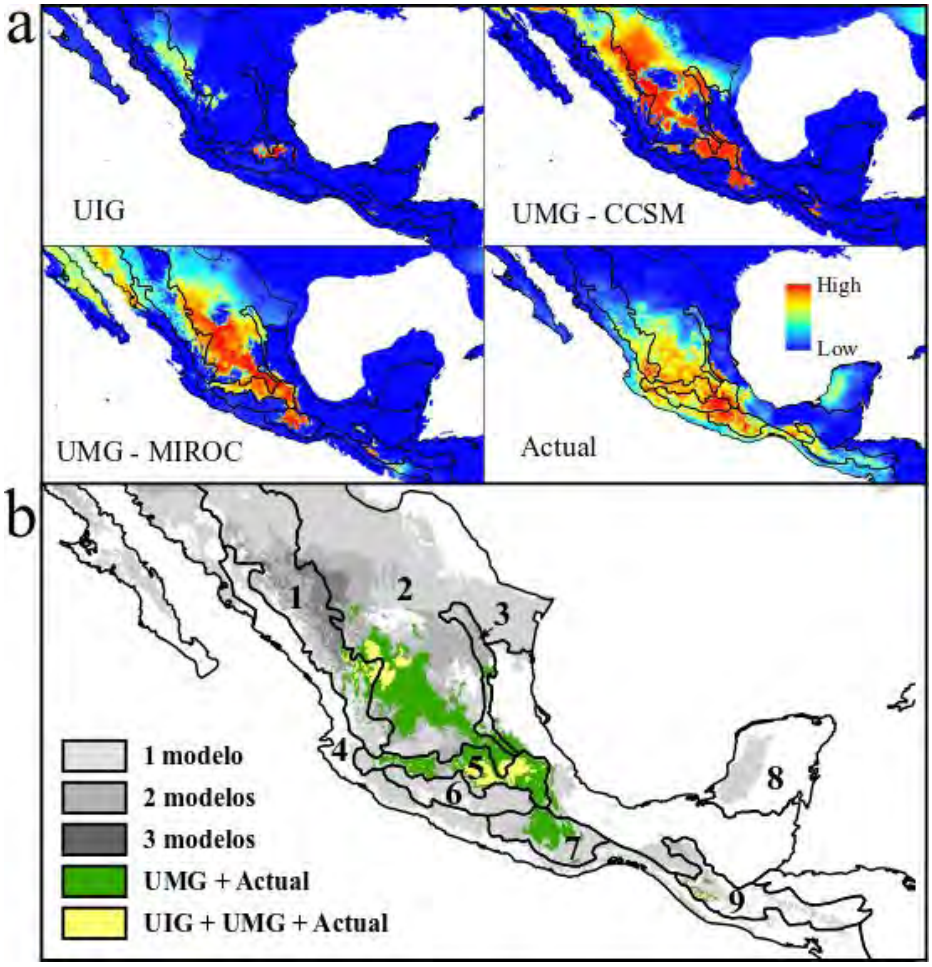


FIGURA 2. Comparación de las zonas de idoneidad climática de *Tithonia tubaeformis* y T. *rotundifolia* durante el Último Interglacial (a), el Último Máximo Glacial bajo el modelo CCSM (b) y MIROC (c), así como en las condiciones acutales. Las zonas de idoneidad climática en común para ambas especies se muestran en color anaranjado. Las líneas representan las provincias biogeográficas de acuerdo a Morrone (2014).

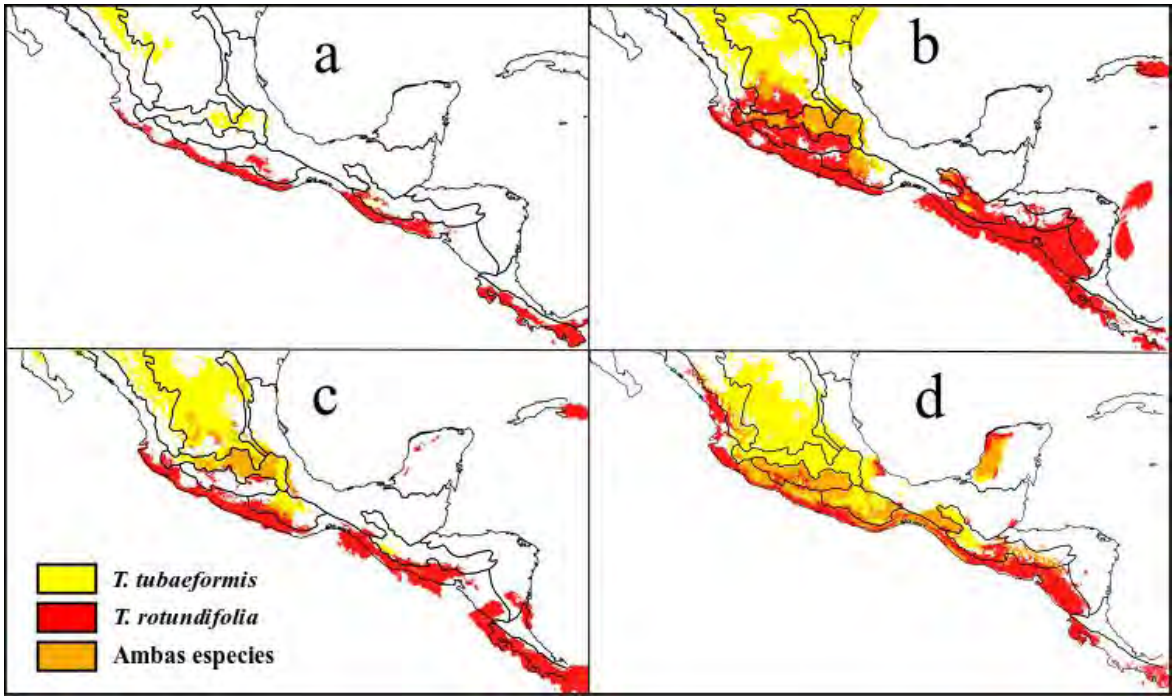
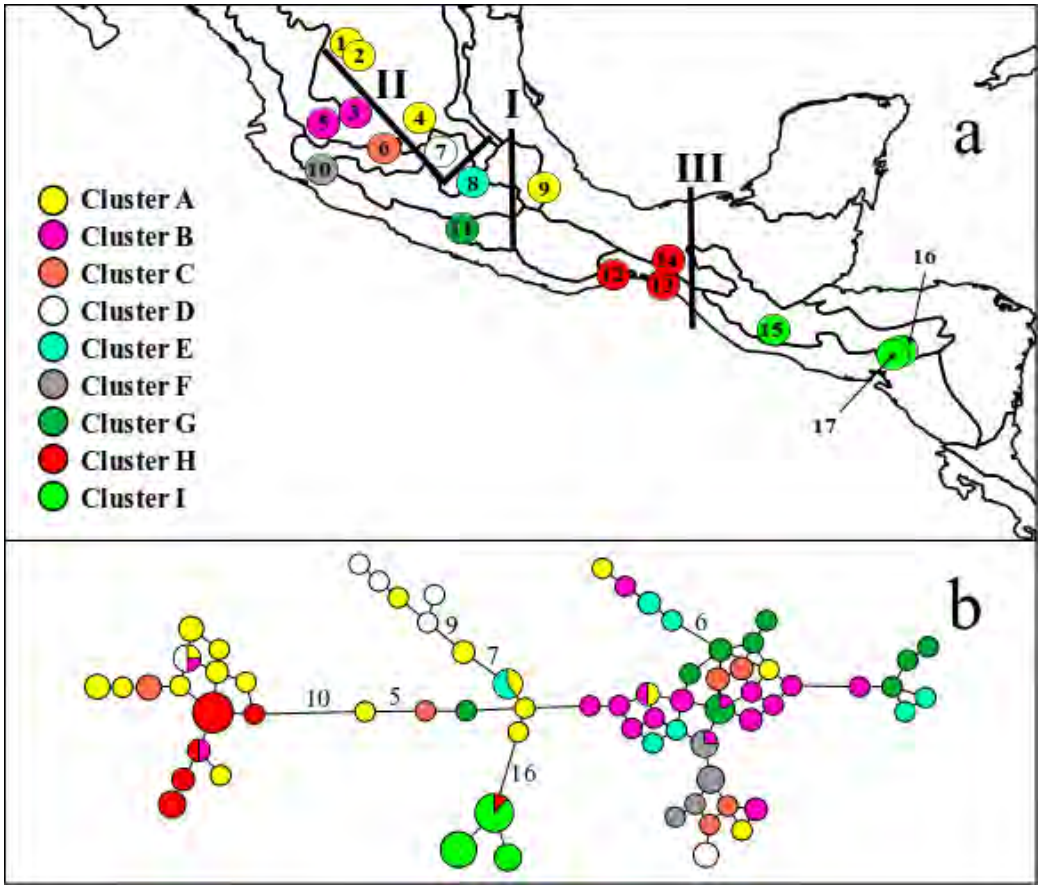


FIGURA 3. Poblaciones de *Tithonia tubaeformis* incluidas en este trabajo (a). Las poblaciones se encuentran enumeradas de acuerdo a la Tabla 1 y los colores representan los clusters genéticos identificados por BAPS. Las líneas gruesas representan a las barreras geográficas identificadas por BARRIER. Barrera I (soporte 72%), Barrera II (soporte 70%), Barrerra III (soporte 69%). La red de haplotipos se muestra en (b). Los colores indican los diferentes clusters genéticos identificados por BAPS, mientras que el tamaño de cada nodo representa la frecuencia de cada haplotipo.



DISCUSIÓN GENERAL

Asteraceae es una de las familias más diversas de las plantas con flor. Se ha calculado que esta familia constituye cerca del 10% de la flora mundial (Funk et al., 2005). Dentro de esta familia, se tienen registros de la ocurrencia de zonas de hibridación contemporáneas (Ferriol et al., 2014; Hülber et al., 2015; Zhang et al., 2017), así como de evidencia de evolución reticulada a través de estudios filogenéticos (Schilling y Panero, 1996, 2011; Vargas et al., 2017), sugiriendo que la hibridación ha jugado un papel importante en la evolución de la familia.

El género *Tithonia* es uno de los más representativos de Asteraceae en México y Centroamérica (La Duke, 1982). Los reportes de hibridación en *Tithonia* son escasos a pesar de que se ha reportado la posibilidad de híbridos entre distintas especies dentro del género así como con especies de géneros relacionados (La Duke, 1982; Reyes-Valdés et al., 2005; Gómez-Martínez et al., 2010). En este trabajo se estudió la dinámica de la hibridación entre las dos especies arvenses y ruderales con la mayor área de distribución del género: *T. tubaeformis* y *T. rotundifolia*. Para este fin, se emplearon marcadores moleculares (microsatélites nucleares) así como marcadores químicos (metabolitos secundarios) con la intención de explorar la incidencia de hibridación en cuatro zonas de hibridación contemporáneas así como del fenotipo químico de los híbridos. Asimismo, se describió la distribución de la variación genética de estas especies mediante el uso de marcadores microsatélites de cloroplasto. Además de inferir los procesos históricos que pudieron dar origen a los patrones de diversidad y estructura genética observados, el uso de cpSSR nos permitió evaluar si existen haplotipos compartidos entre las especies, lo que podría indicar eventos históricos de hibridación entre estas especies. Los patrones de la hibridación contemporáneos explorados en el capítulo II y la estructura geográfica de las especies (capítulos III y IV) se discuten de manera general en las siguientes secciones.

Hibridación entre *Tithonia tubaeformis* y *T. rotundifolia*

La hibridación natural es una importante fuerza evolutiva en plantas vasculares con una amplia variedad de consecuencias para los taxa participantes así como para los ambientes en los que se establecen (Payseur y Rieseberg, 2016). En este trabajo, analizamos la hibridación entre dos especies de malezas anuales ampliamente distribuidas en México y Centroamérica. *Tithonia tubaeformis* y *T. rotundifolia* se establecen en ambientes altamente perturbados por actividades humanas (cultivos, orilla de caminos). Sin embargo, ambas especies presentan una importante divergencia en su preferencia de hábitat como sugiere La Duke (1982). Los modelos de distribución de especies descritos para cada una de las especies en los capítulos III y IV confirman esta observación. *Tithonia tubaeformis* se establece preferentemente en ambientes mésicos en el centro del país, mientras que *T. rotundifolia* lo hace en las costas del pacífico, asociada a ambientes perturbados en bosques tropicales secos. A pesar de la evidente divergencia en sus preferencias de hábitats, fue posible encontrar zonas mixtas en las que se presentan individuos atípicos producto de la hibridación entre estas especies (capítulo II). Por lo que es interesante discutir, ¿cuáles son los factores que favorecen el establecimiento de individuos híbridos entre estas especies?

Desde el inicio del estudio formal de la hibridación, autores como Anderson y Stebbins han sugerido que los híbridos pueden presentan una mayor adecuación en sitios con algún tipo de disturbio. Este patrón ha sido observado en un buen número de trabajos (Todesco et al., 2016). Sin embargo, muchos de éstos tratan con elementos de vegetación primaria que exhiben un estrés fisiológico bajo condiciones de disturbio (p. ej. Lamont et al., 2003). Las especies de *Tithonia* estudiadas en esta tesis son elementos de vegetación secundaria propios de sitios con algún tipo de disturbio, por lo que éstos pueden ser menos importantes en el establecimiento de híbridos. Esto no significa necesariamente que los disturbios no se encuentren implicados en el proceso de hibridación. Al ser elementos de vegetación secundaria, los hábitats disponibles para *T.*

tubaeformis y *T. rotundifolia* se encuentran distribuidos en parches a lo largo de su área de distribución geográfica. A pesar de que los modelos de distribución sugieren un continuo en la distribución de estas especies, es evidente que esto no ocurre así. Las localidades en las cuales se pueden encontrar estas especies generalmente están sujetas a las actividades humanas en los bordes de caminos y cultivos. Por lo tanto, a pesar de que los disturbios podrían no estar implicados en el establecimiento de individuos híbridos (sensu Anderson, 1948), podrían tener un papel importante al crear ambientes en los cuales ambas especies pueden establecerse y entrar en simpatría.

Los modelos de distribución de especies sugieren áreas de simpatría en la periferia de las potenciales zonas de distribución para ambas especies (Cuenca del Balsas, algunas porciones de la Sierra Madre del Sur y de la costa del Pacífico). Las sitios mixtos de *T. tubaeformis* y *T. rotundifolia* encontradas en el Capítulo II ocurren en estas zonas. No obstante, el encontrar dichas zonas de simpatría fue más difícil de lo que sugieren los modelos de distribución. La distribución de los hábitats en parches arriba mencionada puede explicar la dificultad para encontrar dichas zonas de hibridación. Adicionalmente, en algunos sitios se encontró que ambas especies coincidían espacialmente, sin embargo, las épocas de floración diferían, reduciendo así la incidencia de individuos híbridos en una localidad dada (observación personal). Lo anterior puede deberse a la acción de los factores ambientales implicados en la fenología floral de varias especies (Hülber et al., 2010).

Un estudio previo encontró individuos híbridos entre *T. tubaeformis* y *T. rotundifolia*, sugiriendo un patrón de flujo unidireccional hacia *T. rotundifolia* mediante el uso de marcadores dominantes (RAPD; Tovar-Sánchez et al., 2012). Asimismo, López-Caamal et al., (2013) encontraron caracteres morfológicos transgresivos en las zonas mixtas con respecto a los sitios puros de cada especie. En el presente trabajo, se revisaron dichas zonas de hibridación utilizando marcadores codominantes (nSSR). El enfoque utilizado nos permitió identificar y agrupar a los

individuos híbridos en distintas clases de acuerdo a su ancestría. Debido a que se utilizaron pocos loci para este fin, no fue posible distinguir a los individuos híbridos F1 de las retrocruzas hacia ambas especies progenitoras. Sin embargo, a diferencia del estudio llevado a cabo por Tovar-Sánchez et al. (2012), en el presente trabajo se lograron identificar retrocruzas hacia ambas especies progenitoras.

Además del uso de marcadores moleculares, en este trabajo se utilizaron marcadores químicos para explorar el fenotipo de los individuos híbridos. Ambas especies progenitoras fueron fácilmente distinguibles en este sentido: *T. tubaeformis* mostró un flavonoide característico (tithonina), mientras que para *T. rotundifolia* se identificaron dos lactonas sesquiterpénicas diagnósticas (3-acetil-15-hidroxileptocarpina y 15-hidroxileptocarpina). De acuerdo a lo esperado por varios autores (Cheng et al., 2011), los individuos híbridos identificados presentaron un mosaico en la expresión de dichos metabolitos. Algunos individuos híbridos presentaron aditividad de estos metabolitos, mientras que otros mostraron dominancia. Otros tantos (retrocruzas hacia *T. tubaeformis*) mostraron un perfil químico diferente al de las especies progenitoras en el que se describió un compuesto nuevo (5,3',4'-trihidroxi-6,7,8-trimetoxiflavanona). A pesar de que no se logró la identificación genética de los individuos híbridos F1, se ha encontrado frecuentemente que la F1 presenta aditividad de los metabolitos secundarios de ambas especies progenitoras (Cheng et al., 2011). Si ese fuera el caso para los híbridos derivados de *T. tubaeformis* y *T. rotundifolia*, podríamos sugerir que la formación de la primera generación híbrida es difícil, ya que existe un bajo porcentaje de individuos híbridos mostrando aditividad (9%). En otros trabajos, se ha encontrado que la formación de la F1 es difícil, ya que en esta generación se pierde la combinación de genes co-adaptados en las especies progenitoras, resultando en individuos con una baja adecuación. En este sentido, Hauser y Morrison (1964) encontraron una reducción en la viabilidad

de polen de un híbrido putativo entre *T. tubaeformis* y *T. rotundifolia*. Estas observaciones sugieren que existe una selección endógena en contra de los individuos híbridos derivados de estas especies.

Dadas las características del flujo génico entre *T. tubaeformis* y *T. rotundifolia*, se sugiere que el modelo de zona híbrida que mejor se ajusta es el de modelo de Mosaico (Harrison, 1986). La discontinuidad de los hábitats disponibles para las especies progenitoras, así como la ausencia de una transición morfológica gradual entre ambas especies son argumentos a favor de dicho modelo. Sin embargo, el modelo de Mosaico cae dentro de la categoría de los modelos dependientes del ambiente. Es decir, bajo este modelo se esperaría que la adecuación de los híbridos sea relativa al ambiente en el que éstos se establezcan. Los resultados obtenidos sugieren cierto grado de selección endógena en contra de los híbridos, por lo que es necesaria la exploración de la adecuación de los híbridos en distintos ambientes con el fin de conocer si la adecuación de éstos difiere entre ambientes contrastantes.

Estructura geográfica e historia poblacional de *T. tubaeformis* y *T. rotundifolia*

En los capítulos III y IV se estudió la distribución de la diversidad genética en *T. tubaeformis* y *T. rotundifolia*. Asimismo, con la ayuda de modelos de distribución de especies se generaron hipótesis para explicar la historia de las poblaciones durante las oscilaciones climáticas Pleistocénicas. Es importante notar que este trabajo es uno de los primeros en explorar los patrones de diversidad en especies anuales Neotropicales. Además, los trabajos sobre estructura geográfica y filogeografía en el Neotrópico se centran en el estudio de especies templadas, de bosque mesófilo o bien, de elementos de selvas tropicales altas. Por su parte, los estudios sobre especies asociadas con el bosque tropical caducifolio son escasos (p. ej. Arias et al., 2010).

Estudios recientes sobre la estructura geográfica y filogeografía de diferentes especies Neotropicales (Ornelas et al., 2013 Bagley et al., 2014; Ramírez-Barahona y Eguiarte, 2014;

Rodríguez-Correa et al., 2017; Rodríguez-Gómez et al., 2017) han mostrado que existe una gran complejidad en cuanto a los patrones geográficos que explican la distribución de la variación genética al norte del Neotrópico. No obstante, estos trabajos nos permiten observar que existen algunos patrones en común para varios organismos. En particular, algunas barreras geográficas recurrentes en estos trabajos son el Istmo de Tehuantepec (IT), la Depresión de Nicaragua así como el arco volcánico centroamericano entre otros. De estos, el IT ha sido una barrera que ha sido frecuentemente reportada en distintos taxa de plantas, aves y mamíferos (Ornelas et al., 2013). De la misma forma, Gutiérrez-García y Vázquez-Domínguez (2013) encuentran que el IT es una barrera recurrente en la biota del Bloque Maya. Sin embargo, es importante notar que el tiempo de divergencia entre los linajes al este y al oeste del IT ha variado para los taxa estudiados, evidenciando la compleja historia geológica alrededor del IT así como las características de historia de vida (en particular la dispersión) en la estructuración genética de las poblaciones (Ornelas et al., 2013).

Como ya se ha hecho notar en varios apartados de esta tesis, *T. tubaeformis* y *T. rotundifolia* presentan una divergencia importante en sus preferencias de hábitat, siendo la primera propia de ambientes mésicos y la segunda de ambiéntes xéricos en bosques tropicales caducifolios. En estos capítulos, se emplearon modelos de distribución de especies para realizar hipótesis acerca de su historia poblacional así como datos genéticos derivados de cpSSR. Los modelos de distribución de especies sugirieron que ambas especies tuvieron historias completamente distintas desde el último máximo glacial. Mientras que *T. rotundifolia* mostró áreas de idoneidad climática en las costas del pacífico, *T. tubaeformis* las tuvo hacia el centro del país y más específicamente en el altiplano. A pesar de que no existen registros palinológicos para las costas del pacífico, los datos que existen para distintas zonas del altiplano concuerdan con la hipótesis de refugios para *T. tubaeformis* hacia el altiplano. Asimismo, los modelos de distribución mostraron una disyunción de las áreas de

idoneidad climática en el IT, lo que sugirió un papel de éste en la estructuración genética de las poblaciones. Dichas hipótesis (la estructuración debida al IT y la presencia de refugios en las zonas de idoneidad climática), fueron puestas a prueba mediante el análisis de microsatélites de cloroplasto.

Al llevar a cabo los análisis genéticos de ambas especies con cpSSR encontramos altos valores de diversidad genética así como una alta proporción de haplotipos privados (ca. 80%) en comparación con otras especies Neotropicales. Solamente un estudio llevado a cabo por Twyford et al. (2013) con *Begonia heracleifolia* encontraron un porcentaje similar de haplotipos privados. Al someter a prueba la estructuración de las poblaciones debido a la barrera geográfica del IT se encontró que, a pesar de que está implicado en la estructuración de ambas especies, no es la principal causa de la estructuración de sus poblaciones. En contraste, la subdivisión de las poblaciones en un mayor número de grupos explica mejor la estructuración genética, sugiriendo una compleja histórica poblacional de *T. tubaeformis* y *T. rotundifolia*. Sin embargo, los análisis genéticos para ambas especies sugieren que las zonas de estabilidad climática identificadas desde el último máximo glacial pudieron fungir como refugios a partir de los cuales se dio una expansión poblacional. Por un lado, *T. rotundifolia* mostró, en general, una estructuración de sus poblaciones de acuerdo a las zonas de estabilidad climática ubicadas en las costas del Pacífico. La red de haplotipos sugiere una migración de Centroamérica hacia la región del IT. En contraste, *T. tubaeformis* mostró una estructuración compleja y una mayor diversidad genética en las localidades ubicadas en las zonas de idoneidad climática desde el último máximo glacial. Sin embargo, *T. tubaeformis* mostró una compleja estructuración en esta zona, posiblemente a la gran heterogeneidad ambiental producto de una intensa actividad volcánica en la región durante el Pleistoceno tardío (Rodríguez-Gómez et al., 2018). A medida que las poblaciones se apartan más

de estas zonas de idoneidad climática se observó un decremento de la diversidad genética, sugiriendo una migración con dirección norte – sur desde el altiplano hacia Centroamérica.

Una vez descrita la estructura geográfica de las especies, se exploró si la hibridación puede explicar en parte los complejos patrones de estructuración poblacional encontrados. En este sentido, existen reportes de un gran número de haplotipos entre especies que compartieron su área de distribución durante el último máximo glacial (Belahbib et al., 2001; Nevill et al., 2014). Para el caso de *T. tubaeformis* y *T. rotundifolia*, los Modelos de distribución sugieren una disyunción total para ambas especies durante el último interglacial. Por su parte, durante el último máximo glacial, las zonas de idoneidad climática compartidas se encontraron en la Faja Volcánica Transmexicana así como en algunas porciones de la Sierra Madre del Sur. A pesar de que se encontraron algunos haplotipos compartidos entre especies, las individuos que los presentaban se encontraban separados por más de 200 km, sugiriendo que la evolución convergente, y no la hibridación, es el causante de dicho patrón. Debido a esto, se sugiere que la hibridación no ha tenido un papel fundamental en la distribución de la variación genética de estas especies. Asimismo, las zonas de hibridación contemporáneas aparentemente son producto de un contacto secundario entre *T. tubaeformis* y *T. rotundifolia*. Los disturbios producto de las actividades humanas pueden ser la principal fuerza que provoca el contacto entre estas especies. Sin embargo, la exploración de un mayor número de localidades e individuos así como el uso de un marcadores nucleares permitirán conocer los efectos de la hibridación sobre la distribución de la variación genética de *T. tubaeformis* y *T. rotundifolia*.

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